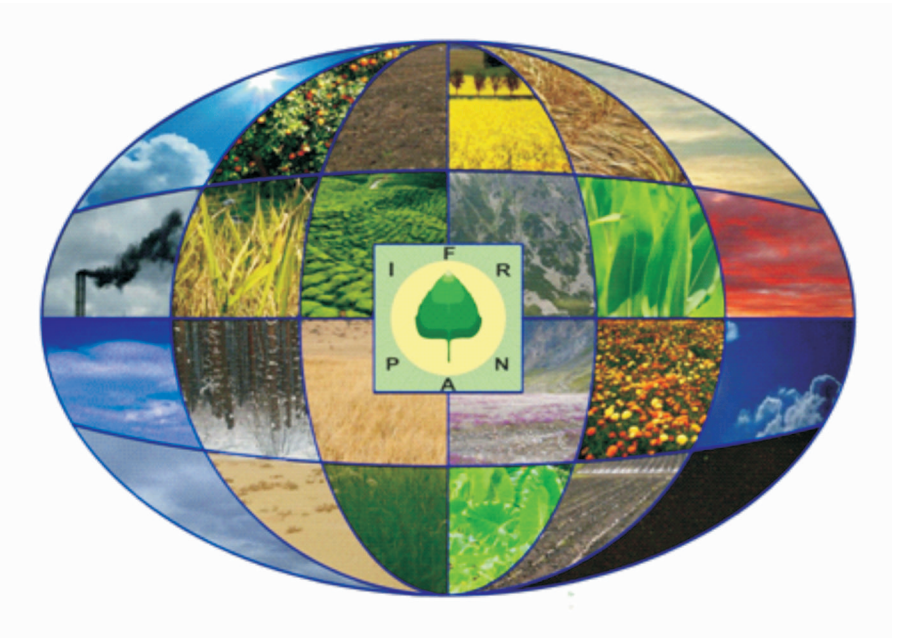
The *F. Górski* Institute of Plant Physiology
Polish Academy of Sciences, Cracow

Cracow-Plant-Stress Conference Series No.9 (problem issues)

Plant Functioning Under Environmental Stress



Edited by

M.T. Grzesiak, A. Rzepka, T. Hura and S. Grzesiak

Cracow 2013

PLANT FUNCTIONING UNDER ENVIRONMENTAL STRESS

EDITED BY

MACIEJ T. GRZESIAK
Department of Ecophysiology, The F. Górski Institute of Plant Physiology, Polish Academy of Sciences, Cracow

ANDRZEJ RZEPKA
Department of Plant Physiology, Institute of Biology, Pedagogical University, Cracow

TOMASZ HURA
Department of Ecophysiology, The F. Górski Institute of Plant Physiology, Polish Academy of Sciences, Cracow

STANISŁAW GRZESIAK
Depatrtment of Ecophysiology, The F. Górski Institute of Plant Physiology, Polish Academy of Sciences, Cracow

THE F. GÓRSKI INSTITUTE OF PLANT PHYSIOLOGY,
POLISH ACADEMY OF SCIENCES,
CRACOW

Published by:
the *F. Górski* Institute of Plant Physiology,
Polish Academy of Sciences,
Niezapominajek street no 2,
PL 30-239 Cracow, Poland

ISBN 978-83-86878-30-7.

Based on the presentations given during the 9th International Conference
"Plant Functioning Under Environmental Stress".
Cracow, 12th to the 15th September, 2012.

This book was financially supported by the Ministry of Sciences
and Higher Education, the Polish Botanical Society, Plant Physiology Branch
and the Committee of Physiology,
Genetics and Plant Breeding, Polish Academy of Sciences.

Cover by Krzysztof Siembiot

Printed by "DRUKROL" LTD. Cracow Poland
Kraków, Ujastek 9
(+48 12) 412-46-50;
http://www.drukrol.pl; e-mail: drukarnia@drukrol.pl

List of Contributors

Cracow, 12th to the 15th September, 2012

PREFACE

Maciej T. Grzesiak

Investigations in the biology of plant stress are the multidisciplinary ones, covering aspects of plant physiology, ecology, biochemistry, biophysics, genetics and breeding. They provide information on physiological processes that are disturbed as a result of the influence of the imposed stress factors. Due to advances in the applied methods and in analytical devices, there exists now a greater possibility to identify and to estimate the reaction of plants to various stresses, at the level of the cell, the tissues, the whole plant, the plant canopy or the ecosystems. In spite of this progress, many problems of stress biology in plants have not yet been solved. The postulate put forward by Professor Jacob Levitt (1911-1990) at the conference in East Lansing in August 1988 entitled „Stress interaction - back to the future", is still a subject of discussion. Professor Levitt postulates that in order to attain further progress it is necessary to reconsider the older, often forgotten research and make an attempt at the synthetic survey of the present state of knowledge related to stress biology in plants. There are many reasons why progress in this area is necessary, considering that about 10 percent of the land surface used for agricultural purposes offers optimal conditions for plant cultivation. The problems of famine in many countries, the losses in food production resulting from drought, floods, too low or too high air temperatures, environmental pollution connected with the industrialization, urbanization, fertilization and chemicalization in agriculture and plant diseases, have become problems of world-wide importance, and they will recur on a global scale because the climate is more and more changeable. The tendency of the climate to warm-up, which has been confirmed by meteorologists, as a result of the "greenhouse effect" does not necessarily imply that there will be no more severe winters.

Higher plants, as sessile organisms, during evolution developed defence mechanisms in order to cope with and function under environmental stresses. Physiologi-

cal basis for plant reaction to environmental stress factors, are despite of this, still the object of researchers interest. Nevertheless, we are far from fully understanding the mechanisms of plant defence against stresses as yet. The modern methodological approach and the applied techniques enable research at various levels of biological organization beginning with field, through single plants, organs, cells and organelles down to the molecular level. The progress in analytical methods allows the investigation of the successive stages of signal transductions or the role of secondary metabolites taking part both in plant defence reactions against stresses and of those responsible for generating stress at the cellular level. The results of these complementary investigations, important from the academic point of view, also provide valuable applicable piece of information, which may be used in agricultural and environmental biotechnology including genetic engineering, selection, and breeding as well as in agronomy.

On a global scale, 90% of all agriculturally used land are affected by abiotic or biotic stress factors and also within the areas that are said to be stress-free, are observed short and of various intensity deviations from the optimum. However, we are still far away from the full and comprehensive knowledge of mechanisms underlying plant acclimatization and adaptation to stress factors. This is due to the complexity of both the reception and transduction of signals, and reactions of plant on various levels of biological organisation, magnified by a multi-gene control of plant responses to stressors, together with the fact, that in many cases there are two or more stress factors appearing together or sequentially. The greater challenge, however, seems to be the proper application of the gained knowledge in practice. Further substantial progress in yielding, both quantitatively and qualitatively, obtained through selection, classical breeding, transgenesis in case of genetically modified organisms or through specially designed agro-technologies, may be effective only if the scientific achievements of physiological, biochemical and molecular bases of plant reactions and tolerance to environmental stress factors are utilized.

Plants, similar to all other living organisms, exist under the influence of the surrounding environment, which in many cases provides conditions far or extremely far from optimal for their growth, development and yielding. Higher plants, as sessile, cannot change their place of existence. Therefore, through evolution, they have developed combinations of molecular, biochemical, physiological, anatomical, morphological, and behavioural features and processes enabling them to adapt to and survive in unfavourable environmental conditions. The processes of acclimatization and adaptation to the changing over time, types and intensity of stressful factors are affected accordingly to the emergence of new threats.

In the popularization of knowledge on plant stress physiology of importance are scientific conferences aiming at presenting the current state of science, exchange of opinions and possibly initiate common scientific projects. International Confer-

ences *„Plant Functioning Under Environmental Stress"* are organized by the *F. Górski* Institute of Plant Physiology of the Polish Academy of Sciences in Cracow under suspicion of Polish Botanical Society and Committee of Physiology, Genetic and Breeding of Plant, Polish Academy of Sciences in co-operation with Slovak Agricultural University in Nitra, Plant Protection Institute, Hungarian Academy of Sciences, University of Life Sciences - SGGW in Warsaw, Pedagogical University in Cracow and Agricultural University in Cracow.

The conference had 176 registered participants including 47 from abroad (Australia, Canada, Iran, Pakistan, England, Denmark, Germany, Portugal, Russia, Slovakia, Turkey, Ukraine, Hungary). Eight plenary lectures were delivered, 35 oral and 134 poster presentation were given. (Abstracts are available at the APP website at www.springer.com DOI 10.1007/s11738-012-1073-0).

There was also a competition for the best presentation of the research results for young scientists. Young scientists were awarded for the best oral presentation: Csilla Juhasz (Hungarian Academy of Sciences), Maciej Maciejewski (Institute of Plant Physiology, Polish Academy of Sciences in Kraków), Michał Dziurka (Institute of Plant Physiology, Polish Academy of Sciences in Kraków), Ernest Bielinis (Poznań University of Life Sciences) and Peter Meszaros (Constantine the Philosopher University in Nitra), and the best poster Agnieszka Wujeska (The University of Melbourne), Svitlana Romanchuk (National Academy of Science of Ukraine), Ernest Bielinis (Poznań University of Life Sciences), Agnieszka Ostrowska (Institute of Plant Physiology, Polish Academy of Sciences in Kraków). Agnieszka Kalandyk (Institute of Plant Physiology, Polish Academy of Sciences in Kraków) and Svitlana Romanchuk (National Academy of Science of Ukraine)

Organizers wish to express their gratitude to the professors Michael B. Jackson (University of Bristol, England), Balazs Barna (Hungarian Academy of Sciences, Budapest), Janusz J. Zwiazek (University of Alberta, Edmonton, Canada), Helena Gawrońska (Warsaw University of Life Sciences), Christian J. Jensen (University of Copenhagen, Denmark), Marcin Rapacz (Agricultural University, Cracow) and Folkard Asch (University of Hohenheim) for their interesting plenary lectures. We are also truly grateful for all chairpersons: Professor Marian Saniewski, Professor Helena Gawrońska, Professor Edward Gwóźdź, Professor Jan Rybczyński and to invited conference guests: Professor Alina Kacperska, Professor Zofia Starck, Professor Angela Filova and Professor Władysław Filek.

We would like to thank for the financial support and for providing some souvenirs to Ministry of Science and Higher Education; Ministry of Agriculture and Rural Development; Ministry of the Environment; Polish Botanical Society; Committee of Physiology, Genetics and Breeding Polish Academy of Sciences; City Council of Cracow; Polish Academy of Arts and Sciences; „Biały Kruk" Publishers, Social Publishing Institute "ZNAK" and the GEOMOR-TECHNIK Ltd. Szczecin.

Cracow, September 12th-15th, 2012

SOME OPTIONS FOR SECURING WATER RESOURCES FOR AGRICULTURAL PRODUCTION

Christian Richardt Jensen
Department of Plant and Environmental Sciences, Faculty of Sciences, University of Copenhagen, Taastrup, Denmark
Corresponding author; email crj@life.ku.dk

Abstract Agriculture is the main user of fresh water resources in the World. Optimisation of agricultural water resources can be obtained by: Selection of water efficient irrigation methods like drip and micro irrigation saving 30-40% water compared to furrow irrigation, and by improving irrigation scheduling using plant and soil sensors and remote sensing based models. Deficit irrigation in the form of regulated deficit irrigation (RDI) and partial root zone drying (PRD) has the potential of raising the water productivity in the range of 10-50%. Use of low quality waters, use of salt tolerant crops, optimising irrigation scheduling are further options for which modelling tools lately have been developed. Using the 'virtual water' principles so that water rich regions secure food supply to dry regions are already tools being exploited. In dry regions small areas with high value crops should be irrigated securing net income and livelihood. In general water can be saved by reducing the overall waste of food throughout the food chain from post-harvest to the end-consumer. Recent findings on these options are discussed.

Key words: Water saving; Pressurized deficit irrigation; Irrigation sheduling; Low quality water; Cropping system 'Virtual' water; Waste of food feed and bio-fuel

Introduction

One third of the total land is situated in arid and semi-arid regions (Flowers et al. 1986). Low precipitation, high evapotranspiration rates and low quality water resources are major problems in these regions. The scarcety of rain water results in the fact that worldwide more than 40% of the food production comes from irrigated land and agriculture is the biggest consumer of water accounting for some 70-90% of all freshwater withdrawals (EIIE 2000; OECD/FAO 2011). Decisions at three

levels can improve awareness about water use (Hoekstra 2003). Firstly the user level where increasing user's awareness, applying water pricing and water saving technology can lead to improvement in local water use efficiency. Secondly at the river basin level evaluation of the value of water for different alternative uses has to be done in order to reallocate water in an economically efficient way. Thirdly at the global level the application of virtual water trade between water scarce and water abundant regions can achieve improvements in the global water use efficiency (Horleman and Neubert 2006). Arid countries can import food instead of extending irrigation; i.e. import 'virtual water' (Chapagain *et al.* 2005a), as a part of Integrated Water Resource Management for these countries (El-Sadek 2011). Therefore water limitation should result in rethinking regional choices of cropping patterns and irrigation systems with consideration of the 'virtual water' concept. Tightly connected to the latter is the water footprint concept, which can be broken down to blue (surface and ground water), green (evaporated from soil stored water) and grey (polluted water) water footprint. The water footprint may be modified and reduced by reducing waste of food and feed (Gustavsson *et al.* 2011) and by rethinking the food composition so that food contain less products of animal origin with high footprint values (Hoekstra 2012). Recently, a Global Water Footprint Standard has been launched (Hoekstra *et al*. 2011).

Better irrigation practise can be obtained by modelling of crop water relations using AquaCrop (Steduto *et al*. 2009) and CropSyst (Stockle *et al.* 2003; Marsal and Stockle 2012) or similar irrigation models for advicing when and how much to irrigate; under different level of nitrogen may be taken into account as done for nitrogen in maize (Abedinpour *et al*. 2012).

As a framework for water saving, (Hsiao et al. 2007) suggested working on a chain of efficiencies, i.e.: Conveyance efficiency (Econv) and farm efficiency (Efarm), until the arrival of the water at the edge of the field. For the different ways of water distribution (furrow, sprinkler, surface drip and subsurface drip, with the latter eliminating most of the soil evaporation) field application efficiencies (Eappl) of 60, 75 and 90% for surface irrigation (border, furrow, basin), sprinkler irrigation and drip irrigation, respectively, have been obtained under European conditions (Dworak *et al*. 2007). Subsurface drip irrigation (SDI) has shown irrigation efficiency (IE) being in the range of 95–100% (Schneider *et al.* 2001). SDI together with fertigation can be used in many crops (Camp 1998; Hagin *et al.* 2003).

The effect of changing irrigation system on water saving

From FAO's AQUASTAT (Siebert and Döll 2007) estimated that 274 million hectares (Mha) are areas equipped for irrigation (AEI), and 315 Mha irrigated areas are harvested (IAH) around year 2000. The main irrigated crops at the global scale are rice (IAH of 103 Mha), wheat (IAH of 67 Mha), maize (IAH of 29 Mha) and cotton (IAH of 16 Mha). More than 50% of the global area with irrigation was

located in India, China and in the United States. The baseline scenario for the International Water Management Institute (IWMI) assumes an increase in AEI of 22% from 1995 to 2025 (Seckler *et al.* 2000). For developing countries FAO estimates AEI to increase by 20% from 202 Mha (1997) to 242 Mha in 2030 (Bruinsma 2003).

Many countries, such as Turkey, India, and China revised their irrigation legislation, subsidizing their farmers for water and energy saving irrigation equipment. At present, the global coverage of sprinkler and micro irrigated areas are 33 and 6 Mha, respectively (Sener, 2010). Hereby the water saving potential is huge if part of the surface irrigated land (estimated surface irrigated land 315-39 = 276 Mha) is reduced and turned into sprinkler and micro irrigated land.

Use of saline water for irrigation

Currently, about 50% of irrigated land in the world, which has at least twice the productivity of rainfed land and may produce one-third of the world's food, is affected by salinization (Hillel 2000). Salinity stress affects crop growth, yield and productivity and sodium (Na^+) and chloride (Cl^-) are the two key ions responsible for both osmotic and ion-specific damage that significantly reduces crop growth and yield (Munns and Tester 2008) as early assessed by (Mass and Hoffman 1977). The complexity of soil salt and soil drying needs modeling and such models are now available. Accucate soil water control is important under saline conditions in order to avoid harmful conctration of in the soil solution when soil water content is reduced (e.g. Razzaghi *et al*. 2011). More mathematical, numerical models such as SaltMod (Oosterbaan 2012) and SALTMED (Ragab *et al.* 2005) have been developed for the simulation and prediction of the salinity of soil moisture, ground and drainage water, the depth of the water table, drain discharge and leaching of salts in irrigated agricultural lands under different geohydrologic conditions, varying water management options, including the use of groundwater for irrigation by pumping from wells , and several crop rotation schedules. Other crop growth models have also been adapted to irrigation with saline water (e.g. Verma et al. 2012).

Controlling salinity by drainage needs a leaching fraction of the total irrigation water to be 10-20% and the salt concentration of the drainage water is normally 5 to 10 times higher than that of the irrigation water resulting in the fact that salt content in the soil is not accumulating (Hoorn and Alphen, 2006). In order to save irrigation water in regions with distinct dry and wet seasons, the drainage system may be operated in the wet season only, reducing environmental hazards from salt in the leaching water. In orchards multiple lines of low-flow drip tape may be used where there is salt accumulation along the tree row, and hereby significant water savings for reclamation leaching may be obtained as opposed to irrigating the whole field (Burt and Isbell, 2005).

By applying the above modelling tools for soil solute concentration control more accurate irrigation scheduling can be obtained for utilisation of this low water quality reserve. Recently Mirlas (2012) presented the model MODFLOW for calculation of water table and soil salinization processes in irrigated fields and it can provide reliable information for the planning of an effective subsurface drainage system to prevent soil salinization. Further, to avoid disposal of saline drainage water to the environment re-use systems of saline drainage water have been developed using robust forages with high water use (Benes *et al*. 2012).

Water saving pressurized irrigation systems

Drip and sprinkler pressurised irrigation systems have in the past been compared with furrow irrigation systems showing distinct differences between drip irrigation, sprinkler irrigation and pivot irrigation (Burt *et al.* 1999; Raine and Foley 2002).

Drip irrigation. Drip irrigation deliver water close to the root systems and hereby it minimizes evaporation and runoff. In most commercial systems computers control the sytem with calibrated emitters supplied from computerized set of valves. By reducing soil evaporation subsurface drip irrigation (SDI) further reduces evaporative losses to the atmosphere and is environmental friendly by causing less soil erosion. SDI has been intensively investigated during the last 30 years (e.g. reviewed by: Ayers *et al.* 1999; Camp 1998; Camp *et al.* 2000). Until now due to high cost of installation SDI has been limited to high value horticultural crops; however, manufactures in cooperation with irrigation system manufacturers are developing more simple and cheap solutions so that SDI is becoming more applicable for field row crops. A review on corn in Kansas (Lamm and Trooinen 2003) indicates that SDI can save as much as 35-55% of the irrigation water compared with common irrigation practices in the region. Further nitrogen fertigation was a very effective management tool in combination with SDI resulting in high nitrogen and water use efficiencies. For cost effectiveness the systems should last 10-20 years except for low cost tape tubing systems lasting 1-2 years.

Sprinkler irrigation. Sprinklers, sprays, or guns can be mounted overhead permanently installed as solid-set irrigation systems or they can be mounted on automatically moving wheeled systems known as traveling sprinklers. The latter system may irrigate unattended.

Pivot irrigation. Center pivot irrigation systems are among the most popular for irrigating field crops and are used on over half of sprinkler irrigated lands in the United States, Brazil, Argentina, and other countries (Allen *et al*. 2000). By 2003 a huge increase in irrigation in USA had occurred to an irrigated area of 21.6 Mha, and in which 51% were irrigated by pivots, 43% by gravity and 6% by other technologies (micro-irrigation). In 2002 there were 170,000 pivot machines, irrigating at least 8 Mha in USA determined by land satellites system. The system also esti-

mated that there were 88,000 pivots outside USA. Altogether, this industry expands between 7 and 11% each year. Between the late 1960s and early 80s there were 80 companies manufacturing pivots worldwide, predominantly in the USA (New Ag International 2008). This major shift was mainly driven by the huge savings in labour force that pivots permit, but also in savings of water and energy. Marginal soils such as sandy soils or soils with slopes cannot be irrigated by gravity, but they might be irrigated by pivots. The biggest problem nowadays for the expansion of this technology is that the farm sizes are either too small or not suited for pivots in many countries. For a 50 ha cotton field size grown under Australian conditions Raine and Foley (2002) estimated that the costs of surface, pivot and drip irrigation is 1.159, 2.300, and 4.000 \$ ha^{-1}, respectively, indicating that the expenses per ha for pivot irrigation is about the half of those of drip irrigation. Crops are often planted in a circle to conform to the center pivot.

Energy is one of the principle costs in irrigation. Recent results on pivot show that the best options are timing irrigation to avoid periods of high energy costs as well as increasing pumping power and pipe size, with a greater system capacity (1.5 L s^{-1} ha^{-1}), and shorter operation time (18 h day^{-1}). The minimum water application cost is obtained for center pivot systems irrigating 75 ha, with lateral pipes of 254 mm (10 in.). (Moreno *et al*. 2012). Many center pivot systems now have drops hanging from a u-shaped pipe attached at the top of the pipe with sprinkler heads that are positioned about 1m (at most) above the crop, thus limiting evaporative losses. Originally, most center pivots were water powered. These were replaced by hydraulic systems (T-L Irrigation) and electric motor driven systems (Reinke, Valley, Zimmatic). Many modern pivots now feature GPS devices. The type of Pivot system known as LEPA (Low Energy Precision Application) has been developed in the USA to conserve both water and energy (Lyle and Bordovsky 1981). This technology has been used primarily for row crop irrigation with application efficiencies typically exceeding 95% (Fipps and New 1990). The mechanical component of a LEPA irrigation system is a moving truss system with water conveyance tubes extending from the system mainline to near the soil surface, where correctly sized orifices control deposition of water to individual soil furrows.

Scheduling irrigation. By irrigation scheduling frequency, quantity and timing of irrigation is undertaken in order to optimize crop growth and production. Worldwide the irrigation is often based on climatic data roughly by calculating accumulated deficit (D) of daily water balance data from evapotranspiration (ET) and rainfall (R) [i.e. $D=\sum(R-ET)$]. During a drying cycle a certain D level will initiate an irrigation event (Hillel et al. 1998). Availability of weather data allows the use of advanced methods for calculation of ET predictions as reviewed by Allan et al. (1999). Thus, the amount of water applied is determined by using a threshold to determine irrigation need and a strategy to prescribe how much water to apply in any situation. Many models is based on knowing the current crop stage,

soil water content and ET (Allen *et al.* 1999) and are therefore able to predict when to irrigate a crop (e.g. Steduto *et al*. 2007). Also crop water status (Jones 2004) and soil water status as reviewed by Jones (2004) and (Evett *et al*. 2012), respectively, are used for irrigation scheduling. Soil moisture sensors buried about 20 cm deep within the crop root zone has been used to maintain soil moisture at a set level. The use of dendrometry, fruit gauges, and other tissue water content sensors, measurements of growth, sap flow, and stomatal conductance are outlined for irrigation scheduling by Jones (2004). Seelig *et al*. (2012) found that in cowpea leaf thickness measurements could be used for irrigation control under green house conditions. Lately, Al-Yahyai (2012) found that in apple trees stem water potential was highly correlated with soil water potential showing potential possibilities of irrigation scheduling from real time measurement of stem water potential. Ballester *et al*. (2012) investigated if transpiration estimated from heat-pulse sap flow measurements using the compensation heat-pulse method can be used as continuous water stress indicators for RDI irrigation of citrus trees. They found that transpiration of well-watered and regulated deficit irrigated citrus trees were in good agreement with differences in stem water potential differences. They suggest the method to be used in relative terms where both sap flow in fully watered and RDI plants are measured and related.

A system with wirelessly transmitted information of several sensors appropriately distributed over various sectors of a round field irrigated by a center-pivot irrigation system could tell the irrigation lever exactly when and which field sector needs to be irrigated. By remote sensing it is possible to obtain information about crop water status. Santos *et al.* (2010) made a remote sensing based assessments of actual ET using METRIC integrated with a water balance model providing accurate estimates of irrigation performance. The proposed methodology allows the estimation of the volume of applied water at the field scale. Comparisons of values obtained from actual applied water records against those calculated with the new methodology resulted in good agreement. METRIC estimates ET as a residual of the energy balance at the surface and is calculated as:

$$LE = Rn - G - H \quad (1)$$

where LE is the latent energy consumed by ET, Rn is net radiation, G is sensible heat flux conducted into the ground, and H is sensible heat flux to the air. Details of the METRIC model are given by Allen *et al.* (2007), and the model was tested by Santos *et al*. (2010). Recently, Ha *et al.* (2012) reviewed downscaling methods to improve spatial resolution of land surface characteristics such as surface temperature and daily ET.

Use of low quality water and fertigation. Low quality waters as waste water often contain nutrients. As irrigation and fertilization are the most important tools for farmers to control crop growth the use of low quality waters for irrigation has to

be seen in the context of fertigation. The control of fertigation is superior under microirrigation (drip and microsprinkler). The principles of fertigation have been thoroughly reviewed by Bar-Yosef (1999). Fewer decision support systems include nutrients and fertigation (e.g. Anastasiou *et al*. 2009; Heidman *et al*. 2008). So far no models for calculation of crop water requirement incorporate the hazards related to the use of wastewater. However, Styczen *et al*. (2010) included fertigation and cleaning techniques and procedures in a management model for decision support when applying low quality waters in irrigation. The cornerstone in the model is the deterministic "Plant–Soil–Atmosphere" model DAISY Abrahamsen and Hansen, (2000), which simulates crop growth, water and nitrogen dynamics and if required heavy metals and pathogen fate in the soil. The irrigation and fertigation module calculates irrigation and fertigation requirements based on DAISY's water and nitrogen demands. A Water Source Administration module keeps track of water sources available and their water quality, as well as water treatments, storage, and criteria for selection between different sources. The management model system can be used for analysis prior to investments or when preparing a strategy for the season.

Deficit irrigation. Deficit Irrigation (DI) is a method of irrigation where the amount of water used is kept below the potential ET and the minor stress that develops has minimal effects on the yield. In this mode of irrigation the entire root-zone is irrigated. Knowing when to apply water is a must for the successful implementation of DI because the sensitivity of the crop to water stress is different at different growing stages. There is, for example, a high sensitivity during flowering with a high risk of a yield decrease via flower or seed abortion (Kirda 2002; Liu *et al.* 2004). Knowing the crop and soil water status in a certain growing environment is important to ensure the correct irrigation practice so water deficits can be imposed at times when it has minimum effect on yield, a practice also termed regulated deficit irrigation (RDI). RDI aims to use drought stress to control vegetative and reproductive growth, and to avoid any luxury water consumption. This requires careful control of the timing and level of water deficit. The response of plants to DI or RDI can be reflected in one factor or in a combination of stimulation of root growth, maintenance of high leaf water potential, osmotic regulation of leaf-turgor pressure, and adaptation to existing soil water status by e.g. reduced leaf elongation and stomatal closure. DI is more common in fruit trees and vines because economic returns in these crops are generally higher than in field crops and less directly related to biomass production (Hsiao *et al.* 2007) than to the quality of fruits and yield (Fereres et al. 2003). Nonetheless, in processing tomatoes DI (or RDI) is widely applied in the Emilia Romagna region, Italy, by irrigation extension service to save water on the basis of local research and using a decision support system (DSS) (Battilani *et al.* 2000; 2003). In drought sensitive species like potatoes and tomatoes extensive field studies showed that 20-25% of the total irrigation water

could be saved by DI (Jensen *et al.* 2010) or about 77 mm savings of water under Mediteranean conditions in early potatoes (Ierna and Mauromicale 2012) without yield depression as compared with fully irrigated plants. The later part of the growing season was the most robust period for saving irrigation water. Under semi-arid conditions mulching with plastic for about 65 d after sowing was ideal to optimize soil moisture and soil temperature and in turn to improve potato productivity (Zhao *et al.* 2012). Comparisons and evaluation of different deficit irrigation techniques have been undertaken by (Sadras 2009). Sepaskhah and Ahmadi (2010) have recently evaluated the PRD technique.

The root chemical signalling mediated by abscisic acid (ABA) for 'isohydric' plants most probably act as a feed-forward mechanism controlling stomatal aperture (Chaves and Oliveira 2004). However, recent investigations in field grapevines suggest if severe soil drying saving 50% water of control plants is applied during deficit irrigation feedback hydraulic signals may play a dominant role (Rodrigues *et al.* 2008). The investigations of drought induced adaptation processes has a potential for understanding the mechanisms of deficit irrigation (Shao *et al.* 2008) and hereby its potential for water saving. In tomatoes and potatoes investigations from several studies showed that gradual soil drying imposed by deficit irrigation (DI) or partial root zone drying irrigation (PRD) applying SDI induced hydraulic and chemical signals from the root system resulting in partial stomatal closure, an increase in photosynthetic water use efficiency, and a slight reduction in top vegetative growth (Jensen *at al.,* 2010). Using SDI Orum *et al.* (2010) under field conditions in Serbia found, that PRD irrigation considerably improved the water productivity of field potatoes (Table 1). In average the water productivity was increased by 50% by using the PRD mode of deficit irrigation as compared to FI.

Table 1. Marketable yield, applied irrigation water (AIW) and irrigation water productivity applying subsurface drip irrigation (SDI) (after, Jonanovic et al. 2010).
(FI: Fully irrigated; PRD: partial root zone drying).

Strategy	Year	Marketable yield ($t\ ha^{-1}$)	AIW (mm)	Water productivity ($kg\ ha^{-1}\ mm^{-1}$)
FI	2007	43.1	188	241
PRD	2007	40.0	125	334
FI	2008	42.5	225	236
PRD	2008	41.3	130	380
FI	Average	42.8	207	239
PRD	Average	40.6	128	357

In maize under semi arid conditions and depending on stress level RDI may increase yield by 10–20% and gross margin up to 167 euroes ha^{-1} compared with an irrigation strategy where the stress´level remains constant during the whole growth cycle. For low water stress conditions, deficit irrigation should be applied during

the initial and vegetative development stages. For medium and high water stress conditions, higher deficit should occur during the ripening and grain filling stages with water savings up to 45%. These studies were obtained as validated simulations (Domínguez *et al*. 2012).

In almond the application of RDI strategy based on an irrigation reduction of 80% during kernel-filling and 50% during post-harvest under SDI conditions, could be profitable economically (even more so than a well- irrigated treatment) and could also optimize water resources under semi arid conditions,both saving significant water quantities and irrigation costs (Romero et al. 2006).

Water footprint and virtual water and land savings. Water productivities, water management and virtual water flows are important to consider in order overcoming scarcity of water for a growing population in the future. Water footprint (WFP) and virtual water have lately been calculated for crops, goods, services, as well as on generic national levels (e.g. Hoekstra and Chapagain, 2007). The WFP of a product is defined as the total volume of fresh water used to produce the product, summed over the various steps in the production chain (e.g. Hoekstra et al. 2009).

From the virtual water standpoint, soybean consumes 3.1 m^3 water kg^{-1} beans while cereals consume around 1.2–1.4 m^3 water kg^{-1} grain and maize 0.7 m^3 water kg^{-1} grain. Tomato and potatoes consume 0.1 m^3 water kg^{-1} fruit (Yang *et al*. 2007). Aldaya *et al.* (2010) found that blue water economic productivity ranges between 0.1–0.2 euros m^{-3} for low-cost cereals and 2–3 euros m^{-3} for vegetables. Vegetables seem to be the most profitable crops, followed by grapes and olives.

About 3000 to 6000 km^3 yr^{-1} of "green" water (precipitation stored in the soil and evapotranspired on cropland) are consumed in addition to sustain rainfed agriculture and parts of irrigated agriculture. The global consumption of "blue" water (taken from rivers, reservoirs, lakes and aquifers and used for irrigation) presently amounts to 927–1660 km^3 yr^{-1} according to recent estimates (Rost *et al*. 2008; Hoff *et al*. 2010). Fader *et al*. (2011) for 11 of the world's major crop species modelled the amount of blue and green water (irrigation and precipitation water) and quantified WFP distinguishing internal WFP (local water) and external WFP (virtual water imported from other countries) and their blue and green components, respectively. Green water flows and footprints show that green water globally dominates both the internal and external WFP (84% of the global WFP and 94% of the external WFP rely on green water). Nevertheless, current trade of the products considered here saves significant water volumes (263 km^3) and land areas (41 Mha), equivalent to 5% of the sowing area of the considered crops and 3.5% of the annual precipitation on this area. Considering the land needed in order to produce imported goods on their own territory, Fader *et al.* (2010) estimate that China and Mexico would need 9 Mha each, North Korea and The Netherlands 7 Mha each, and Japan 16 Mha. Relating these needs to the current cropland extent dem-

onstrates that many countries – 40 in total, especially in North Africa and Latin America – would have to more than double the current cropland to produce their imports on their own territory. However, recently Boelens and Vos (2012) argues allocation of water sought through virtual water flows is embedded in agricultural commodities trade and policies which may deprive farming societies for water locally needed in water and resource weak regions; e.g. cotton produced in and exported from India, Pakistan and China contributes to the over-exploitation of water sources, affecting local water user communities (Chapagain *et al.* 2005b).

Food, feed, bio-fuel production and waste. Worldwide there is a growing demand for grain for satisfying the need for food, feed, and fuel. By increasing the production on the existing farmland it avoids greenhouse gas production by bringing new land and ecosystems into production. In USA average corn yield has increased from 1.6 ton ha^{-1} in the first third of the 20^{th} century to today's 9.5 ton ha^{-1}. This increase is due to new farming technologies such as hybrid corn, use of fertilizers and machinery (Edgerton, 1999). With an increase in prosperity people consumes more meat and dairy products every year. Global meat production is projected to more than double from 229 Mt in 2000 to 465 Mt in 2050 and a similar increase is projected for milk from 580 to 1043 Mt (OECD/FAO, 2011). The increase in middle class consumer demand shifts from stable foods towards processed food products with more animal protein. Based on OECD/FAO predictions global consumption of wheat is expected to decline over the next 10 years, rice production is still to be increased for use in Asia. However grains for feed (poultry, pork), and sugar cane and oil crops for bio-fuel is expected to increase (Table 2).

Table 2. Predicted growth in *per capita* consumption of food products (2008-10 to 20). (After OECD/FAO 2011)

Product	Percent	Product	Percent
Wheat	-0.3	Sheep	8.7
Rice	6.0	Fish	4.8
Coarse grains	6.0	Milk	9.4
Beef	1.0	Veg. oils	10.6
Pork	6.3	Sugar	14.4
Poultry	14.4		

In 2012 cereal consumption for food, feed, biofuel and other purposes are c. 1150, 800, 170, and 200 Mt, respectively; thus grains for food and feed still dominates cereal production and is anticipated to increase by 25% within the next decade (OECD/FAO, 2011) . The demand for grain is a result of the increasing world population (Friend, 2009). Due to climate change the areas useful for grain production is expected to change in the coming years especially expanding into the

northern part of the world with sufficient rain fall (see Plates K and L given by Fischer *et al*. 2002).

Large losses and waste occur from food and feed products from the farmer's field until it reaches its final destination; in storage, transportation, processing, wholesale and retail (Table 3).

Table 3. Waste percentages for each commodity group in each step of the food supply chain for Europe incl. Russia. (Partly after Gustavsson et al., 2011).

	Agricultural production	Postharvest handling and storage	Processing and packaging	Distribution: Supermarket Retail	Consumption	Total
Cereals	2	4	0.5	2	25	33.5
Roots and tubers	20	9	15	7	17	68.0
Oilseeds and pulses	10	1	5	1	4	21.0
Fruit and vegetables	20	5	2	10	19	56.0
Meat	3.1	0.7	5	4	11	23.8
Fish and seafood	9.4	0.5	6	9	11	35.9
Milk	3.5	0.5	1.2	0.5	7	12.7

In 2011 it was estimated to be 1.3 billion tons of food or about 1/3 of the global food production was wasted (Gustavsson *et al.* 2011). Furthermore, vast amounts of food are then wasted in households and restaurants. Every loss or waste of food is equivalent to loss and misuse of water and waste of land (Table 4).

Table 4. Estimated water lost (km^3) due to waste percentages for each commodity group in each step of the food supply chain (FSC) for Europe incl. Russia. (Partly after Gustavsson et al., 2011* and Hoestra 2012**)

	*Commodity (million tonnes)	**Water footprint (m^3/tonnes)	Water (km^3)
Cereals	400	1640	656
Roots and tubers	180	390	70
Oilseeds and pulses	175	3200	560
Fruit and vegetables	201	900	181
Meat	45	8300	374
Fish and seafood	Not estimated	Not estimated	
Milk	232	3000	696
Total			2537

Animal-based food requires a larger amount of water per kilo ready-to-eat product than do fruits and vegetables increasing the amount of water wasted. A 50 percent reduction of food losses and waste at the global level would save 1 350 km^3 of water a year (OECD/FAO, 2011).

Cropping systems. Recently, Jacobsen *et al*. (2012) reviewed how different agronomic measures can be used to support plant production under semi arid conditions. Supplemental irrigation such as deficit irrigation has the potential to overcome periods of low rainfall or high temperatures. It is suggested that improvements in crop production may arise from several strategies such as early sowing enabled by minimum tillage, increased use of organic manure, and an efficient weed control. Further, crop rotations will play an important role in improving weed control, minimizing disease risk, and increasing nitrogen availability. Introduction of drought and salt tolerant crop species such as quinoa and amaranth may result in more resilient crop rotations and high value cash crop products. Genotypic increases of water use efficiency may arise from selection for early vigour, deep roots, increased transpiration efficiency, improved disease resistance, and high assimilate storage and remobilization. A range of crop and management strategies might be combined for a specific target environment in order to optimize crop productivity.

Conclusive remarks. As presented and discussed above a range of options are available for securing water resources for agricultural production. Moving from surface (furrow; border) irrigation to sprinkler and drip irrigation are already taken place as several countries are now implementing more drip and sprinkler (mainly pivot) irrigation on small and large sized areas, respectively. Both methods have the potential to save in the range of 30-40% of the water used under surface irrigation. Water saving might also be obtained by more precise irrigation scheduling supported by models, remote sensing-based assessments of actual ET, and plant and soil water status sensors. These tools are also needed when water saving deficit irrigation is used in the form of RDI and PRD. Use of waste and saline water for irrigation is complex, however new decision support systems and model developments might ease the use of low quality water as cleaning procedures and supplemental fertigation may be counteracting nutritional imbalances. Further use of salt resistant crops together with well managed drainage systems might support crop production under saline conditions. The water foot print and 'virtual' water concept is now common politically recognized as tools for both production of goods and for redistribution of water from rain rich to rain poor areas. The danger of exporting water from rain poor low income areas are lately discussed (e.g Boelens and Vos 2012). Reduction of waste in food, feed and bioful production are new emerging areas of recognition as a world wide water saving option and has the potential of significant water and land savings (Gustavsson *et al*. 2011).

From the above it can be concluded that the options for optimizing agricultural water resources are:

– More surface irrigated land is turned into sprinkler, pivot, or drip irrigated land.
– Irrigation scheduling, supplemental and deficit irrigation are taken into account and become common practice.

– Use of waste and saline water for irrigation is further developed.
– Improvement of cropping systems and inclusion of saline soils for crop production using salt resistant crops and drainage systems for leaching of salts.
– Using the principles of water footprint and virtual water concepts for inter-regional planning of utilization of water resources.
– Reduction of waste of food, feed and bio-fuel.

References

Abedinpour M., Sarangi A, Rajput T BS., Man Singh, Pathak H., Ahmad T. (2012) Performance evaluation of AquaCrop model for maize crop in a semi-aridEnvironment. Agric Water Manage 110: 55–66.

Abrahamsen P., Hansen S. (2000) Daisy: an open soil–crop–atmospheresystem model. Environ Model Softw 15: 313–330.

Aldaya MM., Santos M., Llamas R. (2010) Incorporating the Water Footprint and Virtual Water into Policy: Reflections from the Mancha Occidental Region, Spain Water Resour Manage 24, 941–958.

Allen RG., Pereira LS., Raes D., Smith M. (1999) Crop evapotranspiration. FAO Irrigation and Drainage Paper 56. Rome: FAO.

Allen RG., Keller J., Martin D. (2000) Centre Pivot System Design. The Irrigation Association, 654 Arlington Boulevard, Falls Church, VA 22042-6638, RiJuPeLo Editions, www.irrigation.org. Assesed 10 April (2012)

Allen RG., Tasumi M., Trezza R. (2007) Satellite-based energy balance for mapping evapotranspiration with internalized calibration (METRIC) – model. J Irrig Drain Eng ASCE 133(4): 380–394.

Al-Yahyai R. (2012). Managing irrigation of fruit trees using plant water status. Agric Sci 3(1): 35-43.

Anastasiou A., Savvas D., Pasgianos G., Sigrimis N., Stangellini C., Kempkes FLK. (2009) Decision support for optimised irrigation scheduling. In: Tuzel Y., Oztekin GB., Meric MK. (eds.), International Symposium on Strategies Towards Sustainability of Protected Cultivation in Mild Winter Climate, ISHS Acta Horticulturae 807, pp 253–258.

Ayars JE., Phene CJ., Hutmacher RB., Davis KR, Shoneman RA., Vail SS., Mead RM. (1999) Subsurface drip irrigation of row crops: a review of 15 years of research at the Water Management Research Laboratory. Agric Water Manage 42: 1–27.

Bar-Yosef B. (1999) Advances in fertigation. Adv Agron 65: 2-77

Ballester C., Castel J., Testi L., Intrigliolo DS., Castel JR. (2012) Can heat-pulse sap flow measurements be used as continuous water stress indicators of citrus trees? Irrig Sci. doi 101007/s00271-012-0386-5

Battilani A., Bussieres P., Dumas Y. (2000) IRRIGERE: an improved version of an irrigation scheduling model for the processing tomato crop. Acta Hort 537: 519-526

Battilani A., Dalla Costa L., Lovatti L. (2003) Potato's efficient use of water and nitrogen in a subhumid area. Acta Hort 664: 63-70

Benes SE., Adhikari DD., Grattan SR., Snyder RL. (2012) Evapotranspiration potential of forages irrigated with saline-sodic drainage water. Agric Water Manage 105: 1–7

Boelens R., Vos J. (2012) The danger of naturalizing water policy concepts: Water productivity and efficiency discourses from field irrigation to virtual water trade. Agric Water Manage 108: 16-26

Bruinsma, J. (ed) (2003) World agriculture towards 2015/2030 – an FAO perspective FAO, Rome, Italy, 444 p

Burt CM., Clemmens AJ., Bliesner R., Merriam JL., Hardy L. (1999) Selection of irrigation methods for agriculture On-Farm Irrigation Committee, Reston, Virginia ASCE, American Society of Civil Engineers, 2000, 129 p

Burt CM., Isbell B. (2005) Leaching of accumulated soil salinity under drip irrigation. ITRC Paper No P 05-001 Transactions of the ASAE 48(6): 1-7

Camp CR. (1998) Subsurface drip irrigation: A review. Trans ASAE 41 (5): 1353–1367

Camp CR., Lamm FR., Evans RG., Phene CJ. (2000) Subsurface drip irrigation: past, present and future In: Proceedings of the fourth decennial irrigation symposium, November 14–16, Phoenix, Arizona American Society of Agricultural Engineers, St Joseph, Mich, USA, pp 363–372

Chapagain AK., Hoekstra AY., Savenije HHG., Gautam R. (2005a) The water footprint of cotton consumption. Value of Water Research. Report Series No 18, UNESCO-IHE, Delft, The Netherlands

Chapagain AK., Hoekstra AY., Savenije HHG. (2005b) Saving water though global trade. UNESCO-IHE, Value of Water Research. Report Series No 17, Delft, The Netherlands

Chaves MM., Oliveira MM. (2004) Mechanisms underlying plant resilience to water deficits:prospects for water-saving agriculture. J Exp Bot 55: 2365–2384

Domínguez A., de Juan JA., Tarjuelo JM., Martínez RS., Martínez-Romero A. (2012)
Determination of optimal regulated deficit irrigation strategies for maize in a semi-arid environment. Agric Water Manage 110: 67– 77

Dworak T., Berglund M., Laaser C., Strosser P., Roussard J., Grandmougin B., Kossida M., Kyriazopoulou I., Berbel J., Kolberg S., Rodriguez-Diaz JA., Montesinos P. (2007). EU Water saving potential (Part 1-Report), ENVD2/ETU/2007/001r

Edgerton MD. (2009) Increasing crop productivity to meet global needs for feed, food, and fuel. Plant Physiol 149: 7-13

EIIE (Environmental Impacts of Irrigation in the European Union), (2000) A report to the Environment Directorate of the EC, March 2000

El-Sadek A.(2011) Virtual water: an effective mechanism for integrated water resources management. Agric Sci 2(3): 248-261

Evett SR., Schwartz RC., Casanova JJ., Heng LK. (2012) Soil water sensing for water balance, ET and WUE. Agric Water Manage 104: 1– 9

Fader M., Gerten D., Thammer M., Heinke J., Lotze-Campen H., Lucht W., Cramer, W. (2011) Internal and external green-blue agricultural water footprints of nations, and related water and land savings through trade. Hydrol Earth Syst Sci 15: 1641–1660

Fereres E., Goldhamer DA., Parsons LR. (2003) Irrigation water management of horticultural crops. HortSci 38: 1036-1042

Fipps G., New LL. (1990) "Six years of LEPA in Texas - less water, higher yields. Visions of the Future. " Proceedings of the Third National Irrigation Symposium Phoenix, USA, pp.115-120

Fisher G., Velthuizen van H., Shah M., Nachtergale F. (2002) Global agro-ecological assessment for agriculture in the 21st century: Methodology and results. IIASA, FAO, Luxemburg, Austria, p. 119

Flowers TJ., Hajibagheri MA., Clipson NJW. (1986) Halophytes Quart Rev Biol 61: 313-336

Friend AD. (2009) Darwin Review. Terrestrial plant production and climate change. J Exp Bot 61 (5): 1293–1309

Gustavsson J., Cederberg C., Sonesson U., van Otterdijk R., Meybeck A. (2011) Global food losses and waste. Extent, causes and prevention. FAO, Rome, Italy, 12 p

Ha W., Gowda PH., Howell TA. (2012) A review of downscaling methods for remote sensing-based irrigation management: part I. Irrig Sci. doi 101007/s00271-012-0331-7, published online

Hagin J., Sneh M., Lowengart-Aycicegi A. (2003) Fertigation. Fertilization through Irrigation. Johnston AE. (ed), IPI Resesarch Topics No3, Internatinal Potash Institute, Basel, 81 p

Heidmann T., Tofteng C., Abrahamsen P., Plauborg F., Hansen S., Battilani A., Coutinho J., Dolezal F., Mazurczyk W., Ruiz JDR., Takac J., Vacek J. (2008) Calibration procedure for a potato crop growth model using information from across Europe. Ecol Model 211: 209–223

Hillel D. (2000) Salinity management for sustainable irrigation. The World Bank, Washington, DC

Hillel D. (1998) Environmental soil physics. Academic Press, New York

Hoekstra AY. (2003) Virtual water: An introduction. Proceedings of the International Expert Meeting on Vitual Water Trade, Delft, Value of Water Research Report Series No 12

Hoekstra AY. (2012) The hidden water resource use behind meat and dairy. Animal Frontiers 2: 3-8

Hoekstra AY., Chapagain AK. (2007) Water footprints of nations: Water use by people as a function of their consumption pattern. Water Resour Manage 21: 35-48

Hoekstra AY., Chapagain AK., Aldaya MM., Mekonnen MM. (2009) Water footprint manual. State of the art 2009; Water footprint Network: Enschede, The Netherlands

Hoekstra AY., Chapagain AK., Aldaya MM., Mekonnen MM. (2011) The water footprint assessment manual: Setting the global standard. Earthscan, London, UK

Hoff H., Falkenmark M., Gerten D., Gordon L., Karlberg L., Rockstrom J. (2010) Greening the global water system. J Hydrol 384: 177–186

Hoorn JW. van, Alphen JG. van (2006) Salinity control. In:, Drainage principles and applications. Ritzema HP (ed), Publication 16, International Institute for Land Reclamation and Improvement (ILRI), Wageningen, The Netherlands ISBN 90 70754 339, pp 533-600,

Horlemann L., Neubert S. (2006) Virtual water trade: A realistic concept for resolving the water crisis? German Development Institute, Bonn, Germany – (Studies / Deutsches Institut für Entwicklungspolitik; 25) ISBN 978-3-88985-335-6

Hsiao TC., Steduto P., Fereres, E. (2007) A systematic and quantitative approach to improve water use efficiency in agriculture. Irrig Sci 25: 209-231

Hu Y., Schmidhalter U. (2005) Drought and salinity: A comparison of their effects on mineral nutrition of plants. J Plant Nutr Soil Sci 168: 541–549

Ierna A., Mauromicale G. (2012) Tuber yield and irrigation water productivity in early potatoes as affected by irrigation regime. Agric Water Manage 115: 276– 284

Jacobsen S-E., Jensen CR., Liu F. (2012) Improving crop production in the arid Mediterranean climate. Field Crops Res 128: 34–47

Jensen CR., Battilani A., Plauborg F., Psarras G., Chartzoulakis K., Janowiak F., Stikic R., Jovanovic Z., Li G., Qi X., Liu F., Jacobsen SE., Andersen MN. (2010) Deficit irrigation based on drought tolerance and root signalling in potatoes and tomatoes. Agric Water Manage 98: 403–413

Jones, HG. (2004) Irrigation scheduling: advantages and pitfalls of plant-based methods. J Exp Bot 55 (407), 2427–2436

Jovanovic Z., Stikic R., Radovic BV., Paukovic M., Brocic Z., Matovic G., Rovcanin S., Mojevic M.

(2010) Partial root zone drying increase WUE, N and antioxidant content in field potatoes. Eur J Agron 33: 124-131

Kirda C. (2002) Deficit irrigation scheduling based on plant growth stages showing water stress tolerance In: Deficit irrigation practices, FAO, Rome, pp3-10

Lamm FR., Trooien TP.(2003) Subsurface drip irrigation for corn production: a review of 10 years of research in Kansas. Irrig Sci 22: 195-200

Liu F., Jensen CR., Andersen MN. (2004) Pod set related to photosynthetic rate and endogenous ABA concentration in soybeans subjected to different water regimes and exogenous ABA and BA at early reproductive stages. Ann Bot 94: 405-411

Lyle WM., Bordovsky JP. (1981) Low energy precision application (LEPA) irrigation system. Transactions of the ASAE 24 (5): 1241-1245.

Marsal J., Stöckle CO. (2012) Use of CropSyst as a decision support system for scheduling regulated deficit irrigation in a pear orchard. Irrig Sci 30:139–147

Mass EV., Hoffman GJ. 1977. Crop salt tolerance – Current assessment. Am Soc Civ Engr Proc J Irrig & Drain 1023 (lr2): 115-134

Mirlas V. (2012). Assessing soil salinity hazard in cultivated areas using MODFLOW model and GIS tools: A case study from the Jezre'el Valley, Israel. Agric. Water Manage. 109: 144–154

Moreno MA., Medina D., Ortega JF., Tarjuelo JM. (2012) Optimal design of center pivot systems with water supplied from wells. Agric Water Manage 107: 112-121.

Munns R., Tester M. (2008) Mechanisms of salinity tolerance. Ann Rev Plant Biol : 651–681

New Ag International (2008) Pivot Irrigation for increasingly sophisticated machines. New Ag International, pp 31-41

OECD/FAO 2011 OECD-FAO Agricultural Outlook 2011-2020, Chapter 1, Overview, OECD Publishing, pp 17-50. http://dxdoiorg/101787/agr_outlook-2011-en. Accessed 25 April 2012

Oosterbann RJ. (2012) SaltMod: agro-hydro-salinity model for drainage, leaching of salts, and land rehabilitation . http://wwwwaterloginfo/ saltmodhtm. Accessed 20 April 2012

Orum, JE. Mads Vejlby Boesen, MV. Jovanovic, Z., Pedersen, SM.. (2010) Farmers' incentives to save water with new irrigation systems and water Taxation-A case study of Serbian potato production. Agric Water Manage 98, 465– 471

Ragab R., Malash N., Gawad A G., Arslan A., Ghaibeh A. (2005). A holistic generic integrated approach for irrigation, crop and field management: 2. The SALTMED model validation using field data of five growing seasons from Egypt and Syria International. J Agric Water Manage 78 (1-2): 89-107

Raine SR., Foley JP. (2002) Comparing application systems for cotton- What is the pros and cons. http:// wwwusqeduau/users/raine/ index_files/Raine and Foley Nat Cotton Conf2002pdf . Accessed 20 April 2012

Rodrigues ML., Santos TP., Rodrigues AP., de Souza CR., Lopes CM., Maroco JP.,
Pereira JS., Chaves MM. (2008) Hydraulic and chemical signalling in the regulation of stomatal conductance and plant water use in field grapevines growing under deficit irrigation. Funct Plant Biol 35: 565–579

Romero P., Garcra J., Botta P. (2006) Cost–benefit analysis of a regulated deficit-irrigated almond orchard under subsurface drip irrigation conditions in Southeastern Spain . Irrig Sci 24: 175–184

Razzaghi F., Ahmadi SH., Adolf VI., Jensen CR., Jacobsen S-E., Andersen MN. (2011) Water relations and transpiration of quinoa (Chenopodium quinoa Willd) under salinity and soil drying. J Agron Crop Sci 197 (5): 348-360

Rost S., Gerten D., Bondeau A., Lucht W., Rohwer J., Schaphoff S. (2008) Agricultural green and blue water consumption and its influence on the global water system. Water Resour Res 44, W09405, 17 p. doi:101029/2007WR006331

Sadras VO., 2009 Does partial root-zone drying improve irrigation water productivity in the field? A meta-analysis. Irrig Sci 27: 183–190

Santos C., Ignacio J., Lorite M., Tasumi, R., Allen G., Fereres E. (2010) Performance assessment of an irrigation scheme using indicators determined with remote sensing techniques. Irrig Sci 28: 461–477

Schneider A D., Howell T A., Evett S R. (2001) Comparison of SDI LEPA and spray irrigation efficiency. ASAE paper No 012019, 12 p

Seckler DU., Amarasinghe U., Mold D., De Silva R., Barker R. (2000) World water demand and supply 1990 to 2025 senarios and issues. IWMI Research Report 19, IWMI, Colombo, Sri Lanka, 40 p

Seelig H-D., Stoner RJ., Linden JC. (2012) Irrigation control of cowpea plants using the measurement of leaf thickness under greenhouse conditions. Irrig Sci 30: 247–257

Sener S. (2010) Global Climate Change and its Effect on Irrigation in Mediterranean Region - Water (Energy Saving Irrigation Systems and Some Relevant Research Results). In: CLIMWATER

2010: Horticultural Use of Water in a Changing Climate. 28th International Horticultural Congress, August 22-27 2010, Lisboa
Sepaskhah AR., Ahmadi SH. (2010) A review on partial root-zone drying irrigation. International J Plant Production 4: 241-258
Shao HB., Chu LY., Jaleel A., Zhao CX. (2008) Water deficit stress induced anatomical changes in higher plants. Comptes Rendus Biol. 331: 215-225
Siebert S., Döll P. (2007) Irrigation water use – A global perspective. In: Lozan JL, Grabl H, Hupfer P., Menzel L., Schönwiese C-D (eds), Global Change: Enough Water for all? Universität Hamburg/ GEO, pp104-107
Steduto P., Hsiao TC., Fereres E. (2007) On the conservative behavior of biomass water productivity. Irrig Sci 25: 189–207
Steduto P., Hsiao T C., Raes D., Fereres E. (2009) Aquacrop-The FAO crop model to simulate yield response to water: I. Concepts and underlying principles. Agron J 101: 426–437
Stockle CO., Donatelli M., Nelson R. 2003 CropSyst a cropping systems simulation model. Eur J Agron 18: 289–307
Styczen M., Poulsen RN., Falk AK., Jorgensen GH. (2010) Management model for decision support when applying low quality water in irrigation. Agric Water Manage 98: 472–481
Yang H., Wang L., Zehnder AJB. (2007)Water scarcity and food trade in the Southern and Eastern Mediterranean countries. Food Policy 32: 585–605
Verma AK., Gupta S K., Isaac RK. (2012) Use of saline water for irrigation in monsoon climate and deep water table regions: Simulation modeling with SWAP. Agric Water Manage 115: 186–193
Zhao H., Xiong Y-C., Li F-M., Wang R-Y., Qiang S-C., Yao T-F., Mo F., (2012) Plastic film mulch for half growing-season maximized WUE and yield of potato via moisture-temperature improvement in a semi-arid agroecosystem. Agric Water Manage 104: 68–78

PLANT FUNCTIONING UNDER ENVIRONMENTAL STRESS P. 27-34

Cracow, 12th to the 15th September, 2012

PHOTOSYNTHESIS PARAMETERS WHICH CAN BE ESTIMATED BY PHOTOACOUSTIC SPECTROSCOPY AND ITS PLACE IN MONITORING THE INFLUENCE OF ENVIRONMENTAL POLLUTION ON PLANTS

Janusz Szurkowski

Institute of Experimental Physics, University of Gdańsk, Wita Stwosza 57, 80-952 Gdańsk, Poland

Corresponding author:fizjws@univ.gda.pl

Abstract At present, photoacoustic spectroscopy is one of the measuring techniques providing detailed information on the photosynthesis system and structure of plants. We describe the possibility of application of the photoacoustic spectroscopy to quantify a lot of parameters characterizing plants; determination of sample chemical composition, efficiency of photosynthetic oxygen evolution, photochemical energy storage, photosynthetic action spectra, the time of photothermal signal creation in a photoacoustic cell, the coefficient of oxygen diffusion through the cell wall and the sample depth profiling on different plants. The aim of the work was to demonstrate an opportunity of application of photoacoustic spectroscopy to the monitoring of the contamination effect on plants and presented according to the current state of knowledge in this field. For all the plants selected as bioindicators (ranging from green alga - *Scenedesmus armatus* to Scots pine – *Pinus silvestris*) and for all the used parameters, the values obtained for the plants exposed to contamination are different, within the experimental error, than the reference ones.

Key words: Photoacoustic spectroscopy; Photosynthesis; Environmental pollution; Bioindicators

Introduction

Photoacoustic spectroscopy in plant science. The photoacoustic effect is the production of pressure modulations with and around a sample when it absorbs the intensity-modulated light. In the case of the photosynthetic samples in a gas volume, thermal expansion of the gas surrounding the sample and the photosynthetic

oxygen evolution are the major contributors to the acoustic wave. The photothermal part of the photoacoustic signal is reduced by a fraction equal to the part of the absorbed energy stored by the photosynthetic process as chemical energy. The oxygen component of the photoacoustic signal consists largely of oxygen evolution at PS II.

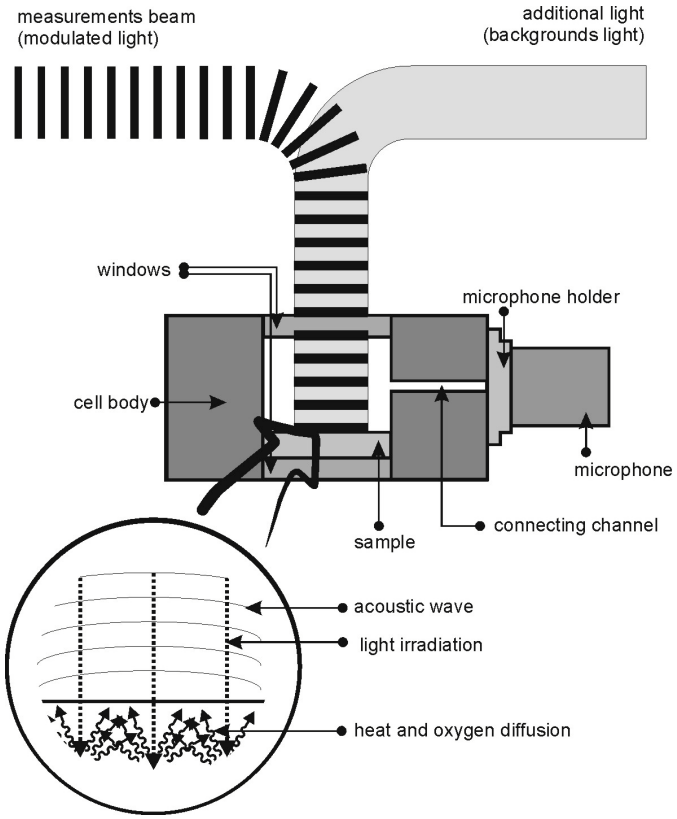


Fig. 1. Schematic view of photoacoustic signal generation in the measuring cell where in studies of plants two light beams are used of differentiated intensities i.e., measuring-modulated and supporting-non-modulated ones

Photoacoustic spectrometers adapted to photosynthesis process studies consist usually of two independent light sources (Fig.1). A measuring light beam, from one of the light sources, can be periodically modulated. It can be realized in two ways by the mechanical chopper (Tukaj and Szurkowski, 1993) or electrical modulation of the light supplier circuit (Szurkowski et al., 2001). The measuring beam, irradiating the sample, has an intensity in the range from 10 to 30 μmol m^{-2} s^{-1}. The sec-

ond light source provides a non-modulated light background to the sample of high intensity attaining at the sample face values from 2000 do 2500 μmol m^{-2} s^{-1}. Nowadays, light guides are very often used to direct and split the both light beams. (Malkin and Canaani 1994; Szurkowski 2001; Szurkowski et al. 2001; Szurkowski 2002). The mixed light beams irradiate the sample in a closed-type cell equipped with a microphone. The acoustic signal is analyzed with a phase-sensitive amplifier (usually of two-phase type allowing the simultaneous measurement of the signal amplitude and its phase shift in relation to the reference signal) (Fig.2).

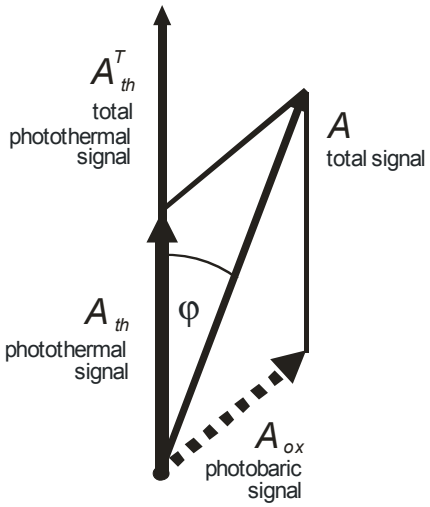


Fig. 2. Photoacoustic signal (A) in a vectorial presentation and its separation into the components: photothermic (A_{th}) and photobaric A_{ox} contributions with φ – phase shift angle (phase of photoacoustic signal); A^T_{th} is the photoacoustic signal in the presence of background light

The photoacoustic signal of the sample can be analyzed as a vectorial summation of photothermal and photobaric contributions, according to the method of Poulet et al. (1983). The amplitude of the oxygen signal (A_{ox}) can be evaluated using equation:

$$A_{ox} = \sqrt{A_{th}^2 + A^2 - 2A_{th}A\cos(\varphi)}$$

where A_{th} and A are the amplitude of photothermal and total signals, respectively, in the absence of background light, and φ – is the phase of the total signal versus the photothermal one. A_{th} can be calculated as follows:

$$A_{th} = k\,A^T_{th}$$

where k is the ratio of the acoustic signal in the absence of background light to the signal in the presence of background light at high frequency, at witch we no observe the photobaric contribution, and A^T_{th} is the signal in the presence of background light at the given frequency.

Methods

Parameters which can be estimated by photoacoustic spectroscopy

Photoacoustic spectra. In photoacoustic spectroscopy we can measure two kinds of photoacoustic spectra i.e. wavelength dependence of the photoacoustic signal amplitude. The first ones, in the presence of additional background light, are the equivalent of absorption spectra. We can observe characteristic maxima of different pigments in the sample and their amplitudes are directly related to their concentration in the measured sample. The second ones, without background light, are rather the "action" spectra, which measure the probability that an absorbed photon of wavelength λ can be re-emitted as a heat (Tukaj and Szurkowski 1993; Szurkowski and Tukaj 1994; Szurkowski and Tukaj 1995).

Depth profiling. One of the advantages of the photoacoustic technique is an opportunity to obtain a depth-profile in the studied sample. By selecting different frequencies (*f*) of the chopped light, one can obtain information from different depths of the sample. In the measurements, the sampling *L* decreases with the modulation frequency increase. The sampling depth is given by equation:

$$L = \sqrt{\frac{D}{\pi \cdot f}},$$

where *D* is the sample's thermal diffusivity (Szurkowski 2001; Szurkowski 2004).

Photoacoustic energy storage. The photothermal part of the signal ($A_{th}^{\cdot}$) is reduced by a fraction equal to that part of the absorbed energy which is stored by the photosynthetic process as chemical energy. By measuring heat emission in the presence or absence of a non-modulated saturating light background, the photosynthetic energy storage (*ES*) is evaluated. *ES* is calculated as

$$ES = \frac{(A_{th}^{T} - A_{th})}{A_{th}^{T}} \cdot 100$$

and expressed as a percentage. In the long chain of events leading to the chemically stable energy form, the only truly light-dependent reaction is that of excitation of the light-absorbing pigment molecule; all of the numerous subsequent processes involve the loss of energy. Therefore, the real efficiency of light energy conversion will be always lower than the measured *ES* (Szurkowski 2001; Szurkowski 2004).

Light-saturation curve. In photoacoustic measurements, the dependence of the relative photosynthetic energy storage (i.e., a product of the energy storage and intensity of incident measuring light) on the intensity of light is an analogous to the classical photosynthesis light-saturation curve (Szurkowski and Tukaj 1995; Szurkowski 1996).

Photobaric signal. The photobaric signal amplitude (A_{ox}) allowed the estimation of both the rate and yield of oxygen evolution. The ratios of photobaric to photothermal signal amplitude corresponds to the yield of oxygen evolution per unit of biomass. This method of oxygen efficiency evolution can be used where the measured photothermal signal (A_{th}) can be a good measure of the applicable biomass – algae deposited on a membrane filter, for example.

It is possible to determine the relative quantum yield of the oxygen evolution on the basis of the oxygen evolution yield and the photosynthetic energy storage. By dividing both the above fractions, we can obtain the relative yield of oxygen evolution in which we only take into account the energy actually consumed in the process of photosynthesis, rather than the total energy absorbed by pigments (Szurkowski et al. 2001; Tukaj at al. 2003; Szurkowski 2004).

Time of the photothermal creation. The application of a two-phase amplifier allows for the measurement of the characteristic time of the photothermal signal creation. For signal analyses at high modulation frequencies, where only the photothermal signal is dealt with, the phase modulation method can be employed. It is widely used in fluorescence decay time studies. By using a single modulation frequency (f) it is possible to determine the characteristic decay time (τ), assuming a one-exponent decay from the equation:

$$\varphi = arctg(2\pi f\tau)$$

Since in photoacoustic measurements the photothermal signal is studied instead of fluorescence signals, the same mathematical approach can be applied (Szurkowski et al. 2001; Szurkowski 2004).

Coefficient oxygen diffusion through the wall cells. Since the amplitudes of the modulated oxygen concentration field and temperature field are attenuated exponentially at the cell boundary, the plot of $\ln(A_{ox}/A_{th})$ versus $(f)^{1/2}$ tends to be a straight line in the low- frequency range, from the slope of which coefficient oxygen diffusion (D_{ox}) can be computed using the formula;

$$ln\frac{A_{ox}}{A_{th}} = -\sqrt{\pi f}\left(\frac{1}{\sqrt{D_{ox}}} - \frac{1}{\sqrt{D_{th}}}\right)l\,,$$

where:
D_{th} – heat diffusion coefficient (diffusivity), D_{ox} – oxygen diffusion coefficient in the cell, l – distance from the source (Poulet et al. 1983; Szurkowski et al. 2001; Szurkowski 2004).

Non-photochemical quenching. The photothermal amplitude of intact leaves was shown to remain constant during induction of photosynthesis at low intensity

of light. Under high-light conditions a noticeable decrease in the heat emission yield was observed resulting from progressive activation of photochemical processes. The photothermal amplitude in methylviologen treated leaves was shown to substantially decrease during induction of photosynthesis. The slow stage of photosynthetic induction observed in this studies is attributed to effect of nonphotochemical quenching on the signal amplitude. The large variability in photoacoustic signal kinetics allowed to determine the "decrease ratio", and the characteristic time of nonphotochemical quenching (Szurkowski and Dobrowolska 2005).

Results and Discussion

Application of photoacoustic spectroscopy in studies of environmental contamination effect on plants. The photoacoustic spectroscopy has been successfully employed to study the influence of stressor, including environmental contamination effect on plants. We can follow changes of the several parameters listed above. The observed variability in the photoacoustic signal spectra and depth profile signatures point to the modification of plant pigments composition as well as to the limitation in the efficiency of energy transfer to the photosynthesis process. Although, the parameter found the widest application is the energy storage. In our research group, for all the plants selected as bioindicators (green algae - *Scenedesmus armatus*, dandelion – *Taraxacum officinale*, Scots pine – *Pinus silvestris*). energy storage values obtained for plants exposed to contamination are lower than or equal to, within the experimental error, those for the reference unaffected ones (Szurkowski 2004).

The best correlation between ES and pollution levels was obtained for a batch culture green microalgae *Scenedesmus armatus* (Szurkowski and Tukaj 1995). After a 24–h culture growth, with increasing contaminant (AFOE – aqueous fuel oil extract) concentration in the culture ES decreased. In these algae, ES is lowered by 41% if a 90% contaminant concentration is used. The effect of AFOE concentration on the photosynthetic ES of the algae after a 24 h is depicted in Fig. 3. Since 24-h period is the generation time for *Scenedesmus armatus* algae, the results should reflect for the most part the adaptive changes that are exhibited by the algae during a one-generation cycle.

A similar relation between a degree of environment pollution and ES variations was already obtained for plants in field conditions. The *dandelion* and *Scots pine* selected for studies were from places where the concentration of atmospheric contaminants such as NO_2, SO_2 and flying dust have been measured for several years (Provincial Inspectorate of Environment Protection in Gdańsk). Energy storage values obtained for *Taraxacum officinale* leaves collected in Tri-city (big cities with a high pollutant's concentration) (ranging from 42 ÷ 54%) are lower than those for leaves collected in a small village (Sominy) (65%) (Szurkowski 2002).

For Scots pine needles collected in the village the energy yield of trapping reaches 54% and decreases with the age to 23% for four-year old needles whereas, for these collected in the municipal areas, the corresponding values are equal to 28÷30% for fresh needles, and about 15÷17% for the 2-3-year old needles (Szurkowski 2001).

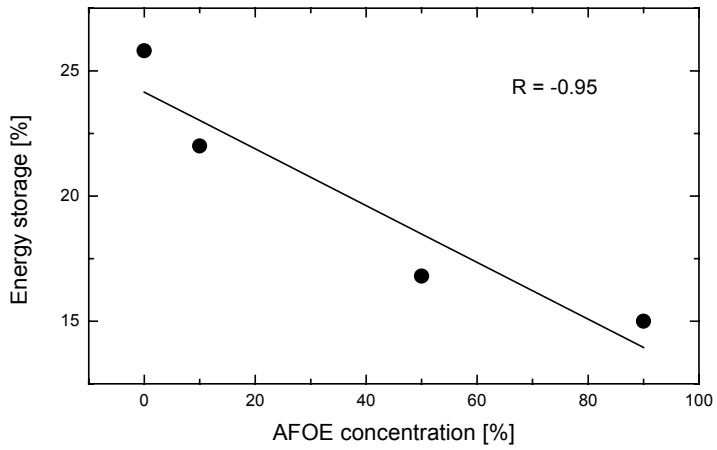


Fig. 3. Influence of different concentrations of AFOE on photosynthetic energy storage of *Scenedesmus armatus* cells after 24 h culture growth with appropriate concentrations of AFOE; R – linear regression coefficient

It is supposed that the currently performed investigations should allow us to take into account the effect of environmental contaminations on the photobaric signal not only for leaves of plants which can be treated, as a first approach, as homogeneous. That results from the concept where the photoacoustic signal is analyzed as a vectorial summation of the photothermal and photobaric contributions. It is assumed that the ratio of the acoustic signal in the absence of background light to the signal in the presence of background light (k) does not depend on the frequency of the modulated light beam. Such an assumption, for leaves of apparent layered structure, is not true (Szurkowski, 2001). The preliminary results already obtained for needles of *Scott pine* are evaluated and prepared for publication.

We believe that the photosynthesis parameters, which can be estimated by photoacoustic spectroscopy, have found the place in monitoring the influence of the environmental pollution on plants in the near future.

References

Malkin S, Canaani O (1994) The use and characteristics of the photoacoustic method in the study of photosynthesis. Annu Rev Plant Mol Biol 45:493-526

Poulet P, Cahen D, Malkin S (1983) Photoacoustic detection of photosynthetic oxygen evolution from leaves. Quantitative analysis by phase and amplitude measurements. Biochim Biophys Acta 724:433-446

Szurkowski J (1996) A model for the relationship between light intensity and the rate of photosynthesis in photoacoustic measurements. Progress in Natural Science 6:554S-557S

Szurkowski J (2001) Application of photoacoustic spectroscopy in studies of environment contamination effect on needles of scots pine (*Pinus silvestris L.*). Bull Environ Contam Toxicol 66:683-690

Szurkowski J (2002) The effect of environmental contamination on the photosynthetic energy storage of *Taraxacum officinale* leaves: photoacoustic characterization. Instrument Sci Technol 30:205-210

Szurkowski J (2004) The possibility to monitoring photosynthesis by photoacoustic spectroscopy and influence of environmental pollution on it (in polish). Wydawnictwo Uniwersytetu Gdańskiego, Gdańsk

Szurkowski J, Baścik-Remisiewicz A, Matusiak K, Tukaj Z (2001) Oxygen evolution and photosynthetic energy storage during the cell cycle of green alga *Scenedesmus armatus* characterized by photoacoustic spectroscopy. J Plant Physiol 158:1061-1067

Szurkowski J, Dobrowolska W (2005) Influence of nonphotochemical quenching in methyloviologen treated dandelion leaves (*Taraxacum officinale* Weber) on energy storage measured by photoacoustic spectroscopy. Bull Environ Contam Toxicol 74:126-132

Szurkowski J, Tukaj Z (1994) Photoacoustic study of the effect of fuel oil on the photosynthetic system of algae *Scenedesmus armatus*. J de Physique 4, C7:535-538

Szurkowski J, Tukaj Z (1995) Characterization by photoacoustic spectroscopy of the photosynthetic *Scenedesmus armatus* system affected by fuel oil contamination. Arch Environ Contam Toxicol 29:406-410

Tukaj Z, Matusiak-Mikulin K, Lewandowska J, Szurkowski J (2003) Changes in the pigment patterns and the photosynthetic activity during a light-induced cell cycle of the green alga *Scenedesmus armatus*. Plant Physiol and Biochem 41:337-344

Tukaj Z, Szurkowski J (1993) Photoacoustic spectra affected by fuel oil in the chlorococcal alga *Scenedesmus armatus*. Acta Physiol Plant 15:219-226

PLANT FUNCTIONING UNDER ENVIRONMENTAL STRESS P. 35-51

Cracow, 12th to the 15th September, 2012

PLANTS TOLERANT TO HEAVY METALS AS TOOLS FOR CLEANING OF THE ENVIRONMENT

Beáta Piršelová*, Patrik Mészáros*,**, Peter Socha**, Ildikó Matušíková**

**Department of Botany and Genetics, Constantine the Philosopher University in Nitra, Nábrežie mládeže 91, 949 74 Nitra, Slovakia,*

***Institute of Plant Genetics and Biotechnology, Slovak Academy of Sciences, Akademická 2, 950 07 Nitra, Slovakia*

Corresponding author: *pmeszaros@ukf.sk*

Abstract. Industrialization, mining and increase of traffic resulted in the release of industrial effluents, often containing heavy-metals and associated contaminants that have adverse to toxic effects on organisms. Deposition of heavy metals into soils went on for decades, resulting in long term exposure of fauna and flora. Though most plants are not able to cope with sudden increased concentrations of heavy metals, genetic diversity and selection pressure dives species evolution conferring them the ability to cope with extreme conditions. Tolerant plants called hyperaccumulators are very promising tools for cost effective and ecologically friendly clean-up of heavy metal contaminated sites. This review is devoted to mechanisms of metal uptake and accumulation, which are prerequisites for ability of such plants to tolerate high concentrations of heavy metals in tissues and cells. Different approaches for genetic engineering of metal tolerance or hyperaccumulation in plants are described, while their potential as well as drawbacks with respect to effective bioremediation of heavy metals are discussed as well.

Key words: Contamination; Heavy metals; Remediation; Plant biotechnology; Environment

Introduction

Metal-exploiting technologies as well as metal pollutants have been a persistent part of human history. However, land and water pollution by heavy metals such as arsenic (As), copper (Cu), cadmium (Cd), lead (Pb), chrome (Cr), nickel (Ni), mercury (Hg) and zinc (Zn) became a worldwide issue especially since the onset of the

industrial revolution. The most important sources of heavy metal pollution are reported to be rapid industrialization, increased anthropogenic activities, modern agricultural practices and faulty waste disposal methods. In addition, in the second half of the 20^{th} century the development of nuclear technology has resulted in contaminations with radionuclides.

It is estimated that the median values of worldwide emissions of Cd, Cu, Pb, and Zn into soils were 22, 954, 796 and 1.372 10^6 $kg.yr^{-1}$, respectively (Nriagu and Pacyna 1988). According to National reports, heavy metals were the most frequent (37.3 %) soil contaminants in 2000 at investigated European countries (European Environment Agency 2000). In Western Europe, out of 1 400 000 sites affected by heavy metals over 300 000 were contaminated (McGrath *et al*. 2001), but pollution problems increasingly occurred in Central and Eastern European countries as well. For example, in Poland, one of the most polluted countries in Central Europe, the annual emission of Cd in the years 1998-2000 was between 50 and 60 tones, Cr close to 90 tones, Cu close to 400 tones, Ni about 250 tones, Pb 650-750 tones, and Zn over 2000 tones (Suchara and Sucharova 2001; Sucharova and Suchara 2004). Concentrations exceeding permitted limits for some toxic elements were found mainly in soils, waters and river sediments. Furthermore, in spite of the relatively low level of industrial activity in less developed regions such as Africa, there is a high potential of toxic heavy metal pollution levels in these regions as well. The Pb emissions in air at ground level in Lagos (Nigeria) are far higher than for cities like London (UK) and New York, but similar to those of Brazil or the Caribbean due to perturbation by roadside traffic (Olade 1987).

The presence of metals in air, soil or water may vary from site to site, depending upon the source of individual pollutant. Uptake of these metals by plants can negatively affect crop growth or even lead to plant death since it interferes with metabolic and physiological processes in plants such as photosynthesis, respiration and development of cell organelles (Schmidt 2003; Schwartz *et al.* 2003; Lone *et al.* 2008). Other negative consequences comprise damages of ecosystems or agricultural productivity, deterioration of food chain, contamination of water resources and economic damage (Raicevic 2005). Soil contamination with heavy metals may also have negative impact on the composition of soil microbial community, adversely affecting soil characteristics (Giller *et al.* 1998; Kozdroj and van Elsas 2001; Kurek and Bollag 2004; Lone *et al.* 2008). Furthermore, excess of metals in the environment and food may produce toxicity in human nutrition, and cause acute or chronic diseases.

Hyperaccumulators

A rapid rate of metal pollution of the environment can be a strong force of selection causing rapid evolutionary changes in organisms including plants. This is manifested as metal tolerance occurring over time scales as hundreds of years and

even decades (Jules and Shaw 1994). Certain plants evolved tolerance to heavy metals in 400 years of mining, but other races became tolerant to copper in 70 years (Bondada and Ma 2003). Evolved metal tolerance has the first time been shown by comparison of the growth of a normal population of *Silene vulgaris* with a *Silene vulgaris* population growing on soil from the copper mine (Prat 1934).

In 1977, the term "hyperaccumulator" was first introduced by (Brooks *et al*. 1977) referring to plants that acquired an inordinately high concentration of nickel. The concept was later extended to other heavy metals such as cadmium, cobalt, copper, lead, selenium and zinc. The values of metal concentration for hyperaccumulators were defined by (Baker *et al*. 2000) as 100 ppm for Cd, 1000 ppm for Ni, Cu, Co, Pb, and 10.000 ppm for Zn and Mn. These values might differ from those defined by some other researchers (Watanabe 1997; Reeves and Baker 2000), nevertheless they are of one order of magnitude greater than those found in non-accumulator species (Salt and Kramer 2000). Currently, 450 species of hyperaccumulators belonging to 45 families are known, of which more than 330 are nickel hyperaccumulators (Reeves and Baker 2000; Reeves 2003; Reeves and Adigüzel 2004; Rascio and Navari-Izzo 2011).

However, many hyperaccumulators are of small size, shallow root systems and grow slowly being unprofitable for remediation purposes. For example, Thlaspi caerulescens removes from soil Cd and Zn, Pb and Mn (Robinson *et al.* 1998; Zhao *et al.* 2003; Nishiyama *et al.* 2005) but not within an economic time frame (< 10 yr). For such reasons, phytoextraction as a technology is not practically used on a large scale (Zhou *et al.* 2004). To achieve efficient remediation, there is a requirement for either high biomass plants (e.g. poplar, willow), or those that have low biomass but high hyperaccumulating characteristics (e.g. Thlaspi species). Identifying new, more pertinent hyperaccumulators and/or elaborating them by means of classical breeding or biotechnology is then a requirement for fast and efficient introduction of phytoremediation into practice.

Cleaning up the environment – phytoremediation

Recognition of the ecological and human health hazards of the heavy metals as pollutants has lead to development of various approaches for decontamination. Various conventional remediation technologies are used to clean heavy metal polluted environments like soil *in situ* vitrification, soil incineration, excavation and landfill, soil washing, soil flushing, solidification and stabilization electrokinetic systems (Marks *et al*. 2000; Mulligan *et al*. 2001). Each of these technologies has its own specific benefits and limitations. However, the enormous cost of most of them has shifted the attention towards alternate and/or complementary technologies such as bioremediation that is based on materials of plant or microbial origin (Schneegurt *et al*. 2001).

The phenomenon of metal hyperaccumulation by plants has considerable importance in phytoremediation (Brooks 1998). Already in 1962, semi-aquatic plants were used for treating radionuclide-contamined waters in Russia (Timofeev-Resovsky *et al*. 1962). The concept of phytoremediation has, however, first been proposed by Utsunamyia in 1980 and later by Chaney (1983) to include a group of technologies using plants to reduce, remove, degrade, or immobilize environmental toxins with the aim of restoring area sites to a condition usable for private or public applications (Peer *et al*. 2005; Lone *et al.* 2008). The first field trial on phytoextraction of zinc and cadmium was conducted by Baker *et al.* (1991).

However, a phytoremediation process is rather time consuming, limited by the rooting depth and the measure of contamination that should not exceed toxicity level to the plants. Even though modern management practice can certainly solve several of these obstacles, a deeper knowledge on principles and basic mechanisms of hyperaccumulation in plants is necessary.

Exploiting biotechnology in heavy metal decontamination

Mechanisms of metal accumulation, exclusion and compartmentation in plants. Hyperaccumulators, that often show increased biomass and root length after allocation to the heavy metal polluted soil (Whiting *et al*. 2000), have evolved increased levels of usually metal specific tolerance (Macnair *et al.* 2000; Verkleij 2008). Hence, these plants constitute an exceptional biological material for understanding mechanisms regulating plant metal homeostasis as well as plant adaptation to extreme metallic environments (Verbruggen *et al.* 2009). Although significant progress has been made in understanding the physiological, genetic and molecular mechanisms of metal uptake, transport, sequestration and tolerance in hyperaccumulator plants (Macnair 1993; Clemens *et al.* 2002; Milner and Kochian 2008; Verbruggen *et al.* 2009), the mechanisms accounting for hypertolerance are still little understood.

Root cell walls bind metal ions from the soil, while the rate of absorption depends on the ionic potential of the elements concerned (Hirsch *et al*. 1998). It has been recently well documented that in the uptake, distribution and detoxification of heavy metals throughout the plant, different metal transport systems play crucial roles while their plasma membrane and/or tonoplast localization in plant cells has been recently confirmed (Migocka and Klobus 2007). Using transcription profiling (Talke *et al.* 2006) identified 29 genes encoding for putative metal homeostasis proteins that were more expressed in the metal-accumulator Arabidopsis halleri than in A. thaliana when exposed to zinc. Out of these, the *ZIP9, ZIP6* and *ZIP3* are members of the ZIP family of metal transporters that are responsible for cytoplasmic metal influx into cells (Pence *et al.* 2000; Assuncao *et al.* 2001). These transporters apparently cooperate with another transporters such as the heavy metal ATPase HMA4 (Hussain *et al.* 2004; Verret *et al*. 2004) proteins of the natural

resistance-associated macrophage protein (Nramp) family (Papoyan and Kochian 2004; Weber *et al*. 2004) and proteins of cation diffusion facilitator (CDF) family (Lasat *et al.* 2000; Williams *et al*. 2000). Higher expression of these genes in metal-tolerant plants is believed to be a consequence of multiplication of these genes within given plant genome (Hanikenne *et al.* 2008) as well as the reason for a more efficient root uptake system and roots-to-shoots translocation of metals.

Because of high reactivity and limited solubility of most metals, chelation by ligands is required once they are taken up into the cell (Clemens *et al.* 2002). Several chelators have been implicated in chaperoning metals including malate, citrate, nicotinamide and free histidine (Rauser 1999; Lasat *et al*. 2000; Sal and Kramer 2000; Williams *et al*. 2000; Ryan *et al.* 2001; Clemens *et al.* 2002; Weber *et al.* 2004; Hanikenne *et al.* 2008). Recently, studies of (Ueno *et al*. 2008) revealed that Cd is translocated from root to xyleme involving an active transport by HMA4, predominantly as aqueous free ions rather than being complexed with citrate. Further metal transporters and metal-binding proteins such as phytochelatins (PC) and metalothioneins (MT) play an important role in metal sequestration. Phytochelatins (PCs) consist of only 3 amino acids in a conformation $(\gamma\text{-GluCys})_n$-Gly and are enzymatically synthesized after exposure of plants to any heavy metal (Harada *et al.* 2004). Metal activates a PC synthetase (glutamicysteine transpeptidase) leading to production of glutathione and finally PCs (Le Faucher and Sigg 2005; Liu *et al.* 2011). The capacity of PCs to bind metals and form a PC-Cd complex, as well as its subsequent transport into the vacuole by either metal/H^+ antiporters or ATP-dependent ABC transporters of tonoplast (Kakinuma *et al*. 1992; Salt and Rauser 1995; Rea *et al.* 1998; Carrier *et al*. 2003) is well documented (Maitani *et al.* 1996; Mehra *et al.* 1996; Rauser 1999; Ma *et al*. 2001; Pittman 2005). The accumulation of PC-metal komplex in vacuole protects the cell from metal toxicity and can undergo long-term distance transport from roots to shoots (Salt and Rauser 1995; Rea *et al*. 1998).

Similarly to PCs, metallothioneins are cysteine rich proteins believed to either chelate the metal ions or transport them to areas where they are needed or less toxic. Furthermore, MTs appear to function as antioxidants (Wong *et al.* 2004) and NO-scavengers involved in plasma membrane repair (Salt *et al*. 1998). Plants have multiple MT gene families responsible for resistance to different metals (Suresh and Ravishankar 2004), however their exact role is still unknown.

Heavy metal tolerance and/or hyperaccumulation could also be related to the ability of plants to induce proteins that are not directly involved in binding to metals. Metal presence evokes elevated level of reactive oxygen species that activate distinct mitogen-activated protein kinases (Jonak *et al*. 2004; Maksymiec 2007). The signal is further transferred through second messengers such as jasmonic acid (Rakwal *et al*. 1996; Maksymiec and Krupa 2002), calcium (Maksymiec and Baszynski 1999) or salicylic acid (Metwally *et al*. 2003) and leads to activation of

genes triggering detoxification processes. Heat shock proteins, for instance, show increased expression in response to stress conditions including heavy metals (Lewis *et al.* 1999). These proteins are known to be involved in normal protein folding but could also be involved in the repair of membrane proteins damaged by different stresses including metals (Lewis *et al.* 1999; Heckathorn *et al.* 2004)**.** Germin-like proteins (Dunwell *et al.* 2008), enzymes of the antioxidative system such as superoxide dismutases, peroxidases (Schützendübel and Polle 2002), and membrane channels controlling the ion and water flux through membrane like aquaporins (Zhang *et al*. 2008) have been shown to eliminate the danger of irreversible damage. Interestingly, various proteins related to plant pathogenesis (PRs) such as the PR-1 have been shown to accumulate in the hyperaccumulator Arabidopsis halleri *(*Becher *et al. 2004)*. In Lupinus luteus a 16 kDa PR-10 family protein was accumulated under Cd, Zn and Cu treatment (Przymusinski *et al*. 2004). More recently, chitinases (PR-3) (Békésiová *et al*. 2008; Kieffer *et al*. 2008) and glucanases (PR-2) (Kieffer *et al.* 2008; Piršelová *et al*. 2011), both previously shown to reveal antimicrobial activity in vitro as well as in planta (Graham and Sticklen 1994; Becher *et al*. 2004), have been suggested to play a more specific role in plants than believed since specific isoforms were induced to different metals applied. This fits with previous observations that hyperaccumulation of metals also provides protection against biotic stresses such as microbes (Boyd *et al*. 1994) or insect (Brooks 1998).

Arbuscular mycorrhizas play also a key role of plant metal tolerance and accumulation (Hildebrandt *et al.* 2007). Several authors showed that plant root colonization by arbuscular mycorrhiza could either reduce the heavy metal content of the plants or increase metal absorption from polluted soils, depending on growth conditions, the fungus and the metal (Heggo *et al.* 1990; Weissenhorn *et al*. 1995).

Though we still know little about the biological and evolutionary significance of metal accumulation in general, hypotheses regarding drought resistance, inadvertent uptake, tolerance or disposal of metal from plants and defence against pathogens have been addressed (Agoramoorthy *et al.* 2009). Apparently, plant adaptation and evolution reverted different molecular mechanisms like ion (iron) homeostasis and different plant defense responses to achieve appropriate survival solutions.

There are variations for mechanisms of metal uptake, translocation, accumulation, and compartmentation of different metals among various plant species, and these variations determine the specific role of given plant species in phytoremediation. For example, contrasting to hyperaccumulators, several metal-tolerant plant species (e.g. grasses) are excluders of metals (Ebbs *et al.* 1997; Baker *et al*. 2000). There are plants like Thlaspi caerulescens in that metal (Zn) is sequestered preferentially in vacuoles of epidermal cells in a soluble form (Frey *et al*. 2000), in other plants metal is accumulated in leaf mesophyll cells (Kupper *et*

al. 2000; Zhao *et al.* 2000; Sarret *et al.* 2002) or in trichomes (Hale *et al*. 2001; Choi *et al.* 2001). In these cell components, metals will not damage the cellular processes and structures. Apparently, metal hyperaccumulators also possess hypertolerance mechanisms to resist the potentially acute cytotoxic effects of the accumulated metals, but the precise relationship between metal accumulation and tolerance has not been resolved.

Genetic engineering of plants for enhanced metabolism of pollutants

A direct method for modifying plant effectiveness of heavy metal uptake and/or tolerance is genetic engineering. Historically, first developed transgenic plants for phytoremediation were tobacco and rapeseed carrying a yeast metallothionein gene for cadmium tolerance (Misra and Gedamu 1989) or Arabidopsis thaliana overexpressing a mercuric ion reductase for tolerance to Hg (Rugh *et al*. 1996). Compared to classical breeding techniques, genetic transformation is a faster and more straightforward way to introduce a desired gene into the plant of interest. The major advantage of this approach is the possibility to overcome evolutionary barriers, hence genes ensuring to microbes the survival in extreme conditions (including metal-toxic environments) are directly amenable for expression in plants.

Many hyperaccumulators are of small stature and therefore are not suitable for field applications. Plants desirable for genetic modification should grow fast, have large biomass, be inherently capable for hyperaccumulation, and amicable for genetic transformation (Eapen and D'Souza 2005; Eapen *et al*. 2007). Most often the soil born Agrobacterium tumefaciens bacteria are used to deliver transgenes into host plants (Gelvin 2000; Chai *et al*. 2003), but for many plants e.g. for trees or different recalcitrant herb species this approach is still challenging (Unterbrunner *et al.* 2007; Dhillon *et al*. 2008; Van Aken 2008).

Classic genetic studies have shown that only a few genes (one to three) are responsible for metal tolerance (Macnair *et al*. 2000). Nevertheless, targeted genome modification might efficiently interfere with the physiology and metal metabolism at several levels. Metal-hyperaccumulating plants and microbes with unique abilities to tolerate, accumulate or detoxify metals and metalloids represent a unique source of candidate genes for genetic transfer. For example, metal uptake can be regulated by designing and using transporters that are responsible for the mineral status and enable metal ions to specifically accumulate or exclude. There are several such transporters identified in a wide variety of organisms including plants. For example, iron uptake was elevated in transgenic tobacco upon transfer of the yeast *FRE1* and *FRE2* genes for ferric reductase (Samuelsen *et al.* 1998; Macnair *et al.* 2000), the iron binding ferritin in rice (Goto *et al.* 1999; Goto *et al*. 2000) and in *Arabidopsis* overexpressing the metal transporter AtNramp1 (Curie *et al.* 2000). Enhanced metal tolerance was also achieved by transferring the vacuolar CAX-2 for transport of Ca, Cd and Mn into tobacco (Hirschi *et al.* 2000) and the yeast

ABC-transporter YCF 1 ensuring cadmium and lead tolerance into *Arabidopsis thaliana* (Song *et al*. 2003). Another strategy to alter metal accumulation in plants relies on genetic manipulation of genes for metallothioneins, phytochelatins or metal chelators. Genes for many MTs of different origin such as the mouse *MT1*, human *MTIA* and *MT II*, chinese hamster *MT II*, yeast *CUP I* and *YCF1*, as well as pea *psMTA* have been introduced to tobacco, *Brassica* species and Arabidopsis thaliana, which resulted in increased tolerance to cadmium (Misra and Gedamu 1989; Maiti *et al*. 1991; Brandle *et al.* 1993; Evans *et al.* 1993; Hattori *et al.* 1994; Pan *et al*. 1994) or mercury (Ruiz *et al.* 2011). Transgenic Brassica juncea and Arabidopsis thaliana overexpressing different enzymes involved in phytochelatin synthesis extracted higher amounts of Cd, Cr, Cu, Pb, Zn, Ag or As (Ow 1996; Zhu *et al.* 1999; Gasic and Korban 2007; Shah and Nongkynrih 2007). The bacterial merA and B genes enabled volatilization of mercury in Arabidopsis thaliana and in transgenic yellow poplar (Meagher *et al.* 2007; Ruiz and Daniell 2009). Furthermore, achieving elevated levels of simple chelators such as nicotinamide or citrate was sufficient for enhanced Fe uptake in rice and increased tolerance to Al (de la Fuente *et al.* 1997).

In addition to the above-mentioned examples there are also some less common strategies focusing on manipulating the rate of metal uptake, accumulation and sequestration. One of them targets oxidative state of the cells, which is a very important component of defense in plants. The so-called oxidative stress is generated very soon upon exposure to both biotic and abiotic stresses and triggers activation of many other defense genes necessary for resistance. Modification of the oxidative stress related enzymes in plants could also result in enhanced metal tolerance. Overexpression of glutathione-S-transferase and peroxidase enhanced Al tolerance of transgenic Arabidopsis plants (Ezaki *et al.* 2000), while the bacterial gene for 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase ensured enhanced accumulation of Cd, Co, Cu, Ni, Pb and zinc (Grichko *et al.* 2000).

Alternatively to introduction of a gene enhancing tolerance, modification of hormone content and biosythesis in a potential hyperacumulator plant was shown to promote biomass production (Eriksson *et al.* 2000). Combination of the two approaches is likely to end up in additive effect on hyperacumulating capacity of generated transgenic plants (Meagher *et al.* 2007).

Other plant-based biotechnology approaches applied for bioremediation

There is a possibility to transfer the trait for metal hyperaccumulation in plants by tissue culture techniques. Somatic hybridization between the hyperaccumulator Thlaspi caerulescens and Brassica napus selected for zinc tolerance ensured that hybrids accumulated amounts of Zn and Cd that are normally toxic for Brassica napus (Brewer *et al.* 1999). Enhanced tolerance to Cr and Ni has been developed in Echinochloa colona hybrids obtained through callus culture and plant regeneration

in both tolerant and non-tolerant calli (Samantaray *et al.* 2001). A classical shoot cloning derived from callus culture of Hybanthus floribundus resulted in plants with elevated nickel uptake (Bidwell *et al.* 2001). Mutants tolerating and/or hyperaccumulaing higher metal concentrations in growth environment are being produced through T-DNA or transposable element mutations, radiation and chemicals as well (Cobbett 2003).

Isolation of the indigenous and presumably stress-adapted arbuscular mycorrhizal fungi can also be a potential biotechnological tool for inoculation of plants for successful resto-ration of degraded ecosystems (Gaur and Adholeya 2004).

Perspectives

It becomes clear that different mechanisms of metal accumulation, exclusion and compartmentation exist in various plant species. Variations exist for tolerance and hyperaccumulation of different metals among various plant species and within populations. Hyperaccumulating plant species represent 0.2% of all angiosperms (Brooks 1998; Baker *et al.* 2000). In order to enhance their exploitability under different conditions, there is a need to develop new (crop) plant species capable of growing on polluted sites and/or accumulating heavy metals. In addition to traditional breeding techniques, hybrids are generated through protoplast fusion, and mutants are obtained through radiation or chemicals. Furthermore, genetic engineering is promising a fast, targeted and efficient way to achieve the desired trait. Candidate genes for enhanced metal tolerance and/or efficient metal accumulation are robustly being identified by novel, high-throughput molecular biology techniques such as transcription profiling (Chakrabarty *et al.* 2009).

Phytoremediation techniques, though still not fully applicable (Fig. 1), are aimed to clean up the environment, the pollution of which is often disastrous. Compared to the classical approaches they offer some advantages e.g. lower costs, less disruption of environment, potential to treat sites with more than one type of pollutant, and promise further perspectives into the future. Furthermore, they provide the possibility to recover valuable metals offsetting the expenses of their implementation (Comis 1996).

After phytoextraction, the harvested plants can also serve as a resource and be transported to areas that are deficient in the given contaminants (e.g. Se), and used as animal feed (Banuelos and Meek 1989; Banuelos *et al.* 1997). Since phytoremediation is relatively positively accepted by public, it is also possible to supplement food for humans with essential nutrients like Zn or Fe. However, attention must be paid to metal transfer factor through food chain (Banuelos *et al.* 1997).

Because of regulations limiting the use of genetically modified organisms especially in Europe, development of phytoremediation techniques are likely to involve genetic use restriction technologies for controlling the dispersion of transgenes into environment. Potential risks ambushed in transgenic plants have to be minimized to

eliminate the concerns of the public. Nevertheless, phytoremediation strategies using both natural and engineered hyperaccumulators represent a very promising tool for relatively cheap, ecological and aesthetic way of cleaning the environment from human-generated pollution.

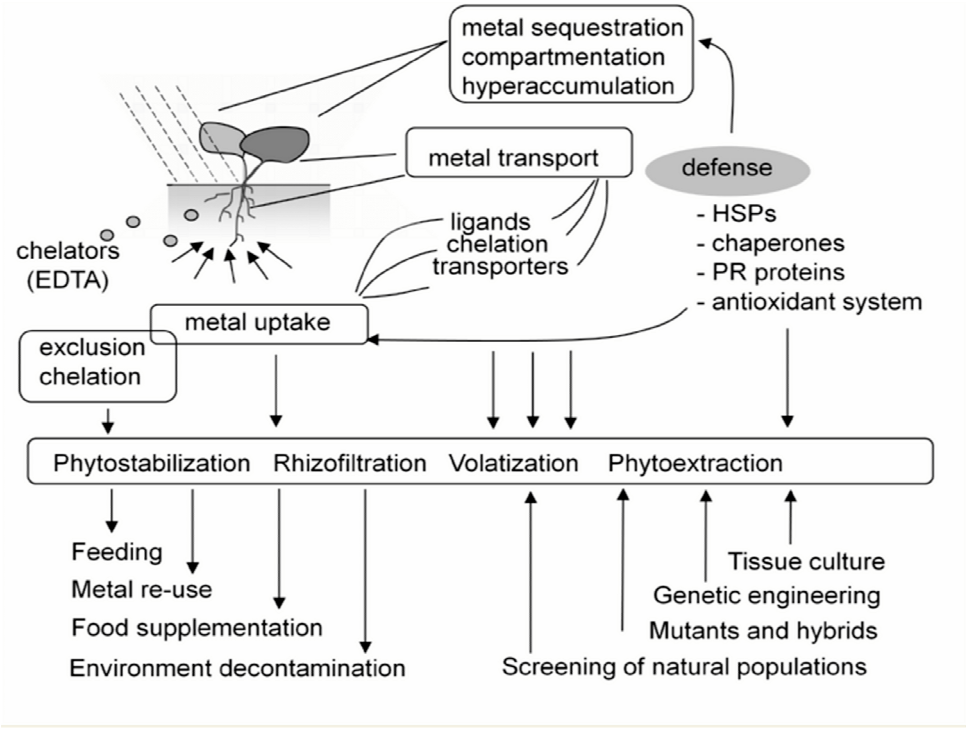


Fig. 1 Decontamination of heavy-metal polluted environment through plant biotechnology. Heavy metals are absorbed from the contaminated environment by roots. Bioavailability and uptake can be increased by presence of chelators such as EDTA (ethylenediaminetetraacetic acid). Metals are chelated and/or transported to the upper parts of the plants, and finally deposited in cell compartments where they cannot be harmful. These changes lead to heavy metal tolerance while hyperaccumulation can occur in certain plant species. Metal-hyperaccumulating plants can be obtained from natural populations or prepared by different biotechnology approaches. Their use shows promising prospects of efficient application for decontamination of the environment and specific enrichment of animal or human diet. PR – Pathogenesis-related, HSPs – Heat shock proteins

Acknowledgments

The work was developed with the support of the grant VEGA 2/0062/11.

References

Agoramoorthy G, Chen FA, Venkatesalu V, Shea PC (2009) Bioconcentration of heavy metals in selected medicinal plants of India. J Environ Biol 30:175–178

Assuncao AGL, Martins PD, De Folter S, Vooijs R, Schat H, Aarts MGM (2001) Elevated expression of metal transporter genes in three accessions of the metal hyperaccumulator *Thlaspi caerulescens*. Plant Cell Environ 24:217–226

Baker AJM, McGrath SP, Reeves RD, Smith JAC. (2000). Metal hyperaccumulator plants: A review of the ecology and physiology of a biological resource for phytoremediation of metal-polluted soils. In: Terry NN, Banuelos GS (eds.) Phytoremediation of Contaminated Soil and Water. Lewis Publishers, Boca Raton, Florida, pp 85–107

Baker AJM, Reeves RD, McGrath SP (1991) In situ decontamination of heavy-metal polluted soils using crops of metal-accumulating plants - a feasibility study. In: Hinchee RE, Oflenbuttel RF (eds.) In Situ Bioreclamation: applications and investigations for hydrocarbon and contaminated site remediation. Battelle Memorial Institute, Boston, pp 600–605

Banuelos GS, Ajwa HA, Mackey B, Wu L, Cook C, Akohoue S, Zambruzuski S (1997) Evaluation of different plant species used for phytoremediation of high soil selenium. J Environ Qual 26:639–646

Banuelos GS, Meek DW (1989) Selenium accumulation in selected vegetables. J Plant Nutr 12:1255–1272

Becher M, Talke IN, Krall L, Kramer U (2004) Cross-species microarray transcript profiling reveals high constitutive expression of metal homeostasis genes in shoots of the zinc hyperaccumulator *Arabidopsis halleri*. Plant J 37:251–268

Békésiová B, Hraška Š, Libantová J, Moravčíková J, Matušíková I (2008) Heavy-metal stress induced accumulation of chitinase isoforms in plants. Mol Biol Rep 35:579–588

Bidwell SD, Pederick JW, Sommer-Knudsen J, Woodrow IE (2001) Micropropagation of the nickel hyperaccumulator *Hybanthus floribundus* (Family *Violaceae*). Plant Cell Tiss Org Cult 67:89–92

Bondada BR, Ma LQ (2003) Tolerance of heavy metals in vascular plants: Arsenic hyperaccumulation by Chinese Brake fern (*Pteris vittata* L.). In: Chandra S, Srivastava M (eds.) Pteridology in the New Millennium. Kluwer Academic Publishers, Netherlands, pp 397–420

Boyd RS, Shaw JJ, Martens SN (1994) Nickel hyperaccumulation defends *Streptanthus polygaloides* (*Brassicaceae*) against pathogens. Am J Bot 81:294–300

Brandle JE, Labbe H, Hattori J, Miki BL (1993) Field performance and heavy-metal concentrations of transgenic flue-cured tobacco expressing a mammalian metallothionein-beta-glucuronidase gene fusion. Genome 36:255–260

Brewer EP, Saunders JA, Angle JS, Chaney RL, McIntosh MS (1999) Somatic hybridization between the zinc accumulator *Thlaspi caerulescens* and *Brassica napus*. Theor Appl Genet 99:761–771

Brooks RR (1998) General Introduction. In: Brooks RR (ed.) Plants that hyperaccumulate heavy metals: their role in phytoremediation, microbiology, archaeology, mineral exploration and phytomining. CAB International, New York, pp 1–14

Brooks RR, Lee J, Reeves RD, Jaffre T (1977) Detection of nickeliferous rocks by analysis of herbarium specimens of indicator plants. J Geochem Expl 7:49–57

Carrier P, Baryla A, Havaux M (2003) Cadmium distribution and microlocalization in oilseed rape (*Brassica napus*) after long-term growth on cadmium-contaminated soil. Planta 216:939–950

Chai T, Chen Q, Zhang Y, Dong J, An CH (2003) Cadmium resistance in transgenic tobacco plants enhanced by expressing bean heavy metal-responsive gene *PvSR2*. Sci China C Life Sci 46:623–630

Chakrabarty D, Trivedi PK, Misra P, Tiwari M, Shri M, Shukla D, Kumar S, Rai A, Pandey A, Nigam D, Tripathi RD, Tuli R (2009) Comparative transcriptome analysis of arsenate and arsenite stresses in rice seedlings. Chemosphere 74:688–702

Chaney RL (1983) Plant uptake of inorganic waste constituents. In: Parr JF, Marsh PB, Kla JM (eds.) Land treatment of hazardous waste. Noyes Data Corporation, Park Ridge, pp 50–76

Choi YE, Harada E, Wada M, Tsuboi H, Morita Y, Kusano T, Sano H (2001) Detoxification of cadmium in tobacco plants: Formation and active excretion of crystals containing cadmium and calcium through trichomes. Planta 213:45–50

Clemens S, Palmgren MG, Kramer U (2002) A long way ahead: Understanding and engineering plant metal accumulation. Trends Plant Sci 7:309–315

Cobbett C (2003) Heavy metals and plants - model systems and hyperaccumulators. New Phytol 159:289–293

Comis D (1996) Green remediation: Using plants to clean the soil. J Soil Water Conserv 51:184–187

Curie C, Alonso JM, Le Jean M, Ecker JR, Briat JF (2000) Involvement of NRAMP1 from *Arabidopsis thaliana* in iron transport. Biochemical J 347:749–755

de la Fuente JM, Ramirez-Rodriguez V, Cabrera-Ponce JL, Herrera-Estrella L (1997) Aluminum tolerance in transgenic plants by alteration of citrate synthesis. Science 276:1566–1568

Dhillon KS, Dhillon SK, Thind HS (2008) Evaluation of different agroforestry tree species for their suitability in the phytoremediation of seleniferous soils. Soil Use Manage 24:208–216

Dunwell JM, Gibbings JG, Mahmood T, Naqvi SMS (2008) Germin and germin-like proteins: evolution, structure, and function. Crit Rev Plant Sci 27:342-375

Eapen S, D´Souza SF (2005) Prospects of genetic engineering of plants for phytoremediation of toxic metals. Biotechnol Adv 3:97–114

Eapen S, Singh S, D´Souza SF (2007) Advances in development of transgenic plants for remediation of xenobiotic pollutants. Biotechnol Adv 25:442–451

Ebbs SD, Lasat MM, Brady DJ, Cornish J, Gordon R, Kochian LV (1997) Phytoextraction of cadmium and zinc from a contaminated soil. J Environ Qual 26:1424–1430

Eriksson ME, Israelsson M, Olsson O, Moritz T (2000) Increased gibberellin biosynthesis in transgenic trees promotes growth, biomass production and xylem fiber length. Nature Biotechnol 18:784–788

European Environment Agency (EEA) (2000) Down to earth: soil degradation and sustainable development in Europe. Environmental Issues Series 16, European Environment Agency, Copenhagen, 32 pp

Evans KM, Gatehouse JA, Lindsay WP, Shi J, Tommey AM, Robinson NJ (1992) Expression of the pea metallothionein-like gene *psmta* in *Escherichia coli* and *Arabidopsis thaliana* and analysis of trace-metal ion accumulation - implications for PsMTA function. Plant Mol Biol 20:1019–1028

Ezaki B, Gardner RC, Ezaki Y, Matsumoto H (2000) Expression of aluminum-induced genes in transgenic *Arabidopsis* plants can ameliorate aluminum stress and/or oxidative stress. Plant Physiol 122:657–665

Frey B, Keller C, Zierold K, Schulin R (2000) Distribution of Zn in functionally different leaf; epidermal cells of the hyperaccumulator *Thlaspi caerulescens*. Plant Cell Environ 23:675–687

Gasic K, Korban SS (2007) Expression of *Arabidopsis* phytochelatin synthase in Indian mustard (*Brassica juncea*) plants enhances tolerance for Cd and Zn. Planta 225:1277–1285

Gaur A, Adholeya A (2004) Prospects of arbuscular mycorrhizal fungi in phytoremediation of heavy metal contaminated soils. Curr Sci 86:528-534

Gelvin SB (2000) Agrobacterium and plant genes involved in t-DNA transfer and integration. Annu Rev Plant Physiol Plant Mol Biol 51:223–256

Giller KE, Witter E, McGrath SP (1998) Toxicity of heavy metals to microorganisms and microbial processes in agricultural soils: A review. Soil Biol Biochem 30:1389–1414

Goto F, Yoshihara T, Saiki H (2000) Iron accumulation and enhanced growth in transgenic lettuce plants expressing the iron- binding protein ferritin. Theor Appl Genet 100:658–664

Goto F, Yoshihara T, Shigemoto N, Toki, Takaiwa F (1999) Iron fortification of rice seed by the soybean ferritin gene. Nature Biotechnol 17:282–286

Graham S, Sticklen MB (1994) Plant chitinases. Can J Bot 72:1057–1083

Grichko VP, Filby B, Glick BR (2000) Increased ability of transgenic plants expressing the bacterial enzyme ACC deaminase to accumulate Cd, Co, Cu, Ni, Pb, and Zn. J Biotech 81:45–53

Hale KL, McGrath SP, Lombi E, Stack SM, Terry N, Pickering IJ, George GN, Pilon-Smits EAH (2001) Molybdenum sequestration in *Brassica* species. A role for anthocyanins? Plant Physiol 126:1391–1402

Hanikenne M, Talke IN, Haydon MJ, Lanz C, Nolte A, Motte P, Kroymann J, Weigel D, Kramer U (2008) Evolution of metal hyperaccumulation required cis-regulatory changes and triplication of HMA4. Nature 453:391–344

Harada E, von Roepenack-Lahaye E, Clemens S (2004) A cyanobacterial protein with similarity to phytochelatin synthases catalyzes the conversion of glutathione to gamma-glutamylcysteine and lacks phytochelatin synthase activity. Phytochemistry 65:3179–3185

Hattori J, Labbe H, Miki BL (1994) Construction and expression of a metallothionein beta- glucuronidase gene fusion. Genome 37:508–512

Heckathorn SA, Mueller, LaGuidice S, Zhu B, Barrett T, Blair B, Dong Y (2004) Chloroplast small heat-shock proteins protect photosynthesis during heavy metal stress. Am J Bot 91:1312–1318

Heggo A, Angle JS, Chaney RL (1990) Effects of vesicular arbuscular mycorrhizal fungi on heavy-metal uptake by soybeans. Soil Biol Biochem 22:865–869

Hildebrandt U, Regvar M, Bothe H (2007) Arbuscular mycorrhiza and heavy metal tolerance. Phytochemistry 68:139–146

Hirsch RE, Lewis BD, Spalding EP, Sussman MR (1998) A role for the AKT1 potassium channel in plant nutrition. Science 280:918–921

Hirschi KD, Korenkov VD, Wilganowski NL, Wagner GJ (2000) Expression of *Arabidopsis* CAX2 in tobacco. Altered metal accumulation and increased manganese tolerance. Plant Physiol 124:125–133

Hussain D, Haydon MJ, Wang Y, Wong E, Sherson SM, Young J, Camakaris J, Harper JF, Cobbett CS (2004) P-type atpase heavy metal transporters with roles in essential zinc homeostasis in *Arabidopsis*. Plant Cell 16:1327–1339

Jonak C, Nakagami H, Hirt H (2004) Heavy metal stress. Activation of distinct mitogen- activated protein kinase pathways by copper and cadmium. Plant Physiol 136:3276–3283

Jules ES, Shaw AJ (1994) Adaptation to metal-contaminated soils in populations of the moss, ceratodon purpureus - vegetative growth and reproductive expression. Am J Bot 81:791–797

Kakinuma Y, Masuda N, Igarashi K (1992) Proton potential-dependent polyamine transport by vacuolar membrane-vesicles of *Saccharomyces cerevisiae*. Biochim Biophys Acta 1107:126–130

Kieffer P, Dommes J, Hoffmann L, Hausman JF, Renaut J (2008) Quantitative changes in protein expression of cadmium-exposed poplar plants. Proteomics 8:2514–2530

Kozdroj J, van Elsas JD (2001) Structural diversity of microbial communities in arable soils of a heavily industrialised area determined by PCR-DGGE fingerprinting and fame profiling. Appl Soil Ecol 17:31–42

Kupper H, Lombi E, Zhao FJ, McGrath SP (2000) Cellular compartmentation of cadmium and zinc in relation to other elements in the hyperaccumulator *Arabidopsis haller*. Planta 212:75–84

Kurek E, Bollag JM (2004) Microbial immobilization of cadmium released from CdO in the soil. Biogeochem 69:227–239

Lasat MM, Pence NS, Garvin DF, Ebbs SD, Kochian LV (2000) Molecular physiology of zinc transport in the Zn hyperaccumulator *Thlaspi caerulescens*. J Exp Bot 51:71–79

Le Faucher S, Sigg L (2005) Phytochelatine als Metallindikatoren? Eawag News 60:22-23

Lewis S, Handy RD, Cordi B, Billinghurst Z, Depledge MH (1999) Stress proteins (hsp's): Methods of detection and their use as an environmental biomarker. Ecotoxicol 8:351–368

Liu GY, Zhang YX, Chai TY (2011) Phytochelatin synthase of *Thlaspi caerulescens* enhanced tolerance and accumulation of heavy metals when expressed in yeast and tobacco. Plant Cell Rep 30:1067-1076. doi 10.1007/s00299-011-1013-2

Lone MI, He ZL, Stoffella PJ, Yang XE (2008) Phytoremediation of heavy metal polluted soils and water: Progresses and perspectives. J Zhejiang Univ-Sc B 9:210–220

Ma JF, Ryan PR, Delhaize E (2001) Aluminium tolerance in plants and the complexing role of organic acids. Trends Plant Sci 6:273–278

Macnair MR (1993) The genetics of metal tolerance in vascular plants. New Phytol 124:541–559

Macnair MR, Tilstone GH, Smith SE (2000) The genetics of metal tolerance and accumulation in higher plants. In: Terry N, Banuelos G (eds.) Phytoremediation of Contaminated Soil and Water. Lewis Publishers, Boca Raton, Florida, pp 235–250

Maitani T, Kubota H, Sato K, Yamada T (1996) The composition of metals bound to class III metallothionein (phytochelatin and its desglycyl peptide) induced by various metals in root cultures of *Rubia tinctorum*. Plant Physiol 110:1145–1150

Maiti IB, Wagner GJ, Hunt AG (1991) Light inducible and tissue-specific expression of a chimeric mouse metallothionein cdna gene in tobacco. Plant Sci 76:99–107

Maksymiec W (2007) Signaling responses in plants to heavy metal stress. Acta Physiol Plant 29:177–187

Maksymiec W, Baszynski T (1999) The role of Ca^{2+} ions in modulating changes induced in bean plants by an excess of Cu^{2+} ions. Chlorophyll fluorescence measurements. Physiol Plant 105:562–568

Maksymiec W, Krupa Z (2002) Jasmonic acid and heavy metals in *Arabidopsis* plants - a similar physiological response to both stressors? J Plant Physiol 159:509–515

Marks RE, Acar YB, Gale RJ, Ozsu-Acar E (2000) In situ remediation of contaminated soils by bioelectrokinetic remediation and other competitive technologies. In: Wise DL, Trantolo DJ, Cichon EJ (eds.) Bioremediation of Contaminated Soils. Marcel Dekker Inc., New York, pp 579–605

McGrath SP, Zhao FJ, Lombi E (2001) Plant and rhizosphere processes involved in phytoremediation of metal-contaminated soils. Plant Soil 232:207–214

Meagher RB, Smith AP, Pischke M, Kim T, Dhankher OP, Heaton ACP (2007) Multigene strategies for engineering the phytoremediation of mercury and arsenic. In: Xu Z, Li J, Xue Y, Yang W (eds.) Biotechnology and Sustainable Agriculture 2006 and beyond, proceedings of the 11th IAPTC&B congress. Springer, Beijing (China), pp 49–60

Mehra RK, Tran K, Scott GW, Mulchandani P, Saini SS (1996) Ag(I)-binding to phytochelatins. J Inorg Biochem 61:125–142

Metwally A, Finkemeier I, Georgi M, Dietz KJ (2003) Salicylic acid alleviates the cadmium toxicity in barley seedlings. Plant Physiol 132:272–281

Migocka M, Klobus G (2007) The properties of the Mn, Ni and Pb transport operating at plasma membranes of cucumber roots. Physiol Plant 129:578–587

Milner MJ, Kochian LV (2008) Investigating heavy-metal hyperaccumulation using *Thlaspi caerulescens* as a model system. Ann Bot 102:3–13

Misra S, Gedamu L (1989) Heavy-metal tolerant transgenic *Brassica napus* L. and *Nicotiana tabacum* L. plants. Theor Appl Genet 78:161–168

Mulligan CN, Yong RN, Gibbs BF (2001) Remediation technologies for metal-contaminated soils and groundwater: An evaluation. Eng Geol 60:193–207

Nishiyama Y, Yanai J, Kosaki T (2005) Potential of *Thlaspi caerulescens* for cadmium phytoremediation: Comparison of two representative soil types in Japan under different planting frequencies. Soil Sci Plant Nutr 51:827-834

Nriagu JO, Pacyna JM (1988) Quantitative assessment of worldwide contamination of air, water and soils by trace-metals. Nature 333:134–139

Olade MA (1987) Heavy metal pollution and the need for monitoring: illustrated for developing countries in West Africa. In: Hutchrson CTC, Mean KM (eds) Lead, mercury, cadmium and arsenic in the environment. John Wiley and Sons, New York, pp 335–341

Ow DW (1996) Heavy metal tolerance genes: Prospective tools for bioremediation. Resour Conserv Recy 18:135–149

Pan AH, Yang MZ, Tie F, Li LG, Chen ZL, Ru B (1994) Expression of mouse metallothionein-I gene confers cadmium resistance in transgenic tobacco plants. Plant Mol Biol 24:341–351

Papoyan A, Kochian LV (2004) Identification of *Thlaspi caerulescens* genes that may be involved in heavy metal hyperaccumulation and tolerance. Characterization of a novel heavy metal transporting ATP-ase. Plant Physiol 136:3814–3823

Peer WA, Baxter IR, Richards EL, Freeman JL, Murphy AS (2005) Phytoremediation and hyperaccumulator plants. In: Tams MJ, Martinoia E (eds.) Molecular biology of metal homeostasis and detoxification: from microbes to man. Top Curr Genet 14:299–340

Pence NS, Larsen PB, Ebbs SD, Letham DLD, Lasat MM, Garvin DF, Eide D, Kochian LV (2000) The molecular physiology of heavy metal transport in the Zn/Cd hyperaccumulator *Thlaspi caerulescens*. Proc Natl Acad Sci USA. 97:4956–4960

Piršelová B, Kuna R, Libantová J, Moravčíková J, Matušíková I (2011) Biochemical and physiological comparison of heavy metal-triggered defense responses in the monocot maize and dicot soybean roots. Mol Biol Rep 38:3437-3446

Pittman JK (2005) Managing the manganese: Molecular mechanisms of manganese transport and homeostasis. New Phytol 167:733–742

Prat S (1934) Die Erblichkeit der Resistenz gegen Kupfer. Berliner Deutsche Botanische Gesellschaft 102:65–67

Przymusinski R, Rucinska R, Gwozdz EA (2004) Increased accumulation of pathogenesis- related proteins in response of lupine roots to various abiotic stresses. Environ Exp Bot 52:53–61

Raicevic S, Kaludjerovic-Radoicic T, Zouboulis AI (2005) In situ stabilization of toxic metals in polluted soils using phosphates: Theoretical prediction and experimental verification. J Hazard Mater 117:41–53

Rakwal R, Tamogami S, Kodama O (1996) Role of jasmonic acid as a signaling molecule in copper chloride-elicited rice phytoalexin production. Biosci Biotechnol Biochem 60:1046–1048

Rascio N, Navari-Izzo F (2011) Heavy metal hyperaccumulating plants: How and why do they do it? And what makes them so interesting? Plant Sci 180:169-181

Rauser WE (1999) Structure and function of metal chelators produced by plants - the case for organic acids, amino acids, phytin, and metallothioneins. Cell Biochem Biophys 31:19–48

Rea PA, Li ZS, Lu YP, Drozdowicz YM, Martinoia E (1998) From vacuolar GS-X pumps to multispecific ABC transporters. Annu Rev Plant Physiol Plant Mol Biol 49:727–760

Reeves RD (2003) Tropical hyperaccumulators of metals and their potential for phytoextraction, Plant Soil 249:57–65

Reeves RD, Adigüzel N (2004) Rare plants and nickel accumulators from Turkish serpentine soils, with special reference to *Centaurea* species. Turk J Bot 28:147–153

Reeves RD, Baker AJM (2000) Metal-accumulating plants. In: Raskin H, Ensley BD (eds.) Phytoremediation of Toxic Metals: Using Plants to Clean Up the Environment. John Wiley & Sons, New York, pp 193–229

Robinson BH, Leblanc M, Petit D, Brooks RR, Kirkman JH, Gregg PEH (1998) The potential of *Thlaspi caerulescens* for phytoremediation of contaminated soils. Plant Soil 203:47–56

Rugh CL, Wilde HD, Stack NM, Thompson DM, Summers AO, Meagher RB (1996) Mercuric ion reduction and resistance in transgenic *Arabidopsis thaliana* plants expressing a modified bacterial *merA* gene. Proc Natl Acad Sci USA 93:3182–3187

Ruiz ON, Alvarez D, Torres C, Roman L, Daniell H (2011) Metallothionein expression in chloroplasts enhances mercury accumulation and phytoremediation capability. Plant Biotech J 9:609–61

Ruiz ON, Daniell H (2009) Genetic engineering to enhance mercury phytoremediation. Curr Opin Biotech 20:213–219

Ryan PR, Delhaize E, Jones DL (2001) Function and mechanism of organic anion exudation from plant roots. Annu Rev Plant Phys 52:527–560

Sal DE, Kramer U (2000) Mechanisms of metal hyperaccumulation in plants. In: Raskin H, Ensley BD (eds.) Phytoremediation of Toxic Metals Using Plants to Clean Up the Environment. John Wiley & Sons, New York, pp 231–246

Salt DE, Kramer U (2000) Mechanisms of metal hyperaccumulation in plants. In: Raskin H, Ensley BD (eds.) Phytoremediation of Toxic Metals Using Plants to Clean Up the Environment, John Wiley & Sons, New York, pp 231–246

Salt DE, Rauser WE (1995) MgATP-dependent transport of phytochelatins across the tonoplast of oat roots. Plant Physiol 107:1293–1301

Salt DE, Smith RD, Raskin I (2004) Phytoremediation. Annu Rev Plant Phys 49:643-668

Samantaray S, Rout GR, Das P (2001) Induction, selection and characterization of Cr and Ni- tolerant cell lines of *Echinochloa colona* (L.) link in vitro. J Plant Physiol 158:1281–1290

Samuelsen AI, Martin RC, Mok DWS, Mok MC (1998) Expression of the yeast free genes in trans-genic tobacco. Plant Physiol 118:51–58

Sarret G, Saumitou-Laprade P, Bert V, Proux O, Hazemann JL, Traverse AS, Marcus MA, Manceau A (2002) Forms of zinc accumulated in the hyperaccumulator *Arabidopsis halleri*. Plant Physiol 130:1815–1826

Scheller HV, Huang B, Hatch E, Goldsbrough PB (1987) Phytochelatin synthesis and glutathione levels in response to heavy-metals in tomato cells. Plant Physiol 85:1031–1035

Schmidt U (2003) Enhancing phytoextraction: The effect of chemical soil manipulation on mobility, plant accumulation, and leaching of heavy metals. J Environ Qual 32:1939–1954

Schneegurt MA, Jain JC, Menicucci JA, Brown SA, Kemner KM, Garofalo DF, Quallick MR, Neal CR, Kulpa CF (2001) Biomass byproducts for the remediation of wastewaters contaminated with toxic metals. Environ Sci Technol 35:3786–3791

Schützendübel A, Polle A (2002) Plant responses to abiotic stresses: heavy metal-induced oxidative stress and protection by mycorrhization. J Exp Bot 53:1351–1365

Schwartz C, Echevarria G, Morel JL (2003) Phytoextraction of cadmium with *Thlaspi caerulescens*. Plant Soil 249:27–35

Shah K, Nongkynrih JM (2007) Metal hyperaccumulation and bioremediation. Biol Plantarum 51:618–634

Song WY, Sohn EJ, Martinoia E, Lee YJ, Yang YY, Jasinski M, Forestier C, Hwang I, Lee Y (2003) Engineering tolerance and accumulation of lead and cadmium in transgenic plants. Nat Biotechnol 21:914–919

Suchara I, Sucharova J (2001) Distribution of 36 element deposition rates in a historic mining and smelting area as determined through fine-scale biomonitoring techniques. Part II: Relative long-term accumulated atmospheric deposition levels. Water Air Soil Pollut 153:229–252

Sucharova J, Suchara I (2004) Distribution of 36 element deposition rates in a historic mining and smelting area as determined through fine-scale biomonitoring techniques. Part I: Relative and absolute current atmospheric deposition levels detected by moss analyses. Water Air Soil Pollut 153:205–228

Suresh B, Ravishankar GA (2004) Phytoremediation - a novel and promising approach for environmental clean-up. Crit Rev Biotechnol 24:97–124

Talke NI, Hanikenne M, Kramer U (2006) Zinc-dependent global transcriptional control, transcriptional deregulation, and higher gene copy number for genes in metal homeostasis of the hyperaccumulator *Arabidopsis halleri*. Plant Physiol 142:148–167

Timofeev-Resovsky EA, Agafonov BM, Timofeev-Resovsky NV (1962) Fate of radioisotopes in aquatic environments. Izv AN SSSR 22:49−67

Ueno D, Iwashita T, Zhao FJ, Ma JF (2008) Characterization of Cd translocation and identification of the Cd form in xylem sap of the Cd-hyperaccumulator *Arabidopsis halleri*. Plant Cell Physiol 49:540–548

Unterbrunner R, Puschenreiter M, Sommer P, Wieshammer G, Tlustos P, Zupan M, Wenzel WW (2007) Heavy metal accumulation in trees growing on contaminated sites in central Europe. Environ Pollut 148:107–114

Utsunamyia T (1980) Japanese Patent Application No. 55-72959

Van Aken B (2008) Transgenic plants for phytoremediation: Helping nature to clean up environmental pollution. Trends Biotechnol 26:225–227

Verbruggen N, Hermans C, Schat H (2009) Molecular mechanisms of metal hyperaccumulation in plants. New Phytol 181:759–776

Verkleij JAC (2008) Mechanisms of metal hypertolerance and (hyper) accumulation in plants. Agrochimica 52:167–188

Verret F, Gravot A, Auroy P, Leonhardt N, David P, Nussaume L, Vavasseur A, Richaud P (2004) Overexpression of ATHMA4 enhances root-to-shoot translocation of zinc and cadmium and plant metal tolerance. FEBS Lett 576:306–312

Watanabe ME (1997) Phytoremediation on the brink of commercialization. Environ Sci Technol 31:182–186

Weber M, Harada E, Vess C, von Roepenack-Lahaye E, Clemens S (2004) Comparative microarray analysis of *Arabidopsis thaliana* and *Arabidopsis halleri* roots identifies nicotianamine synthase, a ZIP transporter and other genes as potential metal hyperaccumulation factors. Plant J 37:269–281

Weissenhorn I, Mench M, Leyval C (1995) Bioavailability of heavy-metals and arbuscular: mycorrhiza in a sewage-sludge-amended sandy soil. Soil Biol Biochem 27:287–296

Whiting SN, Leake JR, McGrath SP, Baker AJM (2000) Positive responses to Zn and Cd by roots of the Zn and Cd hyperaccumulator *Thlaspi caerulescens*. New Phytol 145:199–210

Williams LE, Pittman JK, Hall JL (2000) Emerging mechanisms for heavy metal transport in plants. BBA-Biomembranes 1465:104–126

Wong HL, Sakamoto T, Kawasaki T, Umemura K, Shimamoto K (2004) Down-regulation of metallothionein, a reactive oxygen scavenger, by the small GTPase OsRac1 in rice. Plant Physiol 135:1447–1456

Yuko N, Junta Y, Takashi K (2005) Potential of *Thlaspi caerulescens* for cadmium phytoremediation: comparison of two representative soil types in Japan underdifferent planting frequencies. Soil Sci Plant Nutr 51:827-834

Zhang Y, Wang Z, Chai T, Wen Z, Zhang H (2008) Indian mustard aquaporin improves drought and heavy-metal resistance in tobacco. Mol Biotechnol 40:280–292

Zhao FJ, Lombi E, Breedon T, McGrath SP (2000) Zinc hyperaccumulation and cellular distribution in *Arabidopsis halleri*. Plant Cell Environ 23:507–514

Zhao FJ, Lombi E, McGrath SP (2003) Assessing the potential for zinc and cadmium phytoremediation with the hyperaccumulator *Thlaspi caerulescens*. Plant Soil 249:37-43

Zhou DM, Akram A, Deng DF, Cang L, Si YB (2004) Electrokinetic removal of chromium and copper from contaminated soils by lactic acid enhancement in the catholyte. J Environ Sc-China 16:529–532

Zhu YL, Pilon-Smits EAH, Jouanin L, Terry N (1999) Overexpression of glutathione synthetase in indian mustard enhances cadmium accumulation and tolerance. Plant Physiol 119:73–79

PLANT FUNCTIONING UNDER ENVIRONMENTAL STRESS P. 53-68

Cracow, 12th to the 15th September, 2012

DETERMINATION OF THE PHYSIOLOGICAL INDICATORS OF PEA (*PISUM SATIVUM* L.) AND YELLOW LUPINE (*LUPINUS LUTEUS* L.) TOLERANCE TO DROUGHT AT SEEDLING STAGE

Agnieszka Płażek*, Franciszek Dubert**, Janusz Kościelniak*

**Department of Plant Physiology, Faculty of Agriculture and Economics, University of Agriculture in Kraków, Podłużna 3, 30-239 Kraków, Poland*

***Institute of Plant Physiology, Polish Academy of Sciences, Niezapominajek 21, 30-239 Kraków, Poland*

Correspondence author: rrplazek@cyf-kr.edu.pl

Abstract The objective of the study was to estimate drought tolerance differentiation of 17 strains/cultivars of pea and 15 strains/cultivars of yellow lupine. A selection was made of the most useful morphological and physiological traits of drought tolerance in these plant species in early stage of their development. Selection made on seedlings allows to accelerate choice of plants more drought tolerant, however it requires confirmation by final crop obtained in the field. Twenty two parameters related to plant growth and photosynthetic apparatus efficiency were measured on control and stressed plants. For each parameter and both plant treatments the drought susceptibility indexes (DSI), their standard deviations and the 's' parameter (the normalized difference between the maximal and minimal DSI value) were calculated. Seedlings of yellow lupine responded more strongly to a water deficit than the field pea. The 's' parameter seemed to be more adequate than the DSI coefficient for an evaluation of differentiation in the pool of genotype features with respect to drought tolerance. NAR (net assimilation rate) was the most representative parameter suitable in the study of plant drought tolerance. Parameters associated with plant growth were more appropriate indicators of plant drought sensitivity than those of photosynthetic apparatus efficiency. Among pea strains/ cultivars: SZD178, 'Wenus' and 'Brylant', while of lupine: 'Greece', Espana KI-2, 'Luno' and 'Wodjil' were the most resistant to drought.

Key words: Chlorophyll-a fluorescence; Parameters of plant growth; Pea; Soil drought; Yellow lupine

Abbreviations ABS: absorption energy flux, CS: excited cross-section of leaf, DIo: dissipation energy flux at the level of the antenna chlorophylls, DW: dry weight; ETo: flux of electrons from Q_A^- into the electron transport chain, FW: fresh weight, LA: leaf area, LWR: leaf weight ratio, , NAR: net assimilation rate, OEC: fraction of O_2 evolving centers PSII in comparison to the control sample, PAR: photosynthetically active radiation, PFD: photon flux density, PIcs: overall performance index of PSII photochemistry, PSII: photosystem II, Q_A: the first stable electron acceptor in PSII, RC: number of active reaction centers in the state of fully reduced PSII, RGR_A: relative growth rate of the leaf area, RGR_W: relative growth rate of the plant biomass, , SLA: specific leaf area, TRo: excitation energy flux trapped by RC and utilized for the reduction of Q_A , RWC: relative water content, MRWC: maximum relative water content, RT: relative turgidity.

Introduction

Plant yield efficiency depends on many physiological processes proceeding on the cell, whole plant and canopy level. On a cell level, the yield is determined by assimilate photosynthetic production depending on the plant physiological state and the efficiency of light receptors. On a plant level, the crop depends on assimilate transport from donors to acceptors and the rate of development and abortion of generative organs. In the field, the relations between plants, such as competition for light, water and nutrients, are important for crop production.

In the field, water availability is the main factor deciding about the yield in water limited environments. Drought is often the major reason of yield loss (Baigorri et al. 1999; Saini and Westgate 2000; Sharp et al. 2004). Considering the climatic changes that are taking place in the world today, drought effects are continually more noticeable. In Poland, large agricultural areas dry up due to erratic precipitation during the growing season, high temperature amplitude and ground water "escape". The consequences of drought are intensified because of the poor structure of soils occurring in many regions of Poland (Martyniak 2008). The abovementioned facts have inclined researchers towards studies related to plant tolerance to drought and the physiological processes determining it.

The results of many studies have indicated that the photosynthetic apparatus and plant growth rate are the most sensitive to a soil water deficit (see Rapacz et al. 2010). The most useful parameters in the selection of drought tolerant plants are usually photosynthetic apparatus efficiency and yield structure elements (for example number of pods, ears, seeds, mass of 1000 seeds etc). Photosynthetic apparatus efficiency depends on the light conditions and water content in leaf mesophyll. The commonly used method in the estimation of photosynthetic efficiency is chlorophyll fluorescence, which allows to estimate the loss of light energy and disorders in electron transfer between the chlorophyll of the PSII reaction centres and Q_A (Fracheboud et al. 1999; Lichtenthaler 1996; Maxwell and Johnson 2000). Chlorophyll fluorescence parameters can also detect injuries of both photosystems caused by drought and other environmental stresses. These measurements are very

fast and are not invasive. Li et al. (2006) stated that chlorophyll content, initial fluorescence (F_o), maximum primary yield of photochemistry of PSII (photosystem II) (F_v/F_o) and maximum quantum yield of PSII (F_v/F_m) might be the indicators of barley drought resistance. It was also found that drought tolerant genotypes demonstrated significantly higher values of chlorophyll content, F_o, F_v/F_o and F_v/F_m than sensitive ones under drought stress. Yang et al. (2006) showed that in water deficit conditions, the changes of maximal efficiency of PSII photochemistry (F_v/F_m), actual PSII efficiency (Φ_{PSII}), photochemical quenching (q_P) and non-photochemical quenching (q_N) were observed, and they correlated with a differentiation of the examined materials with regards to drought sensitivity.

Recently, the growing interest of Polish plant breeders in legumes has been increased. In the word the most commonly used leguminous plant is soybean, however, in Poland climatic conditions limit the cultivation of this species. An alternative to soybean is the field pea (*Pisum sativum* L.) and yellow lupine (*Lupinus luteus* L.) because they are more cold tolerant, and their cultivation is less risky. Moreover, pea and lupine are characterized by the favorable amino acid content in seeds. Taking these facts into consideration, the studies related to drought tolerance of legumes seem to be well-grounded.

The objective of the study was to estimate drought tolerance differentiation at seedling stage of 32 strains and cultivars of field pea and yellow lupine. An attempt was made to select the most useful morphological and physiological characteristics of drought resistance in these plant species. The parameters related to growth rate, biomass accumulation, chlorophyll and water content and photosynthetic apparatus efficiency were measured.

Material and methods

Plant material. The investigation was performed on total 32 strains/cultivars of field pea and yellow lupine as presented in Table 1. Pea seeds were obtained from Plant Breeding Szelejewo (Poland), while the lupine seeds were obtained from Poznań's Plant Breeding (Tulce, Poland).

Plant growth and drought treatment. The seeds germinated on wet paper in Petri dishes in the dark and at 25 °C. As antifungal seed dressing 75% tiurame (Zaprawa nasienna T, Organika-Azot SA, Jaworzno, Poland) was used. Five seedlings were planted in boxes (4.5 dm^3 volume) containing a mixture of garden soil and sand (2 : 0.5 v/v). The plants were grown in a glasshouse at 20/17°C day/night in a 16h-photoperiod, using 400 W sodium lamps (Philips SON-T AGRO, Brussels, Belgium) yielding photosynthetically active radiation (PAR) of 400 μmol m^{-2} s^{-1}. The seedlings were watered and fertilized with Hoagland medium (Hoagland and Arnon 1938) as required. Soil moisture was maintained to the 3-leaf phase at a level of 70% of maximum relative water content (MRWC) by adding an appro-

priate amount of water every day. Then the plants were divided into two groups: control and drought stressed. Soil drought was induced gradually by limiting watering and after 7 days moisture level decreased to 20% MRWC (it was equaled to about –2.0 MPa). That was maintained for the following 7 days, while the control plants were watered normally.

Table 1 Cultivars and strains of field pea and yellow lupine used in the study

Field pea					
No	Name	Country of origin	No.	Name	Country of origin
1	Wenus	Poland	10	SZD 175	Poland
2	Kuroch	Poland	11	SZD 178	Poland
3	Brylant	Poland	12	SZD 240	Poland
4	Turkus	Poland	13	SZD 205	Poland
5	Tarchalska	Poland	14	SZD 227	Poland
6	Hubal	Poland	15	SZD 165	Poland
7	Boruta	Poland	16	SZD 188	Poland
8	SZD 1106	Poland	17	SZD 190	Poland
9	SZD 1005	Poland			
Yellow lupine					
1	Talar	Poland	9	Sweet Yellow	South Africa
2	Taper	Poland	10	Yorlupine	Netherlands
3	Mister	Poland	11	Morocco-4	Morocco
4	Luno	Poland	12	Greece	Grece
5	Wodjil	Austria	13	Lubljana	Slovenia
6	Amulet	Poland	14	Espana KI-2	Spain
7	Kastricznik	Russia	15	Portugalia KI-2	Portugal
8	Kormowoj	Ukraine			

Measurements and Analyses

*Growth indexes.*The height of seedling, FW (fresh weight), LA (leaf area), DW (dry weight) of leaves collected from one plant, DW of stem, DW of seedling, RWC (relative water content) in leaves and in whole seedling were estimated. On the basis of these parameters the RGR_W (relative growth rate of the plant biomass), RGR_A (relative growth rate of the leaf area), LWR (leaf weight ratio), NAR (net assimilation rate) and SLA (specific leaf area – of all leaves from one seedling) were calculated (Květ et al. 1971). DW was estimated after drying of plant samples at 70°C for 48 h. RWC was calculated as follows:

$$RWC = [(FW - DW)/FW] \times 100$$

Leaf area was measured using a scanner (ScanMaker 3880, Microtek, Hsinchu, Taiwan) and Delta-T Skan 2.03 software (Delta-T Devices, Cambridge, UK).

These all measurements were done on 15 plants from each treatment.

Chlorophyll content. Chlorophyll content was measured in 3 upper, fully expanded leaves using a portable chlorophyll meter (SPAD-502, Minolta). SPAD-readings were calibrated (Markwell et al., 1995) using the spectrophotometric analysis of extracts according to the Lichtenthaler and Wellburn method (1983). Chlorophyll (a+b) content was expressed as g m^{-2}. The measurements were done in 15 replications for each treatment.

Electrolyte leakage. Ion leakage determining the plasma membrane's integrity was measured in 3 upper, well-developed leaves. Leaf discs (Ø = 1cm) were washed in deionised water, put into plastic vials containing 10 cm^3 deionised water and shaken for 24 h (50 rpm) at 20 °C. Next, ion conductivity was measured (EL_1) using a conductometer (CI 317, Elmetron, Poland). The samples were frozen at –40°C for 24 h, after thawing they were shaken again for 24 h and next total ion leakage (EL_2) was measured. Membrane permeability was expressed as a percentage of total electrolyte leakage (EL_1 x 100/EL_2). The measurements were done in 6 replications for each treatment.

Relative turgidity. Relative turgidity (RT) was measured according to Barrs (1968). The 3 upper fully expanded leaves were cut, weighed to estimate fresh mass (FW_1), floated on water surface and left to attain full turgidity. Next, they were weighed again (FW_2 in full turgidity) and dry weight (DW) was determined. RT was calculated according to the formula:

$$RT = [(FW_1 - DW) / (FW_2 - DW)] \times 100.$$

Photochemical activity of PSII. Chlorophyll fluorescence was measured in the upper well-developed leaf using the Plant Efficiency Analyser PEA (Hansatech Ltd. Kings Lynn, UK). Before measurements, the LED-light source of the fluorometer was calibrated using an SQS light meter (Hansatech Ltd, Kings Lynn, UK). The excitation irradiance had an intensity of 3 000 μmol m^{-2} s^{-1} (peak at 650 nm). Measurements were taken after 30 min of leaf adaptation to darkness (clips with a 4-mm diameter hole). Fluorescence intensity was measured with a PIN-photodiode after being passed through a long-pass filter. Changes in fluorescence were registered during irradiation between 10 μs and 1 s. During the initial 2 ms, the data were collected every 10 μs with 12 bit resolution. After this period, the frequency of measurements was reduced automatically. On the basis of these measurements, the parameters (per excited leaf cross-section; CS) ABS/CS (light energy absorption), TRo/CS (amount of excitation energy trapped in PSII reaction centers), ETo/CS (energy amount used for electron transport), DIo/CS (energy amount dissipated from PSII), RC/CS (number of active reaction centres), PIcs (overall performance index of PSII photochemistry) and OEC (fraction of O_2 evolving centres PSII in comparison to the control sample) were calculated based on the theory of energy flow in PSII and using the JIP test as it was described el-

sewhere (Lazár 1999; Lazár and Pospíšil 1999; Srivastava and Strasser 1977; Strasser and Strasser 1995; Strasser et al. 2000). The measurements were performed on 20–25 plants.

Drought susceptibility index (DSI). All measurements were done on plants grown under drought stress (X_1) and on control plants (X_2). From these results for each parameter and for each strain/cultivar the drought susceptibility index (DSI) was calculated as: DSI = (X_1/X_2) x 100 (Fisher and Mauer 1978). The more DSI is different from 100%, the stronger the drought influences the studied parameter, so it can be seen as a physiological indicator of plant response to a water deficit. However, to comparison various strains/cultivars with respects to their degree of drought susceptibility, their DSI values should be strongly differentiated. Thus, apart from commonly used the standard deviation of DSI (σ), the 's' parameter being the "normalized" difference between the highest and the smallest value of DSI was also calculated, according to the formula: s = [(DSImax–DSImin) x 100]/DSIaverage. The "s" value should be relatively high.

Statistical analyses

All results were tested with the *F*-test (ANOVA/MANOVA) using STATISTICA 7.1 software (Statsoft, Tulsa, OK, USA).

Results

An analysis of variance showed that both factors as pea and lupine strains/cultivars and soil drought strongly differentiated values of most of physiological parameters under study (Table 2). The interaction between plant and drought treatment was significant for 13 parameters in the case of pea and for 17 parameters in the case of lupine.

Field pea. In Table 3 the physiological parameter values carried out on control and drought treated plants were demonstrated. On that basis the DSI coefficients, their standard deviation (σ) and the 's' parameter were calculated. For some parameters the DSI values differ considerably from 100%, for example for SLA (DSI=73.6) or height of seedling (DSI=62.8), however their 's' parameters are to low (23.3 and 22.6 respectively). It is observable that individual DSI values weakly differentiate the parameters under drought stress, while the 's' parameter gives this possibility. Its values, presented in Table 3, are strongly diverse (6.2−192), therefore it could be useful to choose the physiological parameters that are the best indicators of drought stress (however to compare of drought tolerance of several strains/cultivars DSI coefficient was used). On that basis, a total of 11 physiological parameters from the initial 22 were selected for further analyses (according to increasing 's' values): RGR_A, PI_{CS}, OEC, NAR, EL, LA, DW of seedlings, RGRw,

RC/CS, RT, and ETo/CS. The parameters: DW of stem and DW of leaves, despite their high values of the 's' parameter, were not included, since neither parameter, associated with the DW of seedling, introduces new elements characterising plant sensitivity to drought.

Table 2 Analysis of variance of the plant object (species) and drought influence on the studied physiological parameters. The values of the *F*-test and the level of their significance were shown; * – P<0.05, ** – P<0.01, *** – P<0.001), n.s. – not significant

Parameter [units]	Field pea			Yellow lupine		
	Plant object (O)	Drought (D)	Interaction (O x D)	Plant object (O)	Drought (D)	Interaction (O x D)
Height of seedling [mm]	7.66^{***}	960.3^{***}	1.81^{*}	58.9^{***}	44.0^{***}	14.2^{***}
Leaf area [mm^2]	4.28^{***}	330.8^{***}	1.98^{*}	25.0^{***}	1278^{***}	2.54^{**}
DW of stem [mg]	6.65^{***}	134.4^{***}	$1.59^{n.s.}$	10.8^{***}	172.5^{***}	3.14^{***}
DW of leaves [mg]	7.18^{***}	189.2^{***}	$1.25^{n.s.}$	5.64^{***}	399.1^{***}	2.34^{**}
DW of seedling [mg]	3.88^{***}	159.1^{***}	$0.10^{n.s.}$	6.39^{***}	321.6^{***}	2.42^{**}
RGR_A [m m^{-2} d^{-1}] x 10^2	5.88^{***}	1743^{***}	1.68^{*}	3.29^{***}	1765^{***}	$1.41^{n.s.}$
RGR_W [g g^{-1} d^{-1}] x 10^2	$1.55^{n.s.}$	225.2^{***}	$1.17^{n.s.}$	4.81^{***}	334.7^{***}	2.34^{**}
LWR [m^2 g^{-1}] x 100	4.94^{***}	53.9^{***}	$1.17^{n.s.}$	5.73^{***}	3319^{***}	0.76^{***}
SLA [m^2 g^{-1}]	$1.32^{n.s.}$	50.8^{***}	$0.95^{n.s.}$	7.06^{***}	1409^{***}	3.78^{***}
NAR [g m^{-2} d^{-1}] x 10	5.34^{***}	28.5^{***}	$1.43^{n.s.}$	4.32^{***}	53.60^{***}	2.74^{***}
chl. (a + b) [g x m^{-2}]	2.71^{***}	196.3^{***}	2.13^{**}	4.91^{***}	$3.58^{n.s.}$	$1.10^{n.s.}$
El [%]	2.95^{***}	9.98^{***}	$0.50^{n.s.}$	3.25^{***}	8.48^{***}	$0.50^{n.s.}$
WC in leaves [%]	1.88^{*}	1286^{***}	6.92^{***}	4.16^{***}	1728^{***}	4.30^{***}
WC in seedling [%]	5.20^{***}	2083^{***}	3.54^{***}	3.47^{***}	2523^{***}	3.62^{***}
RT [%]	10.71^{***}	4987^{***}	6.10^{***}	3.43^{***}	1881^{***}	$1.63^{n.s.}$
ABS/CS	2.24^{**}	16.35^{***}	1.89^{*}	3.46^{***}	561.2^{***}	9.38^{***}
TRo/CS	2.20^{**}	12.65^{***}	2.32^{**}	6.13^{***}	805.1^{***}	8.27^{***}
ETo/CS	5.78^{***}	4.03^{*}	4.18^{***}	9.66^{***}	705.3^{***}	2.40^{**}
DIo/CS	23.22^{***}	2.58^{***}	$1.22^{n.s.}$	4.70^{***}	$2.65^{n.s.}$	3.20^{***}
OEC [%]	11.04^{***}	375.7^{***}	6.30^{***}	6.14^{***}	89.86^{***}	9.37^{***}
RC/CS	2.77^{***}	62.95^{***}	4.35^{***}	7.74^{***}	792.1^{***}	1.79^{*}
PI_{CS}	6.18^{***}	$1.89^{n.s.}$	5.50^{***}	6.27^{***}	467.9^{***}	$1.28^{n.s.}$

The selected parameters were related to plant growth, the relative increase of plant mass, LA, photosynthetic rate, EL, RT and chlorophyll fluorescence. It is surprising to note that such typical parameters associated with drought influence as chlorophyll content, and most parameters of chlorophyll fluorescence, demonstrated such small differentiation. Conversely, it seems that in the pea strains chlorophyll content stayed unchanged, therefore during recovery after the drought period the plants could regain their whole photosynthetic efficiency.

Table 3 The influence of drought on the studied parameters of field pea plants. For each parameter the mean, average DSI, its standard deviation (σ) and s (s = [(DSI max – DSI $_{min}$) x 100]/DSI average) are shown. In the last column a ranking (R) of parameters from the strongest (R = 1) to the weakest (R = 25) responding to drought is presented

Parameter	Control	Drought	DSI	σ	s	R
RGRa	6.05	1.20	19.6	25.0	192	1*
PI_{CS}	47279	45781	98.1	14.2	72.4	2*
OEC	100	78.2	78.2	8.26	44.8	3*
NAR	43.55	36.49	84.4	3.57	39.4	4*
EL	3.69	4.44	121	8.26	39.2	5*
LA	5527	2819	51.0	10.5	38.5	6*
DW of stem	235.8	166.9	71.7	4.35	38.3	7
DW of seedling	336.5	236.0	71.0	5.79	36.8	8*
RGR_w	8.35	5.86	70.6	7.89	36.4	9*
RC/CS	962.7	1055	110	10.7	35.1	10*
DW of leaves	100.7	69.16	69.4	9.89	34.5	11
RT	90.72	55.92	61.6	1.21	34.2	12*
ETo/CS	973.0	950.6	98.0	6.27	31.8	13*
SLA	5.56	4.09	73.6	4.36	23.3	14
Height of seedling	280.2	175.4	62.8	6.34	22.6	15
TRo/CS	1758	1818	104	7.23	21.7	16
LWR	30.13	29.49	97.9	5.54	21.4	17
ABS/CSm	2218	2297	104	6.25	18.0	18
DIo/CS	459.8	478.2	104	2.45	15.0	21
Chl. (a + b)	1.72	1.58	91.8	1.85	11.7	22
WC in leaves	87.57	80.40	91.8	0.64	8.0	23
WC in seedling	87.89	80.29	91.4	1.86	6.2	24

* – Parameters used to compare drought tolerance of the studied strains/cultivars.

Selected parameters (marked with asterisk in Table 3) were used to compare the drought tolerance of pea strains/cultivars and to identify most resistant or sensitive ones. The results of this analysis were presented in Table 4, which shows in column No. 14 a final ranking of the studied strains and cultivars of pea according to their increasing drought tolerance estimated on the basis of all 11 selected parameters (columns 2−12), where position 1 means the most sensitive strain/cultivar (minimal value of DSI) and 17 the most resistant one (maximal DSI). It should be marked that DSI detailed value for each strain/cultivar and each measured parameter have not been presented. According to final ranking we can see that strain no. 11 (SZD178), and cultivars no. 1 (‘Wenus’) and no. 3 (‘Brylant’) were recognised as the most resistant to a water deficit in the seedling phase, while strains no. 15 (SZD165), no. 9 (SZD 1005) and cultivar no. 5 (‘Tarchalska’) were recognised as the most sensitive.

Table 4 Rankings (using a scale of 1-17) of the studied strains/cultivars of field pea (numbers of strains/cultivars in column 1 as presented in Table 1) according to increasing drought tolerance (on the basis of increasing DSI value) for the chosen parameters (Table 3): 1 – the most sensitive, 17 – the most tolerant to drought. Detailed DSI value for each strain/cultivar and each measured parameter have not been presented. In column 13 a sum (∑) of the values (ranking position) from columns 2-12 is shown. In column 14 a final ranking of the studied cultivars and strains according to increasing resistance to drought is presented

No.	RGR_A	PIcs	OEC	NAR	EL	Leaf area	DW of seedling	RGR_W	RC/ CS	RT	ETo/ CS	∑	R
1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	17	9	16	16	7	17	16	17	3	13	8	139	16
2	14	1	4	12	9	11	14	14	1	3	1	84	5
3	16	11	15	15	13	15	15	15	8	1	13	137	15
4	15	6	6	11	6	7	9	13	9	2	4	88	8
5	13	4	3	5	1	13	4	7	5	8	5	68	3
6	5	17	13	9	4	5	8	8	17	5	16	107	11
7	1	13	7	2	14	1	1	1	16	6	11	73	4
8	6	16	17	3	16	2	3	3	14	12	17	109	12
9	11	2	5	4	11	16	5	4	2	4	2	66	2
10	12	5	11	8	5	12	10	10	7	17	6	103	10
11	7	10	1	17	17	14	17	16	15	16	10	140	17
12	8	7	9	13	10	8	12	12	4	11	9	103	9
13	10	8	10	10	15	10	11	11	10	10	7	112	13
14	9	14	14	1	8	4	2	2	11	7	15	87	7
15	3	3	2	6	2	9	7	6	6	9	3	56	1
16	4	12	8	7	3	3	6	5	12	14	12	86	6
17	2	15	12	14	6	6	13	9	13	15	14	119	14

If two or more plant objects obtained the same values of the sum (column 13), it was assumed that the object with a greater value of interval between the maximal and minimal ranking was located at a higher position in the final ranking (column 14).

Next attempt was made to select the parameter giving the ranking the most adequate to that obtained on the basis of all 11 parameters (Table 4, column 14). For each of the parameters the differences between the final ranking shown in column 14 of Table 4 and strain/cultivar ranking on the basis of individual parameters (Table 4, columns 2−12) were calculated and presented in Table 5. Next, for each parameter these differences for all strains/cultivars were summed up (row marked as ∑). It was assumed that the parameter characterized by the lowest value of ∑ is

more representative for all of the measured parameters. Table 5 demonstrates that the ranking of DW of seedling, NAR and OEC chosen among the 11 parameters was the most adequate to the average ranking, so that they can be used as the best indicators of the field pea's drought sensitivity.

Table 5 The choice of best parameters determining the ranking of the studied cultivars and strains of the field pea according to the degree of drought resistance. The differences between strain/cultivar ranking position evaluated for each studied parameter (2–12 columns in Table 4) and average ranking position calculated on the basis of all parameters (column 14 in Table 4) are shown. In the last two rows, a sum (∑) of these differences and a final ranking (R) of the parameters useful for drought resistance evaluation are presented. The following scale was applied: 1 – the parameter giving the plant object ranking the most adequate to the ranking presented in column 14 in Table 4; 11 – the most inadequate parameter. The number of studied cultivars/strains of pea in column 1 as presented in Table 1. If two or more parameters obtained the same values of the sum (∑), it was assumed that the parameter with the greater value of an interval between the maximal and minimal ranking was located at a higher position in the final ranking (R).

No.	RGR_A	PIcs	OEC	NAR	EL	Leaf area	DW of seedling	RGR_W	RC/CS	RT	ETo/CS
1	1	7	0	0	9	1	0	1	13	3	8
2	9	4	1	7	4	6	9	9	4	2	4
3	1	4	0	0	2	0	0	0	7	14	2
4	7	2	2	3	2	1	1	5	1	6	4
5	10	1	0	2	2	10	1	4	2	5	2
6	6	6	2	2	7	6	3	3	6	6	5
7	3	9	3	2	10	3	3	3	12	2	7
8	6	4	5	9	4	10	9	9	2	0	5
9	9	0	3	2	9	14	3	2	0	2	0
10	2	5	1	2	5	2	0	0	3	7	4
11	10	7	16	0	0	3	0	1	2	1	7
12	1	2	0	4	1	1	3	3	5	2	0
13	3	5	3	3	2	3	2	2	3	3	6
14	2	7	7	6	1	3	5	5	4	0	8
15	2	2	1	5	1	8	6	5	5	8	2
16	2	6	2	1	3	3	0	1	6	8	6
17	12	1	2	0	8	8	1	5	1	1	0
∑	86	72	48	48	70	82	46	58	76	70	70
R	11	8	3	2	6	10	1	4	9	7	5

Yellow lupine. Table 6 presents the mean values of all measured parameters for control and drought stressed plants of the studied strains/cultivars of lupine: the average DSI values, their standard deviations (σ), values of the 's' parameter and the ranking (R) of parameters from the strongest (maximal 's' value) to the weakest

(minimal 's' value) responding to drought. On the basis of the 's' parameter values, the following 9 parameters were chosen for further analyses: RGR_A, EL, RGRw, NAR, height of seedling, DW of seedling, LA, OEC and PI_{CS}. All of them, except of height of seedling, were common for both species under study. Also, in the case of lupine, the DW of stem and DW of leaves were not chosen for further analyses in spite of their high 's' parameter values. Among the chosen parameters are those concerning vegetative plant growth, relative increase of mass, leaf area and photosynthesis rate, EL and two parameters of chlorophyll fluorescence. Similarly as in the pea plants, changes in chlorophyll content in lupine under drought influence did not differentiate plant objects.

Table 6 The influence of drought on the studied parameters of yellow lupine plants. For each parameter the mean, average DSI, its standard deviation (σ) and s (s = [(DSI max – DSI min) x 100]/DSI average) are shown. In the last column a ranking (R) of parameters from the strongest (R = 1) to the weakest (R = 25) responding to drought are presented

Parameter	Control	Drought	DSI	σ	s	R
RGR_A	6.03	1.81	22.8	2.51	147	1*
EL	2.97	26.84	934	357.4	133	2*
RGRw	6.30	2.90	46.2	3.67	92.5	3*
NAR	27.52	18.64	68.2	6.85	82.2	4*
Height of seedling	70.2	41.9	63.5	7.96	68.3	5*
DW of seedling	311.4	174.1	56.8	0.70	64.2	6*
DW of stem	120.0	79.7	67.9	2.92	63.0	7
DW of leaves	191.4	94.5	50.3	1.16	62.0	8
LA	7019	1851	26.6	0.92	46.4	9*
OEC	100	83.9	84.0	10.62	42.4	10*
PI_{CS}	111	613	54.9	2.17	37.5	11*
SLA	3.71	1.97	53.4	0.66	37.3	12
DIo/CS	377.3	387.5	102.8	2.63	36.4	13
RT	91.3	54.4	59.6	3.87	29.5	14
ABS/CS	2084	1830	88.1	4.00	21.6	15
RC/CS	1347.7	990.8	73.6	0.16	21.5	16
TRo/CS	1706	1442	84.8	4.15	19.6	18
Chl.(a + b)	0.96	0.89	92.6	10.10	18.5	20
LWR	61.6	54.4	88.4	0.99	18.1	21
ETo/CS	1076	863	80.3	2.94	15.4	22
WC in leaves	90.4	76.9	85.0	1.90	10.4	23
WC in seedling	90.3	79.3	87.8	1.15	6.9	24

* – Parameters marked with a star were used to compare the drought tolerance of the studied cultivars and strains.

Selected parameters were used to estimate drought sensitivity of the studied strains/ cultivars. In Table 7 they were ranked according to their drought tolerance: position 1 means the most sensitive strain/cultivar, and position 15 means the most

resistant one. The final ranking of lupine strains/cultivars was obtained on the basis of all 9 chosen physiological parameters. Cultivars no.12 (‘Greece’), no. 14 (Espana KI-2), no. 4 (‘Luno’) and no. 5 (‘Wodjil’) were the most resistant, while cultivars no. 6 (‘Amulet’), no. 2 (‘Taper’) and no. 13 (‘Lubljana’) were the most sensitive to soil drought in the seedling phase.

Table 7 A ranking (using a scale of 1–15) of the studied strains and cultivars of yellow lupine (numbers of strains/cultivars in column 1 as presented in Table 1) according to an increasing drought tolerance (on the basis of increasing DSI values) for the chosen parameters (Table 6): 1 – the most sensitive, 15 – the most tolerant to drought). In column 11 a sum (∑) of the values (ranking position) from columns 2–10 is shown. In column 12 a final ranking of the studied cultivars and strains according to increasing resistance to drought is presented

No.	RGR_A	El	RGR_W	NAR	Height of seedling	DW of seedling	LA	OEC	PI_{CS}	∑	R
1	2	3	4	5	6	7	8	9	10	11	12
1	12	7	8	8	10	7	5	13	8	78	9
2	3	1	5	5	1	2	4	10	13	44	2
3	4	5	13	13	4	12	12	9	12	84	10
4	14	2	15	14	3	15	14	11	5	93	13
5	7	12	11	12	7	10	10	15	9	93	12
6	10	3	2	2	2	1	1	8	3	32	1
7	5	4	7	7	6	5	8	14	11	67	5
8	15	9	1	1	14	4	3	6	14	67	5
9	11	6	3	3	8	6	9	3	10	59	4
10	1	8	12	11	12	9	13	12	6	84	11
11	13	11	9	9	5	11	7	2	2	69	7
12	2	13	14	15	15	13	11	7	7	97	15
13	8	14	4	4	9	3	2	1	1	46	3
14	6	10	10	10	11	14	15	4	15	95	14
15	9	15	6	6	13	8	6	5	4	72	8

If two or more plant objects obtained the same values of the sum (column 11), it was assumed that the object with a greater value of interval between the maximal and minimal ranking was located at a higher position in the final ranking (column 12).

In the further stage of investigation an evaluation was done as to which individual parameter gives strain/cultivar ranking the most adequate to that obtained on the basis of all chosen parameters. For each of the 9 chosen parameters the differences between the final ranking (Table 7, column 12) and the ranking done on the basis of a single parameter (Table 7, columns 2–10) were calculated and shown in Table 8. Next, these differences were summed up (row marked as ∑) and it was assumed that the lowest sum the most representative is parameter. Table 8 shows that NAR, RGR_W and LA allow for an evaluation of lupine plant response to drought instead of other parameters.

Table 8 The choice of best parameters determining the ranking of studied strains and cultivars of yellow lupine according to the degree of drought resistance. The differences between strain/cultivar ranking position evaluated for each studied parameter (Table 7, columns 2–10) and the average ranking position calculated on the basis of all parameters (Table 7, column 12) are shown. In the last two verses the sum (∑) of these differences and the final ranking (R) of parameters useful for drought resistance evaluation are presented. The following scale was applied: 1 – the parameter giving the object ranking the most adequate to the ranking calculated on the basis of all studied parameters (Table 7, column 12); 9 – the most inadequate parameter. The number of the studied strains/cultivars of lupine is in column 1 as presented in Table 1

No.	RGR_A	El	RGR_W	NAR	Height of seedling	DW of seedling	LA	OEC	PI_{CS}
1	3	2	1	1	1	2	4	4	1
2	1	1	3	3	1	0	2	8	11
3	6	5	3	3	6	2	2	1	2
4	1	11	2	1	10	2	1	2	8
5	5	0	1	0	5	2	2	3	3
6	9	2	1	1	1	0	0	7	2
7	0	1	2	2	1	0	3	9	6
8	10	4	4	4	9	1	2	1	9
9	7	2	1	1	4	2	5	1	6
10	10	3	1	0	1	2	2	1	5
11	6	4	2	2	2	4	0	5	5
12	13	2	1	0	0	2	4	8	8
13	5	11	1	1	6	0	1	2	2
14	8	4	4	4	3	0	1	10	1
15	1	7	2	2	5	0	2	3	4
Σ	85	59	29	25	55	19	31	65	73
R	9	6	3	2	5	1	4	7	8

Discussion

One of the aims of the work was to find the most representative physiological parameters of drought tolerance of the field pea and yellow lupine. In experiments on plant response to water deficit usually drought susceptibility indexes are calculated (Grzesiak et al. 2007; Rapacz et al. 2010). In the presented study it was shown that a single DSI coefficient is not sufficient enough to make a selection of parameters characterized by high differentiation in the degree of drought response. The ‘s’ parameter seemed to be more adequate to select for both the best physiological indicators of drought resistance and the most tolerant or most sensitive to water deficit parameters. On the basis of this ‘s’ parameter in the case of the field pea, 11 physiological parameters responded most to drought, while in the case of yellow lupine, 9 such parameters were selected. These parameters were associated with plant growth, DW content, membrane integrality (EL) and chlorophyll fluo-

rescence. Among the latter, in the case of the pea these are RC/CS, ETo/CS, PI_{CS} and OEC. The same parameters, except OEC, were also recognized by Rapacz et al. (2010) as parameters diversifying drought sensitivity of malting and fodder spring barley and other plant species (Canaani et al. 1986; Pinior et al. 2005). Moreover, similarly as in our work, EL was also found to be a good indicator of drought response of these plants (Rapacz et al. 2010). Grzesiak et al. (2007) proved that drought had an influence on the damage caused to cell membranes, however EL depended strongly on leaf age. Correlations between some physical parameters of cell membranes and drought tolerance were also observed in wheat plants (Benveniste-Levkovitz et al. 1993).

In the presented investigation both pea and lupine plants demonstrated significant changes in Chl a content under drought conditions, however the DSI coefficient showed small differentiation between the strains/cultivars. Also, other authors showed a significant decrease of this parameter in drought treated plants (Richards 1978, Ali 1997). Rapacz et al. (2010) stated that both studied groups of barley require different drought indicators. Our experiment demonstrated that most parameters chosen for lupine response to water deficit were similar to that as in the case of pea. Among numerous chlorophyll fluorescence parameters only OEC and PI_{CS} were recognized as parameters which differentiate studied strains/cultivars of pea and lupine. Common parameters for both studied plant species were also EL, NAR, RGR_A, RGR_W, and DW of seedling. Finally, for the pea, the most representative physiological indicators differentiating the strain/cultivar response to drought were DW of seedling, NAR and OEC, while for lupine NAR, RGR_W and LA. For both species these are mainly growth parameters, so it could be supposed that photosynthetic efficiency is not so sensitive to rehydration as plant growth. This result confirms earlier observations of Hura et al. (2007), which stated that the faba bean and maize showed better tolerance of the photosynthetic apparatus to long-term water deficit due to activation of the mechanism connected with the synthesis of phenolic compounds playing the role of photoprotectors. Growth parameters, especially NAR, strongly responded to water limitation in soil. It can be assumed that a decrease in the NAR values (connected with a decrease in CO_2 assimilation) in drought treated plants was the result of stomata closure observed in water deficit. Yellow lupine responded more strongly to the water deficit in the seedling phase than the field pea, which was stated on the basis of the DSI values. In the case of the pea, the DSI of 16 physiological parameters out of 22 studied at the beginning of the investigation amounted to values close to 100% (value of control plants), while in the case of lupine the DSI of only 6 parameters do not differ significantly from the control.

Conclusions

1. The ‘s’ parameter seems to be more appropriate than the DSI coefficient to choose the most representative parameter differentiating the drought tolerance of various plant strains/cultivars.

2. NAR is the most representative parameter suitable in the studies of drought tolerance of the field pea and yellow lupine.
3. Parameters associated with seedling growth and membrane permeability (NAR, DW of seedlings, LA, $RGR_{A,}$ RGRw, EL) are more proper as indicators of plant drought sensitivity than the parameters of photosynthetic apparatus efficiency (only two: OEC and PI_{CS} from the numerous parameters measured could be used as indicators).
4. Among the 17 studied strains/cultivars of field pea, SZD178, 'Wenus' and 'Brylant' are the most resistant, while SZD165, SZD1005 and 'Tarchalska' are the most sensitive to soil drought in the seedling phase.
5. Among the 15 strains/cultivars of yellow lupine, 'Greece', Espana KI-2, 'Luno' and 'Wodjil' are the most resistant, while 'Amulet', 'Taper' and 'Lubljana' are the most sensitive to soil drought in the seedling phase.

Acknowledgements

This study was supported by the Polish Ministry of Agriculture and Rural Development HOR hn – 4040 dec – 14/08

References

Ali HC (1997) Comparison of chlorophyll content and stomatal size of inbred lines and their hybrids of corn (*Zea mays* L.). Z Acker-Pflanzenbau 145: 166–170

Baigorri H, Antolín MC, Sánchez-Díaz M (1999) Reproductive response of two morphologically different pea cultivars to drought. Eur J Agron 10: 119–128

Barrs HD (1968) Determination of water deficits in plant tissues. In: Kozlowski TT (ed) Water deficits and plant growth, Acad. Press, N-Y. and London, vol. I: pp. 235–368

Benveniste-Levkovitz P, Canaani O, Gromet-Elhanan Z, Atsmon D (1993) Characterization of drought resistance in a wild relative of wheat, *Triticum kotschyi*. Photosynth. Research 35: 149–158

Canaani M, Havaux M, Malkin P (1986) Hydroxylamine, hydrazine and methylamine donate electrons to the photooxidizid side of PSII in leaves inhibited in oxygen evolution due to water stress. Ciochim. Biophys. Acta 851: 151–155

Fischer RA, Maurer R (1978) Drought resistance in spring wheat cultivars. I. Grain yield responses. J. Agr. Res. 29: 897–912

Fracheboud Y, Haldimann P, Leipner J, Stamp P **(**1999) Chlorophyll fluorescence as a selection tool for cold tolerance of photosynthesis in maize (Zea mays L.). J. Exp. Bot. 50: 1533–1540

Grzesiak MT, Rzepka A, Hura T, Hura K, Skoczowski A (2007) Changes In response to drought stress of triticale and maize genotypes differing in drought tolerance. Photosynth. 45: 280–287

Hoagland DR, Arnon DI (1938) The water-culture method for plants without soil. Univ. Calif. Agr. Exp. Stn. Cir. 347, 29–32

Hura T, Hura K, Grzesiak M (2007) Effect of long-term drought stress on Lear gas Exchange and fluorescence parameters in C_3 and C_4 plants. Acta Physiol. Pant. 29: 103–113

Květ J, Ondok JP, Nečas J, Jarvis PG (1971) Methods of growth analysis. In: Šesták Z, Čatský J, Jarvis PG (eds) Plant Photosynthetic Production. Manual of Methods, 343–Dr W. Junk Publ., The Hague, pp. 391

Lazár D (1999) Chlorophyll *a* fluorescence induction. Biochim. Biophys. Acta 1412: 1–28

Lazár D, Pospíšil P (1999) Mathematical simulation of chlorophyll *a* fluorescence rise measured with 3-(3',4'-dichlorophenyl)-1,1 dimetylurea-treated barley leaves at room and high temperatures. Eur. Biophys. J. 28: 468–477

Li R, Guo P, Baum M, Grando S, Ceccarell S (2006) Evaluation of chlorophyll content and fluorescence parameters as indicators of drought tolerance in barley Agricultural Sciences in China. 5: 751– 57

Lichtenthaler HK (1996) Vegetation stress: An introduction to the stress concept in plants. J. Plant Physiol. 148: 4–14

Lichtenthaler HK, Wellburn A (1983) Determination of total carotenoids and chlorophyll a and b of leaf extracts in different solvents. Biochem. Soc. Trans. 603: 591–592

Markwell J, Osterman JC, Mitchell L (1995) Calibration of the Minolta SPAD-502 leaf chlorophyll meter. Photosynth, Res. 46: 467–472

Martyniak L (2008) Response of spring cereals to a deficit of atmospheric precipitation in the particular stages of plant growth and development. Agricultural water management 95:171–178

Maxwell K, Johnson GN (2000) Chlorophyll fluorescence – a practical quide. J. Exp. Bot. 51: 659–668

Pinior A, Grunewald-Stöcker G, von Alten H, Strasser RJ (2005) Mycorrhizal impact on drought stress tolerance of rose plants probed by chlorophyll *a* fluorescence, proline content and visual scoring. Mycorrhiza 15: 596–605

Rapacz M, Kościelniak J, Jurczyk B, Adamska A, Wójcik M (2010) Different patterns of physiological and molecular response to drought in seedlings of malt and feed-type barleys (*Hordeum vulgare*). J. Agron. Crop Sci. 196: 9–19

Richards RA (1978) Variation between and within species of rape-seed (*Brassica campestris* and *B. napus*) in response to drought stress. III. Physiological and photochemical characters. Aust. J. Agr. Res. 29: 495–501

Saini HS, Westgate ME (2000) Reproductive development in grain crops during drought. Advan. Agron. 68: 59–96

Sharp RE, Poroyco V, Hejlek LG, Spollen WG, Springer GK, Bohnert HJ (2004) Root growth maintenance during water deficits: physiology to functional genomics. J. Exp. Bot. 55: 2343–2351

Srivastava A, Strasser RJ (1977) Constructive and destructive actions of light on the photosynthetic apparatus. J. Sci. Industr. Research 56: 133–148

Strasser BJ, Strasser RJ (1995) Measuring fast fluorescence transients to adress environmental questions: the JIP test. In: Mathis P (ed) Photosynthesis: from light to biosphere, Kluwer Academic, Dordrecht, pp. 977–980

Strasser RJ, Srivatava A, Tsimilli-Michael M (2000) The fluorescence transient as tool to characterize and screen photosynthetics samples. In: Yunus M, Pathre U, Mohanty P (eds) Probing photosynthesis: mechanism, regulation and adaptation, Taylor and Francis, Bristol, pp. 45–483

Suryadevara SR, Hildebrand D (2009) Changes in oil content of transgenic soybeans expressing the yeast SLC1 gene. Lipids 44: 945–951

Thakur M, Hurburgh CR (2007) Quality of US soybean meal compared to the quality of soybean meal from other origins. J. Am. Oil Chem. Soc. 84: 835–843

Yang X, Chen X, Ge Q, Li B, Tong Y, Zhang A, Li Z, Kuang T, Lu C (2006) Tolerance of photosynthesis to photoinhibition, high temperature and drought stress in flag leaves of wheat: A comparison between a hybridization line and its parents grown under field conditions. Plant Sci. 171: 389– 97

PLANT FUNCTIONING UNDER ENVIRONMENTAL STRESS P. 69-83

Cracow, 12th to the 15th September, 2012

RESPONSES OF MOSSES SPECIES ON ENVIRONMENT STRESS FACTORS

Agata Stokłosa-Wojtaś, Andrzej Rzepka, Grzegorz Rut

Department of Plant Physiology, Institute of Biology,Pedagogical University, Podchorążych 2, 30-084 Kraków, Poland

Corresponding author*: agatastoklosawojtas@op.pl*

Abstract In this reviewed the influence of abiotic stress factors on selected species of mosses was analyzed. Mosses which live in the natural habitat are exposed to many stress factors and should, therefore, posses protective systems against oxidative stress. One of the primary antioxidant enzymes is the superoxide dismutase (SOD). Bryophytes are characterised by a very broad ecological amplitude of occurrence. Most moss species are highly resistant to desiccation. The process of drying and rehydration can be repeated several times without causing major changes in the functioning of the organism. Exposure of mosses gametophores with hypoxic stress makes the intensity of photosynthesis to decrease. Results of this study indicate that hypoxia does not affect dramatically the intensity of dark respiration, what helps to prolong the activity of plants under a long-term stress. Studies on the response of mosses to UV-B radiation, especially in the field, show that under the influence of this type of radiation smaller mosses gametophores are produced. The results of these experiments can be helpful in entire understanding of the evolutionary aspects of tolerance and acclimation to UV-B radiation, which gave the plants an opportunity to control the land. Bryophytes are characterised by tolerance to salinity, which can be determined through analysis of the various metabolic pathways. ABA and stress factor, in this case salt, affect the expression of genes which participate in the protection of plants, and ABA may be responsible for the ability of bryophytes to tolerate stress. Research on moss species showed a far lower photosynthesis rate when plants were cultured in an atmosphere of elevated CO_2 concentration. Results of this study may indicate that carbon dioxide can be in some cases, a stress factor, both when considered in terms of concentration and time of exposition. Recent research on the physiology of bryophytes suggests, that physiological processes are variable in nature among different populations and species of bryophytes. While vascular plants evolved in the morphological adaptation to environmental conditions, it seems that the evolution of bryophytes was associated with improvement of physiological and biochemical strategies.

Key words: Moss; Stress factors

Introduction

Living organisms are characterized by a state of internal balance within the system of life processes and physicochemical parameters of tissues, organs known as homeostasis. In natural environment the ideal conditions rarely exist, because the organisms are affected by many factors, which often modify the course of life processes. As sedentary organisms, plants are not able to move and therefore cannot escape from the source of negative stimuli. Factors that interfere with plant growth and development, and thus disrupt their homeostasis are known as stressors.

Different plant species vary in resistance: the ability to preserve the integrity, threatened by the potentially dangerous factors external or internal in origin. For this reason, factor of the same intensity, for some plants will be referred to as the stressor for others not. The impact of stressors depends not only on the properties of the plant itself, but also on its age and stage of development, time of stimulation, intensity and rate of change of stress, (Starck, 1995; Grzesiak, 1996; Kacperska, 2002) and the possibility of hormesis. Phenomenon of the hormesis, the excitation, is to mobilize the effects of small amounts of toxic substances or agents. It may include stimulating the cells, to build proteins. For example a long-term duration of high temperature causes a change in photosynthesis, and short-term one production of protective proteins (Dobrzyński, 2006).

Oxidative stress. Abiotic and biotic stress factors affect the balance between the production of reactive oxygen species (ROS, AOS) and processes for their removal by a special antioxidant systems. Stress factors, irrespective of the initial place of effect, affect the balance between reducing-oxidizing cellular buffers called redox homeostasis (Foyer, Noctor, 2003; Buchanan, Balmer, 2005; Dietz, 2008). This phenomenon is a common background for most of the environmental stress seen not only as a source of oxidative stress, but also as a mechanism to control major aspects of plant adaptation to different environmental conditions. Numerous studies have shown that ROS and antioxidants may act as an intracellular system of informing not only about the impact of stress, but also normal plant growth (Pfannschmidt *et al.*, 2001).

In recent years, knowledge about the role of oxidative stress and redox regulation in the functioning of cells, grown to the extent that the 1985 Sies ideas were rejected in assuming that oxidative stress is the imbalance of oxidants and antioxidants with a predominance of the latter leading to potential damage. It has been replaced by the theory of Jones in 2006. The new definition emphasizes the importance of redox regulation and oxidative stress is described as a disorder of redox signalling and control (Kornaś *et al.*, 2010; Grzenkowicz *et al.*, 2002). Reactive oxygen species are produced in cells by the action of many different factors such as: pathogen attack, wounding, UV light, an excess of photosynthetic active radia-

tion (PHAR), water deficit, osmotic stress and salt, too low or high temperature, high availability of oxygen after a period of hypoxia, atmospheric pollutants, excess of metal ions, the deficit of some mineral salts, or herbicides. It also accompanies the natural physiological processes, such as the formation of papillae on the roots of legumes, lignin biosynthesis or the aging of cells. Active oxygen forms ROS or AOS (reactive / active oxygen species) are produced during certain redox reactions and also as a result of incomplete oxygen reduction or water oxidation by the chain transport of an electron in mitochondria and chloroplasts (De Las Rivas *et al.*, 2004; Halliwell, 2006; Shaw, 2008).

In the plant organisms antioxidant mechanisms enabling the elimination of ROS and preventing oxidative stress in cells exposed to various stressors are present. Among these antioxidants, non-enzymatic and enzymatic are listed. Non-enzymatic antioxidants include, among others: Glutathione (GSH) (Schafer, Buettner, 2001), tocopherols (vitamin E) (Janas, 2005), carotenoids, ascorbic acid (vitamin C) and flavonoids. Antioxidant enzymes involved in the elimination of ROS are: superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbic reductase (DHARA) and glutathione reductase (GR) (Śmiechowska et al, 2008).

Mosses which live in the wild are exposed to many stress factors and must, therefore, have protective systems against oxidative stress (Rzepka *et al.*, 2005). One of the primary antioxidant enzymes is the superoxide anion radical (SOD). In *Mnium undulatum* gametophores five isoforms of SOD: a manganese (Mn SOD), one iron-(Fe SOD) and three forms of copper zinc: Cu / Zn SOD I, Cu / Zn SOD II and Cu / Zn SOD III were identified. In *Polytrichum commune* and *Polytrichum piliferum* gametophores no iron oxide forms of SOD were detected.(Rzepka, 2008).

Water stress. The ability of plants to survive periods of water scarcity in the environment are described as a resistance to drought. This phenomenon underlies the evolution of land plants. The most common effect of water stress in cells is to reduce the water potential and activity of cellular water. Reduction in cell volume after the loss of turgor contributes to increase concentrations of low and high molecular weight compounds, salts and ions. As a result of water loss the cell volume changes, further more a change of spatial relations between plasmalemma, tonoplast and organelle membranes occurs. After removal of hydration water a change in structure and conformation of macromolecules is observed (Olszewski *et al.*, 2007).

Bryophytes are characterised by a very broad ecological amplitude of distribution. Habitats occupied by them differ radically in degrees of water supply. Among the species of bryophytes we are finding those typically aquatic, occupying a very moist habitats and species associated with extremely dry environments (rocks, sand dunes). Most moss species are organisms with very high resistance to desiccation (Bewley, 1978; Bewley *et al.*, 1993; Bartošková *et al.*, 1999; Proctor, 2001). The

process of drying and rehydration can be repeated several times without causing major changes in the functioning of the body (Krupa, 1974).

Reducing the contents of water leads to a reduction in the intensity of the gas changes. The process of photosynthesis occurs in the moss, when the leaves are in a state of full turgidity. Some moss species are resistant to extreme dryness, and after partial dehydration reduction in photosynthesis occurs, but also periodic intensification of respiration was found (Krupa, 1974; Beweley *et al.*, 1978). This high resistance to desiccation of mosses and their ability to reactivate the physiological processes after re-hydration is a characteristic feature of these organisms and differed them from the majority of vascular plants.

Oxygen deficiency.Plants are mostly aerobic organisms, and therefore reduce of the compactness of oxygen (hypoxia) or lack (anoxia) of it can lead to stress, which manifested in plant cell metabolism changes (Rzepka *et al.*, 2005). Under the conditions of our climate happens, that plants are a subject to periodic flooding due to heavy rains or spring thaws (Jackson *et al.*, 1993, 2003; Drew, 1997; Chung *et al.*, 2001). Some species of higher plants adapted to survive anaerobic conditions that occur periodically, for example, caused by flooding with water root system or even aerial parts (Sedbrook *et al.*, 1996; Crawford and Brraendle, 1996). Prolonged flooding causes the plants usually to die, because of the suspension of oxygen respiration processes and under cooling, when flooding occurs in early spring, and poisoning with products of anaerobic processes taking place in soil. At higher temperatures the intensity of respiration increases, and so hypoxia or anoxia is for them especially dangerous. In such conditions, even more abundant rainfall particularly in poorly permeable soils cause lack of oxygen. Most plants get the oxygen needed from the soil air. Only certain plants are able to take in oxygen through the stem to the roots such as rice and marsh plants, which enable to normal development, even when soil is fully covered with water (Bohnert et al, 1995; Asada, 2000; Chang *et al.*, 2000). When excess water in the soil occurs its biocenotic balance is compromised. Filling compartments with stagnant water creates anaerobic conditions (Kacperska, 2002). Lack of oxygen in the soil causes the shift of a processes of aerobic microbial organic matter decomposition to anaerobic digestion, which is several times slower, but as a direct product decomposition yields alcohols, organic acids and other active compounds, which can have harmful effects on plants are produced. When oxygen deficiency in plant tissues occurs an alcoholic fermentation process can take place. Arises an alcohol - the final product of the process, and the amount of energy released is several times lower than during the oxygen respiration. The energy dissipated in the process of fermentation is often not enough for the necessities of life, and often after some time spent in anaerobic conditions plants die. Death is also speed up by the fact that alcohol accumulating in the fermentation process is poisonous (Kato-Naguchi, 2000). In a completely anaerobic conditions vegetation of higher plants is impossible.

Many of the literature data concerns the adaptation of plants to conditions associated with a deficiency of oxygen caused by the periodic flooding of the roots with water (Vartepian and Jackson, 1997; Visser *et al.*, 2003; Bragina *et al.*, 2002). Tolerance to anaerobic conditions is different in different species, but always associated with synthesis of an oxidative shock protein (De Maio, 1999; Wu, 1995; Vinocur, Altman, 2005).

Treatment of mosses gametofores with hypoxic stress makes the intensity of photosynthesis to fall. Results of this study indicate that it does not, however, influence dramatically to change the intensity of dark respiration, which helps to prolong the activity of plants under long-term stress (Rzepka, 2008). This may be due to increased levels of transcription of alcohol dehydrogenase, pyruvate decarboxylase and aldolase as well as enolase (Feenov and Bailey-Serres, 1995). This indicates the launch of the fermentation process (Drew, 1997). Fluorometric methods enable to quickly and with high efficiency and sensitivity evaluate both, the plant responses to disturbance of photosynthesis by stress factors, as well as the effectiveness of the corrective mechanisms and an integral capability of plants to maintain homeostasis under adverse environmental conditions (Krause and Somersalo, 1989; Lichtenthaler *et al.*, 1986; Murkowski, 2004; Schapendonk *et al.*, 1992).

For the higher plants not subjected to stress, the optimal value of the parameter Fv / Fm is about 0.83. The Proctor (2001) study showed that under optimal conditions bryophytes are often characterized by lower values of Fv / Fm than 0.83. For mosses gametofores, such as Mnim undulatum, the value of the test conditions Fv / Fm was 0.62. Marked reduction in this parameter in hypoxic conditions, could evidenced about a disturbance of electron transport within PSII. However, prolonged immersion in water does not cause any further changes, which indicates the adaptation of PSII to the conditions of hypoxia (Deltaro *et al.*, 1998). Hypoxia leads to production of the reactive oxygen species (Blokhina *et al.*, 2001; Alscher 2002; Mittler 2002; Bartosz 2008). It is likely that one of the reason for the increased production of ROS is the disrupted mitochondrial electron transport in the respiratory chain (Blokhina *et al.*, 2000,2001,2003). It can be concluded that the gametofores photosynthetic activity is strongly inhibited under conditions of hypoxia-induced by a complete flooding of gametofores.

Radiation stress. Normal growth and development of plants, and thus their ecological success largely depends on the quantity and quality of light absorbed. Plants in addition to light in the PAR (photosynthetically active radiation) can absorb and utilize a broad spectrum of light waves from the UV to RF, because of the need to optimize their metabolism in a constantly changing environment (Kuźniak *et al.,* 2009). PAR spectrum light allows the process of photosynthesis, and ultimately is converted to chemical energy (ATP and reduced equivalents as NADPH). This process is associated with the process of formation of molecular oxygen from the

water. Redox status of certain components and products of PET (photosynthetic electron transport), such as plastochinon, pyridine dinucleotide, glutathione maintain the stoichiometric ratio of PET (by adjusting the pH trans-thylakoid) and cellular redox status, which plays an important role in the regulation of acclimation and defence responses in plants. Plants have the ability to absorb more light energy than they need to drive photosynthetic CO_2 assimilation (S. Karpiński *et al.*, 1999; Karpiński *et al.*, 2003; Noctor, Foyer, 1998; Asada *et al.*, 1999; Mullineaux, Karpiński, 2002). Different components absorb light and transfer electrons from water splitting complex onto ferredoxin, which has the ability to generate major forms of ROS such as: $\bullet O_2$- superoxide anion, H_2O_2 hydrogen peroxide, •OH hydroxyl radical. Production of ROS above a certain level may lead to different damage (photoinhibition and photo-oxidation), and may awaken in some cases, programmed cell death (PCD), as a result of defensive reactions or acclimation reactions (Karpiński *et al.*, 1999; Karpińska *et al.*, 2000; Mateo *et al.*, 2004; Mühlenbock *et al.*, 2008). As a result of a radiation stress the photoinhibition of photosynthesis reaction occurs, which mainly manifests in the destruction of photosystem II (PS II) reaction center and slowing down the transport of electrons in both photosystems. Due to long photoinhibition oxidative stress in the tissues can be induced, which manifests in the initiation of free radical oxidation of chloroplasts membranes lipids and oxidation of photosynthetic pigments. Plants have specialized repair mechanisms, which in the process of restitution may fully or partially restore the lost efficiency of the photosynthesis reaction (Foyer *et al.*, 1994; Murkowski and Skórska, 2004). Both, photoinhibition effects and also phenomena accompanying the disintegration of the photosynthetic apparatus are reflected in changes in the values of chlorophyll fluorescence (FL) (Maxwell and Johnson 2000; Murkowski and Skórska, 1997; Schreiber *et al.*, 2000). The long-term acclimation responses concern nuclear and chloroplast gene regulation, controlled by the (specific) changes in redox potential and by proteolytic degradation of the existing light-absorbing complexes and other proteins (Pfannschmidt *et al.*, 2001). Potentially damaging ROS are also necessary to start protective responses, such as lowering the activity of PSII. In this way, ROS are cellular and systemic signals that can affect other signalling systems and induce defence in chloroplasts, including redox changes in the vicinity of PSII (Karpiński *et al.*, 1999; Mullineaux *et al.*,2000).

Studies on the response of mosses to radiation especially in the field of UV-B, show that under the influence of this type of radiation produce smaller mosses gametofores occurs. It was also found that the mosses spores are very sensitive to radiation. Ripe gametophytes are however less sensitive than their protonemas. It turns out that *Arabidopsis* seedlings are more vulnerable than moss *Polytrichum patens* gametofores. In addition, this species is capable of regeneration after irradiation of UV-B, even after the onset of visible chlorosis of tissues. The results of these experiments can provide a starting point for a thorough understanding of the

response, function and regulation of UV-B radiation in moss and can be helpful in fully understanding the evolutionary aspects of tolerance and acclimation to UV-B radiation, which gave the plants an opportunity to control the land (Lindeberg, 1996; Johanson *et al.*, 1995; Caldwell,1971; Gabriel and Bates, 2005).

Salt stress. High concentration of salts in the soil causes reduced water availability for plants. Salt ions interact specifically with water molecules, which change the physical state of water and its interactions with proteins and membranes in the cell. Harmful effects on plants of saline soils, results therefore from excessive suction of the soil and the deadly effect of high concentrations of mineral salts in the protoplasm. High suction force of the soil solution causes physiological drought. Higher concentrations of magnesium salts (especially high ratio of Mg : Ca), and the salt of carbonic acid act as a poison, causing the soil to over-alkalize and prevent iron uptake. Excess of sodium chloride is also harmful to plants (Bilski, 1988; Kacperska,1996; Starck, 1983). At the time of exposure of plant to the stress factor such as elevated salt concentrations in soil solution, the mechanisms of receiving signals and transmitting them to the cell, which activates adaptive responses, can be observed. Bryophytes are characterised by tolerance to salinity, which can be determined through analysis of the various metabolic pathways. The main role is played by proteins that mediate the transmembrane transport of ions and allow maintenance of ionic and osmotic homeostasis while under salt stress. These proteins protect cells from denaturation and degradation as well as remove the effect of oxidative stress in the course of salt stress. ABA and stress factor, which is salt, affect the genes expression, that participate in the protection of plants, and ABA may be responsible for the ability of bryophytes to tolerate stress (Richardt *et al.*, 2010). Salt excess inhibits the growth of plants and causes large losses in agricultural production in the world. Therefore, understanding the mechanisms that trigger adaptive responses are fundamental to biology. Response of higher plants to salt stress was investigated at the cellular, molecular as well as physiological and biochemical level. However, little is known about the mechanisms underlying this type of reaction to stress factors in mosses (Bilski J. 1988).

Bryophytes are known for their importance in research on plants systematic and evolution, as well as tolerance to difficult living conditions (Bilski J. 1990). Studies have shown that in signal transduction and transcriptional regulation proteins - phytochromes, kinases, Mcamb1, Mcamb2 and proteins such as 14-3-3 and PpDBF1 are involved (Zhu, 2001; Starck 1983). Depending on the type of salt, its concentration, plant species, the environment and other associated factors, the effect of salinity on plants can vary. It can cause reversible or irreversible disturbance in their functioning (Bilski, 1990; Kalaji and Pietkiewicz,1993). Under the influence of salanity inhibition of growth and development of plants follows (Bilski,1988; Bilski, 1990; Starck *et al.,* 1995). Too intense salt stress can lead to death, while for example under a relatively small NaCl concentrations even growth of whole plants may be strongly stimulated (Starck 1983).

Elevated of carbon dioxide concentrations. Plants have evolved in the course of evolution the ability to bind carbon dioxide in photosynthesis. This is one of the most important biochemical processes on Earth. In literature we can find varies specific data about the impact of elevated CO_2 concentrations on the photosynthesis rate (Ainsworth *et al.*, 2003; Allen, 1996; Minorsky, 2006; Ziska, 2003) and the intensity of CO_2 assimilation by plants. The reason for these studies is the need to determine the changes in physiology and morphology of plants, due to increased CO_2 concentrations in the atmosphere. The measure of the process of photosynthesis is the plant biomass (Allen *et al.*, 1996; Narbutt *et al*., 1990; Jach *et al.*, 2000; Leakey, 2006; Stover, 2007) and their rate of growth (Grant , 2004; Voelker, 2006). The degree of reduction in the intensity of photosynthesis is dependent on the time of exposure to elevated CO_2 concentration (Idso *et al*., 1991), and may be due to the functioning of photosynthetic apparatus. Elevated CO_2 concentration causes stomatal closure (Ainsworth *et al.*, 2003; Lecain *et al.*, 2003). An increased production of assimilates could lead to their excessive accumulation in chloroplasts (Bunce, 1993; Mousseau and Saugier, 1992; Woźny and Przybył, 2004). Elevated CO_2 concentration is also associated with the occurrence of oxidative stress, which leads to abnormal sequestration of carbon (Miszalski, 1998, 2001; Niewiadomska, 1999, 2004; Rzepka, 2005). In natural environment plants are affected by a number of different stress factors, their overlapping effects on the elevated CO_2 concentration occurs. These factors include: the intensity of light (Hand *et al.*, 1993; Wang *et al.*, 2003), temperature (Allen *et al.*, 1996; Reddy *et al.*, 1995), soil moisture (Fleischer *et al.*, 2008; Lecain *et al.*, 2003), and the availability of nitrogen (Ollinger *et al.*, 2002 ;Reddy *et al.*, 2004) or ozone (Kerstiens, 1995).

Elevated levels of carbon dioxide promote photosynthesis. However, an increase of more than 50%, compared to the current level, begins to exert the opposite effect - the plants produce less protein and grow worse. This is because the higher concentration of carbon dioxide impairs photorespiration plants - a process in which they combine atmospheric oxygen with carbohydrates. Increased photosynthesis initially makes up for these losses, but with the increase of CO_2 concentration plants adapt and slow down growth. Another problem is the absorption of nitrogen, an element necessary for the production of proteins and plant growth. Most of the nitrogen is absorbed by plants through the roots from the soil, in the form of nitrates. These are the main component of natural and artificial fertilizers. This mechanism has not yet been thoroughly studied, but it is known that this photorespiration impairment inhibits the plant uptake of nitrogen. In the latest studies show that the impact of elevated carbon dioxide concentration, in some cases of low levels of atmospheric oxygen inhibits nitrogen uptake in wheat and *Arabidopsis* (Bloom *et al.*, 2010). Experimental results indicate that elevated CO_2 causes greater changes in the intensity of photosynthesis and biomass increment in C3 plants (Lecain, 2003; Wilsey *et al.*, 1997). Changes observed in C4 plants do not show such

significant impact or they not observed at all. However, the rate of photosynthesis in C4 plants reached the highest intensity at lower concentrations of CO_2, but at relatively high temperatures. Sometimes the results can be reversed, and the study of photosynthesis to elevated CO_2 concentrations in C3 and C4 shows that results obtained are higher for C3 species (Garbutt *et al.*, 1990).

Mosses have a simple body structure; do not have stomata or tissues transporting water or asymilates, and their physiological activity is closely associated with the presence of water (Krupa, 1978, Rzepka, 1990). Mosses life habitats vary, in comparison to the concentration of CO_2 in the atmosphere. Simple anatomical structure affects the CO_2 collection and transport of assimilates and limits their photosynthetic activity (Hebant. 1977). It was shown that, as in higher plants, the intensity of photosynthesis in mosses depends on the concentration of CO_2 in the atmosphere (Krupa and Rzepka, 1995). Research moss species showed far lower rate of photosynthesis when cultured in an atmosphere of elevated CO_2 concentration. Results of this study may indicate that carbon dioxide can be in some cases, stress factor, both when considered in terms of concentration and time interaction (Rzepka 2008).

Temperature stress. The effect of temperature is observed in all physiological processes, and are closely dependent on it. Temperature is undoubtedly one of the most important environmental factors that determine not only the intensity but also the possibility of life processes. In comparison with other plants, bryophytes temperature tolerances appear to be greatest. Bryophytes have a unique physiology that allows them to survive adverse environmental conditions (eg. extreme temperatures) (Gabriel de Almeida, 2000).They can be found in the caves, the geothermal wells or in areas of permafrost in the tundra (Jägerbrand *et al.*, 2003). Bryophytes have the ability to perform photosynthesis at relatively low temperatures, some (eg *Lanuginosum racomitrim*) to -10 ° C (Kallio and Heinonen, 1973), but photosynthesis occurs rarely above 25 ° C. Even in tropical species, the rate of photosynthesis decreases above this temperature (Frahm, 1990), and respiratory processes are intense (Frahm, 1987). At higher temperatures, in the majority of bryophytes, a reversible inhibition of photosynthesis is observed (Weis *et al.*, 1986), sometimes it can lead to irreversible damage to photosynthetic apparatus due to damage of photosystem II (Weis *et al.*, 1986). Bryophytes have mechanisms that protect them from excessive absorption of sunlight. The leaves of some species of mosses (*Sphagnum, Leucobryum*) have what we call hyalocysts (Allen *et al.*, 1984), which reflect light, and thus assist in cooling the body. It's faulty to assume that all species of bryophytes have the same kind of basic physiological mechanisms. Recent research on the physiology of bryophytes suggests, that physiological processes are variable in nature among different populations and species of bryophytes. While vascular plants evolved in the morphological adaptation to environmental conditions, it seems that the evolution of bryophytes was associated with improvement of physiological and biochemical strategies (Glime, Janice, 2007).

Conclusions

Mosses are the simplest land plants, characterized by a simple anatomy and morphology. They occur in all climates, from cold Polar Regions, to the hot equatorial lands. They are characterized by a high degree of adaptation to the terrestrial environment. Selected studies analysis of physiological responses to abiotic stress factors of different species of mosses allows an approximation of interactions between complex environmental conditions and the functioning of these simply constructed organisms. It seems wrong to assume that all species of bryophytes have the same kind of basic physiological mechanisms. The results of recent research on the physiology of bryophytes suggest that physiological processes are variable in nature among different populations and species of bryophytes.

It can be concluded that vascular plants evolved in the morphological adaptation to environmental conditions, and the evolution of bryophytes was associated with improvement of the physiological and biochemical strategies.

References

Ainsworth E.A.. Rogers A., Blum H., Nosberger J., Long S.P.. (2003). Variation in Acclimation of Photosynthesis in Trifolium repens after Eight Years of Exposure to Free Air CO_2 Enrichment (FACE), Journal of Experimental Botany, Vol. 54 (393): 2769-2774.

Allen R.D., Nessler C.L., Galewsky S., Neumann A.J. (1984). The Role of Cellulase in Hyalocyst Pore Formation in the Moss *Syrrhopodon texanus.* Ann Bot 53 (3): 431-438.

Allen L.H., Baker J.T., Boote K.J.. (1996). The CO_2 Fertilization Effect: Higher Carbohydrate Production and Retention as Biomass and Seed Yield., W: Global climate change and agricultural production. Direct and indirect effects of changing hydrological, pedological and plant physiological processes Copyright by FAO, Rome, Italy.

Alscher R.G., Erturk N., Heath L.S.. (2002). Role of superoxide dismutases (SOD) in controlling oxidative stress in plants. J. Exp. Bot. 53: 1331-1341.

Asada M., Uchibe E., Hosoda K.. (1999). Cooperative behavior acquisition for mobile robots in dynamically changing real worlds via vision-based reinforcement learning and development. *Artificial Intelligence*, 110(2): 275-292.

Asada K.. (2000). The water-water cycle as alternative photon and electron sinks. Philos. Trans. R. Soc. London B. Biol. Sci. 355: 1419-1431.

Bartošková H., Komenda J., Naus J.. (1999). Functional changes of photosystem II in the moss Rizomnium punctatum (Hedw.) Induced by different rates of dar desiccation. J. Plant Physiol. 154: 597-604.

Bartosz G.. (2008). Co to są reaktywne formy tlenu?. W: Druga twarz tlenu. Wyd. 2. Warszawa: Wydawnictwo Naukowe PWN.

Bewley J.D., Pacey J.. (1978). Desiccation-induced ultrastructural changes in drought- sensitive and drought-tolerant plants. W: J.H. Crowe and J.S. Clegg (red.) Dry biological systems, Academic Press, New York: 53-73.

Bewley J.D., Reynolds T.L., Oliver M.J.. (1993). Evolving strategies in the adaptation to desiccation. W: T.J. Close and E.A. Bray (red.). Plant responses to cellular dehydration during environmental stress. Corrent topics in plant physiology: American Society of Plant Physiologists Series, American Society of Plant Physiologists, Rockville, Maryland. Vol. 10: 193-201.

Bilski J.. (1988). Reakcja roślin na stresy mineralne powodowane zakwaszeniem i zasoleniem środowiska. Część IV. Wpływ NaCl i Na_2 SO_4 na wzrost i skład chemiczny siewek jęczmienia, pszenicy i owsa. Biuletyn IHAR: 165: 75-83.

Bilski J.. (1990). Zakwaszenie i zasolenie podłoża jako czynniki stresowe dla roślin. Rocz. Nauk Roln., seria D, t. 222.

Blokhina O.B., Vrolainen E., Fagerstedt K.V., Hoikkala A., Wähäla K., Chirkova T.V. (2000). Antioxidant status of anoxia – tolerant and intolerant plant species under anoxia and reaeration. Physiologiae Plantarum 109: 396-403.

Blokhina O.B., Chirkova T.V., Fagerstedt K.V.. (2001). Anoxis stress leads to hydrogen peroxide formation in plant cells. J. Exp. Bot. 52: 1179-1190.

Blokhina O.B., Vrolainen E., Fagerstedt K.V.. (2003). Antioxidants, oxidants damage and oxygen deprivation stress: a review. Ann. Bot. 91: 179-194.

Bohnert H.J., Nelson D.E., Jensen R.G.. (1995). Adaptations to environmental stresses. The Plant Cell, 7: 1099-1111.

Bragina T.V., Drozdova I.S., Ponomareva Y.V., Alekhin V.I., Grineva G.M.. (2002). Photosynthesis, respiration and transpiration in maize seedlings under hypoxia induced bycomplete flooding. Biol. Sci. 384: 274-277.

Buchanan B.B., Balmer Y.. (2005). Redox Regulation: A Broadening Horizon. Annu Rev Plant Biol 56: 187-220.

Bunce J.A.. (1993). Effects of doubled atmospheric carbon dioxide concentration on the responses of assimilation and conductance to humidity. Plant Cell Environ. 16: 189-197.

Caldwell M.M.. (1971). Solar UV radiation and the growth and development of higher plants. Str.: 131-177 in A. C. Giese, editor. Photophysiology. Volume 6. Academic Press, New York, USA.

Chang W.P., Lang Huang, Min Shen, Webster C., Burlingame A.L., Roberts J.K.M.. (2000). Patterns of protein synthesis and tolerance of anoxia in root tips of maize seedlings acclimated to a low-oxygen environment and identification of proteins by mass spectrometry. Plant Physiol. 122: 295-317.

Chung-Ta L., Chin-Ho L.. (2001). Physiological adaptation of crop plants to flooding stress. Proc. Natl. Sci. Counc. ROC (B). 25:148-157.

Crawford R.M.M., Brraendle R.. (1996). Oxygen deprivation stress in changing environment. J. Exper. Botany 47: 145-159.

De Las Rivas J., Balsera M., Barber J.. (2004). Evolution of oxygenic photosynthesis: genome-wide analysis of the OEC extrinsic proteins. Trends Plant Sci. 9: 18–24.

Deltaro V.I., Calatayud A., Gimeno G., Barreno E.. (1998). Water relations, chlorophyll fluorescence, and membrane permeability during desiccation in bryophytes from xeric, mesic and hydric environments. Canadian Jounal of Botany. 76: 1923-1929.

De Maio A. (1999). Heat shock proteins: facts, thoughts, and dreams. Shock (Augusta, Ga.) 11 (1): 1– 12.

Dietz K-J. (2008). Redox signal integration: from stimulus to networks and genes. Physiol. Plant. 133 (3): 459-468.

Dobrzyński L. (2006). Hormeza zjawisko powszechne i powszechnie nieznane. W: Postępy Techniki Jądrowej: 9-15.

Drew M.C. (1997). Oxygen deficiency and root metabolism: Injury and acclimation under hypoxia and anoxia. Annu. Rev. Plant Physiol. Plant mol. Biol. 48:223-250.

Feenov S.L., Bailey-Serres J.. (1995). Post transcriptional regulation of gene expression I oxygen deprived roots of maize. Plant J. 7: 287-295.

Fleisher D.H., Timlin D.J., Reddy V.R.. (2008). Interactive Effects of Carbon Dioxide and Water Stress on Potato Canopy Growth and Development, Agronomy Journal 100: 711-719.

Foyer C.H., Lelandais M., Kunert K.J.. (1994). Photooxidative stress in plants. Physiologia Plantarum 92(4): 696–717.

Foyer C.H., Noctor G.. (2003). Redox sensing and signaling associated with reactive oxygen in chloroplasts, peroxisomes and mitochondria. Physiol Plant 119: 355–364.
Frahm J-P.. (1987). Which factors control the growth of epiphytic bryophytes in tropical rainforests? Symp. Biol. Hung. 35: 639-648.
Frahm J-P.. (1990). Bryophyte phytomass in tropical ecosystems. J. Linn. Soc. Bot. 104: 23-33.
Gabriel R.M. de Almeida.. (2000). Ecophysiology of Azorean Forest Bryophytes. Ph.D. thesis, Department of Biology, Imperial College of Science, Technology and Medicine, Silwood Park, England: 308.
Gabriel R., Bates J.W.. (2005). Bryophyte community composition and habitat specificity in the natural forests of Terceira, Azores. Plant Ecology 177 (1): 125-144.
Garbutt K., Williams W.E., Bazzaz F.A.. (1990). Analysis of the Differential Response of Five Annuals to Elevated CO_2 during Growth, Ecology Volume 71 (3): 1185–1194.
Glime J.M.. (2007). *Bryophyte Ecology*. Vol. 1. Physiological Ecology. Ebook sponsored by Michigan Technological University and the International Association of Bryologists.
Grant R.F., Kimball B.A., Wall G.W., Triggs J.M., Brooks T.J., Pinter P.J., Conley M.M., Ottman M.J., Lamorte R.L., Leavitt S.W., Thompson T.L., Matthias A.D.. (2004). Modeling Elevated Carbon Dioxide Effects on Water Relations, Water Use, and Growth of Irrigated Sorghum. Agronomy Journal, Vol. 96: 1693-1705.
Grzenkowicz J., Wojtkowiak D., Podhajska A.J.. (2002). Reaktywne formy tlenu jako cząsteczki sygnałowe. Pol J Cosmetol; 2: 90-106.
Grzesiak S. (1996). Stres środowiskowy – wprowadzenie do konferencji. W: Grzesiak S., Miszalski Z. (red.). Ekofizjologiczne aspekty reakcji roślin na działanie abiotycznych czynników stresowych. Zakład Fizjologii Roślin im. F. Górskiego PAN, Kraków: 15-18.
Halliwell B. (2006). Reactive species and antioxidants. Redox biology is a fundamental theme of aerobic life. Plant Physiol., 141: 312–322.
Hand D.W., Wilson J.W., Acock B.. (1993). Effects of Light and CO_2 on Net Photosynthetic Rates of Stands of Aubergine and Amaranthus . Annals of Botany, Vol. 71: 209-216.
Hebant C. (1977). The conducting tissues of bryophytes J. Cramer. In der A.R. Gantner Verlag Kommanditgesellschaft.
Idso S.B., Kimball B.A. (1991). Downword Regulation of Photosynthesis and Growth at High CO_2 Levels. No Evidence of Either Phenomenon in Three-Year Study of Sour Orange Trees.. Plant Physiology, Vol. 96: 990-992.
Jach M.E., Laureysens I., Ceulemans R. (2000). Above- and Below-ground Production of Young Scots Pine (Pinus sylvestris) Trees after Three Years of Growth in the Field under Elevated CO_2. Annals of Botany, Vol. 85: 789-798.
Jackson M.B., Black C.R. (1993). Interacting stresses on plants in changing climate. Berlin: Springer-Verlag: 123-134.
Jackson M.B., Ram P.C. (2003). Physiological and molecular basis of susceptibility and tolerance of rice plants to complete submergence. Annals of Botany 91: 227-241.
Janas K., Szafrańska K., Posmyk K. (2005). Melatonina w roślinach. Kosmos. 54: 251-258.
Jägerbrand A. K., Molau U., Alatalo J. M.. (2003). Responses of bryophytes to simulated environmental change at Latnjajaure, northern Sweden. J. Bryol. 25: 163-168.
Johanson U., Gehrke C., Bjorn L.O., Callaghan T V.. (1995). The effects of enhanced UV-B radiation on the growth of dwarf shrubs in a subartic heathland. Functional Ecology 9: 713-719.
Kacperska A.. (1996). Czy można mówić o wspólnym podłożu odpowiedzi roślin na działanie stresowych czynników środowiska. Konf. „Ekofizjologiczne aspekty reakcji roślin na działanie abiotycznych czynników stresowych", Kraków: 49-59.
Kacperska A.. (2002). Reakcje roślin na abiotyczne czynniki stresowe. W: Kopcewicz J., Lewak S. (red). Fizjologia roślin. Wydawnictwo Naukowe PWN. 612-667.

Kalaji M. H., Pietkiewicz S.. (1993). Salinity effects on plant growth and other physiological processes. Acta Physiol. Plant., 15 (2): 89-124.

Kallio P., Heinonen S.. (1973). Ecology of Rhacomitrium lanuginosum (Hedw.). Brid. Rept. Kevo Subarct. Res. Stat. 10: 43-54.

Karpinska B., Wingsle G., Karpinski S.. (2000). Antagonistic effects of hydrogen peroxide and glutathione on acclimation to excess excitation energy in Arabidopsis. IUBMB Life. 50(1): 21-6.

Karpiński S., Reynolds H., Karpińska B., Wingsle G., Creissen G. Mullineaux P.. (1999). Systemic signaling and acclimation in response to excess excitation energy in Arabidopsis. Science, 284, 654-657.

Karpiński S., Gabrys H., Mateo A., Karpinska B., Mullineaux P.M.. (2003). Light perception in plant disease defence signalling. Current Opinion in Plant Biology, 6:390-396.

Karpiński S., Reynolds H., Karpińska B., Wingsle G., Creissen G. Mullineaux P.. (1999). Systemic signaling and acclimation in response to excess excitation energy in Arabidopsis. Science, 284, 654-657.

Kato-Naguchi H.. (2000). Abscisic acid and hypoxic induction of anomia tolerance in roots of lettuce seedlings. J. Exp. Of Botany 51(352): 1939-1944.

Kerstiens G., Townend J., Heath J., Mansfield T.A.. (1995). Effects of Water and Nutrient Availability on Physiological Responses of Woody Species to Elevated CO_2. Forestry, Vol. 68: 303-315.

Kornaś A., Kuźniak E., Ślesak I., Miszalski Z.. (2010). The key role of the redox status in regulation of metabolism in photosynthesizing organisms. Acta Biochimica Polonica. 2/2010 143–151.

Krause G.H., Somersalo S.. (1989). Fluorescence as a tool in photosynthesis research: application in studies of photoinhibition, cold acclimatisation and freezing stress. Phil. Trans. R. Soc. Lond., B. 323: 281-293.

Krupa J.. (1974). Struktura anatomiczna liści mchów a ich aktywność fizjologiczna. Wyd. Nauk. WSP, Kraków: 5-58.

Krupa J.. (1978). Photosynthesis rate in moss leaves of various anatomical structure. Acta. Soci. Bot. Pol. 4: 391-402.

Krupa J., Rzepka A.. (1995). Responses of moss gametophores to elevate concentration of CO_2. Biological Biulletin of Poznań. 32: 71-71.

Kuźniak E., Niewiadomska E., Miszalski Z., Karpinski S.. (2009). The role of chloroplasts and redoks status in holistic regulation of stress responsem in plants. W: Compartmentation of Responses to Stresses in Higher Plants, True or False. Maksymiec W. (red.) Transworld Research Network, 163-192.

Leakey A.D.B., Uribelarrea M., Ainsworth E.A., Naidu S.L., Rogers A., Ort D.R., Long S.P. (2006). Photosynthesis, Productivity, and Yield of Maize Are Not Affected by Open-Air Elevation of CO_2 Concentration in the Absence of Drought. Plant Physiology, 140(2): 779–790.

Lecain D.R., Morgan J.A., Mosier A.R., Nelson J.A.. (2003). Soil and Plant Water Relations Determine Photosynthetic Responses of C3 and C4 Grasses in a Semi-arid Ecosystem under Elevated CO_2. Annals of Botany, Vol. 92: 41-52.

Lichtenthaler H., Buschmann C., Rinderle U., Schmuck G.. (1986). Application of chlorophyll fluorescence in ecophysiology. Radiat. Environ. Biophys. 25: 297-308.

Lindeberg J.. 1996. Effects of UV-B radiation on Sphagnum growth and pigmentation and the role of UV-A radiation. Thesis. Department of Ecology, Plant Ecology, Lund University, 223 62 Lund, Sweden.

Mateo A., Mühlenbock P., Rusterucci C., Chang C.C-C., Miszalski Z., Karpińska B., Parker J.K., Mullineaux P., Karpiński S. (2004). Lesion Simulating disease 1 is required for acclimation to conditions that promote excess excitation energy. Plant Physiol. 136, 2818-2830.

Maxwell K., Johnson G.N.. (2000). Chlorophyll fluorescence a practical guide J. Exp. Bot. 51 (345): 659-668.

Minorsky P.V.. (2006). No Direct CO_2 Fertilization Effect in Field-grown Maize. Plant Physiology 140(2): 397–398.

Miszalski Z., Ślesak I., Niewiadomska E., Bączek-Kwinta R., Lüttge U., Ratajczak R.. (1998). Subcellular localization and stress responses of superoxide dismutase isoforms from leaves in the C_3-CAM intermediate halophyte Mesembryanthemum crystallinum L. Plant Cell & Environ. 21: 169-179.

Miszalski Z., Niewiadomska E., Ślesak I., Lüttge U., Kluge M., Ratajczak R.. (2001). The effect of irradiation on carboxylating/decarboxylating enzymes and fumarase activities in Mesembryanthemum crystallinum L, exposed to salinity stress. Plant Biology 3: 17-23.

Mittler R.. (2002). Oxidative stress, antioxidants and stress tolerance. Trends Plant Sci. 7: 405-410.

Mousseau M., Saugier B.. (1992). The Direct Effect of Increased CO_2 on Gas Exchange and Growth of Forest Tree Species. Journal of Experimental Botany, Vol. 43: 1121 – 1130.

Mullineaux P., Ball L., Escobar C., Karpińska B., Creissen G., Karpiński S.. 2000. Are diverse signalling pathways integrated in the regulation of arabidopsis antioxidant defence gene expression in response to excess excitation energy? Philos. Trans R. Soc. Lond. B Biol. Sci. 355(1402): 1531–1540.

Mullineaux P., Karpiński S.. (2002). Signal transduction in response to excess light: getting out of the chloroplast. Curr. Opin. Plant Biol. 5: 43–48.

Murkowski A.. (2004). Zastosowanie luminescencji chlorofilu do badania reakcji aparatu fotosyntetycznego roślin pomidora na stres świetlny oraz chłód. Acta Agrophysica. 4(2): 431-439.

Murkowski A., Skórska E.. (1997). Chlorophyll a luminescence - an index of photoinhibition damages. Currents Topics in Biophysics, 21(1): 72-78.

Murkowski A., Skórska E.. 2004. Chlorophyll fluorescence in research of chill and light stress in cucumber plants from *in vitro* culture during acclimation. Horticulture and Vegetable Growing, 23 (2): 192-198.

Niewiadomska E., Miszalski Z., Ślesak I., Ratajczak R.. (1999). CAT activity during C3-CAM transition in Mesembryanthemum crystallinum L. leaves. Free Rad. Res. 31: 251-256.

Niewiadomska E., Karpińska B., Romanowska E., Ślesak I., Karpiński S.. 2004. A salinity- induced C3-CAM transition increases energy conservation in the halophyte Mesembryanthemum crystallinum L.. Plant Cell Physiol. 45: 789-794.

Noctor G., Foyer C.H.. (1998). A re-evaluation of the ATP: NADPH budget during C_3 photosynthesis. A contribution from nitrate assimilation and its associated respiratory activity? Journal of Experimental Botany 49, 1895–1908.

Ollinger O.V., Aber J.D., Reich P.B., Freuder R.J.. (2002). Interactive effects of nitrogen deposition, tropospheric ozone, elevated CO_2 and land use history on the carbon dynamics of northern hardwood forests, Global Change Biology Volume 8 (6): 545-562.

Olszewski J., Pszczółkowska A., Kulik T., Fordoński G., Płodzień K., Okorski A., Wasielewska J. (2007). Wpływ deficytu wodnego na wskaźniki wymiany gazowej, produkcyjność i zdrowotność ziarna odmian pszenicy ozimej. Acta Sci. Pol., Agricultura 6(4), 33-42.

Pfannschmidt T., Allen J.F., Oelmüller R. (2001). Principles of redox control in photosynthesis gene expression. Physiologia Plantarum 112 (1): 1–9.

Proctor M.C.F.. (2001). Patterns of desiccation tolerance and recovery in bryophytes. Plant Growth Regul. 35:147-156.

Reddy V.R., Reddy K.R., Hodges H.F.. (1995). Carbon Dioxide Enrichment and Temperature Effects on Cotton Canopy Photosynthesis, Transpiration and Water-Use Efficiency, Field Crops Research, Vol. 41: 13-23.

Reddy K.R., Koti S., Davidonis G.H., Reddy V.R.. (2004). Interactive Effects of Carbon Dioxide and Nitrogen Nutrition on Cotton Growth, Development, Yield, and Fiber Quality. Agronomy Journal, Vol.96: 1148-1157.

Richardt S., Timmerhaus G., Lang D., Qudeimat E., Corrêa L.G., Reski R., Rensing S.A., Frank W.. (2010). Microarray analysis of the moss Physcomitrella patens reveals evolutionarily conserved transcriptional regulation of salt stress and abscisic acid signalling. Plant Mol. Biol. 72(1-2): 27-45.

Rzepka A.. (1990). Zależność natężenia wymiany gazowej gametoforów i sporogonów wybranych gatunków mchów w zależności od stężenia CO2. Zeszyt Badań Tarnobrzeskich PAN, Kraków: 38-65.

Rzepka A., Krupa J., Ślepak I.. (2005). Effect of hypoxia on photosynthetic activity and antioxidate response in gamatophores of Mnium undulatum. Acta Physiol. Plant (27) No.2:205-212.

Rzepka A.. (2008). Ekofizjologiczne aspekty reakcji różnych gatunków mchów na abiotyczne czynniki stresowe. Wydawnictwo Naukowe AP Kraków.

Schafer F.Q., Buettner G.R.. (2001). Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. Free Radic. Biol. Med.: 1191–212.

Schapendonk A.H.C.M., Van der Putten P.E.L., Dolstra O., Tonk W.I.M.. 1992. Chlorophyll fluorescence: a non-destructive method for detecting damage in the photosynthetic apparatus in plants. Acta Hort. 304: 61-70.

Schreiber J., Hilpert U., Höring L., Worschech L., König B., Ossau W., Waag A., Landwehr G.. (2000). Luminescence Studies on Plastic Stress Relaxation in ZnSe/GaAs(001). Physica Status Solidi (b) 222(1), 169–177.

Sedbrook J.C., Kronebusch P.J., Borisy G.G., Trewavas A.J., Masson P.H.. (1996). Transgenic Auquorin reveals organ+specific cytosolic Ca $^{2+}$ responses to anoxia in Arabidipsis thaliana seedings. Plant Physiol. 111:243-257.

Shaw G.H. (2008). Earth's atmosphere — Hadean to early Proterozoic.Chem Erde-Geochem, 68: 235–264.

Sies H. (1985). Oxidative Stress. London: Academic Press: 1-7.

Starck Z. (1983). Fizjologiczne aspekty reakcji roślin na zasolenie. Post. Nauk Roln., 2: 17-26.

Starck Z. (1995). Współzależność między fotosyntezą i dystrybucją asymilatów, a tolerancją roślin na niekorzystne warunki środowiska. Zeszyty Problemowe Postępów Nauk Rolniczych (3): 19-35.

Stover D.B., Day F.P., Butnor J.R., Drake B.G.. (2007). Effect of Elevated CO_2 on Coarse-root Biomass in Florida Scrub Detected by Ground-penetrating Radar, Ecology Vol. 88, 1328–1334.

Śmiechowska A., Kusznierewicz B., Bartoszek A., Namieśnik J.. (2008). Badania właściwości przeciwutleniających związków pochodzących z najczęściej spożywanych owoców i warzyw. Analityka; 4: 26-30.

Vartepian B.B., Jackson M.B.. (1997). Plant adaptations to anaerobic stress. Ann Bot. 79: 3-20.

Vinocur B., Altman A.. (2005). Recent advances in engineering plant tolerance to abiotic stress: achievements and limitations. Current opinion in biotechnology 16 (2): 123–32.

Visser E.J.W., Voesnek A.C.J., Vartapetia B.B., Jackson M.B.. (2003). Flooding and plant growth. Ann. Bot. 91: 107-114.

Voelker S.L., Muzika R-M, Guyette R.P., Stambaugh M.C.. (2006). Historical CO_2 Growth Enhancement Declines with Age in Quercus and Pinus. Ecological Monographs, Vol. 76 (4): 549–564.

Wang K., Kellomaki S., Li C., Zha T.. (2003). Light and Water-use Efficiencies of Pine Shoots Exposed to Elevated Carbon Dioxide and Temperature, Annals of Botany, Vol. 92: 53-64.

Weis E., Wamper D., Santarius K.A.. (1986). Heat sensitivity and thermal adaptation of photosynthesis in liverwort thalli. Oecologia 69: 134-139.

Wilsey B.J., Coleman J.S., McNaughton S.J.. (1997). Effects of Elevated CO_2 and Defoliation on Grasses: a Comparative Ecosystem Approach. Ecological Applications Vol. 7 (3): 844–853.

Woźny A., Przybył K.. (2004). Komórki roślinne w warunkach stresu. Tom I-II. Wydawnictwo Naukowe UAM, Poznań.

Wu C.. (1995). Heat shock transcription factors: structure and regulation. Annual review of cell and developmental biology 11: 441–69.

Zhu J.-K.. (2001). Plant salt tolerance. Plant Sci Trends. 6: 66-71.

Ziska L.H.. (2003). Evaluation of the Growth Response of Six Invasive Species to Past, Present and Future Atmospheric Carbon Dioxide, Journal of Experimental Botany, Vol. 54: 395-404.

PLANT FUNCTIONING UNDER ENVIRONMENTAL STRESS P. 85-94

Cracow, 12th to the 15th September, 2012

THE ROLE OF OVER EXPRESSION OF P5CS GENE ON PROLINE, CATALASE, ASCORBATE PEROXIDASE ACTIVITY AND LIPID PEROXIDATION OF TRANSGENIC TOBACCO (*NICOTIANA TABACUM* L.) PLANT UNDER *IN VITRO* DROUGHT STRESS

Somayeh Zarei, Ali Akbar Ehsanpour, Jallil Abbaspour

Department of Biology, Faculty of Science, University of Isfahan, Isfahan, Iran

Correspondending author : ehsanpou@sci.ui.ac.ir

Abstract. In this study proline content and activity of catalase (CAT), and ascorbate peroxidase (APX) and level of lipid peroxidation in terms of malondialdehyde (MDA) content were measured in transgenic tobacco (*Nicotiana tabacum* cv. Wisconsin) over expressing Δ-1-pyrroline-5-carboxylate synthase (P5CS) gene and non transgenic plants as control. Drought stress was applied using polyethylene glycol (PEG) 6000 at concentration of 217, 264, 320, 637, 1292 mmol/kg (0, 5, 10, 20, 30% respectively). Proline content especially in transgenic plants were increased in leaves and roots significantly. CAT and APX activities increased under drought stress and the highest activity was observed in 10 and 20%. MDA content was increased by increasing of PEG and the highest MDA content was revealed in transgenic and non transgenic plants at 20% and 30%, respectively. Our results suggest that P5CS is an inducible gene and over production of proline and induction of CAT and APX activity are involve in drought tolerance mechanism.

Key words: Tobacco; Drought stress; Proline; Catalase; Ascorbate peroxidase; P5CS gene

Introduction

One of the most important abiotic stress is drought, which results in the disruption of water potential slopes, loss of turgor and decreasing of pressure potential. Abiotic stresses and osmotic adjustment contributes to pressure potential maintenance and stress tolerance of plants (Cherian *et al.*, 2006). In response to water stress, plants accumulate osmolytes and protect themselves against drought stress.

Proline is one of the most common compatible osmolytes and plays an overriding role in osmotic pressure adjustment (Yamch *et al.*, 2005). Proline allows many plant species to survive under stress without interfering with normal biochemical reactions (Stewart, 1981). The most important function of proline is turgor maintenance and scavenging the excess reactive oxygen species (ROS). It stabilizes protein, enzyme and other subcellular structures such as membranes. Proline also act as an antioxidant, and regulates cellular redox status under stress condition (Chinnusamy *et al.* 2005). The first response of plants to drought stress is closure of stomata and a decrease in CO_2 concentration in leaf mesophyll tissue. Duo to decrease in CO_2 concentration, consequently, accumulation of NADPH and loss of $NADP^+$ occur and oxygen accepts electrons and leads to ROS formation (Sairam *et al.*, 1998; Asada, 1999). ROS results in injure to vital molecules such as nucleic acids, proteins, structural carbohydrates, and lipids (Mittler, 2002; Daveis, 1987). Lipid peroxidation of cellular membranes is the most important effects of ROS that finally leads to disruption of plant growth and development (Chen *et al.* 2000, Sreenivasulu *et al.* 1999).

Plants prevent or alleviate ROS damages by antioxidants protection system which provides protection against oxidative stress. Antioxidants are composed of non enzymatic (glutathione, ascorbate and carotenoids) and enzymatic such as catalase (CAT), ascorbate peroxidase (APX), superoxide dismutase (SOD) and glutathione reductase (GR) (Apel and Hirt, 2004; Bhardwaj *et al.,* 2007).

Ascorbate peroxidases have been found in higher plants, algae and some cyanobacteria (Sano *et al.*, 2001; Sharma and Dubey, 2004). Ascorbate, as an electron donor, is utilized in reduction of hydrogen peroxide (Shigeoka *et al.*, 2002). CAT and APX reduce H_2O_2 to water and O_2 (Gratao *et al.* 2005). Unlike APX, CAT acts without any electron donor or reducing agent (Mallick and Mohn, 2000).

Some plants such as *Vigna aconitifolia* and *Arabidopsis thaliana* reduction of glutamate to its semialdehyde intermediate is catalyzed by a single bifunctional enzyme, Δ-1-pyrroline-5-carboxylate synthase (P5CS), whose transcription is induced in plant subjected to salt and drought stress (Yoshiba *et al.*, 1995). Furthermore, data indicated that, over expression of *V. aconitifolia* P5CS (VaP5CS) in transgenic tobacco plants increased proline level and rendered plants less sensitive to osmotic stress (Kishor *et al.*, 1995). However, there is a controversy discussion in balance activity of P5CS and P5CR enzyme (Verbruggen, 1995). It has been documented that transgenic plants over expressing P5CS gene increases concentration of proline and resulted in more resistance to both drought and salt stress (Kishore *et al*, 1995). The objective of this work was to understand better the relationship between drought tolerance and proline content, the CAT and APX activity and lipid peroxidation in transgenic tobacco plants over expressing P5CS gene.

Material and Methods

Plant materials and treatment. Transgenic tobacco plants (*Nicotiana tabacum* cv. Wisconsin) (T1 seeds) carrying P5CS gene were surface sterilized in 70 % ethanol and then were grown on MS medium (Murashige & Skoog, 1962) and kept in the growth chamber (16/8 h light and dark respectively, with approximately 40 µM photon $m^{-2}s^{-1}$ light density) at 25°C. After 18-20 days, seedlings then were transferred to MS medium supplemented with PEG (217, 264, 320, 637, 1292 mmol/kg) After 4 weeks post treatment, proline content, CAT and APX activities and lipid peroxidation were measured in leaves and roots.

Proline measurement. Free proline accumulation was estimated using ninhydrin reaction based on method described by Bates (1973). A small portion (0.04 g) of leaves or roots was homogenized with 1.7 ml of 3% (w/v) sulphosalicylic acid (Merk). The homogenate was centrifuged at 13000 rpm for 20 min. Then ninhydrin reagent (1 ml) (Sigma) and glacial acetic acid (1 ml) were added to 1 ml of the centrifuged extract. The mixture was boiled for 1 h in a water bath and then cooled on ice. Then 2 ml toluene was added to each tube, and tubes were placed in the dark for 1h. Absorption of chromophore was determined at 520 nm by spectrophotometer (Shimadzu UV-160, Japan). Toluene was used as blank. Proline content was calculated using L-proline (Sigma) as a standard curve.

Enzyme assays. For enzyme extraction, fresh samples of leaves from control and stressed seedlings (0.1 g) were homogenized in an ice bath in 1 mL of phosphate buffer saline (PBS, pH:7.4) containing NaCl (8 g/l), KCl (0.2 g/l), Na_2HPO (41.44 g/l), 0 KH_2PO_4.(24 g/l) and polyvinylpyrrolidone (PVP, 1%). The homogenate was centrifuged at 14000 rpm at 4 °C for 20 min. The supernatant was collected and used for enzyme (CAT and APX) activity analysis.

Catalase (EC 1.11.1.6).The CAT activity was determined by measuring the decomposition of H_2O_2. The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.4), 10 mM H_2O_2, and 0.05 ml of the enzyme extract (Aebi, 1984). Then the absorbance at 240 nm was recorded every 10 sec. up to 1 min. The CAT activity was calculated and expressed as U/g FW^{-1} min^{-1} (One unit of CAT activity is defined as the amount of enzyme required to consume 1 µmole H_2O_2 min–1). CAT activity was calculated using the coefficient of absorbance of 0.0394 mM^{-1} cm^{-1} by spectrophotometer (Shimadzu).

Ascorbate peroxidase (EC 1.11.1.11). APX was estimated by slightly modified procedure of Nakano and Asada (1981). APX activity was determined by measuring the consumption of ascorbate. Reaction mixture contained 25 mM potassium phosphate buffer (pH 7.0), 0.5 mM ascorbic acid, 0.2 mM EDTA-$_4$H, 0.1 mM H_2O_2, 50 µl of BSA, and 0.05 ml of the enzyme extract. The absorbance at 290 nm was recorded every 10 sec up to 1 min. One unit of APX activity was defined as the amount of enzyme required to consume 1 µmole ascorbate min^{-1}. Ascorbate

peroxidase activity was calculated using the coefficient of absorbance of 2.8 mM^{-1} cm^{-1}.

Lipid peroxidation. Fresh plant material (0.1 g) was homogenized in 2.5 ml 0.1% (w/v) trichloroacetic acid (TCA). The level of lipid peroxidation was measured using the malondialdehyde (MDA), thiobarbituric acid (TBA) reaction based on method of Heath and Packer (1968). Lipid hydroperoxides resulting from peroxidation of the cell membrane react with thiobarbituric acid (TBA) to form MDA, which is a crystalline pink pigment with absorption from 525 to 535 nm (Persky *et al.* 2000). The absorbance of extract was measured at 532 and 600 nm. The amount of MDA-TBA complex was calculated from the coefficient of absorbance 155 $mM^{-1}cm^{-1}$.

Statistical analysis

All experiments were carried out in a Completely Randomized design. The mean values of proline, CAT and APX activity and lipid peroxidation level were taken from the measurements of four replicates and the "Standard Error" of the means was calculated. Two-way ANOVA was applied to determine the mean between different treatments and then Tukey test was performed and significance was determined at $P < 0.05$. All statistical analyses were carried out using SPSS Software program version 10.

Results

Proline content. As the PEG concentration increased, proline level of shoot in transgenic and non transgenic plants, was increased significantly. In transgenic plants, either in leaf or root the proline content was significantly higher than non transgenic (Fig.1A, B). In both plant types, 20 and 30% PEG showed the highest level of proline content. In the root, proline content was increased (1.3 and 1.2 fold) by increasing of PEG particularly in the higheast level at 20 and 30% PEG compare to non transgenic plants.

*APX activity.*The APX, showed a high significant activity by increasing of PEG up to 20 % either in transgenic or non transgenic plants. Transgenic plant showed higher APX activity than non transgenic plants in all PEG concentrations. In transgenic both plant type, the APX activity decreased at 30 % PEG. (Fig. 2).

*CAT activity.*Results indicated that the CAT activity was increased by increasing of PEG concentration. In transgenic plants, CAT activity at 5, 10 and 20% PEG while in non transgenic plants at 10 and 20% PEG increased significantly however, at 30% the activity of CAT decreased compared to the control plants (Fig. 3).

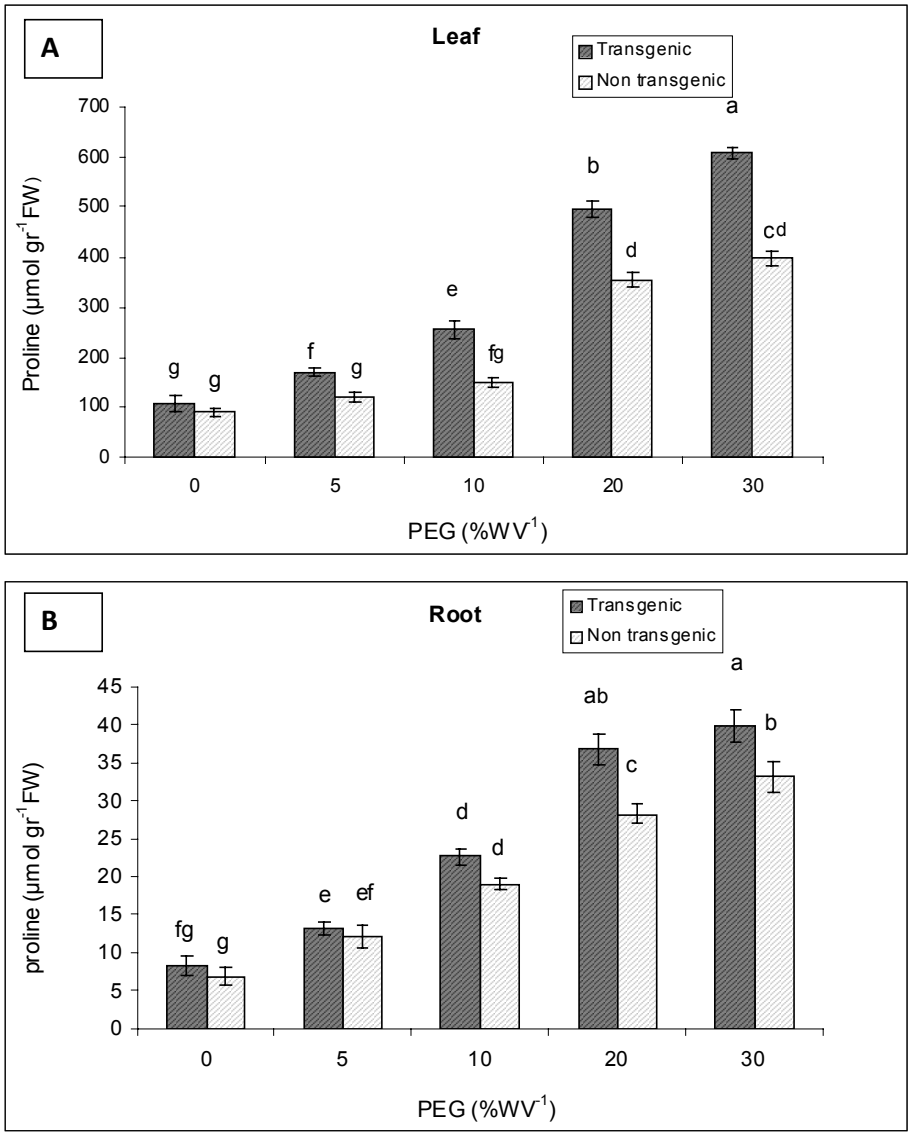


Fig. 1. Proline content in shoot (A) and roots (B) of transgenic and non transgenic tobacco seedlings. Values are means ± Sd. Uncommon letters are significant based on Tukey test ($P < 0.05$). (0, 5, 10, 20 30% of PEG are equal to 217, 264, 320, 637, 1292 mmol/kg respectively)

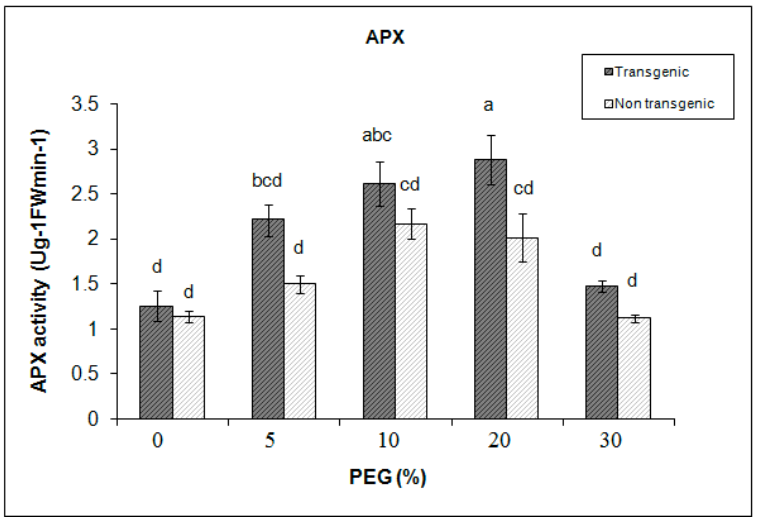


Fig. 2. Ascorbat peroxidase (APX) activity of leaves of tobacco seedlings in response to drought stress. Values are means ± SE. Uncommon letters are significant based on Tukey test (P < 0.05). (0, 5, 10, 20 30% of PEG are equal to 217, 264, 320, 637, 1292 mmol/kg respectively)

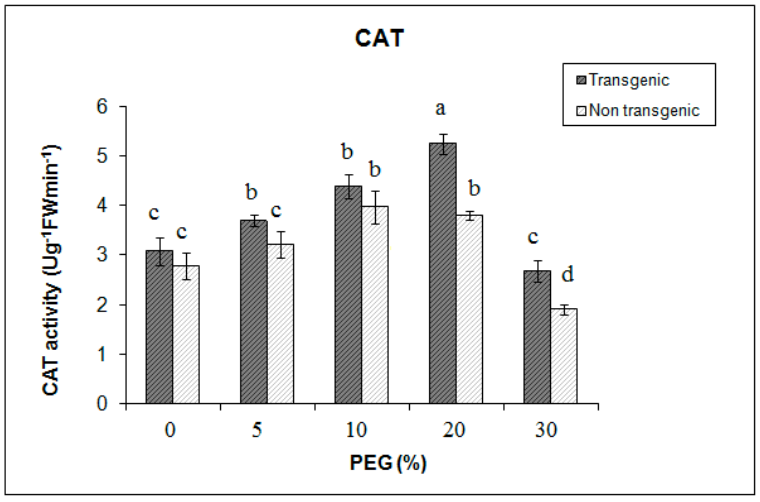


Fig. 3. Catalase (CAT) activity of leaves of tobacco seedlings. Values are means ± SE. Uncommon letters are significant based on Tukey test (P < 0.05). (0, 5, 10, 20 30% of PEG are equal to 217, 264, 320, 637, 1292 mmol/kg respectively)

Lipid peroxidation. Lipid peroxidation data (measured as MDA content), showed a significant increase in both plant types in 10, 20 and 30% of PEG. MDA was increased significantly in comparison to control plants. In non transgenic plants at 10 and 20% PEG it was significantly higher than transgenic ones (Fig.4).

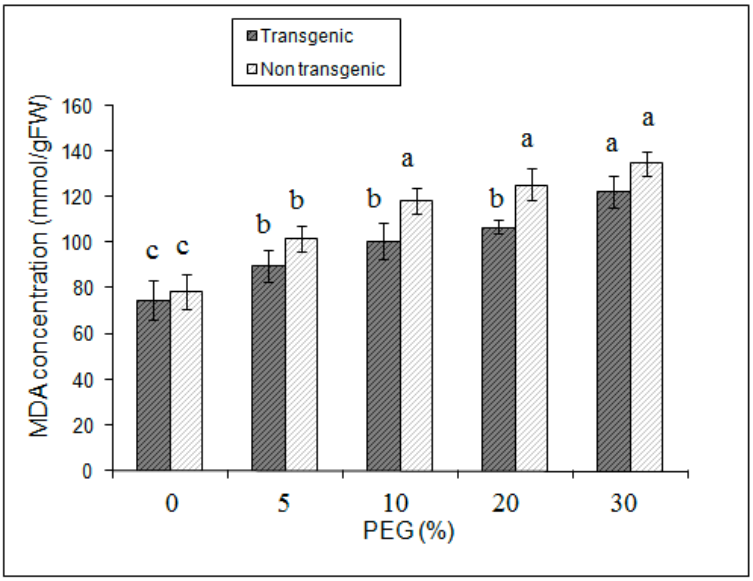


Fig. 4. Effect of PEG on MDA content in transgenic and non transgenic tobacco plants. Values are means ± SE. Similar letters indicate not significant based on Tukey test ($P < 0.05$). (0, 5, 10, 20 30% of PEG are equal to 217, 264, 320, 637, 1292 mmol/kg respectively)

Discussion

Results of this study showed that proline was accumulated in shoot and roots of transgenic and non transgenic plants when plants subjected to drought stress. The accumulation of proline in shoot and roots of transgenic plants was much higher than in non transgenic plants. It seems that over expression of P5CS gene in transgenic plants, have a remarkable role in proline synthesis and accumulation. As P5CS gene is a rate-limiting enzyme in the proline biosynthetic pathway in plants and has an important role in transgenic plants over expressing P5CS gene, it is expecting that it is responsible for more proline accumulation in transgenic plants. As proline plays an important role in osmotic adjustment as well as membrane protection, free radical scavenging, and redox buffering (Verbruggen and Hermans,

2008; Kishor et al, 2005), high level of proline, accumulation by over expression of P5CS, can tolerate plants against osmotic stress (Hank & Hwang, 2003). Similar data was obtained when our transgenic and non transgenic tobacco plant exposed to osmotic (PEG) stress. These results are agreement with the report from Kishore *et al.* (2005) transgenic tobacco in the present study over expressing P5CS gene produced a high level of the antioxidant enzymes and synthesized more proline than the controls.

Our results showed that drought stress changed antioxidant enzymes activity in leaves of both plant types. APX and CAT activity have been induced in leaves of transgenic plants higher than non transgenic ones. These finding are similar to results obtained by Khedr *et al.* (2003) and Ghorbanli *et al.* (2004). On the other hand, it has been known that abiotic stresses, including drought stress, induce ROS accumulation, resulting oxidative damage to membrane lipids, proteins, and nucleic acids (Smirnoff, 1993). Plants increase activity of detoxifying enzymes such as superoxide dismutase, catalase, and ascorbat peroxidase to combat oxidative stress. Proline has a role in scavenging ROS and protect proteins against denaturation (Alia *et al.* 1991), increasing of CAT and APX activity may cooperates with other antioxidant enzymes for more tolerance to drought stress in our tobacco plants.

It is well known that ROS induce lipid peroxidation of membranes. The change in MDA content is often used as an indicator of oxidative damage (Sung, 1996; Goel and Sheoran, 2003). Our data showed that MDA content enhanced by increasing of drought stress intensity. It has been reported that MDA content, as lipid peroxidation criteria, in transgenic plants was lower than non transgenic plants (Smirnoff and Cumbes, 1989; Matysik *et al.* 2002). It has been shown that proline can reduce lipid peroxidation in alga cells exposed to heavy metals (Mehta and Gaur, 1999), it can be speculating that similar role might be considered for proline accumulation due to P5CS over expression in tobacco plants too. The lower level of lipid peroxidation in leaves of transgenic plants suggests that, these plants are better protected from oxidative damage under drought stress than non transgenic ones.

Conclusion

It can be concluded that over expression of P5CS gene in tobacco plants and proline accumulation in cooperation with CAT and APX activities increase drought tolerance in tobacco plants.

Acknowledgment: Authors wish to thank University of Isfahan and Plant Stress Center of Excellence (PSCE) for their support.

References

Aebi, H. (1984). Catalase in vitro. Methods of Enzymology. 105: 121-126.

Alia, P. S. P., Pardha, S. P. and Mohanty, P. (1991). Proline enhances primary photochemical activities in isolated thylakoid membranes of *Brassica juncea* by arresting photoinhibitory damage. Biochemical and Biophysical Research Communications. 181: 1238–1244.

Apel, K. and Hirt, H. (2004). Reactive oxygen species: metabolism, oxidative stress, and signal transduction. Annual Review of Plant Biology. 55: 373-399.

Asada, K. (1999). The water- water cycle in chloroplast; scavenging of active oxygen and dissipation of excess photons. Annual Review of Plant Physiology and Plant Molecular Biology. 50: 601- 639.

Bates, L. S. (1973). Rapid determination of free proline for water-stress studies. Plant and Soil. 39: 205-207.

Bhardwaj, R, N. Arora, P. Sharma, H and Arora, K. (2007). Effects of 28-homobrassinolide on seedling growth, lipid peroxidation and antioxidative enzyme activities under nickel stress in seedlings of *Zea mays* L. Asian Journal of Plant Science. 6: 756-772.

Chen, W. P., Li, P. H. and Chen, T. H. H. (2000). Glycinebetaine increases chilling tolerance and reduces chilling-induced lipid peroxidation in *Zea mays* L. Plant Cell Environment. 23: 609-618.

Cherian, S., Reddy, M. P. and Ferreira, R. B. (2006). Transgenic plants with improved dehydration-stress tolerance: Progress and future Prospects. Biologia Plantarum. 50: 481- 495.

Chinnusamy, V., Jagendorf, A.and Zhu, J. K. (2005). Understanding and improving salt tolerance in plants. Crop Science. 45: 437–448.

Davies, K. J. (1987). Protein damage and degradation by oxygen radicals. I. General aspects. Journal of Biological Chemistry. 262: 9895-9901.

Goel, A. and Sheoran, L. S. (2003). Lipid peroxidation and peroxide scavenging enzymes in cotton seeds under natural ageing. Biologia Plantarum 46: 429–434.

Ghorbanli, M., Ebrahimzadeh, H. and Sharifi, M. (2004). Effects of NaCl and mycorrhizal fungi on antioxidative enzymes in soybean. Biol. Plant. 48: 575-581.

Gratao, P. L., Polle, A., Lea, P. J. and Azevedo, R. A. (2005). Making the life of heavy metal- stressed plants a little easier. Functional Plant Biology. 32: 481–494.

Hank, H. and Hwang, C. H. (2003). Salt tolerance enhanced by transformation of a P5CS gene in carrot. Journal of Plant Biotechnology. 5: 149–153.

Heath, R. L. and Packer, L. (1969). Photoperoxidation in isolated chloroplast. I. Kinetics and stoichiometry of fatty acid peroxidation. Archives of Biochemistry and Biophysics. 125: 189-198.

Kavi Kishor, P. B., Sangam, S., Amrutha, R. N., Laxmi, P. S., Naidu, K. R., Rao, K. R. S. S., Rao, S., Reddy, K. J., Theriappan, P. and Sreenivasulu, N. (2005). Regulation of proline biosynthesis, degradation, uptake and transport in higher plants: its implications in plant growth and abiotic stress tolerance. Current Science. 88: 424-38.

Khedr, A. H. A., Abbas, M. A., Wahid, A. A. A., Quick, W. P. and Abogadallah, G. M. (2003). Proline induces the expression of salt-stress-responsive proteins and may improve the adaptation of *Pancratium maritmum* L. to salt stress. Journal of Experimental Botany 54: 2553-2562.

Kishore, B., Hong, Z., Miao, G., Hu, C. and Verma, D. (1995). Over expression of Δ- Pyrroline-5-carboxylate synthetase increase proline production and confers osmotolerance in transgenic plants. Plant Phsiology. 108: 1387-1394.

Kishore P.B.K., Sangam S., Amrutha R.N., Laximi P., Naidu, K., Rao, K.R., Rao, S., Reddy, K.J., Theriappon, P. and Sreenivasula, N. (2005). Regulation of proline biosynthesis, degredation, uptake and transport in higher plants: its implication in plant growth and abiotic stress tolerance. Current Science. 88: 427-438.

Mallick, N. and Mohn, F. H. (2000). Reactive oxygen species: Response to algal cells. Journal of Plant Physiology 157: 183-193.

Matysik, J., Alia, B. B. and Mohanty, P. (2002). Molecular mechanisms of quenching of reactive oxygen species by proline under stress in plants. Current Science. 82: 525-532.

Mehta, S. K. and Gaur, J. P. (1999). Heavy metal-induced proline accumulation and its role in ameliorating metal toxicity in *Chlorella vulgaris*. New Phytologist 143: 253-259.

Mittler, R. (2002). Oxidative stress, antioxidntas and stress tolerance. Trends in Plant Science 7: 405-410.

Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiologia Plantarum. 15: 473-497.

Nakano, Y. and Asada, K. (1987). Purification of ascorbate peroxidase in spinach chloroplasts: its inactivation in ascorbate-depleted medium and reactivation by monodehydroascorbate radical. Plant and Cell Physiology. 28: 131-140.

Sairam, R. K., Deshmukh, P. S. and Saxna, D. C. (1998). Role of antioxidant systemes in wheat genotype tolerance to water stress. Biologia Plantarum 41: 387-394.

Savouré, A., Jaoua, S., Hua, X.J., Ardiles, W., Van Montagu, M. and Verbruggen, N. (1995). Isolation, characterization, and chromosomal location of a gene encoding the 11- pyrroline-5-carboxylate synthase in *Arabidopsis thaliana* . FEBS Letter 372: 13–19

Sano, S., Ueda, M., Kitajima, S., Takeda, T., Shigeoka, S., Kurano, N., Miyachi, S., Miyake, C., Yokota, A. (2001). Characterization of ascorbate peroxidases from unicellular red alga Galdieria partita. Plant and Cell Physiology 42: 433–440.

Sharma, P. and Dubey, R. S. (2004). Ascorbate peroxidase from rice seedlings: properties of enzyme isoforms, effects of stresses and protective roles of osmolytes. Plant Science 167: 541–550.

Shigeoka, S., Ishikawa, T., Tamoi, M., Miyagawa, Y., Takeda, T., Yabuta, Y. and Yoshimura, K. (2002). Regulation and function of ascorbate peroxidase isoenzymes. Journal of Experimental Botany 53: 1305–1319.

Smirnoff, N. and Cumbes, Q. J. (1989). Hydroxyl radical scavenging activity of compatible solutes. Phytochemistry 28: 1057-1060.

Smirnoff, N. (1993). The role of active oxygen in the response to plants to water deficit and desiccation. New Phytologist. 125: 27-58.

Sreenivasulu, N., Ramanjulu, S., Rmachandra-Kini, K., Prakash, H.S., Shekar-Shetty, H., Savithri, H.S., Sudhakar, C. (1999). Total peroxidase activity and peroxidase isoforms as modified by salt stress in two cultivars of fox-tail millet with differential salt tolerance. Plant Scienc. 141: 1-9.

Stewart, C. R. (1981). Proline accumulation: Biochemical aspects. In: Paleg, L. G., Aspinall, D (Eds), Physiology and Biochemistry of drought resistance in plants pp 243-251.

Sung, J. M. (1996). Lipid peroxidation and peroxide-scavenging in soybean seeds during aging. Physiologia Plantarum. 97: 85–89.

Verbruggen, N. and Hermans, C. (2008). Proline accumulation in plants: a review. Amino Acids. 35: 753-759.

Yamchi, A., Rastgar Jazii, F., Ghobadi, C., Mousavi, A. and Karkhanehee, A. A. (2005). Increasing of tolerance to osmotic stresses in tobacco *Nicotiana tabacum* cv. xanthi through overexpression of p5cs gene. Journal of Science and Technology of Agriculture and Natural Resources. 8: 40- 49.

Yoshiba Y, Kiyosue T, Katagiri T, Ueda H, Mizoguchi T, Yamaguchi-Shinozaki K, Harada Y, Shinozaki K (1995). Correlation between the induction of a gene for 11-pyrroline-5- carboxylate synthase and the accumulation of proline in *Arabidopsis thaliana* under osmotic stress. Plant Journal 7: 751–760

PLANT FUNCTIONING UNDER ENVIRONMENTAL STRESS P. 95-114

Cracow, 12th to the 15th September, 2012

CHANGES IN PHYSIOLOGICAL TRAITS OF POPLAR (POPULUS SSP) HYBRIDS INOCULATED WITH *H. CRUSTULINIFORME*

Maciej T. Grzesiak*, Janusz J. Zwiazek**

*The Franciszek Górski Institute of Plant Physiology, Polish Academy of Sciences, Niezapominajek 21, PL 30-239 Cracow, Poland.

**Department of Renewable Resources, University of Alberta, 4-42 Earth Science Building, Edmonton, AB, T6G 2E3, Canada.

Corresponding author: e-mail m.grzesiak@ifr-pan.krakow.pl

Abstract Effects of ectomycorrhizal fungus *H. crustuliniforme* on growth and physiological traits (leaf water potential, gas exchange, leaf chlorophyll concentration and fluorescence, leaf and root hydraulic conductivity) were studied in several greenhouse-grown cultivars of hybrid poplars. Mycorrhizal structures were present only in inoculated roots and the frequency of mycorrhiza and the intensity of mycorrhizal colonization varied between the cultivars. Mycorrhizal frequency reached about 75%, and the intensity of mycorrhizal colonization in roots ranged from 30 to 44%. Compared to non-inoculated plants, mycorrhizal plants had greater height, lower leaf water potential, higher leaf chlorophyll concentrations, and higher gas exchange rates as well as higher photochemical efficiency. Inoculation of poplar clones with *H. crustuliniforme* resulted in increases in root hydraulic conductance in Berlin, WP69, P38P38, and Assiniboine hybrid poplar clones. Inoculated balsam poplar plants had also higher root volume, leaf areas and leaf lamina conductance compared with non-inoculated controls. If persistent over a long-term, the observed differences between inoculated and non-inoculated plants could significantly alter plant growth and survival. Long-term growth studies would help clarify the long-term impact of ectomycorrhizal associations on poplar growth.

Key Words: poplar; leaf and root hydraulic conductance; water potential; gas exchange; chlorophyll content; chlorophyll *a* fluorescence; mycorrhiza; root colonization

Abbreviations:

C – non–inoculated plants,
M – inoculated plants,
K_{leaf} – leaf hydraulic conductance,
K_{lam} – leaf lamina hydraulic conductance,
K_{pet} – petiole hydraulic conductance,
LA – leaf area,
K_{root} – root hydraulic conductance,
RV – root volume,
H – plant height,
ψ – leaf water potential,
Pn – Net photosynthesis,
E – transpiration rate,
g_S – stomatal conductance,
WUE – water use efficiency index,
F_0 – minimum fluorescence,
F_M – maximum fluorescence,
F_V – variable fluorescence
F_V/F_M – potential quantum yield of PSII,
MF_R – frequency of mycorrhiza in the root system,
MC_R – intensity of the mycorrhizal colonization in the root system,
MC_{RF} – intensity of mycorrhizal colonization in the root fragments.

Introduction

The genus *Populu*s include 35 deciduous tree species largely found in temperate regions of the northern hemisphere. Many poplars can form hybrids either in nature or through breeding programs which take advantage of their fast growth (as much as 7 m per year) and tolerance of adverse environmental conditions. Poplars can be colonized by both ectomycorrhizal (ECM) and vesicular-arbuscular mycorrhizal (VAM) fungi (Hooker *et al*. 1992, Smith and Read 1997, Siemens and Zwiazek 2008), which help supply the trees with water and mineral nutrients and protect the roots against diseases (Pendelton and Smith 1983, Armstrong *et al*. 1992, Martin *et al*. 2001).

Hebeloma crustuliniforme is a common ECM fungus found in temperate and boreal forest ecosystems in North America and is often present in sand dunes that are adjacent to natural water reservoirs (Gryta *et al.* 1997), a common natural habitat for many willows and poplars (Sell *et al*. 2005). A characteristic feature of the ECM fungi is the presence of a mantle and Hartig net. ECM associations are formed predominantly on the fine root tips of the host plant, which are unevenly

distributed in the soil profile (Meyer 1973). During the symbiotic phase, ECM fungi form the mantle sheath that surrounds the root and progress into the apoplastic space of the rhizodermic and cortical cells producing the Hartig net (Martin *et al*. 2001). In greenhouse-grown balsam poplar seedlings, *H. crustuliniforme* was found to develop a mantle layer characteristic of ECM associations, but no distinct Hartig net was present (Siemens and Zwiazek 2008).

Plant responses to mycorrhizal fungi depend on numerous factors including host plant and fungal species, soil conditions, time of inoculation and morphological and anatomical characteristics of plant roots and fungi mycelia. There are divergent opinions concerning advantages resulting from the symbiosis between vascular plants and mycorrhizal fungi (Johnson *et al.* 1997, Martin *et al*. 2001, Brundrett 2002, Kottke 2002). Plants with roots colonized by ECM fungi showed improved water uptake (Landhausser *et al*. 2002), salt stress resistance (Nguyen *et al.* 2006, Calvo-Polanco *et al.* 2008), and low temperature resistance (Anderson and Coats, 1994, Turnau *et al.* 2008). However, numerous studies have also reported no changes (Calvo-Polanco 2009) or even detrimental effects (Lehto and Zwiazek 2011) of ECM associations on plant stress resistance.

The importance of ECM for plant water relations remains largely unresolved (Lehto and Zwiazek 2011). ECM associations can increase root hydraulic conductivity in poplars (Marjanović *et al*. 2005, Siemens and Zwiazek 2008), likely by inducing the expression of root aquaporins (Marjanović *et al.* 2011). However, the increase in root hydraulic conductivity in ECM balsam poplar (*Populus balsamifera*) was found not to be the factor involved in the observed increase in shoot growth rates in ECM plants (Siemens and Zwiazek 2008).

There is a growing interest in the intensive plantations of hybrid poplars and maximizing their growth and yield. ECM associations could play an important part in this process. However, little is known about the effects of ECM fungi on the physiology of hybrid poplars. In the present study, we examined the effects of root inoculation with *H. crustuliniforme* on growth and physiological processes (gas exchange, leaf water potential, chlorophyll content and fluorescence, leaf and root water flow and hydraulic conductance) in fast-growing hybrid poplar cultivars and in the native balsam poplar (*Populus balsamifera*) rooted cuttings. We hypothesized that the inoculation of fast growing hybrid poplar clones with ECM fungi would increase the hydraulic conductivity of the root system which may be a factor limiting fast growth of plants under favorable environmental conditions.

Materials and methods

Plant material. We used shoot cuttings of six poplar cultivars for Study 1 and three poplar cultivars for Study 2. Cuttings of poplar hybrids for Study 1 were obtained from the Alberta-Pacific Forest Industries Inc. (Al-Pac), Canada and those

of cultivars for Study 2 from the *Arboretum* of the Institute of Dendrology, Polish Academy of Sciences, Kórnik near Poznań.

The following poplars were used for Study 1: balsam poplar (*P. balsamifera)* clone AP1004, Berlin (*P. x berolinensis* cv. Berlin poplar), Walker (*P. deltoides* x *P. x petrowskyana*), Assiniboine (*OP Walker* cv. Assiniboine), WP69 *(Walker* progeny), and P38P38 *(P. balsamifera* x *P. simonii*). The following cultivars were used for Study 2:

P. petrovskyana (P. × *petrowskyana)*, P. deltoides "Plantierensis" (*P. deltoides* x *P. nigra* 'Plantierensis') , and P.balsamifera.

Soil substrate. In Study 1, the plants were growing in Sushine Mix #4 Aggregate Plus (Sun Gro Horticulture, Vancouver, Canada) horticultural soil (Formulated with Canadian sphagnum peat moss, coarse perlite, starter nutrient charge (with Gypsum) and dolomitic limestone) and in Study 2 a mixture of clay, garden substrate and sand (2:2:1, by volume). Before filling the pots, soil substrate was autoclaved for 72 h at 105^{o}C. In Study 2, the analysis of sterilized soil was conducted on PDA (Potato Dextrose Agar, Sigma Aldrich) medium, and showed a complete effectiveness of the sterilization process. For Study 1, fungal culture was prepared in modified Melin Norkrans nutrient liquid medium (Hutchison 1991) . Plants were inoculated by injecting into the soil 5 ml of the fungal culture two and four weeks after placing the stems in the soil for rooting. For Study 2, biopreparation containing *H. crustuliniforme* mycelium (5 g of the biopreparation per 1 kg of the soil substrate) was added to the soil substrate that was designated for plants belonging to the mycorrhizal treatment. Biopreparation was obtained from the Forest Gene Bank in Kostrzyca, Poland. The analysis carried out on the PDA medium shown the presence and good vigor of the *H. crustuliniforme* mycelium.

Rooting and Growth Conditions. In both studies, 30-40-cm-long stem segments were placed in containers and rinsed for two days with gentle stream of water. Stem cuttings (18-cm in length) were excised under water and placed in rooting-pots filled with autoclaved soil for 4 weeks in the greenhouse. After 4 weeks of growth in rooting-pots, plants were transferred to 1100 cm^3 pots in both experiments. Plants were placed in a greenhouse with air temperature (day/night) of 23/18^0 C, 65-70% relative humidity. During the experiments plants were watered once a day and fertilized once a week with 25% modified Hoagland's solution. In Study 2, pots were weighted before watering to calculate the amount of water to be added, to keep the water content in soil at the level of 65-70% FWC (field capacity).

Measurements

In Study 1, gas exchange parameters and leaf hydraulic conductance were measured in plants 11 weeks after rooting and root hydraulic conductance measurements were carried out in plants rooted for 13 weeks. In Study 2, plant growth, leaf water potentials, gas exchange parameters, chlorophyll fluorescence, and chlorophyll concentrations were measured in plants rooted for 11 weeks.

Leaf hydraulic conductance. Leaf hydraulic conductance (K_{leaf}) was measured using the high pressure flow meter - HPFM (Dynamax Inc., Houston,USA). Leaves were connected to the HPFM through their petioles using compression couplings, and were perfused with water at constant pressure that ranged from 350 to 450 kPa. Computer software recorded the water flow, applied pressure and computed K_{leaf} as flow (Q) to pressure (P) ratio every 2 s, saving mean values every 30 s. The flow stabilized after 20 min. Petiole hydraulic conductance (K_{pet}) was calculated as the mean of the six successive readings. Leaf blade area (LA) was measured with a leaf area meter (LI-3100, Li-Cor Biosciences, Lincoln, NE, USA). Leaf lamina hydraulic conductance (K_{lam}) was calculated according to Sack *et al*. (2002) as:

$$K_{lam} = 1/(1/K_{leaf}–1/K_{pet})/LA$$

Root hydraulic conductivity. Similarly to leaf hydraulic conductance, a high pressure flow meter – HPFM (Dynamax Inc., Houston, USA) was used for the root hydraulic conductance (K_{root}) measurements. The measurements of root hydraulic conductance (K_{root}) were taken as previously described (Tyree *et al*. 1995, Muhsin and Zwiazek 2002). To prevent water leak through the stem cutting, pressure-resistant silicone sealant was applied to the upper and lower surface of the cuttings 5 days before measurements. For K_{root} measurements, the shoots of plants were excised 3 cm above the root collar and the roots were connected through the cut stem to the high pressure flow meter via a piece of flexible high-pressure tubing. The measurements were carried out in the transient mode with the roots pressurized up to 0.5 MPa (Calvo-Polanco *et al*. 2008). For each plant, root volumes (RV) were measured using the volume displacement method (Voicu and Zwiazek 2004) after removing the roots from pots and washing them in water. Root hydraulic conductivity (L_{root}) was calculated as:

$$L_{root}=K_{root}/RV.$$

Measurements of leaf and root hydraulic conductivity were carried out in 6 plants from each poplar clone.

Plant height (H) was measured from the base of the shoot at the soil level to the shoot apex. There were 10 plants taken for height measurements from each of the three cultivars and two treatments (n = 10).

Leaf water potential (ψ) was measured using a thermocouple psychrometer HR 33T (WESCOR, Inc., USA) equipped with C-52 SF (WESCOR) sample chambers and a digital multimeter Metex M-3640D (Metex, Korea). All measurements were taken in a „dew point" mode on leaf discs (7-mm diameter). Measurement was carried out on leaves from the bottom, median and upper part of the shoot. For each cultivar (3) inoculation treatment (2) and leaf position (3), the measurements were taken in 5 replications (n =5).

Gas exchange parameters: Net photosynthesis (Pn) transpiration rate (E) and stomatal conductance (gs) measurements were taken between 9:00 and 12:00. On the basis of measurements of Pn and E Water Use Efficiency Index (WUE) was calculated (WUE=Pn/E). In Study 1, gas exchange measurements were taken with the LCA4 portable infra-red gas analyzer (ADC Limited, Hoddesdon, UK) equipped with the LED light. In Study 2, CIRAS 2 (PP Systems, Herts, UK) was used with Parkinson`s sample chamber and light attachment. During the measurements, leaves were exposed to 1000 μmol m^{-2} s^{-1} photosynthetically active radiation. In Study 1, the measurements were carried out on leaves from the upper part of the shoot (5-6 from the top) in six plants (n=6) and in Study 2, on the leaves from the bottom, median and upper part of the plant in 5 replications (n=5).

Chlorophyll concentrations were measured using the Konica Minolta SPAD-502 (Konica Minolta, Sensing, Inc, Japan) (Study 1) and Cl-01 (Hansatech, UK) chlorophyll analyzer (Study 2). Measurements were carried out on fully-elongated leaves from the upper part of the plant (Study 1) and from the bottom, median and upper parts (Study 2). In Study 1, the measurement were made in six plants (n=6), and in Study 2 for each cultivar (3) and inoculation treatment (2), the measurements were taken in 5 replications (n =5).

Chlorophyll a fluorescence was measured with the Handy PEA (Plant Efficiency Analyser) analyzer (Hansatech, England). Intact leaves were dark-adapted for 30 min using leaf clips. The ratio F_V/F_M was calculated, where F_V is the difference between the maximum fluorescence F_M and minimum fluorescence F_0. For each cultivar (3) inoculation treatment (2) and leaf position (3), the measurements were taken in 5 replications (n =5).

Fungal colonization. In Study 2, root samples were collected from five inoculated plants after 11 weeks of growth. Determinations of fungal inoculums in soil which were carried out on the PDA (Potato Dextrose Agar, Sigma Aldrich medium), showed that *H. crustuliniforme* was absent from the non-inoculated treatment group.

For root mycorrhizal analyses, root samples were placed in polyethylene bags and stored at 4°C for about 2 weeks. Staining of the mycorrhizal structures was done according to the method by Phillips and Hayman (1970). Samples were washed in water and cut into 1-cm segments before placing in plastic vials filled with 50% ethanol. To remove ethanol, root samples were washed in distilled water and cleared for 24 h in 7% KOH solution. Afterwards, the roots were placed for 24 h in 5% lactic acid and stained with 0.05 % aniline blue in mixture of lactic acid, glycerol and water 1:1:1 (by volume). Root specimens were mounted on microscope glass slides and examined under microscope OLYMPUS SZ-PT–SZ-60, Japan at 400 magnification using 30 root samples from each plant. Frequency of mycorrhiza in the root system (MF_R), intensity of the mycorrhizal colonization in the root system (MC_R), intensity of mycorrhizal colonization in the root fragments (MC_{RF}), were estimated with application of method by Trouvelot *et al*. (1986) using the Mycocalc computer program.

Statistical analysis. The statistically significant differences in root colonization parameters were evaluated by the analysis of variance in a completely randomized design using a Duncan's multiple range test. The effects on leaf water potential, gas exchange, chlorophyll content and chlorophyll fluorescence from Study 2 were analyzed by a paired t-test.

Results

Root colonization. Changes in mycorrhizal traits (MF_R, MC_R, MC_{RF}) were observed in roots inoculated with *H. crustuliniforme*. There were small, but statistically significant differences between poplar cultivars in the frequency of MF_R and intensity MC_R with the highest values measured in balsam poplar (*P. balsamifera*). However, the intensity of mycorrhizal colonization of the root segments (MC_{RF}) was not statistically significant between the cultivars (Fig. 1). The frequency of mycorrhiza (FM_R) was high with the values of about 75%, and MC_R MC_{RF}) by fungal hyphae was lower and ranged from 30 to 44%.

Leaf and root hydraulic conductance. In Study 1, in non-inoculated hybrids (C), leaf areas (LA) ranged from 92.7 to 120 cm^2. Inoculation with *H. crustuliniforme* resulted in a significant increase in LA only in balsam poplar, but not in other poplars(Fig. 2a). Similarly, there were no significant differences in leaf lamina hydraulic conductance (K_{lam}) (Fig. 2b) between non-inoculated and inoculated plants with the exception of K_{lam} in balsam poplar which was higher in inoculated compared with non-inoculated plants.

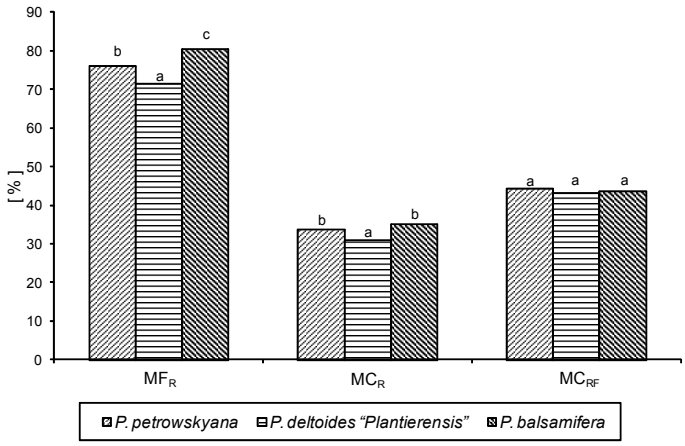


Figure 1. Mycorrhizal frequency (MF_R), , intensity of the mycorrhizal colonization(MC_R), and intensity of mycorrhizal colonization (MC_{RF}) in roots of three poplar cultivars. Same letter indicates insignificant difference according to Duncan test (P=0.05)

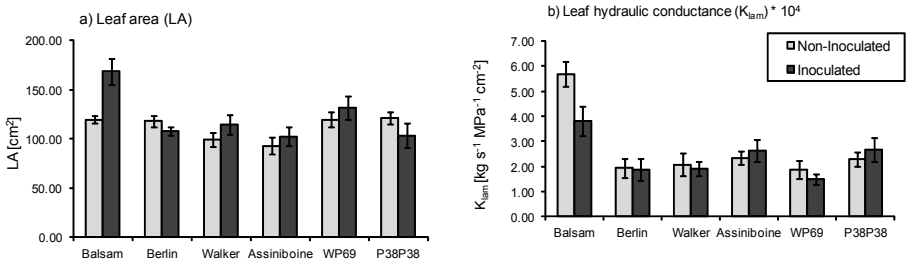


Figure 2. Leaf area and leaf lamina conductance in six poplar hybrids non-inoculated (C) and inoculated (M) with *Hebeloma crustuliniforme*. Mean values ± standard error (n=6)

Root volumes (RV) in non-inoculated plants varied from 23 cm^3 in Walker to 94 cm^3 in P38P38 and in inoculated plants from 27 cm^3 in Walker to 106 cm^3 in P38P38. Statistically-significant differences in RV between non-inoculated and inoculated plants were observed in Berlin and P38P38 (increase in RV due to inoculation) and WP69 (decrease in RV due to inoculation). For Walker and Assiniboine, there were no significant differences in RV between inoculated and non-inoculated plants (Fig. 3a). In measurements of K_{root} in case of non-inoculated plants values varied from 0.0153 for Walker to 0.0340 kg s^{-1} MPa^{-1} for WP69. In

inoculated plans for Berlin, Assiniboine, WP69 and P38P38 there were statistically significant increase in root water flow (K_{root}) and in Balsam and Walker the differences were non-significant (Fig. 3b). Root hydraulic conductance (L_{root}) in case of non-inoculated plants ranged from 0.165 for P38P38 to 0.648 kg s^{-1} MPa cm^{-3} for Walker. A significant increase in L_{root} was observed for Assiniboine and WP69 and in Balsam a decrease was observed. In Berlin, Walker and P38P38 differences between inoculated and non-inoculated plants were insignificant.

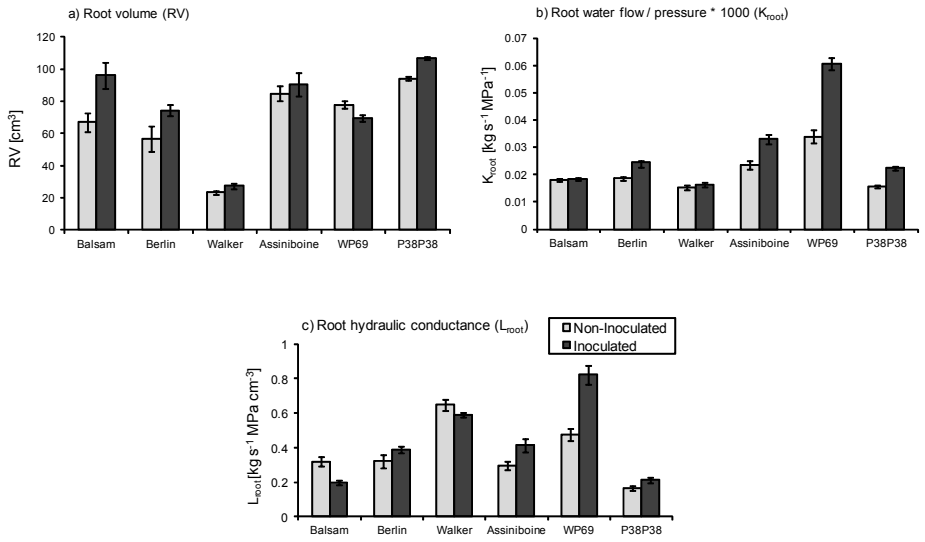


Figure 3. Leaf water flow / pressure, leaf area and leaf hydraulic conductance in six poplar hybrids non-inoculated (C and inoculated (M) with *Hebeloma crustuliniforme*. Mean values ± standard error (n=6).

Plant height, leaf water potential, chlorophyll content and chlorophyll fluorescence. Inoculation with H. crustuliniforme resulted in Study 2 in an increase in plant height (ΔH) for cultivars P. petrovskyana, P. deltoides Plantierensis, P. balsamifera by 19, 15 and 25% compared with non-inoculated plants, respectively (Table 1).

Leaf water potential (ψ) in both in non-inoculated and inoculated plants increased from the lower to upper leaves (Fig. 4). Statistically significant differences in ψ between non-inoculated and inoculated plants were observed only for *P. balsamifera* for the upper and middle leaves. (Table 2, Fig. 4).

Table 1. Plant height (Δ H-cm) after 3 and 11 weeks of growth in 3 cultivars of poplar mycorrhizal with *H. crustuliniformem.* Mean values ± standard error. (Exp.2).

Treatment	Week				Δ H [cm]	%C
	3	%C	11	%C		
P. petrowskyana						
C- Control	23,7 ± 1.4		62,5 ± 1,8		38,8 ± 2,0	
M- Mycorrhiza	22,9 ± 1,1	94,1	68,5 ± 1,5	109,6	46,2 ± 2,7	119,1
P. deltoides„Plantierensis"						
C- Control	15,1 ± 0,9		54,3 ± 1,3		39,2 ± 1,1	
M- Mycorrhiza	16,4 ± 0,8	108,6	61,5 ± 0,8	113,2	45,1 ± 1,1	115,1
P. balsamifera						
C- Control	24,7 ± 1,2		67,8 ± 1,1		42,4 ± 1,9	
M- Mycorrhiza	27,4 ± 1,0	110,9	80,4 ± 0,9	119,8	53,0 ± 1,4	125,0

In Study 1, chlorophyll concentration was measured after 11 weeks of plant growth only in the upper leaves (5th leaf from the top). For non-inoculated plants, leaf chlorophyll concentrations ranged from 33.0 in Walker to 50.7 in balsam poplar (Fig. 5d). Statistically significant increases in leaf chlorophyll concentrations of inoculated plants were observed in Berlin and WP69 (Fig. 5d). In Study 2, chlorophyll concentrations were higher in the upper compared with the lower leaves (Table 2, Fig. 4). Compared to non-inoculated plants, the leaves in inoculated plants showed an increase in chlorophyll concentration in young leaves. In the lower leaves (middle and bottom parts) of balsam poplar, chlorophyll concentrations were also significantly higher in inoculated compared with non-inoculated plants (Table 2, Fig. 4).

Table 2. Changes of water potential (ψ – MPa) and chlorophyll content (SPAD) of leaves taken from different part of plant stem in 3 cultivars of poplar mycorrhizal with *H. crustuliniforme.*(Exp. 2).

Leaf position	Leaf water potential [MPa]		Chlorophyll content [SPAD]	
	C	M	C	M
P. petrowskyana				
Bottom	-0,507	-0,508 ns	7,25	7,21 ns
Median	-0,539	-0,538 ns	8,38	9,09 ns
Upper	-0,663	-0,588 *	8,40	10,68 *
P. deltoides„Plantierensis"				
Bottom	-0,511	-0,508 ns	7,38	7,39 ns
Median	-0,541	-0,535 ns	8,41	9,07 ns
Upper	-0,632	-0,565 *	8,76	10,42 *
P. balsamifera				
Bottom	-0,513	-0,511 ns	7,75	8,08 ns
Median	-0,565	-0,548 *	9,01	11,11 *
Upper	-0,657	-0,578 *	10,18	13,20 *

ns, * - differences between C and M are not significant or significant at P<0,05 according to t-test, respectively.

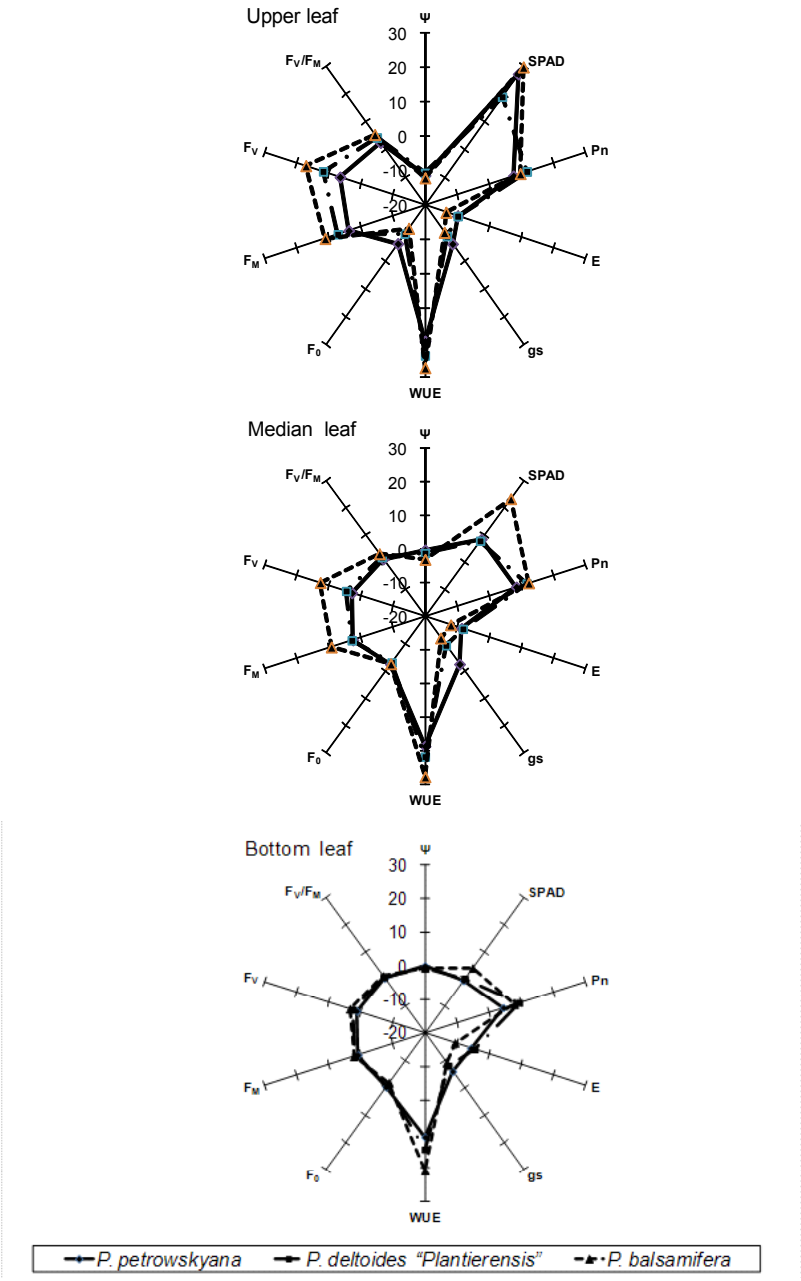


Figure 4. Radar graph of changes in physiological characters in different poplar cultivars. Results presented as a deviation from control plants (non-inoculated) in percent

Table 3. Changes of gas exchange parameters (Pn- Net photosynthesis, E- transpiration rate, g_S - stomatal conductance, WUE- water use efficiency index) of leaves taken from different part of plant stem in 3 cultivars of poplar mycorrhizal with *H. crustuliniforme.* (Exp. 2).

Leaf position	Gas exchange parameters							
	Pn [µmol (CO_2) $m^{-2} \cdot s^{-1}$]		E [mmol (H_2O) m^{-2} s^{-1}]		g_S [mmol (CO_2) m^{-2} s^{-1}]		WUE [µmol (CO_2) mmol (H_2O) $^{-1}$]	
	C	M	C	M	C	M	C	M
P. petrowskyana								
Bottom	8,9	9,3 ns	5,3	5,0 ns	168,4 ns	158,3	1,68	1,86ns
Median	9,4	10,2 ns	5,8	5,3 ns	189,3 ns	184,5	1,62	1,92 ns
Upper	11,9	12,8 *	6,1	5,5 *	209,3 *	196,4	1,95	2,33 *
P. deltoides„Plantierensis"								
Bottom	9,6	10,5 ns	4,5	4,3 ns	162,4 ns	149,3	2,13	2,44 ns
Median	10,2	11,4 *	4,9	4,5 *	179,2 *	162,3	2,08	2,53 *
Upper	11,0	12,3 *	5,1	4,6 *	189,0 *	172,1	2,16	2,67 *
P. balsamifera								
Bottom	10,1	10,9 ns	4,7	4,2 ns	142,3 ns	129,3	2,15	2,60 ns
Median	10,5	11,8 *	5,0	4,4 *	159,3 *	140,0	2,10	2,68 *
Upper	11,3	12,4 *	5,2	4,5 *	165,0 *	148,0	2,17	2,76 *

ns, * - differences between C and M are not significant or significant at $P<0,05$ according to T-test, respectively

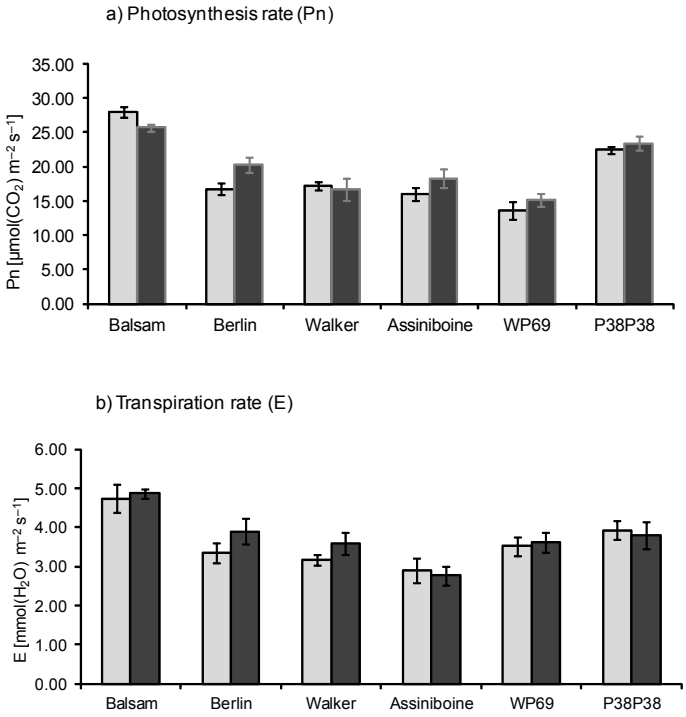
In Study 2, there was a tendency in both non-inoculated and inoculated plants to a decrease of initial fluorescence (F_0) values between the bottom and upper leaves, but there was an increase in other parameters including. maximal fluorescence Fm, variable fluorescence Fv, and maximum quantum yield of PS2 Fv/Fm (Table 4, Fig. 4). However, statistically significant differences between non-inoculated and inoculated plants were observed partly for the leaves from the upper part, and for *P. balsamifera* also for the middle part (Table 4).

*Gas exchange.*In Study 1, there was little effect of inoculation with *H. crustuliniforme* on gas exchange parameters in plants. Statistically significant increase in net photosynthesis (Pn) was observed only in Berlin, and that of stomatal conductance (gs) in Berlin and P38P38 clones (Fig. 5 abc). In Study 2, upper leaves in both inoculated and non-inoculated *P. petrovskyana, P. deltoides "Plantierensis", P. balsamifera* had higher Pn, transpiration rates (E), stomatal conductance (gs) and water use efficiency index (WUE) compared with the middle and lower leaves (Table 3). Significantly higher Pn, gs and WUE and lower E were measured in the upper leaves of inoculated compared to non-inoculated plants of all cultivars and in the middle leaves of *P. deltoides "Plantierensis"*and *P. balsamifera* (Table 3, Fig 4).

Table 4. Changes of chlorophyll *a* fluorescence parameters of leaves taken from different part of plant stem in 3 cultivars of poplar mycorrhizal with *H. crustuliniforme.* (Exp. 2).

Leaf position	Chlorophyll *a* fluorescence parameters							
	F_0		F_M		F_V		F_V/F_M	
	C	M	C	M	C	M	C	M
P. petrowskyana								
Bottom	262,4	261,5 ns	1139,2	1149,6 ns	876,8	888,1 ns	0,770	0,773 ns
Median	260,9	254,3 ns	1139,5	1168,4 ns	888,6	914,1 ns	0,773	0,782 ns
Upper	246,8	231,4 *	1163,2	1207,8 ns	916,4	976,4 *	0,788	0,808 *
P. deltoides„Plantierensis"								
Bottom	267,5	265,3 ns	1135,1	1155,2 ns	867,6	889,9 ns	0,764	0,770 ns
Median	262,8	254,6 ns	1165,1	1198,7 ns	902,3	944,1 ns	0,774	0,788 ns
Upper	248,7	224,3 *	1170,3	1255,3 *	921,6	1031,0 *	0,787	0,821 *
P. balsamifera								
Bottom	269,4	264,3 ns	1154,3	1179,5 ns	884,9	915,2 ns	0,767	0,776 ns
Median	264,3	257,3 ns	1180,0	1289,6 *	915,7	1032,3 *	0,776	0,800 *
Upper	247,3	218,3 *	1195,6	1330,0 *	948,3	1111,7 *	0,793	0,836 *

ns, * - differences between C and M are not significant or significant at P<0,05 according to t-test, respectively



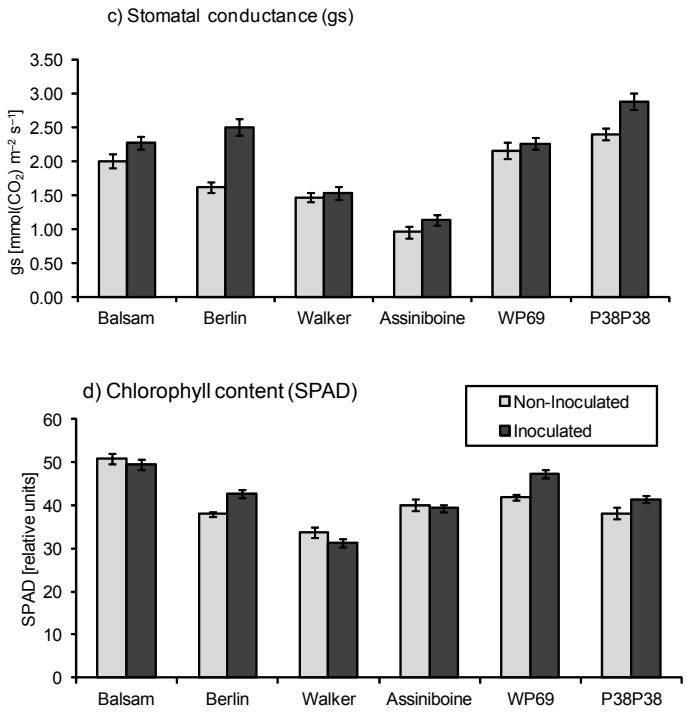


Figure 5. Gas exchange parameters and leaf chlorophyll concentrations in six poplar hybrids non-inoculated (C) and inoculated with *Hebeloma crustuliniforme* (M). Mean values ± standard error (n=6)

The F-values of two-way ANOVA expressing the influence of the factors implemented it experiments on the measured traits in poplar plants are presented in table 5 (experiment 1) and in table 6 (experiment 2). In first experiment there is a significant influence of genotype on all measured parameters. Influence of treatment is distinct in gs, root volume, root hydraulic conductance and root hydraulic conductivity. In the experiment 2 influence of treatment and the leaf position on all measured values was observe and in most cases in case of genotype. Also there is influence of treatment x leaf position on leaf water potential, chlorophyll content, Fv and Fv/Fm.

Table 5. The F-values of two-way ANOVA expressing the influence of the factors implemented it the experiment 1 on the measured traits in poplar plants

	Pn	E	gs	chl	LA
Treatment	2.57	1.01	34.758 ***	5.99 *	3.581
Genotype	41.06 ***	11.76 ***	69.518 ***	64.31 ***	6.768 ***
Genotype x treatment	2.04	0.55	5.220 ***	5.30 ***	3.443 **
	K_{leaf}	K_{lam}	RV	K_{root}	L_{root}
Treatment	0.0650	1.7180	13.081 ***	115.280 ***	20.008 ***
Genotype	25.8135 ***	15.8462 ***	58.450 ***	148.990 ***	104.807 ***
Genotype x treatment	0.5051	2.1400	3.572 **	24.921 ***	24.185 ***

* - p<0.05, ** - p<0.01, *** - p<0.001.

Table 6. The F-values of two-way ANOVA expressing the influence of the factors implemented it the experiment 2 on the measured traits in poplar plants

	ψ	Pn	E	gs	WUE
Treatment	40.05 ***	13.057 ***	7.372 **	21.60 ***	65.814 ***
Genotype	2.66	2.710	11.014 ***	52.15 ***	38.572 ***
Leaf position	141.95 ***	21.463 ***	3.141 *	30.77 ***	6.393 **
Genotype x treatment	0.32	0.353	0.096	0.55	2.293
Treatment x Leaf	29.24 ***	0.228	0.278	0.13	1.618
Genotype x Leaf position	0.62	2.258	0.095	1.22	1.215
Genotype x treatment x Leaf position	0.83	0.021	0.008	0.12	0.061
	SPAD	F_0	F_M	F_V	F_V/F_M
Treatment	39.795 ***	5.64 *	13.10 ***	34.20 ***	55.4 ***
Genotype	22.869 ***	0.01	5.56 **	10.04 ***	5.9 **
Leaf position	72.555 ***	12.70 ***	6.83 **	24.36 ***	67.5 ***
Genotype x treatment	2.843	0.03	1.47	2.86	1.6
Treatment x Leaf	11.493 ***	1.37	1.77	5.17 **	8.8 ***
Genotype x Leaf position	2.411	0.17	0.52	1.22	1.5
Genotype x treatment x Leaf position	0.406	0.15	0.27	0.53	0.5

* - p<0.05, ** - p<0.01, *** - p<0.001.

Discussion

Divergent opinions on advantages of ECM association, which can be found in the literature, likely reflect differences in factors such as plant and fungal species, soil environmental conditions, time of inoculation as well as morphological and anatomical characteristic of plant root and fungal mycelium (Koide 1985, Johnson *et al*. 1997, Martin *et al*. 2001, Brundrett 2002, Jifon *et al.* 2002, Kottke 2002).

In Study 2, there were statistically significant differences in the frequency (MF_R) and intensity (MC_R) of mycorrhizal colonization between the studied poplar clones (Fig.1). The frequency of mycorrhizae was high, reaching the values of 71 to 80% while intensity of colonization was lower and ranged from 30 to 35%.

In study 2, inoculated plants had significantly greater height (H), chlorophyll content (SPAD), and in leaf water potential (ψ) (Table 1, 2, Fig. 4). Frequently, plant growth depression following mycorrhizal colonization can be attributed to the carbohydrate drain by the fungus, while growth stimulation may occur when the benefits of increased nutrient uptake exceed the carbon cost of the association (Schroeder and Janos 2004, Correa *at al.* 2006). Similarly to plant growth, there are various opinions on the benefits of mycorrhizas for the host plant water relations (Muhsin and Zwiazek 2002, Correa et al. 2006, Lehto and Zwiazek 2011). It could be speculated that the effects of mycorrhizas on water uptake may also be linked to the plant nutritional status (Lehto and Zwiazek 2011). The concentration of nutrients may affect root hydraulic conductivity through the effects on aquaporins (Muhsin and Zwiazek 2002) and it has been long known that mycorrhizal associations may increase phosphorus and nitrogen uptake by plants (Smith and Read 1997). Also the form of nitrogen may affect root water transport (Steudle and Meshcheryakov). Ammonium is predominant form of mineral nitrogen in the forest and ECM fungi increase ammonium uptake by woody plants (Rudawska *et al*. 1994, Javelle *et al*. 1999, Correa *et al*. 2006).

In our study, inoculation with *H. crustuliniforme* increased root hydraulic conductance in balsam poplar, Assiniboine and WP69 and leaf hydraulic conductance in *P. balsamifera* (Fig. 2,3). Numerous studies have reported an increase in root hydraulic conductance by ectomycorrhizas which may be caused by the increase in the transmembrane (Marjanović *et al*. 2005, Lee *et al*. 2010) or apoplastic (Muhsin and Zwiazek 2002) water transport. However, ectomycorrhizal associations do not always stimulate root hydraulic conductivity (Nardini *et al*. 2000, Siemens and Zwiazek 2008, Yi *et al*. 2008) and the reasons for these differences in plant responses remain unclear. The effects of mycorrhizal associations on leaf water transport have been little studied, however, the indirect effect through plant nutritional status could be expected (Calvo-Polanco *et al*. 2008).

In Study 1, the differences in gas exchange parameters between non-inoculated and inoculated plants were small and mostly statistically insignificant (Fig. 5). However, in Study 2, ectomycorrhizal associations triggered an increase in net photosynthesis of leaves from the upper plant part, likely due to higher stomatal conductance facilitating absorption of CO_2 (Table 3, Fig. 4).

The results of this study showed that leaves from the upper plant part in non-inoculated plants had higher value of minimal florescence (F_0) and lower value of maximal fluorescence (F_M) compared with inoculated plants. Also F_V/F_M in inoculated plants was significantly higher than in non-inoculated plants but only in the

upper leaves (Table 4, Fig 4). The ratio of F_V/F_M is a measure of the capacity of the primary photochemistry of photosystem II which itself is sensitive to a changes of environmental growth conditions (Lichtenthaler 1992, Lichtenthaler *et al*. 2005). According to Lichtenthaler 1992 and Strasser *et al.* 2000 parameters of chlorophyll a fluorescence may be used as a direct indicator of plant photosynthetic activity.

In conclusion, the effects of inoculation on the physiological responses of the studied poplars were relatively minor and varied depending on the clone and leaf age (position on the stem). However, even small differences in physiological parameters between mycorrhizal and non-mycorrhizal poplars may become highly significant if they persist over the longer time. More distinct responses could occur if the mycorrhizal inoculation is accompanied by a stress factor *e.g.* drought. Walker, Assiniboine and Northwest are among the hybrid poplar clones which are moderately drought tolerant (Silim et al. 2009). Moderately tolerant clones maintained higher net photosynthesis at lower leaf water potential than the other clones. Stomatal closure was gradual in tolerant and in moderately clones e.g. Northwest. Arango-Velez *et al.* (2011) studied influence of drought on stomatal factors in hybrids P38P38, Okanese, Walker, Assiniboine, Berlin and in balsam poplar (*Populus balsafimera*). They showed differences in stomatal sensitivity to drought and vulnerability to stem xylem cavitation. P38P38 reduced stomatal conductance in response to mild stress while the balsam poplar clone maintained high leaf stomatal conductance under more severe drought conditions. Differences between the severely stressed clones were also observed in leaf water potentials with no or relatively small decreases in case of Assiniboine, P38P38, and Walker. Tschapliski *et al.* (1998) showed that *Populus deltoides x P. nigra* shows better drought resistance in comparison to *Populus trichocarpa x P. deltoides* and maintained higher midday leaf water potentials, suggesting better stomatal control of water loss. Therefore, longer-term studies may be needed in poplars to evaluate the significance of physiological changes due to ECM fungal inoculation for tree growth and survival.

Acknowledgements. We thank the Natural Sciences and Engineering Research Council of Canada (NSERC) and AlbertaPacific Forest Indusries (AL-Pac) for funding of this study. We are grateful to Al-Pac for providing hybrid poplar cuttings and the *Arboretum* of the Institute of Dendrology, PAS in Kórnik for cuttings of poplar cultivars. Also we thank Kostrzyca Forest Gene Bank for mycorrhizal biopreparation of *Hebeloma crustuliniforme.* We are indebted to Dr. Katarzyna Hura and dr. Barbara Wojciechowska for their assistance with mycorrhizal analysis.

References

Anderson T.A., Coats J.R. 1994. Bioremediation through Rhizosphere Technology.Washington D.C.

Arango-Velez A., Zwiazek J.J., Thomas B. R., Tyree M. T. 2011 Stomatal factors and vulnerability of stem xylem to cavitation in poplars. Physiol. Plant. 143: 154–165.

Armstrong R.D., Helyar K.R., Christie E.K. 1992. Vesicular-arbuscular mycorrhiza in semi-arid pastures of soth-west Queensland and their effect on growth responses to phosphorus fertilizers by grasses. Aust.J.Agr.Res. 43: 1143-1155.

Brundrett M. 2002. Coevolution of roots and mycorrhizas of land plants. New Phytologist. 154: 275–304.

Calvo-Polanco M., Zwiazek J.J., Voicu. M.C. (2008) Responses of ectomycorrhizal American elm (*Ulmus americana*) seedlings to salinity and soil compaction. Plant and Soil 308:189-200.

Calvo-Polanco M., Jones M.D.,Zwiazek J.J. 2009 Effects of pH on NaCl resistance of American elm (*Ulmus americana*) seedlings inoculated with *Hebeloma crustuliniforme* and *Laccaria bicolor*. Acta Physiol. Plant. 31:515-522

Cochard H., Guillot A., Tyree M.T., Sakr S., Herbette S., Venisse J., Barigah T.S., Correa A., Strasser R.J., Martins-Loucao M.A. 2006. Are mycorrhiza always beneficial. Plant and Soil. 279: 65-73.

Gryta H., Debaud J.C., Effosse A., Gay G., Marmeisse R. 1997. Fine-scale structure of populations of the ectomycorrhizal fungus *Hebeloma crustuliniforme* in costal sand dune forest ecosystems. Mol. Ecol. 6-353.

Hooker J. E., Munro M., Atkinson D. 1992 Vesicular-arbuscular mycorrhizal fungi induced alteration in poplar root system morphology. Plant and Soil 145: 207-214.

Hutchison, L.J. 1991 Description and identification of cultures of ectomycorrhizal fungi found in North America, Mycotaxon 42: 387-504

Javelle A., Chalot M., Sonderström B., Botton B. 1999. Ammonium and methylamine transport by the ectomycorrhizal fungus *Paxillus involutus* and ectomycorrhizas. FEMS Microbiol Ecol, 30: 355-366.

Johnson NC., Graham J.H. Smith F.A. 1997. Functioning of mycorrhizal associations along the mutualism-parasitism continuum. New Phytol. 135-575.

Jifon J.L., Gracham J.H., Droillard D.L., Syversen J.P. 2002. Growth depression of mycorrhizal Citrus seedlings grown at high phosphorus supply is mitigated by elevated CO_2. New Phytol. 155: 133-142.

Koide T. 1985. The nature of growth depression in sunflower caused by vesicular-arbuscular mycorrhizal infection. New Phytol. 99: 449-462.

Kottke I. (2002). mycorrhiza-rhizosphere determinants of plant communities. In: Plant roots – the hidden half. Waisel Y. Eshel A. Kafkafi U. (ed). Marcel Decker Inc. New York, Basel. 919-932.

Landhausser S.M., Muhsin T.M., Zwiazek J.J. 2002. The effect of ectomycorrhizae on water relations in aspen (*Populus tremuloides*) and white spruce (*Picea glauca*) at low soil temperatures. Can. J. Bot. 80(6): 684–689.

Lee S.H., Calvo-Polanco M., Chung G.C. , Zwiazek J.J. 2010. Cell water flow properties in root cortex of ectomycorrhizal (*Pinus banksiana*) seedlings. Plant Cell and Envir. 33:769-780.

Lehto T., Zwiazek J.J. 2011 Ectomycorrhizas and water relations of trees: a review. Mycorrhiza 21:71–90

Lichtenthaler H.K.1992. The Kautsky Effect: 60 years of chlorophyll fluorescence induction kinetics. Photosynth. 27, 45-55.

Lichtenthaler H.K., Buschmann C., Knapp M. 2005. How to correctly determine the different chlorophyll fluorescence parameters and the chlorophyll decrease ratio R_{Fd} of leaves with the PAM fluorometer. Photosynth. 43, 379-393.

Marjanović Ž., Uehlein N., Kaldenhoff R., Zwiazek J.J., Wieß M., Hammp R. 2005 Aquaporins in poplar: what a difference a symbiont makes!. Planta 222:258–268

Martin F.M., Perotto S., Bonfante P. 2001. Mycorrhizal fungi: A fungal community at the interface between soil and roots. In: The rhizospfere biochemistry and organic substances at the soil-plant interface. Pinton R, Varanini Z, Nannipieri P. (ed). Marcel Decker Inc. New York, Basel. 263-296.

Meyer FH. 1973 Distribution of ectomycorrhizae in native and man-made forests. In: Marks GC, Kozlowski TT (eds) Ectomycorrhizae. Their Ecology and Physiology. Academic Press, London, pp 79–105.

Muhsin T.M., Zwiazek J.J. 2002 Ectomycorrhizas increase apoplastic water transport and root hydraulic conductivity in *Ulmus americana* seedlings. New Phytol 153:153–158.

Nardini A., Salleo S., Tyree M.T., Vertovec M. 2000 Influence of the ectomycorrhizas formed by Tuber melanosporum Vitt. on hydraulic conductance and water relations of Quercus ilex L. seedlings. Ann. For. Sci. 57:305–312.

Nguyen H, Calvo-Polanco M, Zwiazek J.J. 2006. Gas exchange and growth responses of ectomycorrhizal *Picea mariana, Picea glauca*, and *Pinus banksiana* seedlings to NaCl and Na_2SO_4. Plant Biol. 5: 646-652.

Pendelton R.L., Smith B.N. 1983. Vesicular-arbuscular mycorrhizae of weedy and colonizer plant species disturbed in Utah. Oecologia 59: 296-301

Phillips J.M., Hayman D.S. 1970. Improved procedures for clearing roots and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. Transactions of British Mycological Society 55: 158-160.

Rudawska M., Kieliszewska-Rokicka B., Debaut J.C., Lewandowski A., Gay G. 1994. Enzymes of ammonium metabolism in ectendomycorrhizal and ectomycorrhizal symbiosis of pine. Physiol. Plant 92: 279-285.

Sack, L., P.J. Melcher, M.A. Zwieniecki, Holbrook N.M. 2002 The hydraulic conductance of the angiosperm leaf lamina: a comparison of three independent measurement methods. J. of Exp. Bot. 53, 2177-2184

Sell J., Kayser A., Schulin R., Brunner I. 2005 Contribution of ectomycorrhizal fungi to cadmium uptake of poplars and willows from a heavily polluted soil. Plant and Soil 277:245–253

Schroeder M.S., Janos D.P. 2004. Phosphorus and intraspecific density alter plant response to arbuscular mycorrhizas. Plant Soil. 264: 335-348.

Siemens J.A., Zwiazek J.J. 2008. Root hydraulic properties and growth of balsam popular (*Populus balsamifera*) mycorrhizal with *Hebeloma crustuliniforme* and *Wilcoxina* var. *mikolea*. Mycorrhiza, 18: 393-401.

Silim S., Nash R., Reynard D., White B., W. Schroeder 2009 Leaf gas exchange and water potential responses to drought in nine poplar (Populus spp.) clones with contrasting drought tolerance. Trees 23: 959–969.

Smith S.E., Read J.W. 1997. Mycorrhizal symbiosis. 2nd ed., Academic, London UK.

Steudle E., Meshcheryakov A. B. (1996) Hydraulic and osmotic properties of oak roots. J. Exp. Bot. 47: 296: 387-401

Strasser R., Srivastava A., Tsimilli-Michael M. 2000. The fluorescence transient as a tool to characterise and screen photosynthetic samples. In Probing Photosynthesis: Mechanisms, Regulation and Adaptation. ed. M U P Yunus, Pathre Mohanty. pp. 445–483. Taylor and Francis, London.

Trouvelot A., Kough J.L., Gianinazzi-Pearson V. 1986. Mesure du taux de mycorhization VA d'un systéme radiculaire. Recherche de méthodes d'estimationayant une signification functionally: Mycorrhizae: physiology and genetics. Proceedings of the 1st ESM, Dijon, INRA: 217-221.

Tschaplinski T. J., Tuskan G. A., Gebre G. M., Todd D. E. 1998 Drought resistance of two hybrid Populus clones grown in a large-scale plantation. Tree Physiology 18: 653-658.

Turnau K., Anielska T., Ryszka P., Gawroněski S., Ostachowicz B., Jurkiewicz A. 2008. Establishment of arbuscular mycorrhizal plants originating from xerothermic grasslands on heavy metal rich industrial wastes - New solution for waste revegetation. Plant and Soil 305: 267-280.

Tyree M.T., Patiño S., Bennink J., Alexander J. (1995). Dynamic measurements of root hydraulic conductance using a high-pressure flowmeter in the laboratory and field. J of Exp Bot 46, 83–94.

Voicu M.C., Zwiazek J.J. (2004). Cyclohexamide inhibits root water flow and stomatal conductance in aspen (*Populus tremuloides*) seedlings. Plant Cell Environ 27:199–208.

Yi H., Calvo-Polanco M., MacKinnon M.D., Zwiazek J.J. (2008) Responses of ectomycorrhizal *Populus tremuloides* and *Betula papyrifera* seedlings to salinity. Environ. Exp. Bot. 62:357–363.

PLANT FUNCTIONING UNDER ENVIRONMENTAL STRESS P. 115-122

Cracow, 12th to the 15th September, 2012

THE ROLE OF 24-EPIBRASSINOLIDE IN THE RESPONSE OF PLANTS SUNFLOWERS TO COPPER STRESS

Angelika Filova*, Oksana Sytar**

** Department of Plant Physiology, Slovak University of Agriculture in Nitra, Tr. A. Hlinku 2, 949 01 Nitra, Slovak Republic*

*** Department of Plant Physiology and Ecology, Taras Shevchenko National University of Kyiv, Volodymyrs'ka St. 64, 01601 Kyiv, Ukraine*

Corresponding author; angela.filova@uniag.sk

Abstract. The purpose of our experiments was to consider sensitivity of the chosen 6sunflower cultivars (Helianthus annuus L. Cv. Belinda, cv. Codiwer, cv. ESPrim, cv. MAS 95, cv. MAS 97 and cv. Spirov) to copper ions with the possibility of elimination by means of brassinosteroids on the ground of physiological characteristics (content of dry basis, amount of assimilatory pigment, praline content, RWC as well as MDA) and to show possible resistance mechanisms of this plant to copper ions. Lipid peroxidation is a biochemical marker for the free radical mediated injury. Character of changes in lipid peroxidation (LP) depends on intensity of influence stressor and from plant sensitivity. In leaves of experimental plants cultivars MAS 97 and SPIROV after Cu treatment by concentration 5 mM the level of malondialdehyde (MDA) content has been increased on 11% and 30% respectively. The higher MDA content has been observed in leaves of cultivar ESPrim. In the other experimental variants under Cu treatment content of MDA was on control level which evidence about more less sensitivity these cultivars to Cu treatment. The combination of Cu+ BRs has been shown MDA content in leaves of all experimental plants on control level which can evidence about protection effect of BRs under Cu treatment on the leaves of sunflower cultivars.

Key words: Brassinosteroids; Chlorophylls; Copper; Helianthus annuus L; MDA; Proline

Introduction

Plants are during their life exposed to the effect of many stressful factors of biotic and abiotic disposition (e.g. heavy metals), which can not only slow down their life functions, but also harm particular organs and in isolated instances it can lead to the plant death. The stress factor influence on plants can cause oxidative

stress, which is typical of rapid temporary production of big amount of active forms of oxygen (AFO), and break a balance between the production and reduction of AFO. Malondialdehyde (MDA) is a product of oxidative harm of lipids and it represents the best explored marker of the oxidative stress (Behnamia *et al.* 2009).

Copper is an activator of the enzymes which participates on a metabolism N and synthesis of proteins. In a case of copper lack the content of free nitrogenous substances in the cells is increasing at the expense of protein production (Haidari 2010). Therefore it is necessary, especially by an intensive fertilization, to ensure that a plant has got enough copper. The biggest supply of copper is in the chloroplasts in which it works as a stabilizer of the chlorophyll. The lack of copper causes a disruption of the photosynthesis process and it leads to a limitation of the growth intensity. In spite of the physiological importance of copper, on the other hand excessive content of this element in the soil becomes the stressful factor for the plants (Divi and Krishna 2000, Hayat et al. 2000). The effect of copper toxicity on the plants is truly provable. However many aspects of the toxic effect of copper on the plants are clarified, the results of several physiological and biochemical analysis are controversial. Changes in the processes of photosynthesis and water regime influenced by the copper effect in the plants from a family Asteraceae are markedly different (Ali et al.2007, Bates et al. 1973). Big variability in seed germination noticed for instance in the various hybrids of sunflower. Big variety in a reaction of plants on the heavy metals is constantly revealing new questions which solution can play very important role from the point of view of preserving pure environment and man's health. A sunflower is thanks to the big biomass an ideal plant for decontamination of soil (phytoremediation), whereas it accumulates notable content of heavy metals in the roots Fariduddin et al. 2009)

Brassinosteroids (BRs) are steroidal phytohormones with a wide scale of effects. By exposing plants to the drought stress as well as to the heavy metals their survival is improved and also resistance and yield are increased. The plant response is regulated directly (by the synthesis of metabolites) or indirectly (by induction of antioxidizing compounds and enzymes), often in the interaction with other phytohormones. Experiments studying an impact of brassinosteroids on the reaction of plants stressed e.g. by water deficit and toxicity of heavy metals are different in various parameters. Therefore their proper interpretation is difficult. Thanks for better understanding of their influential mechanisms, it is possible to discover new possibilities for their usage in agricultural biotechnology. Meanwhile, a research of other aspects like reception, transport and stability of Brs, as well as development of Brs analogues with high activity should continue. Only with the knowledge of unique mechanisms of plant resistance to stress and with the predictable effects in the field conditions, it will be possible to use a whole potential of BRs in the practise. In the majority of studies demonstrating anti-stress effects of BRs it is used an exogenous treatment. Nowadays the exogenous application of BRs is common

field method in Japan, China, Russia and Belarus (Bajygus and Hayet 2009, Divi and Krishna 2000).

We tested the hypothesis that application of EBL will alleviate the toxic effect of copper applied through shotgun approach on sunflower growth and metabolism and will ultimately result in improved yield at harvest and give the farmers a better option to grow their crops under stress condition. The purpose of our experiments was to consider sensitivity of the chosen sunflower cultivars to copper ions with the possibility of elimination by means of brassinosteroids on the ground of physiological characteristics (content of dry basis, amount of assimilatory pigment, praline content, RWC as well as MDA) and to show possible resistance mechanisms of this plant to copper ions.

Material and methods

There were used 6 hybrids of Common Sunflower (Helianthus annuus L): Belinda (FRA), Codiwer (FRA), ESPrim (FRA), MAS 95 (SVK), MAS 97 (HUN) a Spirov (BUL) which were provided by the company FINAGRO spol. s.r.o. Bratislava. Sterilized seed were budding for 24 hours in the distilled water and consequently were sprouting on Petri dish (Ø 15 cm) with wetted filter paper in darkness. After 3 – 4 days of sprouting were approximately equally sprouted seeds sown into 15 l plastic bowls with a mixture of peat and perlite (in a ratio of 4:1), poured with distilled water which corresponds to maximum soil sorption capacity (~1000 ml). Plants were left to grow to the beginning phase of butonization – stars (32 day). Afterwards they were poured with distilled water (control variant plants) or with the solution enriched with copper with concentration 80 μM $CuSO_4$.$5H_2O$ and the last chosen combination was Cu +EBL (80 μM $CuSO_4$.$5H_2O$ + 100 μM EBL). Waterings in the next phases of the experiment did not contain metal or brassinosteroids. According to the needs distilled water was applied which quantity corresponded to the half of dose of maximum sorption substrate capacity (500 ml). After 10 days sprouts were separated from the roots, roots were cleaned off soil and thoroughly washed with water. Consequently, we measured their length, fresh and dry weight. The experiment was realized in three repetitions. The gained data was analysed by mathematical-statistical methods of programe MS Excel. Meaning of the differences by comparing the sets were determined by the Student test.

Hormone preparation Brassinolide 24-epibrassinolide was purchased from Sigma-Aldrich, USA. Stock solution (10^{-4}M) was prepared by dissolving the hormone in 5 ml of ethanol in a 100 ml volumetric flask. Five milliliters of 0.5% surfactant "Tween-20" was added to it, and the final volume was made up to the mark by using double distilled water (DDW). The 10^{-8}M concentration of EBL was prepared by dilution of the stock solution.

Analysis of lipid peroxidation. Malondialdehyde (MDA) was measured spectrophotometrically by the reaction with Thiobarbituric acid. Plant material (roots 500mg and leaves 200mg) was homogenized in 3 ml 0.1 M Trichloroacetic acid (TCA). The mixture was warmed up to temperature 95 ^{o}C for 30 min and afterwards was centrifugated by 10,000 × g per 5 min. Quantity of MDA was determined according to Heath and Packer [10] by wave length 532 nm and conversion using molar extinction coefficient 155 $m.M^{-1}.cm^{-1}$.

Chlorophyll and photosynthesis measurements The chlorophyll content in fresh leaf samples was measured by using a SPAD chlorophyll meter (SPAD-502, Konica Minolta Sensing, Inc., Japan).

Determination of proline content The proline content in fresh leaf and root samples was determined by the method of Bates *et al.* (1973). Samples were extracted in sulfosalicylic acid. To the extract, an equal volume of glacial acetic acid and ninhydrin solutions was added. The sample was heated at 100°C, to which 5 ml of toluene was added. The absorbance of the aspired layer was read at 528 nm on a spectrophotometer.

RWC was calculated by placing the values in the following formula:

$$RWC = (\text{fresh mass} - \text{dry mass})/(\text{turgor mass} - \text{dry mass}) * 100\ \%$$

Results and Discussion

Tested cultivars of Common Sunflower showed according to the tested parameters, such as length and weight of sprouts and fresh weight of roots, relatively high rate of tolerance. In spite of the fact that no significant visual symptoms of toxic effect of metal were markedly noticeable, we detected decrease in the content of dry basis in the roots (less than 25 – 39 % in comparison with the control of two tested cultivars treated by Cu cv. MAS 97 and cv. SPIROV). Cv. Belinda, cv. Codiwer a cv. MAS 95 tends to be the most resistant or tolerant to Cu toxicity from the point of view of evaluation of morphological parameters of particular cultivars.

BRs treatment completely compensated reduction of biomass caused by Cu toxicity. In the comparison with the untreated plants (control and Cu stress) there was noticed an improvement of root growing in the treated plants. Yu *et al.* (2004) and Zhang *et al.* (2008) observed that BRs application improved assimilation of carbon and nitrogen by the stabilization of membrane structures in the stressful conditions and also improved general growth and plant photosynthesis. Photosynthetic apparatus cv. MAS 97, cv. SPIROV and cv. ESPrim reacted the most sensitive to the dose of copper what expresses in the content reduction especially of chlorophyll a (by 41%), chlorophyll b (by 22%) and carotenoids (by 29%). Negative impact on the photosynthetic apparatus efficiency is one of the typical signs of the effect of various kinds of abiotic stress such as drought, high temperature, and also heavy metals.

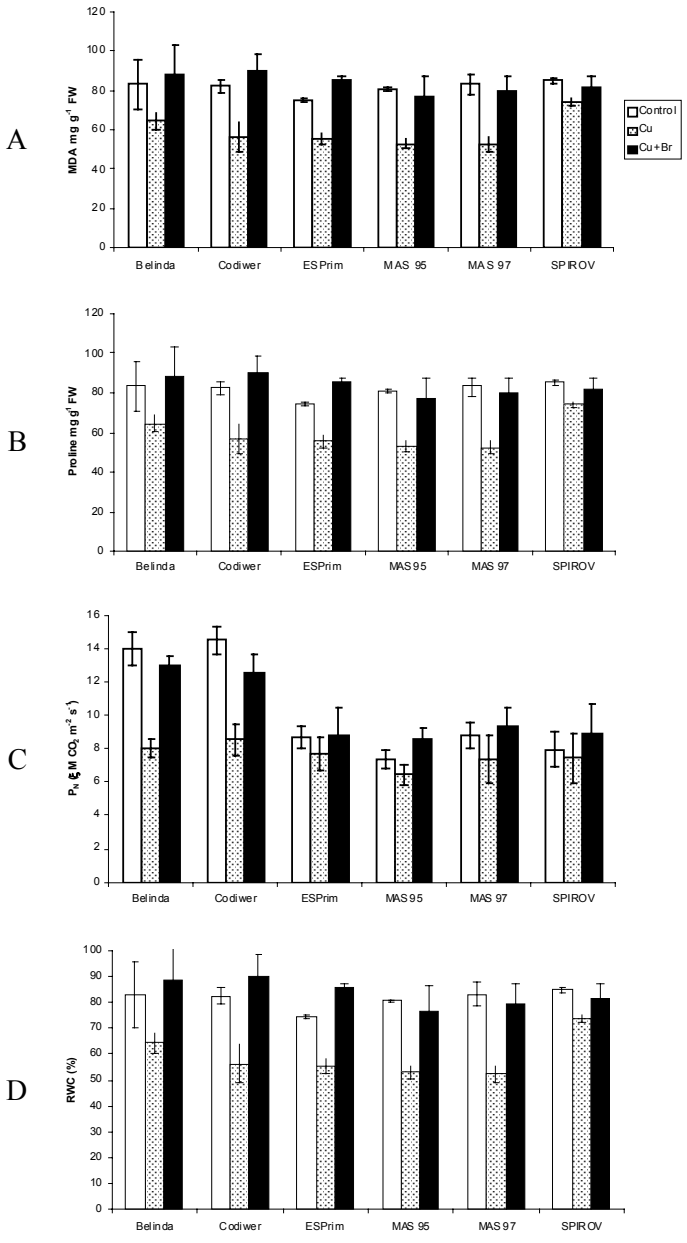


Fig 1. Effect of EBL on A: MDA content, B: proline content, C: net photosynthetic rate and D: relative water content in sunflower plants subjected to Cu stress

Brassinosteroids induced an improvement in photosynthesis efficiency which can be caused by stomatal or non-stomatal factors or by their combination. Influence of BRs on a conductivity of stomas was noticed by Hayat *et al.* (2000, 2010) and Fariduddin *et al.* 2009). Non-stomatal efficiency limitations of photosynthesis can be related with the photosynthetic pigments, concentration and activity of enzyme Ribulose-1,5-bisphosphate carboxylase oxygenase (RuBisCo) and use of assimilative products. (Yuan *et al.* 2010) consider as an acceptable explanation that exogenous application of BRs is increasing the capacity of carbon oxide assimilation in Calvin cycle using increased initial activity of RuBisCo. In the following experiments the BRs treatment reduced the decreasing of chlorophyll content and assimilation speed, increased efficiency of photosystem II and activity of enzymes, such as RuBisCo, nitroreductase and glutamine synthase (Yuan *et al.* 2010). Carotenoids are pigments protecting chlorophylls from the photo-oxidative damage [11]. Their content is decreasing with the increasing level of the oxidative stress caused by the Cu toxicity in all cultivars of Common Sunflower and is significantly higher in the plants treated by BRs, in the comparison with the controlled ones.

According to the measurement of MDA level it is likely that BRs help the effective catching of ROS by increased activity of the antioxidative enzymes system. Production of MDA and other aldehydes in the plants exposed to the Cu toxic stress is a dependable indicator of the production of free radicals in the plant tissue and today it serves as the indicator of lipid peroxidation and meanwhile of stress damage, such as heavy metals at the cell level (Heideri 2010). The cultivars resistant to the copper toxicity are able to catch the free radicals and less MDA is produced with the decreasing content of hydrogen peroxide in a comparison with the sensitive cultivars [5]. On the basis of gained data we can claim MAS 97, SPIROV and ESPrim belong to the sensitive cultivars of sunflower as they produced increased amount of MDA (more than 11 – 30% in a comparison with the control). On the second hand, among tolerant to Cu toxicity sunflower cultivars belong Belinda, Codiwer and MAS 95. Stated cultivars produced lower part of MDA in a comparison with the controlled variant.

With the Cu + BRs combination we noticed decreased MDA content in the leaves of all common Sunflower cultivars, what indicates possible elimination of copper toxicity in the sunflower plants using the exogenous application of brassinosteroids.

Plants raised from the seeds given presowing seed soaking treatment in 80 μM$CuSO_4.5H_2O$ showed significant reduction in all measured growth parameters (i.e., length, fresh and dry mass of root and shoot, and leaf area) irrespective of varietal difference. However, treatment with EBL to the foliage favored growth and neutralized the negative effects generated by Cu more effectively in all cultivars. Significant reduction in photosynthetic parameters occurred from Cu application, net photosynthetic rate (PN), and relative water capacity (RWC) of six cul-

tivars, given presowing seed soaking treatment in CuSO4, as compared to their respective controls.

All varieties showed significantly different responses to the spray treatments. Enzyme activity increased in response to both metal and hormone treatment in six cultivars, but Codiwer, showed higher enzymatic activity than did Belinda, ESPrim, MAS 95 and MAS 97 in response to the treatments. The foliar spray of either with 24-epiBL significantly enhanced the growth, photosynthesis, antioxidant enzymes and proline content in copper stressed sunflower plants.

The exogenous application of plant hormones has been found to counter toxic effects of various abiotic stresses. Brassinosteroids (BRs) are a new type of phytohormones that elicit a wide range of physiological processes in plants (Bajguz and Hayet 2009, Behnamia *et al.* 2009, Hayat *et al.* 2010). At present, 70 analogs have been identified, and among these, three natural brassinosteroids (brassinolide, 24-epibrassinolide (EBL), and 28-homobrassinolide (HBL)) are known to have an economic impact on plant metabolism, growth and productivity, and experience high stability under field conditions (Ali *et al.* 2007).

Conclusion

Many aspects of the copper toxic efficiency on the plants are clarified, however results of several physiological and biochemical analysis are controversial. At the same time, high variability of plant reaction to the heavy metal ions depending up the genotypes complicates unambiguity of the conclusions. Deeper biochemical and molecular-biological analysis can contribute to revealing of other possible mechanisms of Common Sunflower resistance to copper ions or other heavy metals. Wide scale of BRs effects on the plants stressed by the heavy metals instigates to search for mechanisms and connections which disproves old concepts and motivates development of new methods.

References

Ali.B, Hayat S, Ahmad A. (2007) 28-Homobrassinolide ameliorates the salt stress in chickpea (Cicer arietinum). Environ Exp Bot 59:33–41.

Bajguz A, Hayat S. (2009). Effects of brassinosteroids on the plant responses to enviromental stresses. Plant Physiology and Biochemistry 47: 1-8

Bates L.S, Waldron R.P., Teaxe I.W. (1973). Rapid determination of free proline for water stress studies. Plant and Soil 39:205–207

Behnamnia M. , Kalantari K.M. Rezanejad F. (2009). Exogenous application of brassinosteroid alleviates drought-induced oxidative stress in Lycopersicon esculentum. General and Applied Plant Physiology 35(1-2): 22-34

Divi U.K, Krishna, P. (2009). Brassinosteroid: a biotechnological target for enhancing cropyield and stress tolerance. New Biotechnology 26: 131-136

Fariduddin Q, Yusuf M., Hayat S., Ahmad A.(2009). Effect of 28-homobrassinolide on antioxidant acapacity and photosynthesis in Brassica napus plants exposed to different levels of copper. Enviromental and Experimental Botany 66: 418-424

Hayat S., Ahmad A., Mobin M., Hussain A., Fariduddin Q. (2000). Photosynthetic rate, growth and yield of mustard plants sprayed with 28-homobrassinolid. Photosynthetica 38: 469-471

Hayat S., Hasan S.A., Yusuf M., Hayat Q., Ahmad A. (2010). Effect of 28-homobrassinolide on photosynthesis, fluorescence and antioxidant system in the presence or absence of salinity and temperature in Vigna radiata. Environ Exp Bot 69:105–112

Heidari M. (2010). Nucleic acid metabolism, proline concentration and antioxidants enzyme activity in canola (Brassica nupus l.) under salinity stress. Agric Sci China 9: 504–511

Heath. RL., Packer L. (1968). Photoperoxidation in isolated chloroplasts. I. Kinetics and stoichiometry of fatty acid peroxidation. Archives in Biochemistry and Biophysics125,189–198

Li G., Wan S., Zhou J., Yang Z., Qin P. (2010). Leaf chlorophyll fluorescence, hyperspectral reflectance, pigments content, malondialdehyde and proline accumulation responses of castor bean (Ricinus communis L.) seedlings to salt stress levels. Ind Crops Prod 31:13–19

Yu J.Q., Huang L.F., Hu W.H., Zhou Y.H., Mao W.H., Ye S.F., Nogues S. (2004). A role of brassinosteroid in regulation of photosynthesis in *Cucumis sativus*. Journal of Experimental Botany 55: 135-143

Yuan G.F., Jia Ch.J., Zhen L., Sun B., Zhang L.P., Liu N., Wang Q.M. (2010). Effect of brassinosteroids on drought resistance and abscisic acid concentration in tomato under water stress. Scientia Horticulturae 126: 103-108

Zhang M. , Zhai Z. , Tian X. , Duan L. , Li Z. (2008). Brassinolide alleviated the adverse effect of water deficit on photosynthesis and the antioxidant of soybean (Glycine max L.). Plant Growth Regulation 56: 257-264

PLANT FUNCTIONING UNDER ENVIRONMENTAL STRESS P. 123-135

Cracow, 12th to the 15th September, 2012

ANATOMICAL, ULTRASTRUCTURAL AND BIOCHEMICAL SIGNS OF *TRAPA NATANS* LEAVES ADAPTATION TO SUBMERGENCE IN NATURAL WATER HABITS

Olena M. Nedukha

Department of Cell Biology and Anatomy, Institute of Botany, National Academy of Sciences of Ukraine, Tereschenkivska Str. 2, 01601, Kyiv, Ukraine

Corresponding author: o.nedukha@hotmail.com

Abstract. The influence of nature flooding on both anatomical structure of floating and submerged leaves and ultrastructure cells of during vegetative stage was studied by light and transmission electron microscopy. It is established that flood effect to change of anatomical structure of *Trapa natans* submerged leaves, their shape, size and structure of leaflet. The cell ultrastructure of floating leaf mesophyll in *T. natans* indicates the typical cellular signs of mesophytes. It is revealed that submergence leads to the change of ultrastructure in undifferentiated mesophyll (parenchyma) cells and causes the decrease of chlorophylls and carotenoids conten in the comparison with those in air leaves.

Key words: Leaf, anatomy; Cell ultrastructure; Flooding; Chlorophyll, *Trapa natans*

Introduction

It is known that soil flooding of plants leads in depend from its short- or long-term to the changes in structural and metabolic levels in both of root system and in over ground organs (Jackson, Drew 1984; Vartapetian, Jackson 1997). In particular, anoxia, hypoxia, aerenchyma formation in root, stem and leaves, the changes of cell structure of these organs, and also the decrease of photosynthetic activity of flooding organs are occurred. Whereas, high aquatic plants in natural conditions are generated of the cellular mechanisms of the tolerance to submerged conditions. These mechanisms helps leaves and stems to adapt for the decrease of light, for the change of its spectral characteristic, and also for the change of CO_2 and O_2 content into water and slimy bottom (Smith, Walker, 1981; Madsen, Maberty, 1991; Lansberg, 2003).

The increase of leaf area, decrease of leaf thickness and cuticle thickness, stomata reduction or their absent, formation of air space (aerenchyma) are the main structural patterns of leaf resistance to flooding effect for many plants (Mommer *et al.*, 2005, a; 2005, b; 2006). In spite of the numerous data of concerning of the study of flood influence to growth and function of high plants, deserve particular attention of the study structural peculiarities of leaves, which are permanently functioned as both under water surface and both above water surface. It is necessary for the understanding of cellular mechanisms of plant adaptation to flooding. The aim of our experiments was to realize comparative study of the structural signs and the distinguished patterns of submerged and floating leaves on light optical, electron microscopic levels, and to determine the content of photosynthesizing pigments in submerged and floating leaves of *Trapa natans*.

Material and methods

Plant material

Floating and submerged leaves of *Trapa natants* L. (water chestnut) were used to study. Water plans were harvested in June at the vegetative stage of plant growth. Plants grew in water along-shore of the Rusanivskiy channel (left Shore of Dnepr River, in Kyiv) on the depth of 80-100 cm. The sun illumination (photosynthetic photon fluency rate (PPFR)) on the floating leaf surface was 1450-1500 μmol quantum·m^{-2}·sec^{-1}, and on the abaxial surface was 800-850 μmol quantum·m^{-2}· sec^{-1}); on the upper surface of the submerged leaves of plants (about 10-12 cm below the water surface) was 14-17 μmol quantum·m^{-2}· sec^{-1}. The measure of PPFR is cannot be done about 80-100 cm below the water surface. PPFR was measure by the means of the Light Meter LI-250 (USA, LI-COR). The temperature of water was + 18°C and the temperature of air was + 20°C.

The first and second floating leaves (which were mature, and they be situated on periphery of rosette) and the first and second submerged leaves, when were near germinate seed (chestnut) were used for the microscopic and biochemical investigations. We are taking the leaves on electromicroscopic study from three plants of the same color and the same size of stem and leaves. The leaves from seven-nine plants were used for the biochemical investigations, and 11-13 plants were used for the phenological study.

Microscopy and biochemical analyses

Middle part of lamina from three plants, notable from floating leaves and from middle part of needle-like round particles of the submerged was fixed at noon (on the beach of Rusanivskiy canal) in the solution of 1% paraformaldehyde and 5% glutaraldehyde (1:1, v) in 0.05 M cacodylate buffer, pH 7.2 for 1 h at 24°C and

then samples were transferred to 4°C for 20 hours. Later the samples were washed in the identical buffer, post fixed with 1% osmium tetra oxide for 12 hours at 4°C, dehydrated in a graded ethanol series, then in a graded acetone series and embedded in epon/araldite resin accordingly to the protocol (Weakley 1975). For transmission electron microscopy ultrathin sections were stained with citrate lead and studied in JEM-1200EX electron microscope. For light microscopy semi thin sections (3 μm) were stained in the Schiffs reagenz (Merck) accordingly to the protocol (Jensen, 1965), then were analyzed with Axioskope (Germany) the light-optical microscope.

Chlorophylls and carotenoides content were determined in floating leaves and submerged leaves from 7-9 plants using the protocol and the formulas described by Robelen and Wetstein (Gavrilenko *et al.*, 1975) on the SP-2000 spectrophotometer. Three replications of the biochemical analysis were made. Values of biochemical and cytological results were expressed at the mean and standard errors.

Results and discussion

Phenological observation and light-microscopical analysis. *Trapa natans* is heterophyllous plant. Shoot of this plant has two distinct leaf types. The floating leaves has solid leaflet, and submerged leaves are highly dissected (Fig. 1A - C).

Fig. 1. General view of *Trapa natans* grown under natural submergence*:*
A – floating leaves; B, C – submerged dissected leaves

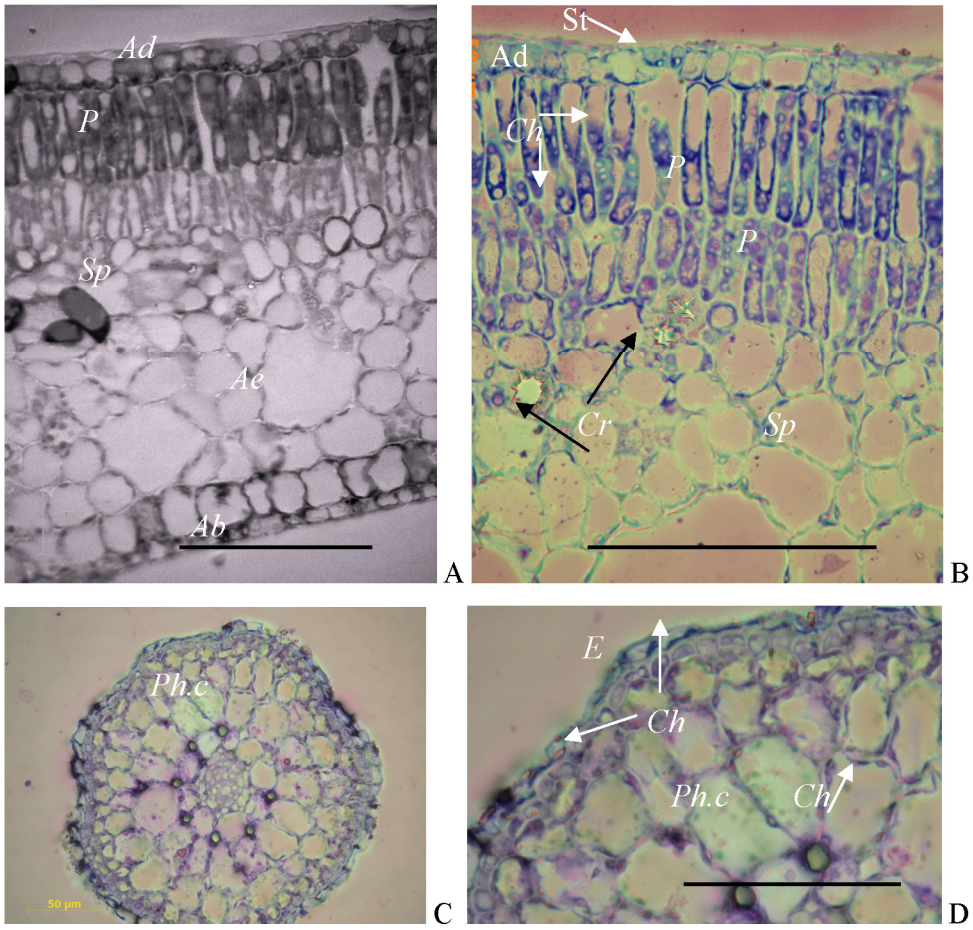


Fig. 2. The cross sections of *Trapa natans* of floating leaf (Fig. 2A) and submerged leaf (Fig. 2B). *Ab* – abaxial epidermis, *Ad* – adaxial epidermis, *Ae* –aerenchyma, *Ch* – chloroplast, *Cr* – crystal of calcium oxalate, *P* – palisade, *Sp* – spongy mesophyll, *St* – stomata, *Ph.c* –photosynthesizing cell. Bar = 100 μm (Fig. A, B), bar = 50 μm (Fig. C, D)

The floating leaves forms the rosette from 13-19 leaves; leaflet has triangular in form, with conspicuously toothed margins along the outside edges. The upper surface of the leaf is glossy. All petioles have the central thickened part (air "cushion"). The submerged leaves has feather-like form, consists from 18 to 40 needle-like particles. Average distance between submerged leaves was different on the shoot (from 40 to 80 mm).

Floating leaves. Light-microscopic analysis of the transverse sections of leaflets revealed a dorsoventral structure (Fig. 2A, B) and air space between cells of pali-

sade and spongy mesophyll. In epidermis one cell layer and in palisade two cell layer were observed. The stomata were in adaxial epidermis. In spongy mesophyll 6-9 cell layers were detected. The dimensions of cells are presented in Table 1.

Table 1. The size of leaflets and cells in *T. natans* floating and submerged leaves. Effect of flooding is significance different (** $p < 0.001$; * $p < 0.05$) from the above-water (air) condition

Parameter	Floating leaf	Submerged leaf
Type of mesophyll	Dorsoventral	Centric
Average size of leaflets (mm)		
long axis	51.3 ± 2.6	50.1 ± 12.3
short axis	64.1 ± 1.5	36.0 ± 2.4**
Average size of petioles (mm)		
long axis	112 ± 10.4	3.5 ± 0.4**
diameter (near attachment to shoot)	3.9 ± 0.1	0.9 ± 0.4**
Average thickness of leaf, μm	300 ± 15	217 ± 10**
Number of palisade parenchyma layers	Two	Absent
Number of spongy parenchyma layers (or layers of photosynthesizing parenchyma in submerged leaves)	6 – 9	5– 7
Average size of palisade mesophyll cell, μm:		Absent
long axis	40.3 ± 2.0	
short axis	9.3 ± 1.4	
Average size of spongy mesophyll cell (or parenchyma cell in submerged leaf), μm :		From 7 ± 0.8 to 33 ± 4.8 μm in a long axis or in a diameter
long axis	18.9 ± 2.8	
short axis	20.8 ± 2.6	
Average size of adaxial epidermal cell (or epidermal cell in the round particles of submerged leaf), μm :		
height	15.3 ± 0.6	7.7 ± 0.5**
width	10.9 ± 0.5	10.3 ± 1.2
Average size of abaxial epidermal cell, μm :		-
height	6.6 ± 0.4	
width	11.3 ± 0.8	
Average number of chloroplast per section:		
in adaxial epidermal cell or epidermis of submerged leaf	2 .3 ± 0.1	3.1 ± 0.1*
in palisade cell	13 .3 ± 2.7	-
in sponge mesophyll cell or photosynthesizing cell of submerged leaf in abaxial epidermal cell	6.7 ± 0.8 1.8 ± 0.2	7.7 ± 0.5 -

More number of chloroplasts was in the palisade cells (Table 1) in comparison with cells of epidermis and spongy mesophyll. The crystal structures (diameter near 10 mkm) observed in cells of spongy mesophyll (Fig. 2B, arrows).

Submerged leaf. Light-microscopic analysis of the transverse sections of particles of dissected leaflet revealed the centric (unifacial) structure (Fig. 2C, D) and

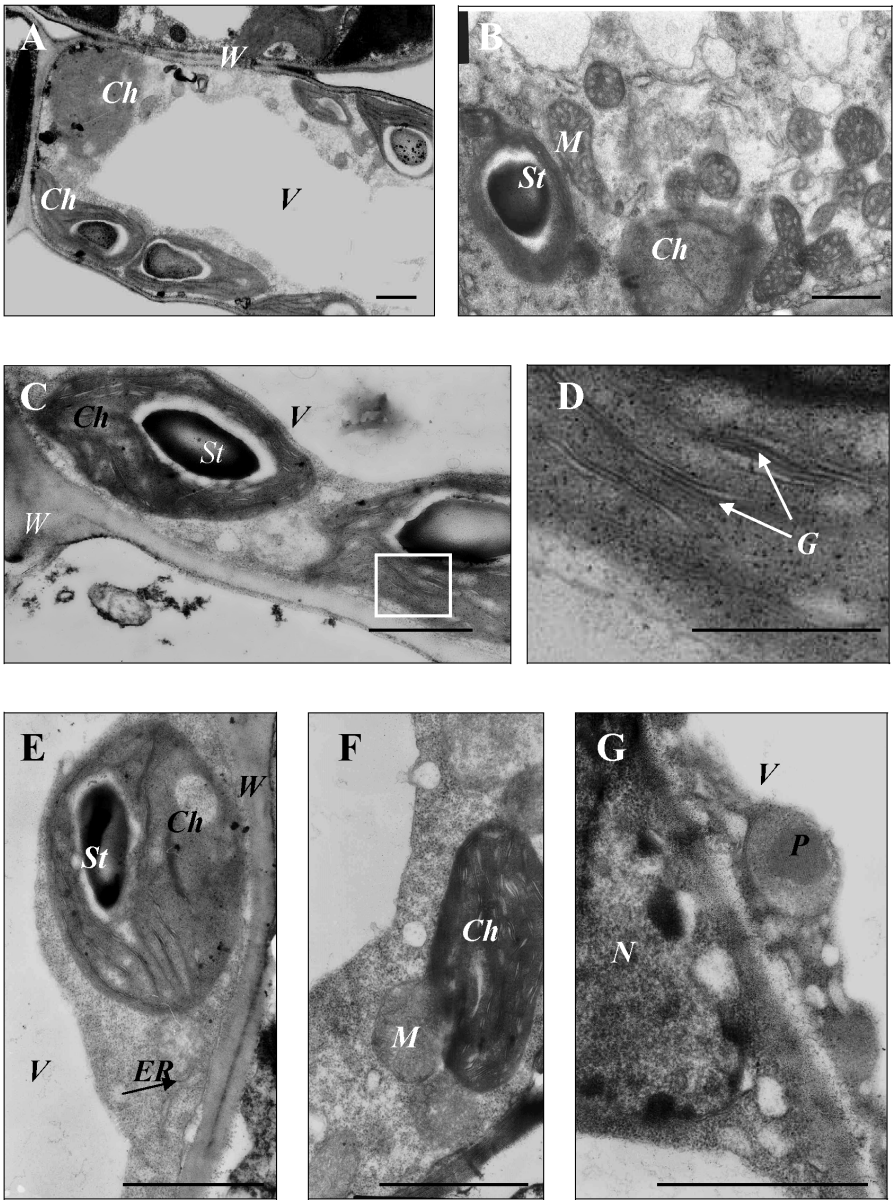
very small air space between cells of 4th and 6th layers of parenchyma. Stomata are absent in the submerged leaves. Vascular bundle is situated in the centre of round leaflets particle. The dimensions of cells are presented in Table 1. It is necessary to note the increase of the size of photosynthesizing parenchyma cells from sub epidermal outer layer to inner layer (near vascular bundle). Chloroplasts observed in the epidermal cells and photosynthesizing parenchyma (Fig. 2D, arrows)

Ultrastructural analysis of Trapa natans leaves

Floating leaves. Ultrastructural analysis of palisade parenchyma and cells of spongy mesophyll indicated that cells had a typical structure that was similar to parenchyma cells of mesophytes (Fig. 3). The cells had a big central vacuole; organelles were situated along cell walls. Chloroplasts of palisade cells (Fig. 3A, C, E, and F) and spongy mesophyll (Fig. 3B) were oval, round or lens like form depending on section plane. Average size of chloroplast in mesophyll was 3.5-4 μm in long axis and 1.2–2 μm in short axis. Chloroplasts contained one big starch grains (average size was 1.7–1.8 x 0.5–1.2 μm); and from time to time, second very small starch grain observed in chloroplast stroma. Large number of grana (up to 15–21) per chloroplast section was characterized for plastids. Thylakoids number in grana varied from 2 to 6 (Fig. 3 D). The diameter of grana is equaled 0.4–0.6 μm. Round, oval and or slightly elongated mitochondria (Fig. 3B, F) were of a condense type and the size of was 0.5–1.3 μm in long axis and 0.3–0.5 μm in short axis. The endoplasmic reticulum (ER) was characterized by short canals of granular type (Fig. 3B, E). An abundance of ribosomes and polysomes were observed in cytoplasm. Round or oval peroxisomes, diameter from 0.5 to 0.7 μm, were also in cytoplasm (Fig. 3G). The part of mitochondria and peroxisomes were in contact with chloroplasts (Fig. 3B, F). Curved shape nucleus has compact chromatin near of lightly swelled envelope (Fig. 3G). Cell walls were 0.3–0.5 μm wide. From time to time, certain organelles, cell walls (Fig. 3A) and middle plate (Fig. 3A, E) were very electron dense.

Results of chlorophylls and carotenoids content measurements are shown in figure 4. The ratio of Ch *a:b* is equaled 3.2 in the floating leaves. Common content of chlorophylls is equaled 2. 626 mg· g^{-1} FW.

Submerged leaves. We found the some common signs in the cell ultrastructure of photosynthesizing parenchyma in comparison with those of the floating leaves in *T. natans.* The cells of parenchyma independently of the layer were greatly vacuolated and organelles were situated near cell walls (Fig. 5). The definite differences were also revealed. There were big elongate (4.5–5.7 μm in long axis and 1.0–1.2 μm in short axis), oval and curved chloroplasts; more number (up to 17) and size thylakoids in grana (Fig. 5A–E). Starch grains observed very seldom.



Scale bar = 1 μm (fig. A–C, E–G), and scale bar = 0.5 μm (fig. D)

Fig. 3. Fragments of mesophyll cell of *Trapa natans* floating leaves: *Ch* – chloroplast, *ER* – endoplasmic reticulum, *G* – grana, *M* mitochondria, *N* – nucleus, *P* – peroxisome, *St* – starch grain, *V* – vacuole, *W* – cell wall. Figure 3D – the increased fragment of chloroplast that outlined by the white square on the figure 3C

The size of one solitary starch grain was five times less than that in the chloroplast of floating leaf. Besides we observed chloroplasts with the increased number of plastoglobules (from 5 to 15 in chloroplast section), diameter from 30 to 50 nm (Fig. 5E). Most mitochondria have developed crista system (Fig. 5F, G, and H) and electron dense matrix. Average size of round and oval mitochondria was 0.5–0.6 μm in long axis and 0.3–0.4 μm in short axis. ER was granular type. Very short dictiosomes are assemble near cell walls (Fig. 5 H), whereas multivesicular bodies are situated near central vacuole (Fig. 5G). Oval nucleus contained diffuse chromatin (Fig. 5F). The width of the cell walls ranged from 0.2 to 0.4 μm in the sections. It is necessary to the presence of the hyper electron density in the cell walls (Fig. 5G, double white arrows), electron dense formations in vacuoles (Fig. 5F), and hyper electron density of cytoplasm and the organelles of some cells (Fig. 5H, the lower cell).

The content of chlorophyll *a* and carotenoids were lower in the submerged leaves of *T. natans* in comparison with that of the floating leaves (Fig. 4). There were the decreased of the common content of chlorophylls and the ratio of chlorophyll *a* : *b* in the submerged leaves.

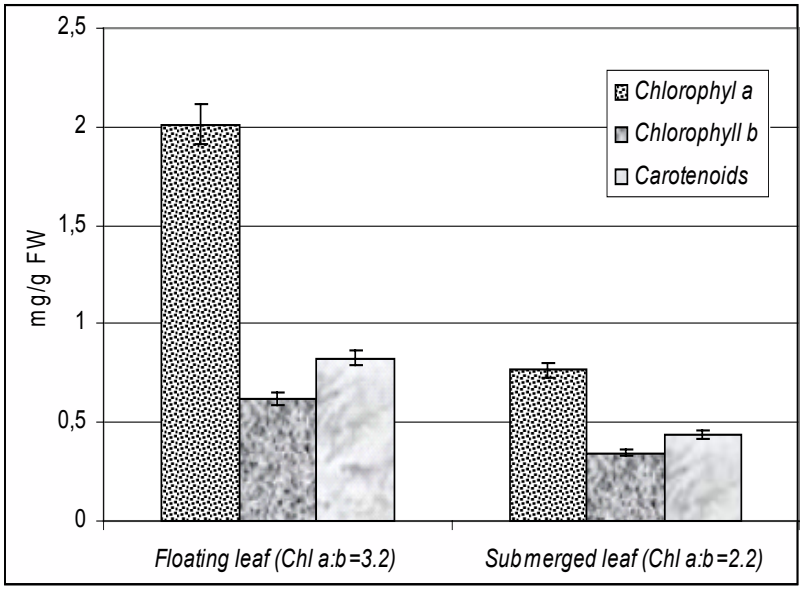
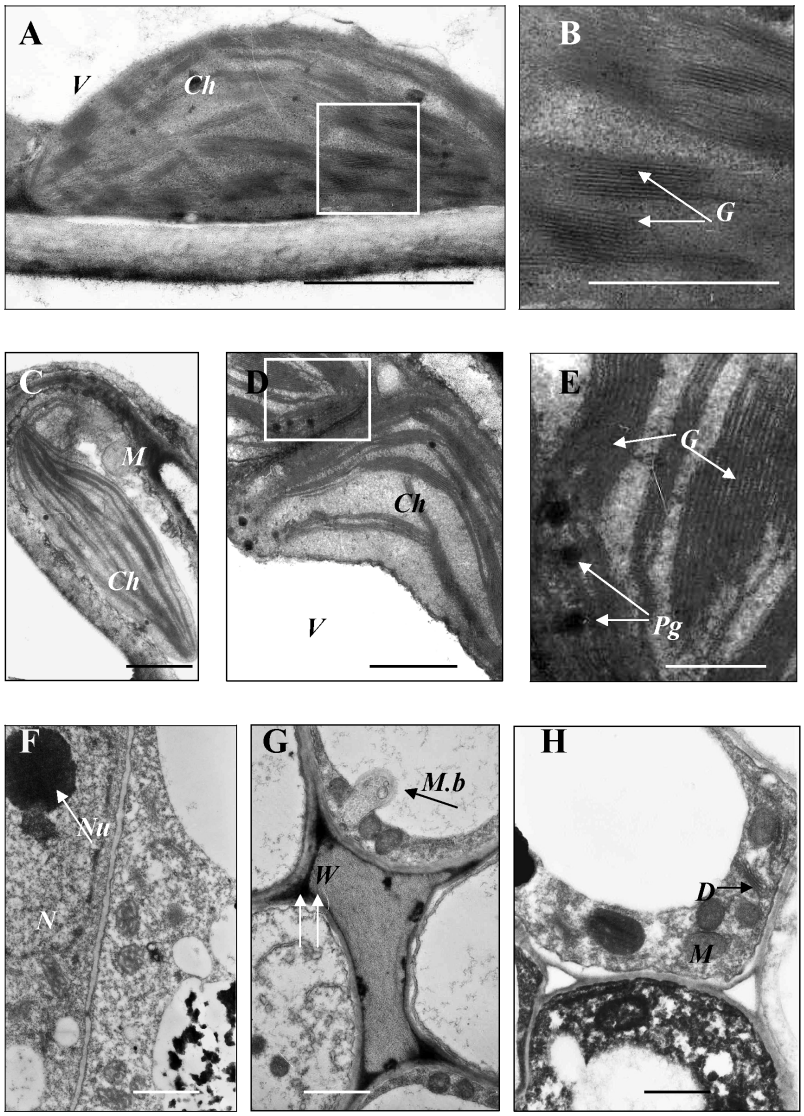


Fig. 4. Effect of natural submergence on the content chlorophylls and carotynoids in leaves of *Trapa natans*. Values (of pigments) are means ± SE in the floating leaves are significantly different from that in the submerged leaves ($p < 0.05$)



Scale bar = 1 μm (fig. A–C, E–G), and scale bar = 0.2 μm (fig. D)

Fig. 5. Fragments of photosynthesizing cells of *Trapa natans* submerged leaves: *Ch* – chloroplast, D – dictiosome of Golgi apparatus, *ER* – endoplasmic reticulum, *G* – grana, *M* – mitochondria, *M.b* – multivesicular body, *N* – nucleus, Nu – nucleolus, *P* – peroxisome, *Pg* – plastoglobule, *St* – starch grain, *V* – vacuole, *W* – cell wall. Figure 5B - the increased fragment of chloroplast that outlined by the white square on the figures 5 A. Figure 5 E - the increased fragment of chloroplast that outlined by the white square on the figures 5D, accordingly

Thus, the phenological observations showed floating leaves triangular in shape and dissected shape of submerged leaves of *Trapa natans* that were analogous to the shape and structure leaf of submerged leaves of *Potamogeton pectinatus, P. amplifolius*, *Myriophyllum spicatum, M. aquaticum* (Allsopp, 1965; Deschamp, Cooke, 1983; Nedukha, 2011), and floating leaves of *Trapa natans* showed solid leaflet and air cushion in petiole by an analogous to that in the air leaves of *Nuphar lutea, Trapa sp.* and *Potamogeton perfoliatus* (Bercu, 2004; Nedukha, 2011). Light-optical analysis revealed the presence of small air space, the presence of chloroplasts in epidermal cells, the absent of stomata, the decrease of leaflet thickness and undifferentiated mesophyll in centric (unifacial) structure submerged leaves of the studied species. The main problems during flooding are the decrease of sun light in water, slow diffusion rates of gases between water and flooding organs and also the depletion of carbohydrates, which is substrate for respiration (Vartapetian, Jackson, 1997; Mommer *et al.*, 2006). It is possible that centric structure and reduced thickness of submerged leaves are serving for uniform light absorption and for uniform gases transport in submerged leaves. The size and form of the revealed the crystal structures in spongy mesophyll cells of *T. natans* floating leaves are like to those of the crystal of calcium oxalate written in the stem and petioles of *Abutilon pictum* (Brander, 1987). The function of such crystals is the participation in mineralization of tissues (Arnott, 1976, cited by: Brander, 1987).

We found the ultrastructure of mesophyll of floating leaves of the investigated plants was a typical for mesophytes plant plants, that were wrote in the papers studied chloroplasts (Silaeva, 1978; Anderson, 1986; Yano, Terashoma, 2001; Przybyl, Idzikowska, 2003). However, we were revealed the specific signs of structure that were only characteristic for submerged leaves. The presence grana with the large number of thylakoids were the first feature of chloroplasts. It is known that this sign is typical for chloroplast of shade plants (Boardman, 1977). This feature is the cause of the influence of faint lighting, change of its spectral characteristic, that lead to intensification of chlorophyll *b* synthesis (Gorishina, 1989; Nikolaeva, Vlasova, 1990). In consideration of that sun illumination in water, where submerged leaves grew 80–120 cm from water surface, was less than on surface of the floating leaves, we can draw the conclusion that the structure of submerged cells of *T. natans* is determined by the reduced illumination. Secondly, it is known that chlorophyll *b* and photosystem II are generally located into grana thylakoids (Gudvin, Merser, 1986; Belsky, 1989), and this permit to propose on the correlation of the chloroplast structure in submerged leaves with the specified composition of photosynthesizing pigments. It is known that the ratio of chlorophylls *a* : *b* is indicated on the relative size of light-harvesting complex, whereas the ratio of chlorophyll *a* to carotenoids is characterized for chlorophylls integrity under photo destruction action (Belsky, 1989).

It is known that carotenoids very quickly put out of superoxide radical of oxygen ($O_2^{\cdot-}$), that are formed during of oxidized/reduced reactions. $O_2^{\cdot-}$ are the sources

of the generation of other toxic form of oxygen, and they provoke the disturbance of thylakoid membranes. In addition, carotenoids can also to adsorb of energy surplus from activated chlorophyll molecules and hereby carotenoids can to prevent $O_2^{\cdot-}$ formation (Merzlyak, 1989; Foyer, Noctor , 2003).

Besides, we revealed almost total absent of starch in chloroplast of the submerged leaves of *T. natans*. According to G. Nekrasova *et al.* (Nekrasova *et al.*, 2003), who examined the photosynthetic substances with using of the ^{14}C (radioactive carbon) of the submerged leaves in some high aquatic plants, the decrease of sucrose and starch synthesis is occurred in the submerged leaves. M. Kasperbauer and J. Gamelton (Kasperbauer, Hamilton, 1984) studied the possible mechanism of starch decrease; they consider the influence of distant red light as the main reason of the decrease of reserve starch synthesis in the submerged leaves, whereas above-wafer leaves are functioned in the red light.

Remarkable the decrease of the ratio of chlorophylls *a : b* and the reduce of the content of chlorophylls (*a* + *b*) in T. natans submerged leaves are typical for leaves grown under conditions of lowering of light (Gorishina, 1989; Nikolaeva, Vlasova, 1990); These signs are shown the significant contribution of chlorophyll *b* for intensification of PS II function under lowering light. Besides, these authors are considered the similar signs as the indicators of the adaptation during shading that helps to preserve of the balance the light absorption between PSI and PSII (Anderson, 1986; Gorishina, 1989; Nikolaeva, Vlasova, 1990).

Besides, we observed the appearance or the presence of hyper electron dense formations in the vacuoles, in connection with the cell walls and in the organelles in the submerged leaves of *T. natans*. It is possible, that similar dense structures are contained the tannins (phenolic compounds), which early wrote by A.Vasilyev (Vasilev, 1972) in the vacuoles of *Pinus sibirica* cortex cells, can complex with numerous types of molecules, including, cellulose, proteins and other (Haslam, 1989). These cellulose-tannin interactions are toxic for a lot microorganisms and alga, that, it is possible, are the protectors for an invasion of microorganisms into submerged organ of plant.

Conclusions

1. Submergence in natural water habitats led to change of anatomical structure of
2. *Trapa natans* submerged leaves, affect the shape, size and structure of leaflet.
3. The ultrastructure of floating leaf mesophyll in *T. natans* at vegetative
4. stage indicates the typical cellular signs of mesophytes.
5. Submergence leads to the change of ultrastructure in undifferentiated mesophyll (parenchyma) cells and causes the decrease of chlorophylls and carotenoids content.

References

Allsopp A. (1965). Land and water forms: physiological aspects. Hadb. Pflanzen physiol. 15: 1236–1255.

Anderson J.M. (1986). Photoregulation of composition, function and structure of thylakoids membranes . Ann. Rev. Plant Physiol. 37: 93–136.

Belsky A., Amudson R., Duxbury J. *et al.* (1989). The effects of trees on their physical, chemical and biological environments in a semi – arid savanna in Kenya. J. Appl. Ecol. 1989.26: 1005–1024.

Bercu Rodica. (2004). Histoanatomy of the leaves of Trapa natans *(Trapaceae)*. Phytol. Balcan. 10: 51-55.

Boardman N.K. (1977). Comparative photosynthesis of sun and shade plants. Ann. Rev. Plant Physiol. 28: 355–377.

Deschamp P.A., Cooke T.J. (1983). Leaf dimorphism in aquatic angiosperms: significance of turgor pressure and cell expansion. Science 219: 506-507.

Foyer C.H, Noctor G. (2003). Redox sensing and signaling associated with reactive oxygen inchloroplasts, peroxisomes and mitochondria. Physiol. Plant. 119: 355-364.

GavrilenkoV., Ladigina M., Khadobina L. (1975). Practical works for plant physiology. Ed. V. Gavrilenko. High School, Moscow, Russia: 390 pp.

Gorishina T.A. (1989). Photosynthetic apparatus of plants and environments. V.Polevoy (Ed.), Publ. Universitet Leningrad: 202 pp. (In Russ.).

Gudvin G., Merser E.(1986). Introduction in Plant Biochemistry. Moscow: Mir. Vol. 1. 322 pp.

Haslam E. (1989). Plant polyphenols. In: Chemistry and significance of condensed tannins. Eds. Hemingway R.W. and Karchesy J.J. N.Y., UK.: Plenum Press, Cambridge University Press.

Jackson M.B., Drew M.C. (1984). Effect of flooding on herbaceous plants. In: Flooding and plant growth . Ed. Kozlowski T.T. London: Academic Press, p. 47–128.

Jensen A. (1965). Botanical Histochemistry. Ed. Tsinger N. Moscow: Mir. 377 pp.

Kasperbauer M.J., Hamilton J.L. (1984). Chloroplast structure and starch grain accumulation in leaves that received different red and far-red levels during development . Plant Physiol. 74: 967–970.

Lansberg G.S. (2003). Optics. – Moscow, Publ.: Physico-Math.literature. – 848 p.

Madsen T.V., Maberty S.C. (1991). Diurnal variation in light and carbon limitation of photosynthesis by two species of submerged freshwater macrophytes with a differential ability to use bicarbonate. Freshwater Biol. 26: 175–187.

Merzlyak M.N. (1989). Activated oxygen and oxidative processes in membranes of plant cell. Itogi of Nayki I Techniki. Ser. Fiziologia Rasteniy, Vol. 6: 1-167 p. (In Russ.).

Mommer L., Visser J.W. (2005), a. Under photosynthesis in flooded terrestrial plants: a matter of leaf plasticity. Ann. Bot. 96: 581-589.

Mommer L., Pons T.L., Wolters-Arts M. *et al.* (2005), b. Submergence-induced morphological, anatomical, and biochemical responses in a terrestrial species affects gas diffusion resistance and photosynthetic performance. Plant Physiol. 139: 497–508.

Mommer L., Pons T.L., Visser J.W. (2006). Photosynthetic consequences of phenotypical plasticity in response to submergence: Rumex palustris as a case study. J. Exp. Bot. 57: 283-290.

Nedukha O.M. (2011). Heterophylly in Plants. Kiyv: Publ. AltPress: 1-191 pp. (Ukr.).

Nekrasova G., Ponzhina D., Maleva M., Pyankov V. 2003. Photosynthetic metabolism and activity of carboxylative enzymes in above-water, floating and submerged leaves of hydrophytes. Fiziologia of Rasteniy. 50: 65–75. (In Russ.).

Nikolaeva M.K., Vlasova M.P. (1990). Anatomical peculiarities, pigments composition and photosynthetic activity of bean leaves grown under different illumination intensity. Fiziologia Rasteniy. 37: 66-68. (In Russ.).

Przybyl K., Idzikowska K. (2003). Ultrastructural changes in chloroplasts of mesophyll cell of chlorotic and prematurely yellowed leaves of *Betulu pendula Rothr*. Acta Soacietatis Botanicorum Poloniae. 72: 289–293

Silaeva A.M. (1978). Structure of chloroplasts and environment. Kiev: Naukova Dumka. 201 pp.

Smith F.F., Walker N.A. (1981). Photosynthesis by Aquatic Plants: Effects of unstirred layers in relation to assimilation of CO2 and HCO3 and isotopic discrimination. New Phytol. 6: 245–259.

Vasilev A.E. (1972). Vacuoles, lisosome aqpparatus. In: Atlas of the ultrastructure of plant cells. Ed. G. Kozubov and M. Danilova. Petrozavodsk. Publ. Anochina. P. 178-189. (In Russ.).

Vartapetian B., Jackson M.B. (1997). Plant adaptation to anaerobic stress // Ann. Bot. 79: 3–20.

Weakley B. (1975). Beginner's Handbook in Biological Electron Microscopy. Ed. V. Polevoy, Mir, Moscow (In. Russ.).

Yano S., Terashoma I. (2001). Separate localization of light signal perception for sun and shade type chloroplast and palisade tissue differentiation in *Chenopodium album L.* Plant Cell Physiol. 42: 1303-1310.

PLANT FUNCTIONING UNDER ENVIRONMENTAL STRESS P. 137-152

Cracow, 12th to the 15th September, 2012

THE PARTICIPATION OF CELL WALL POLYSACCHARIDES IN CELLULAR MECHANISMS OF LEAF TOLERANCE TO NATURE FLOODING OF PLANTS

Olena M. Nedukha, Elizabeth L. Kordyum

Department of Cell Biology and Anatomy, Institute of Botany, National Academy of Sciences of Ukraine, Kyiv, Ukraine

Corresponding author: o.nedukha@hotmail.com

Abstract The comparative analysis of the polysaccharides composition of cell walls and cellular distribution of lignin and cellulose in epidermis and mesophyll of *Trapa natans* and *Sagittaria sagittifolia* above-water and submerged leaves was carried out with the standard biochemical methods and the laser confocal microscopy. The images of quantitative distribution of lignin and cellulose in the cell walls were obtained on cellular level depending on the type of leaf tissue and on environment conditions of leaf growth. The increase of lignin, and hemicelluloses were established in submerged leaves of two studied species, whereas the change of pectin and protopectin content are characterized by the species dependent. The role of lignin and cellulose in the cellular mechanisms of adaptation to nature flooding is discussed.

Key words: Cell wall; Polysaccharides; Lignin; Cellulose; Laser confocal microscopy; Trapa natans; Sagittaria sagittifolia

Introduction

Flooding is the one of the major abiotic stresses for plants. This stress provokes the appearance of a serious problem for agriculture and a major determinant of species preservation and distribution in natural populations. Soil waterlogging identified as a major abiotic stress and the constraints it imposes on roots and shoots have marked effects on plant growth and development (Armstrong, Drew, 2002; Jackson, Colmer, 2005; Dat *et al.*, 2006; Parent *et al.*, 2008). The main problem during flood is the shortage of oxygen and CO_2 due to the change diffusion rates of gasses in water. The outer cell walls of submerged leaves and shoots

are the one and the main barriers between plant and water environment. The recent data shown the presence of cuticle pores in the outer cell walls of leaves as in the terrestrial plants (Schonherr, 2006), so and in the submerged leaves of some hydrophytes and rice seedlings (Rose-John, Kende, 1985; Nedukha, 2010) with the using of the standard biochemical methods and transmission electron microscopic method. Besides, it was established the effect of submergence on lignin and cellulose content in the submerged shoots of *Ludwigia repens* (Little, 2003). An integrated study of biochemical composition and content of the main polysaccharides of cell wall in submerged leaves and distribution of these polysaccharides in epidermal and mesophyll cells of typical hydrophytes are not studied enough. The aim of our experiments was to investigation the localization and distribution of lignin and cellulose in the cell walls of different tissues, and also to determine the content of the main polysaccharides in submerged and air leaves of *Trapa natans* L. and *Sagittaria sagittifolia* L. water plants.

Material and methods

Plant material *Trapa natants* (**water chestnut) and *Sagittaria sagittifolia* above-water and submerged leaves** were used to study. Water plans were harvested in June at the vegetative stage of plant growth. Plants grew in water alongshore of the Rusanivskiy canal (left Shore of Dnepr River, in Kyiv) on the depth of 80-100 cm. The sun illumination (photosynthetic photon fluency rate (PPFR)) on the floating (of *T. natans*) and air (above-water of *S. sagittifolia*) leaf surface was 1450-1500 μmol quantum·m^{-2}·sec^{-1}, and on the abaxial surface was 800-850 μmol quantum·m^{-2}· sec^{-1}; on the upper surface of the submerged leaves of *S. sagittifolia* plants (about 10-12 cm below the water surface) was 14-17 μmol quantum·m^{-2}· sec^{-1}. The measure of PPFR is cannot be done about 80-100 cm (where were collected submerged leaves of *T. natans*) below the water surface. PPFR was measure by the means of the Light Meter LI-250 (USA, LI-COR). The temperature of water was + 18°C and the temperature of air was + 20°C. For the microscopic investigations we used leaves by three plants from each species: (1) Floating leaves of *T. natans*, which were mature, and they be situated on periphery of rosette, and also the first and second submerged dissected leaves (Fig. 1*a*); and (2) Air (sagittate form) (Fig. 1b) leaves and elongated submerged (Fig. 1b, c) from three *S. sagittifolia* plants. The leaves from 9-11 plants of the each species were used for the biochemical investigations.

Microscopy and biochemical analyses The cytochemical method accordingly to C. Smith *et al.* (1986) was used for the study of both distribution lignin and relative content of this polysaccharide in walls. The live samples leaflets were stained for 5-10 min in 0,001% solution of auramine-*O* (Sigma) dissolved in water (on alongshore of the Rusanivskiy canal), intensively washed with H_2O, then in 0.05 M pho-

sphate buffer, post fixed in the solution of 1.0 % paraformaldehyde in 0.05 M phosphate buffer, pH 7.2 et +4°C; then the samples were examined with laser scanning confocal microscope LSM5 (Zeiss, Germany). For detection of complex auramine-*O*–lignin fluorescence a laser was excited at 543 nm, and the fluorescence emission detected at 598 nm wave length using using an x 10, x 20 and x 40 objectives the PASCAL program. Chlorophyll auto fluorescence was excited at 440 nm and fluorescent emission detected et 660 nm. Three replications of cytological study of leaves were made.

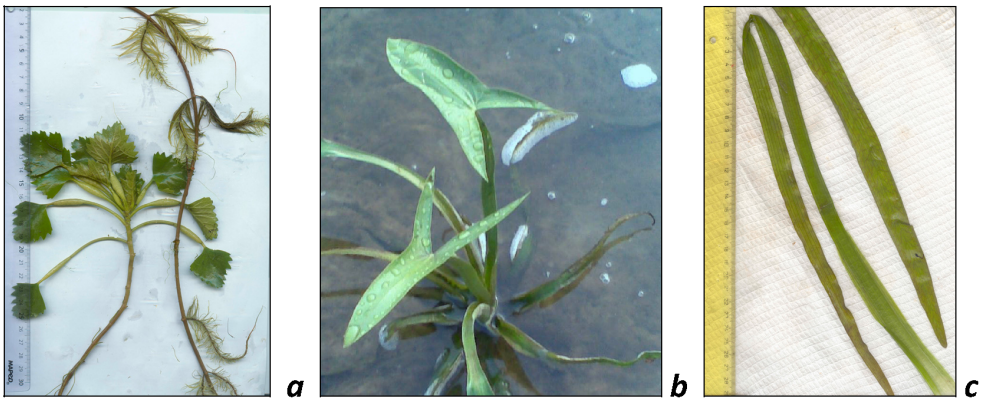


Fig. 1. General view of Trapa natans (a) and Sagittaria sagittifolia (b, c) leaves

The cytochemical method accordingly to W. Herth (1980) was used for the study of both distribution cellulose and relative content of this polysaccharide in walls. The samples of leaflets were fixed in the solution of 1.25% paraformaldehyde in 0.05 M phosphate buffer, pH 7.4 at +4°C; washed with the buffer and stained for 5-10 min in 0,001% solution of calcofluor white RW (Sigma) dissolved in the buffer. The samples were examined with laser canning confocal microscope LSM5 (Zeiss, Germany). Complex calcofluor-cellulose fluorescence intensity was measured in the cell walls as a function of emissions wave length using the PASCAL program. Calcofluor-cellulose was excited at 494 nm and fluorescence emission detected at 516 nm using an x 10, x 20 and x 40 objectives. Chlorophyll auto fluorescence was excited at 440 nm and fluorescent emission detected at 660 nm. Three replications of cytological study of leaves were made.

Common cellulose, lignin, hemicelluloses, pectin and protopectin content were determined in 9-11 leaves (without middle midrib) using the standard protocols as described by V. Arasimovich and A. Ermakov (1987). The dry samples were used for the biochemical study. Three replications of each biochemical analysis were made. Values of biochemical and cytochemical results were expressed as the mean

and standard errors. Statistical significance of relative content of lignin and cellulose in cells was determined using the BIO software (Institute of Botany, Kiyv, Ukraine) and a Student's test ($P < 0.05$).

Results and discussion

Trapa natans. Lignin investigation

Floating leaves. Cytochemical analysis of lignin in the leaves are shown as the yellow fluorescence in the walls of adaxial and abaxial epidermis, and also in palisade and spongy mesophyll (Fig. 2a-c). But the fluorescence intensity of auramine-*O*-lignin complex was different in the tissues (Tab. 1; Fig. 2e, f). Outer wall of epidermis had the greatest fluorescence intensity of auramine-*O*-lignin complex in the comparison with other walls of epidermis and mesophyll. It is revealed that maximum frequency for lignin in the abaxial epidermal cell was 25200 (pixels, yellow line) at intensity (Fig. 2h), the maximum frequency for auto fluorescence of chlorophyll in that abaxial epidermal cell was 600000 (pixels, red line) at intensity (Fig. 2h),

The content of lignin in the floating leaves was high (Tabl. 2), and it was amount to 68.6 ± 4.9 mg/g DW.

Submerged leaves. Similarly, cytochemical analysis of the complex auramine-O-lignin in submerged leaves of *T. natans* showed yellow fluorescence of lignin in cell walls of epidermis (Fig. 2 e) and photosynthesizing cells. There were some differences in fluorescence intensity of lignin in cell walls of epidermis and cell wall inner parenchyma. The level of fluorescence intensity of lignin is presented in the Table 1 and Fig. 2g, g′ (diagram). Luminescense intensity changes in submerged leaves: 1.52 times increased in anticlinal walls, 1.2 times – in periclinal wall of epidermis; and decreased 1.66 times in cell wall of photosynthesizing parenchyma (in comparison with that in cell walls of epidermis and spongy mesophyll of swimming leaf, accordingly). It is necessary to note that independently from the condition of leaf growth the relative content of lignin in the anticlinal walls in epidermis always was more than in periclinal cell walls. It is revealed that maximum frequency for lignin in the epidermal cell of submerged leaf was 16 times more (400000 pixels, yellow line) at intensity (Fig. 2g), whereas the maximum frequency for auto fluorescence of chlorophyll in epidermal cell of submerged leaves was three times less (= 200000 pixels) than that in floating cells (Fig. 2g).

The content of lignin in submerged leaves was increases 1.65 times in the comparison with that in the swimming leaves (Tab. 2).

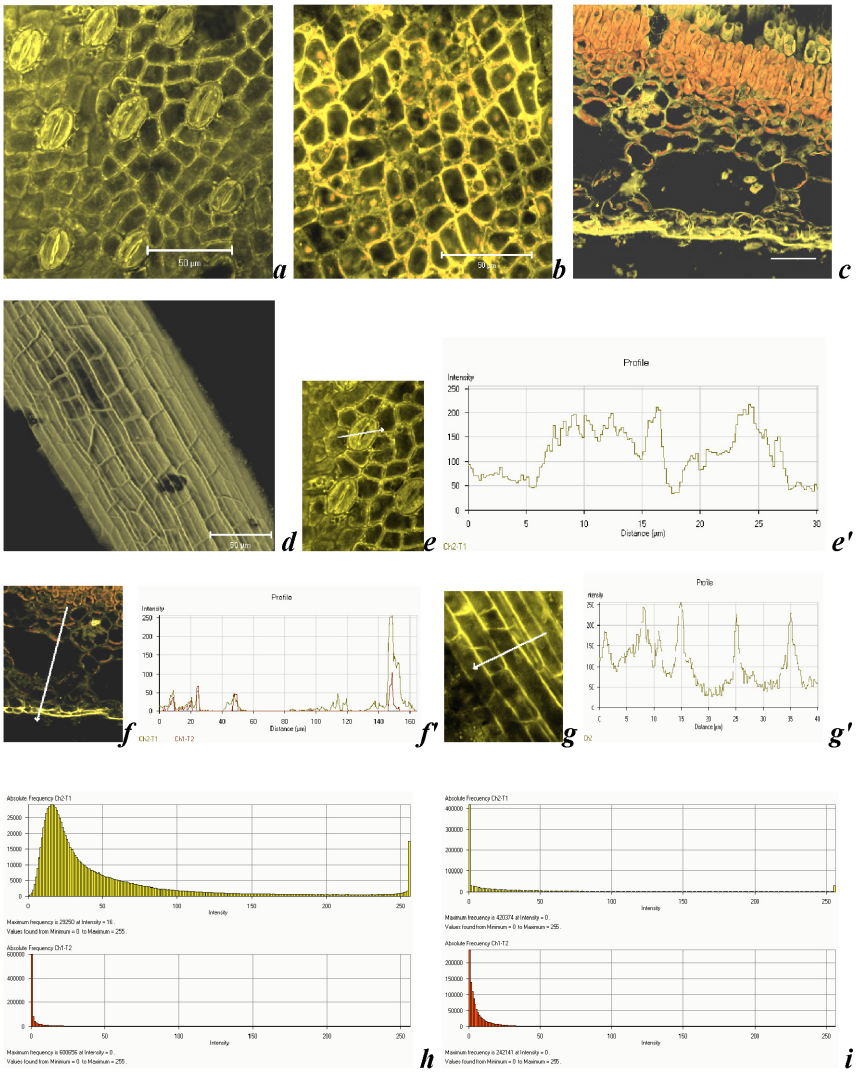


Fig. 2. Micrographs of cytochemical fluorescence of lignin in the floating (Fig. *a-c*) and submerged leaf cells (*d*) of *Trapa natans*. Localization of lignin has yellow fluorescence. Tissue types are noted: *a* - adaxial epidermis, *b* - abaxial epidermis, *c* - palisade and spongy mesophyll; *e, e′, f, f′, g, g′* - histograms of fluorescence intensity of lignin (yellow line) and chlorophyll auto fluorescence intensity (red line). Ordinate –Fluorescence intensity, relative units (pixels). Abscissa – Distance (μm), which was scanned on the figure *e, f, g*. This distance is shown as white line on the figure *e′, f′, g′*. On Figure *d* – 3D structure. On Figures *h* and *i* – absolute frequency of pixels for lignin (yellow graph) and for auto fluorescence of chlorophyll (red graph). Bars = 50 μm

Table 1. The intensity of auramine-*O*-lignin complex fluorescence in above-water and submerged leaves of *T. natans* and *S. sagittifolia* at vegetative stage

Species/tissue/cell wall	Floating (or above-water) leaf	Submerged leaf
Trapa natans:		
Adaxial epidermis (or epidermis of round particles of submerged leaf)		
– outer (periclinal) wall	69 ± 4.3	81 ± 2.7*
– anticlinal wall	152 ± 12	231 ± 19***
Stomata		Absent
– cell wall of pore	207 ± 11	
– outer (periclinal) wall of guard cell	113 ± 10	
– anticlinal wall of guard cell	135 ± 17	
Abaxial epidermis		
– outer (periclinal) wall	86 ± 7.9	
– anticlinal wall	155 ± 11	
Palisade mesophyll	173 ± 15	Absent
Spongy mesophyll (or photosynthesizing cell of submerged leaf	68 ± 7	41 ± 5**
Sagittaria sagittifolia:		
Adaxial epidermis (or epidermis of submerged leaf)		
– outer (periclinal) wall	90 ± 5.9	242 ± 11***
– anticlinal wall	160 ± 11	191 ± 9.1*
Stomata		
– cell wall of pore	171 ± 14	Absent
– outer (periclinal) wall of guard cell	83 ± 7	
– anticlinal wall of guard cell	136 ± 12	
Abaxial epidermis		
– outer (periclinal) wall	27± 3.9	
– anticlinal wall)	70 ± 5.7	
Stomata		
– cell wall of pore	207 ± 21	
– outer (periclinal) wall of guard cell	53 ± 5.3	
– anticlinal wall of guard cell	65 ± 5.2	
Palisade mesophyll	55 ± 3.7	
Spongy mesophyll (or photosynthesizing cell of submerged leaf	34 ± 2.1	50 ± 3.7***

*, **, *** indicated values that differ significantly from the fluorescence intensity of auramine-lignin complex of above-water leaves at $p = 0.05$; $p = 0.01$; $p = 0.001$, respectively

Table 2. The some biochemical characteristics of *T. natans* and *S. sagittifolia* leaves at vegetative stage. Effect of nature flooding is significantly different (*P < 0.05, **P < 0.01 and ***P < 0.001) from air-water conditions

Parameter	Trapa natans		*Sagittaria sagittifolia*	
	Floating leaf	Submerged leaf	Above-water (air) leaf	Submerged leaf
Middle content of water in leaf, %	81,4 ± 0.9	90,5 ± 0.8**	88,7 ± 0.7	93,8 ± 0.9***
Content of lignin, mg/g DW	68.6 ± 4.9	113 ± 14***	25.6 ± 3.1	58.6 ± 4.9***
Content of cellulose, mg/g DW	196 ± 11	147 ± 10*	86 ± 5.9	71 ± 3.7*
Content of hemicellulose, mg/g DW	165 ± 13	241 ± 12***	357 ± 11	397 ± 12*
Content of pectin, mg/g DW	118 ±10	117 ± 11	210 ± 14	50.4 ± 4.7***
Content of protopectin, mg/g DW	101 ± 9.5	45.7 ± 3.7***	22.7± 9.1	86.3 ± 7.1***

Sagittaria sagittifolia. Lignin investigation

Above-water (air) leaves. Cytochemical analysis of lignin in arrow-like leaves of *S. sagittifolia* showed bright yellow fluorescence of lignin in the cell wall of epidermis and mesophyll cells, similar to that observed in floating leaves of *Trapa natans* (Fig. 3a, b). The level of luminescence intensity is presented in Table 1 and Fig. 3e′. The analysis of lignin luminescence intensity showed that relative content of lignin in walls of adaxial epidermis was more than that in cell walls of lower epidermis. The relative content of lignin in cell walls of mesophyll was decreased by 2-4 times in the comparison with that in walls of an adaxial epidermis. It is revealed that maximum frequency for lignin in the abaxial epidermal cell, and mesophyll cell was to 100000 (pixels, yellow line) at intensity, the maximum frequency for auto fluorescence of chlorophyll in that mesophyll cell was 800000 (pixels, red line) at intensity (Fig. 3f).

The content of lignin in the air leaves of *S. sagittifolia* was 2.6 times less than in the floating leaves of *T. natans* (Tabl. 2), and it was amount to 25.6 ± 3.1 mg/g DW.

Submerged leaves. Cytochemical analysis of the complex auramine-O-lignin in submerged leaves of of *S. sagittifolia* showed yellow fluorescence of lignin in cell walls of epidermis and photosynthesizing cells (Fig. 3c, d). There were some differences in fluorescence intensity of lignin in cell walls of epidermis and cell walls of photosynthesizing parenchyma. The level of fluorescence intensity of lignin is presented in the Table 1 and Fig. 3f, f' (diagram). It is revealed that maximum frequency for lignin in the epidermal cell of submerged leaf was 3 times more (300000 pixels, yellow line) at intensity (Fig. 3h), whereas the maximum frequency for auto fluorescence of chlorophyll in epidermal cell of submerged leaves was considerably less than that in air-water leaves of *S. sagitiffolia*. The content of lignin in submerged leaves was increases 2.28 times in the comparison with that in the air-water leaves (Tab. 2).

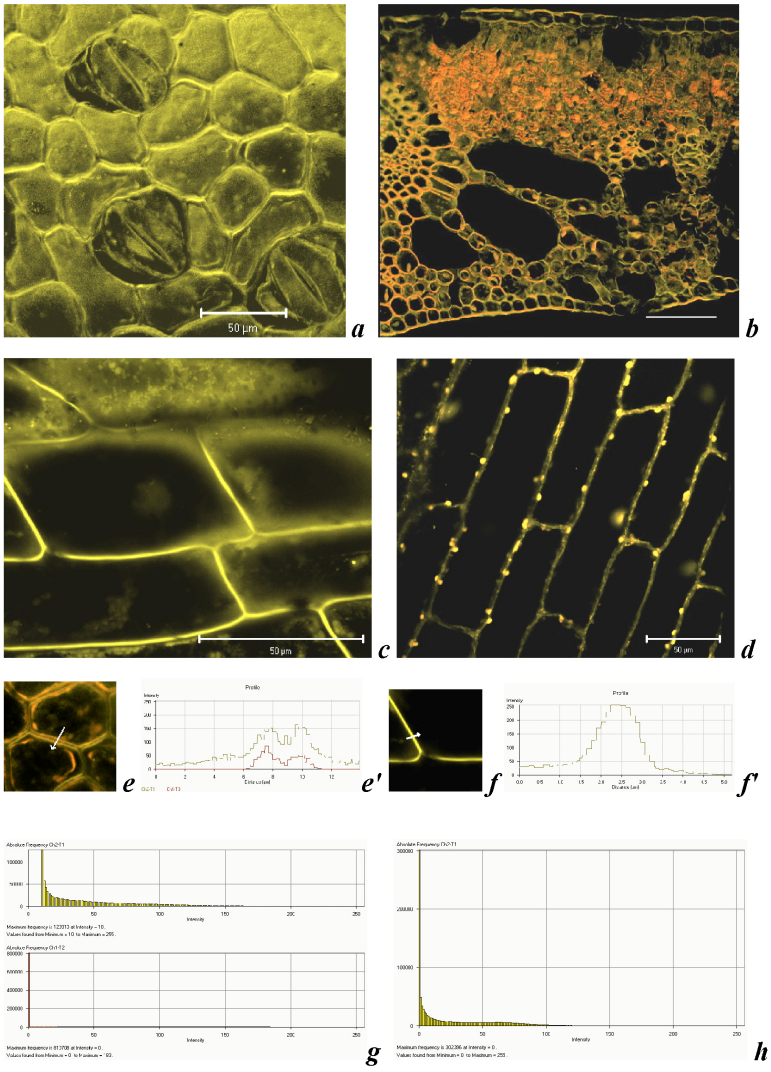


Fig. 3. Micrographs of cytochemical fluorescence of lignin in the air (Fia. ***a, b***) and submerged leaf cells (***c, d***) of *Sagittaria sagittifolia*. Localization of lignin has yellow fluorescence. Tissue types are noted: ***a*** - adaxial epidermis, ***b*** – transverse section across leaf, ***c*** – epidermis, ***d*** – photosynthesizing parenchyma; ***e, e', f, f'*** - histograms of fluorescence intensity of lignin (yellow line) and chlorophyll auto fluorescence intensity (red line). Ordinate –Fluorescence intensity, relative units (pixels). Abscissa – Distance (μm), which was scanned on the figure ***e,*** and ***f***. This distance is shown as white line on the figure ***e*** ***'***and ***f'***. On Figure ***a*** – 3D structure. On Figures ***g*** and ***h*** – absolute frequency of pixels for lignin (yellow graph) and for auto fluorescence of chlorophyll (red graph). Bars = 50 μm

Trapa natans. Cellulose investigation

Floating leaves. Cytochemical analysis of cellulose in the leaves are shown as the regular green fluorescence in the walls of adaxial and abaxial epidermis, and also in palisade and spongy mesophyll (Fig. 4a-c). Ehe fluorescence intensity of calcofluor-cellulose complex was different in the tissues (Table 3; Fig. 4 g). Outer wall of epidermis had the greatest fluorescence intensity of calcofluor-cellulose complex in comparison with other cell walls of mesophyll. The maximum frequency for cellulose in the abaxial epidermal cell was 20000 (pixels, green line) at intensity (Fig. 4 i).

The content of cellulose in the floating leaves was 2.9 times more than the content of lignin in those leaves (Tab. 2).

Submerged leaves. Similarly, cytochemical analysis of the complex calcofluor-cellulose in submerged leaves of *T. natans* showed green fluorescence of cellulose in cell walls of epidermis and photosynthesizing cells (Fig. 4d, e, f). There were some differences in fluorescence intensity of cellulose in cell walls of epidermis and inner photosynthesizing parenchyma. The level of fluorescence intensity of cellulose is presented in the Table 3 and Figure 4h, h′ (diagram).

Luminescense intensity changes in submerged leaves: two times decreased in anticlinal cell walls of epidermis in comparison with those in air leaves. Whereas, luminescense intensity in walls of photosynthesizing parenchyma was lightly more in comparison with that in mesophyll in arrow-like leaves. The maximum frequency for cellulose in the epidermal cell of submerged leaf was near 25000 pixels, green line) at intensity (Fig. 4j), whereas the maximum frequency for auto fluorescence of chlorophyll in epidermal cell of submerged leaves was three times less than that in floating cells (Fig. 4 j), its was equaled 40000 pixels.

The content of cellulose in submerged leaves is decreasd in the comparison with that in the floating leaves (Tab 2).

Sagittaria sagittifolia. Cellulose investigation

Air leaves. Cytochemical analysis of cellulose in the leaves are shown as the regular green fluorescence in the walls of adaxial and abaxial epidermis, and also in palisade and spongy mesophyll (Fig. 5a-c). Ehe fluorescence intensity of calcofluor-cellulose complex was different in the tissues (Tab. 3; Fig. 5g). Outer wall of epidermis had the greatest fluorescence intensity of calcofluor-cellulose complex in comparison with other cell walls of epidermis and mesophyll. The maximum frequency for cellulose in the abaxial epidermal cell was 25000 (pixels, green line) at intensity (Fig. 5i), and frequency for chlorophyll was 150000 (pixels, red line) at intensity.

Table 3. The intensity of calcofluor-cellulose complex fluorescence in above-water and submerged leaves of *T. natans* and *S. sagittifolia* at vegetative stage

Species/tissue/cell wall	Floating (or above-water) leaf	Submerged leaf
Trapa natans:		
Adaxial epidermis (or epidermis of round particles of submerged leaf)		
– outer (periclinal) wall	158 ± 12	129 ± 11*
– anticlinal wall	150 ± 13	72 ± 6.3***
Stomata		Absent
– cell wall of pore of guard cell	42 ± 3.2	
– outer (periclinal) wall of guard cell	108 ± 10	
– anticlinal wall of guard cell	63 ± 5.5	
Abaxial epidermis		
– outer (periclinal) wall	142 ± 13	
– anticlinal wall	95 ± 7.1	
Palisade mesophyll	51 ± 4.8	Absent
Spongy mesophyll (or photosynthesizing cell of submerged leaf	44 ± 2.7	54 ± 2.1**
Sagittaria sagittifolia:		
Adaxial epidermis (or epidermis of submerged leaf)		
– outer (periclinal) wall	99 ± 9	41 ± 3.7***
– anticlinal wall	146 ± 13	69 ± 5.7***
Stomata		
– cell wall of pore of guard cell	45 ± 3.9	Absent
– outer (periclinal) wall of guard cell	52 ± 3.7	
– anticlinal wall of guard cell	113 ± 11	
Abaxial epidermis		
– outer (periclinal) wall	30 ± 3.7	
– anticlinal wall)	112 ± 11	
Stomata		
– cell wall of pore of guard cell	49 ± 3.8	
– outer (periclinal) wall of guard cell	36 ± 3.1	
– anticlinal wall of guard cell	147 ± 17	
Palisade mesophyll	106 ± 11	
Spongy mesophyll (or photosynthesizing cell of submerged leaf	56 ± 3.5	69 ± 3.9*

*, **, *** indicated values that differ significantly from the fluorescence intensity of calcofluor-cellulose complex of above-water leaves at $p = 0.05$; $p = 0.01$; $p = 0.001$, respectively

The content of cellulose in the air leaves *T. natans* was 3.4 times more than the content of lignin in those leaves (Tab. 2).

Submerged leaves. Similarly, cytochemical analysis of the complex calcofluor-cellulose in submerged leaves of *S. sagittifolia* showed green fluorescence of cellulose in cell walls of epidermis and photosynthesizing cells (Fig. 5d, e, f). There were some differences in fluorescence intensity of cellulose in cell walls of epidermis and cell walls of inner parenchyma. The level of fluorescence intensity of cellulose is presented in the Table 3 and Figure 5h, h′ (diagram).

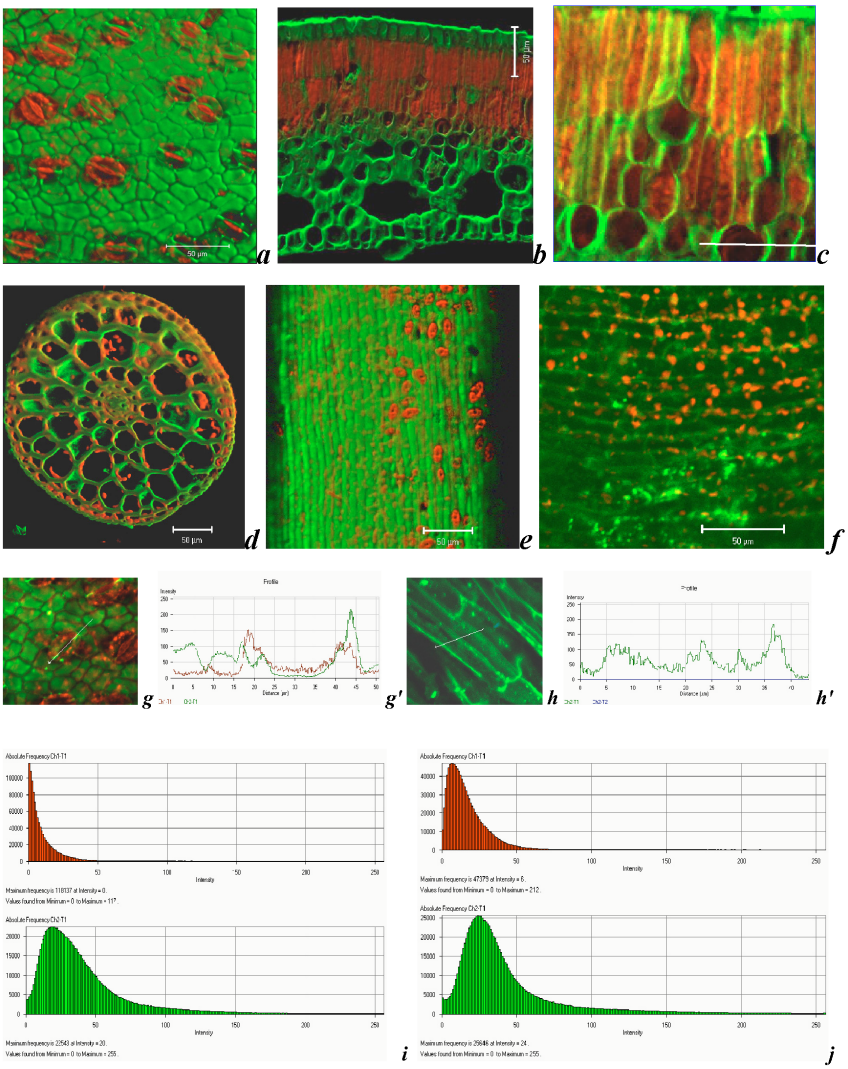


Fig. 4. Micrographs of cytochemical fluorescence of celulose in the floating (Fia. *a-c*) and submerged leaf cells (*d-f*) of *Trapa natans.* Localization of cellulose has green fluorescence. Tissue types are noted: *a* - adaxial epidermis, *b* – transverse section across leaf, *c* – mesophyll, *d* —1st layer of parenchyma in submerged leaf; *g, g′, h, h′* - histograms of fluorescence intensity of cellulose (green line) and chlorophyll auto fluorescence intensity (red line). Ordinate –Fluorescence intensity, relative units (pixels). Abscissa – Distance (μm), which was scanned on the figure *g,* and *h*. This distance is shown as white line on the figure *g′* and *h′*. On Figures *a, b , c ,d* – 3D structure. On Figures *j* and *h* – absolute frequency of pixels for cellulose (green graph) and auto fluorescence of chlorophyll (red graph). Bars = 50 μm

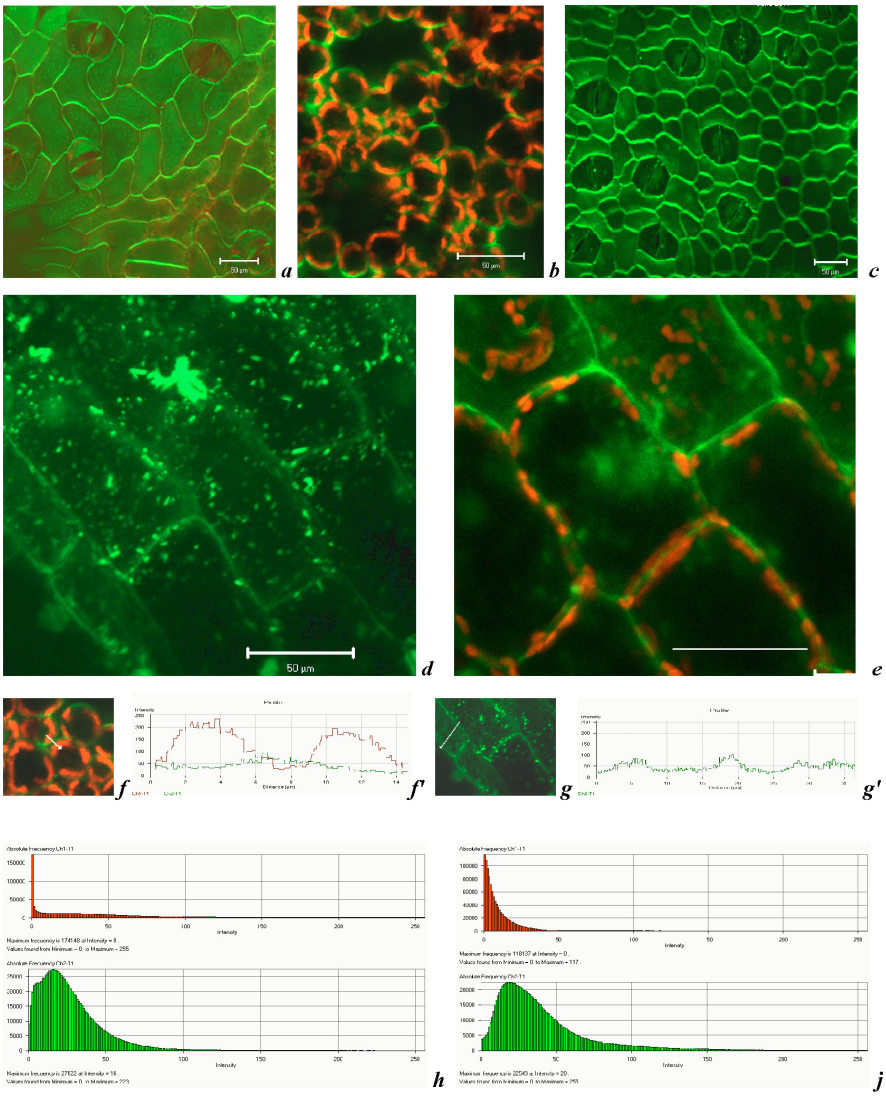


Fig. 5. Micrographs of cytochemical fluorescence of celulose in the air (Fia. ***a-c***) and submerged leaf cells (***d, e***) of *Sagittaria sagittifolia.* Localization of cellulose has yellow fluorescence. Tissue types are noted: ***a*** - adaxial epidermis, ***b*** – spongy mesophyll, ***c*** – abaxial epidermis, ***d, e*** - photosynthesizing parenchyma of submerged leaf; ***f, f′, g, g′*** - histograms of fluorescence intensity of cellulose (green line) and chlorophyll auto fluorescence intensity (red line). Ordinate –Fluorescence intensity, relative units (pixels). Abscissa – Distance (μm), which was scanned on the figure ***f,*** and ***g***. This distance is shown as white line on the figure ***f′***and ***g′***. On Figures ***a*** and ***c*** – 3D structure. On Figures ***h*** and ***j*** – absolute frequency of pixels for cellulose (green graph) and for auto fluorescence of chlorophyll (red graph). Bars = 50 μm

Luminescence intensity changes in submerged leaves: two times decreased in cell walls of epidermis in comparison with those in air leaves. Whereas, luminescence intensity in walls of photosynthesizing parenchyma did not differ from that in cell walls of mesophyll in arrow-like leaves. It is necessary to note that independently from the condition of leaf growth the relative content of cellulose in the anticlinal walls in epidermis always was more than in periclinal cell walls. It is revealed that maximum frequency for cellulose in the epidermal cell of submerged leaf was less (20000 pixels, yellow line) at intensity (Fig. 5j), the maximum frequency for auto fluorescence of chlorophyll in epidermal cell of submerged leaves was also less than in air leaves, its account 100000 pixels (Fig. j).

The content of cellulose in submerged leaves is lightly decreased in the comparison with that in the air leaves (Tab 2).

Discussion

Thus, we showed the presence of lignin in submerged and above-water leaves of two species hydrophytes (*Trapa natans* and *Sagittaria sagittifolia*) and also the increased content of lignin in the submerged leaves are like to that in submerged shoots of *Ludwigia repens* (Little, 2003). S. Little shown that in submerged shoots of *L. repens* contained lignin 1.6 times more than this polysaccharide in the above-water shoots. Why it is occurs? It is known that lignin is highly branched polymer of phenylpropanoid compounds formed through a complex biosynthesis route (Rastogi, Dwivedi, 2008), synthesis of lignin is depend on the several endogenic and exogenic factors, including light, temperature, gases and biotic stresses (Moura *et al.*, 2010). In consideration of functional significance of lignin as mechanical support allowing plant to stand (Niklas, 1992), and that during submergence leaves and shoots must to oppose water pressure (weigh) and action of wave (Tyree, Cheung, 1977; Niklas, 1992). Submerged leaves are losing trichomes, stomata and thicker cuticle (Nedukha, 2011); therefore the surface of epidermal cells in submerged leaves become more accessible for an invasion of pathogens. It is known that in cells of leaves and shoots are occurs an increase in lignifications in responses to attack by pathogens (bacteria, fungi) (Hano *et al.*, 2006; Moura *et al.*, 2010). It is known that lignin polymer is synthesized by random radical coupling of hydroxycinnamyl subunits called monolignols, mainly conyferyl (CA), sinapyl (SA) and *p*-coumaryl alcohols (*p*-CA) (Boerjan *et al.*, 2003.). Besides, the water pressure and the speed of Stream River also can to effect on the growth and structural-functional parameters of tissue and cell in the *Veronica anagallis-aquatica* submerged leaves (Boeger, Paulson, 2003). The latter data did not exclude as the influence water environment and the activation of monolignols (CA, SA, *p*-CA) synthesis on lignin content in *T. natans* and *S. sagittifolia* leaves.

The cytochemical analysis of lignin in different tissues of air leaves of *T. natans* and *S. sagittifolia* revealed the increase content of lignin in cell walls of adaxial epidermal tissue in comparison with walls of mesophyll and abaxial epidermis. It is known that adaxial epidermis is the one barrier and protection of leaf from action of ultraviolet, which is provoke the intensified synthesis of lignin (Hilal *et al.*, 2004; Moura *et al.*, 2010). Taking into account the above and the results of our experiments the greatest fluorescence of lignin in the adaxial epidermis cell of leaves that situated above water surface of *T. natans* and *S. sagittifolia*, it is suggested that outer epidermis of studied leaves of hydrophytes protects of floating leaves from the increased UV radiation so the part of it is reflected from ambient water surface.

Cytochemical analysis of epidermis in *T. natans* and *S. sagittifolia* leaves showed that the fluorescence intensity of cellulose dependents from wall type: in the periclinal epidermal walls of submerged leaves (independently of the plant species) its content was less than that in above-water leaves. Whereas the relative content of cellulose in walls of photosynthesizing cells was more than in that in cells of spongy parenchyma air leaves. It is known that polysaccharide cellulose forms semi crystalline microfibrils that impart considerable mechanical strength, and it's can certainly be considered to be a major contributor to overall plant strength and the same tensility in secondary walls (Delmer, Amor, 1995). Based on the data of these authors the following suggestion could be made; in the underwater environment cellulose of outer periclinal walls in epidermal cells of the studied plants are more flexible, whereas cell walls of parenchyma cells in submerged leaves, it is possible, are capable more mechanical strength. For the present these questions leave open.

Biochemical analysis revealed differences in the values of cellulose in the submerged and above-water leaves of *T. natans* and *S. sagittifolia:* the light lessening in leaves of *T. natans* and significant decrease in leaves of *S. sagittifolia.* The data relative to cellulose content in plants grown at flooding are diverse. Rose-John and Kende (1984) did not revealed the difference in rice air-grown and submerged internodes in content of cellulose, whereas Little (2003) shown decrease 1.5 times cellulose content in *Ludwigia repens* submerged shoots in comparison with above-water shoots. It is possible; it is depend from species, tissue, and stage of plant growth. We suggest that the decrease cellulose content in submerged leaves of the studied species is caused by the inhibition of *CesA* genes (cellulose synthase catalytic subunit), which encodes the enzyme responsible for cellulose synthesis in primary (Arioli *et al.*, 1998; Taylor *et al.*, 2000) and secondary wall (Tanaka *et al.*. 2003).

Besides, biochemical analysis revealed differences in the values of hemicelluloses and pectin in submerged and above-water leaves of *T. natans* and *S. sagittifolia.* These polysaccharides are *the* components of wall matrix, from which depend of

cell wall ultrastructure, cross-link of cellulose microfibrils and binding of pectin with metal ions (Delmer, Amor, 1995).

Thus, the obtained results revealed the particularities of polysaccharides of cell walls of *T. natans* and *S. sagittifolia* submerged leaves, which can be used as a basis for future study of both adaptation mechanisms of agricultural plants during flood, and possible for gene-engineering of plant stability in flooded area.

References

Arioli T., Peng L, Betzner AS, Burn J, Wittke W, Herth W, Camilleri C, Höfte H, 1998. *Molecular analysis of cellulose* biosynthesis in Arabidopsis. Science. 279: 717–720.

Armstrong W., Drew M.C. 2002. Root growth and metabolism under oxygen deficiency. In: Wasel Y et al. Plant Roots: The Hi The Hidden Yalf, 3rd edn. New York and Basel: 729–761.

Arasimovich V., Ermakov A. 1987. The determination of polysaccharides and lignin, in: Methods of Biochemical Study of Plants. Ed. Ermakov. A. Agropromizdat, Leningrad, Russia: 143–172.

Boeger M.R.T., Poulson M.E. 2003. Morphological adaptations and photosynthesis rates of amphibious *Veronica anagallis-aquatica (Scrophulariaceae)* under different flow regimes. Aquat. Bot. 75: 123–135.

Boerjan W., Ralph J., Baucher M. 2003. Lignin biosynthesis. Annu Rev Plant Biol. 54: 519–546.

Dat J., Folzer H., Parent C., Badot P.-M., Capelll N. 2006. Hypoxia stress: current understanding and perspectives. In: Teixeira da Silva J.A.. Ed. Floriculture. Ornamental and plant biothechnology. Advances and Topical Issues. Vol. 3. Global Science Books. Isleworth, UK, 664–674.

Delmer D.P., Amor Y. 1995. Cellulose biosynthesis. The Plant Cell. 7: 987–1000.

Hano C., Addi M., Bensaddek L., Cronier D., Baltora-Rosset S., Doussot J., Maury S., Mesnard F.,Chabbert B., Hawkins S., Laine E. 2006. Differential accumulation of monolinol-derived compounds in elicited flax *(Linum usitatissimum*) cell suspension cultures. Planta. 223: 975–989.

Herth W. 1980. Calcofluor white and congo-red inhibit microfibril assembly of Poteriochromonas: evidence for a gap between polymerization and microfibril formation. J. Cell Biology. 84: 642–658.

Hilal M., Parrado M., Rosa M., Gallardo M., Orce L., Massa M., Gonzabel J., Prado F. 2004. Epidermal lignin deposition in quinoa cotyledons in response to UV-B radiation. Photochemistry and Photobiology. 79: 205–210.

Jackson M.B., Colmer T.D. 2005. Response and adaptation by plants to flooding stress. Annals of Botany. 96: 501–505.

Little S.T. 2003. Adaptation and acclimaton of populatons of *Ludwigia repens* to growth in high- and lower–CO2 springs. A Dissertation presented to the graduate school of the University of Florida in partial fulfillment of the requirements for the degree of doctor of philosophy. – USA: Univ. Florida: 1–157.

Moura J.C., Bonine C.A., Viana J., Dornelas M.C., Mazzafera P. 2010. Abiotic and biotic stresses and changes in the lignin content and compositon in plants.. JIPB (J. Integrative Plant Biology). 52, N 4: 360–376.

Nedukha O.M. 2010. Epidermis leaf structural responses of some aquatic plants to constant water environment. Advances of Agricultural Sciences. Problem Issues. Issue. 545: 169–178.

Nedukha O.M. 2011. Heterophylly in Plants. Kiyv: Publ. Press: AltPress. 1-191 pp. (In Ukr.).

Niklas K.J. 1992. Plant Biomechanics: An Engineering approach to Plant Form and Function. Univ.of Chicago Press Amazon. com. 622 p.

Parent C., Capelli N., Berger A., Crevecoeur M., Dat J. 2008. An overview of plant responses to soil waterlogging. Plant Stress. 20, N1: 20–27

Rastogi S., Dwivedi U. 2008. Manipulation of lignin in plants with special reference to O-methyltransferase. Plant Science. 174: 264–277.

Rose-John S., Kende H. 1984. Effect of submergence on the cell wall composition of deep-water rice internodes . Plant Physiology. 76: 106–111.

Schonherr, 2006. Characterization of aqueous pores in plant cuticles and permeation on ionic solutes. J. Exp. Bot. – 2006. – 57. – P. 2471–2491.

Smith C.A., Skvirsky R.C., Hirsch A. 1986. Histochemical evidence for the presence of a suberinlike compound in Rhizobium-induced nodules of the nonlegume *Parasponia rigida.* Can. J. Bot. 64(7): 1474–1483.

Tanaka K., Murata K., Yamazaki M., Onosato K., Miyao A., Hirochika H. 2003. Three distinct rice cellulose synthase catalytic subunit genes required for cellulose synthsis in the secondary wall. Plant Physiology. et al. 133: 73–83.

Taylor N., Laurie S., Turner S. 2000. Multiple cellulose synthase catalytic subunits are required for cellulose synthesis in Arabidopsis . Plant Cell. 12: 2529–2540.

Tyree M.T., Cheung Y. N. S. 1977. Resistance to water flow in *Fagus grandifolia* leaves. Can. J. Bot.. 55, No. 20: 2591–2599.

PLANT FUNCTIONING UNDER ENVIRONMENTAL STRESS P. 153-163

Cracow, 12th to the 15th September, 2012

CHANGES OF MAIZE AND TRITICALE ROOT SYSTEM STRUCTURE AFFECTED BY DIFFERENT SOIL COMPACTION

Piotr Szczyrek, Maciej T. Grzesiak

The Franciszek Górski Institute of Plant Physiology, Polish Academy of Sciences, Niezapominajek str.21, PL 30-231 Cracow, Poland

Corresponding autor: pszczyrek@ifr-pan.krakow.pl

Abstract. The response of growth characteristics (shoots and roots dry matter, number and length of particular components of the root system) of maize and triticale to different soil compaction levels were investigated. A experiment was conducted with plants grown in a root-box with soil bulk densities throughout the 0-40 cm soil profile of 1.10, 1.34 and 1.58 g cm $^{-3}$ corresponding to a soil strength of 0.73, 1.05 and 1.68 MPa, respectively. Measurement of growth characteristics were carried out 21 and 49 days after sowing. Plants grown under the moderate (M) or severe (S) level of soil compaction in comparison to treatment with low soil compaction (L), showed a decrease in leaf number, dry matter of stem and leaves (DM_S) and roots (DM_R), and an increase in the shoot to root (DM_S/DM_R) ratio. Distinct differences in distribution of roots in the soil profile of root boxes were observed. In moderate (M) and severe (S) soil compaction levels in comparison to low level (L), a slight increase in dry root matter in the top level (0-15 cm) of the soil profile was observed, as was a drastic decrease in root biomass in M and S treatments in the soil profile from 15 to 30 cm and from 30 to 40 cm. Different soil compaction levels did not influence the number of seminal and seminal adventitious roots but decreased their length, as well as their number and total length in nodal roots. Future studies of the response of plants to growth in soil compaction should examine in more detail, chlorophyll fluorescence, hormonal signals from roots and the relationship between sink and source with regard to water and carbon supply.

Key words: Soil compaction; Maize; Triticale; Biomass; Root architecture

Abbreviations:

DM_S- shoot dry matter, DM_R- root dry matter, L, M, S- low, medium, severe soil compaction, respectively.

Introduction

The growth of plants roots is affected by physical factors in the soil and soil compaction is an abiotic stress that damages crop plants. Soil mechanical impedance is caused mainly by the use of heavy machinery in soil cultivation and by natural processes. Soil compactions as well as changes in soil water potential are two major factors that cause high mechanical impedance or excessive soil strength (Henderson 1991, Yamauchi 1993, Masle 2002). A change in growth traits was found in wheat, cotton and peanuts which were caused by high soil compaction. Growth of the above ground part of a plant is greatly reduced, compared to the root, when plants are grown with a restricted rooting volume or on compacted soils (Masle and Passioura 1987, Masle et al. 1990, Bingham and Bengough 2003). Slower leaf growth of young seedlings grown on compacted soil and plants were still in the grain reserve-dependent early growth stage. At later growth stages, plants grown on compacted soils were found to have a higher photosynthetic rate and total leaf surface had been substantially reduced. These results suggested that carbon supply was not a major factor limiting growth and there is no evidence that a lack of nutrients or water were limiting growth (Castillo et al. 1982, Hoffmann and Jungk 1995, Ishaq et al. 2001, Williams and Weil 2004).

Typical responses of a plant root system structure to soil compaction include reduction in number and length of roots, restriction of downward penetration of the main root axes, decrease in leaf thickness, increase in the dry matter shoot-to-root ratio and a decrease in crop grain yield (Clark et al. 2003, Fageria et al. 2006). The degree of restriction of root growth in compact soil depends also on the species and the age of the plants (Yamauchi 1993, Masle 2002). Inhibited plant growth is mostly attributed to reduced rooting volume (Iijima and Kono 1991, Yamauchi 1993, Masle 2002, Fageria et al. 2006, Grzesiak 2009). Sometimes during short-time growth under high soil impedance, a temporary increase in the number and length of laterals roots was observed (Yamauchi 1993, Iijima et al. 2004). A hormonal signal from the roots is the cause of shoot growth reductions on compacted soils and a hormonal mechanism may play a role in controlling root growth response to mechanical impedance on compacted soils (Jackson 2002, Else et al. 2009).

The restrictive effect of soil compaction can be physically and physiologically constraining to overall plant growth and yield through poor development of the root system as high soil impedance influences root elongation and proliferation (Tu and Tan 1991, Lipiec et al. 1993, Iijima et al.1994, Grzesiak 2009). In wheat, root length, root density and grain yield can be reduced in compacted soil compared to the non-compacted (Oussible et al. 1993). The effects of soil compaction are emphasized in drought stressed plants. In limited-rainfall areas, compacted soil can amplify these effects by reducing the ability of plants to exploit ground soil water

reservoirs. Water use is primarily determined by root system density and depth during periods of soil drought (Thangraj et al. 1990). Increased root density and depth may be responsible for drought avoidance in some rice genotypes. Therefore, identification of genotypes with a greater ability to penetrate compacted soil layers is important in developing superior drought-resistant cultivars (O'Toole and DeDatta 1983). In the study by Yu *et al.* (1995), rice cultivars from dry-land origins had greater root penetration ability than cultivars from wetland origins.

As acquisition of water and mineral nutrients is primarily determined by the dimension of the root zone and distribution of root density, root proliferation and elongation are therefore closely related to soil water content and photosynthesis of the plants in exposure to soil compaction (Iijima and Kono 1991, Oussible et al. 1993, Yamauchi 1993). Although it is reported that the responses of growth in each root type and root system architecture to compacted soil are different among plants with concentrated and scattered root systems (Iijima and Kono 1991), there is limited knowledge regarding relations between the modification in root system architecture and gas exchange and water regime of leaves (Yamauchi 1993, Grzesiak et al. 2002). Some researches indicate that there are genotypic differences of root growth tolerance to soil compaction and it is important to know the characteristics of roots associated with high tolerance to soil compaction for plant breeding (Iijima and Kono 1991, Grzesiak 2009). According of studies by Materechera et al. (1992), the size of the root has a significant effect on the root ability to penetrate a strong soil compaction layer. Vocanson et al. (2006) observed in *Pea* cultivars that differed in sensitivity to soil compaction, displayed reduced root distribution in the soil profile which had a direct impact on the final depth reached by the roots.

According to Kono et al. (1987) and Yamauchi (1993), the root system structure of cereal plant consists of seminal, seminal adventitious, nodal and lateral roots. The cereal species develop two types of root system, depending on the angle of growth of branches (lateral roots) and their distribution in a soil profile. A root system of a "concentrated type" has a greater number of nodal roots densely distributed. Another type designated as "scattered", has fewer but longer nodal roots, many of which run obliquely and vertically in the soil profile. Maize root systems belong to the "scattered" type, and triticale to the "concentrated" type (Yamauchi et al. 1987, Kono et al. 1987).

The aim of this study was to examine the effect of growth response of maize and triticale seedling to different levels of soil compaction, in relation to the compaction effects on the dry matter of shoot and roots, number and length of all components of a root system. Also the root system structure was analyzed by separately determining traits for each component, rather than dealing with the entire roots mass. Maize and triticale have different types of photosynthesis (C3-triticale, C4-maize) and a root system structure, which is "concentrated" for triticale and "scattered" for maize. The responses of these two species to soil compaction would explain how these species manage their growth.

Materials and methods

Plant material. The research was carried out on plant material obtained from a breeding station in Choryn, Poland (triticale-breeding strain, CHD-147) and from SAMPLO–Holding Tristina, Slovakia (maize single-cross hybrids, Nova).

Growth conditions. Plants were grown in air-conditioned growth cabinets under the following day/night conditions: temperature 23/18^{o}C (± 2.5^{o}C), relative humidity (RH) 70/60% (± 5%) and during a 14 h day from 7:00 to 21:00 (artificial irradiance from high pressure sodium lamps, *Philips SON-TAGRO,* 400 W) PAR was equal to about 350 μmol m^{-2} s^{-1}.

Plants were grown in root boxes, which enabled the non-destructive isolation of all compartments of the root system and the pin board set was used according to Kono et al. (1987). The set of a "root pin board method" consists of a plexiglass root box (width – 0.25 m, depth – 0.40 m, thickness – 0.02 m), a pin board for sampling the root system, and a polyethylene sheet (envelope) for handling and preserving the root system. Root boxes were filled with a mixture of soil, peat and sand (1:1:3 – v:v:v). Air-dried soil substrate was sieved in a 0.25 cm mesh and mixed with a compound fertilizer (N - 28 mg, P - 18 mg, K - 14 mg) at the rate per 1 kg of the soil substrate. Three treatments were used to compare the effect of soil compaction on low seedling growth (low- L, moderate- M and severe- S). For those treatments, the soil bulk densities throughout the 0-40 cm soil profile was set at 1.10, 1.34 and 1.58 (g cm^{-3}) corresponding to a soil strength of 0.73, 1,05 and 1.68 (MPa), respectively. Prior to sowing, root boxes were soaked with water for 30 minutes and dried for 48 hours. Eight hours after the soaking of a root-box, volumetric soil water content in treatments: L, M and S, was 0.47, 0.43 and 0.39 (cm^3 cm^{-3}) respectively. After 48 hours the volumetric soil water content decreased to 0.25, 0.21 and 0.18 respectively. According to Hillel and van Bavel (1976) these values were assumed to be 100% of soil field water capacity (FWC). A single pre-germinated grain was planted at a depth of 3 - 4 cm. The root-boxes were weighted every day, and the amount of the water loss through transpiration was refilled to keep the constant mass of root-boxes in each treatment at a level of 65-70% FWC.

Mean mechanical impedance in soil substrate profile from 5 to 35 cm for treatments L, M and S of air-dried soil were 0.84, 1.23 and 1.99 MPa, respectively and for wet soil (65–70% FWC), were 0.73, 1.05 and 1.68 MPa, respectively. For all treatments soil mechanical impedance for air-dried soil were consistently higher in comparison with wet soil. Differences between L, M and S treatments were 0.11 0.18 and 0.31 MPa, respectively. On average, in treatment S mechanical impedance of air-dried and wet soil were 2.4 and 1.6 times higher than treatments L and M, respectively (Fig. 1).

Before measurements the sampled seedling was cut into shoots and roots. The roots were sampled after the soil in the pot had been washed away by a gentle

stream of water. The sample of root was preserved in a FAA (formalin, acetic acid, and ethanol) solution.

Measurements

For measurements of number and length of seedling root components (seminal, seminal adventitious, nodal), the DELTA T SCAN (England) analyzer was used. The dry matter of the above-ground part (DM_S) and root (DM_R) were determined 21 and 49 days after plant sowing. A sample of roots was divided into 3 parts, grown in different soil profiles of the root-box (0-15, 15-30 and 30-40 cm). Dry matter was measured after drying at 65^{o}C for 72 h. Determination of dry matter and root traits was made in 5 replications for each treatment and day of harvest.

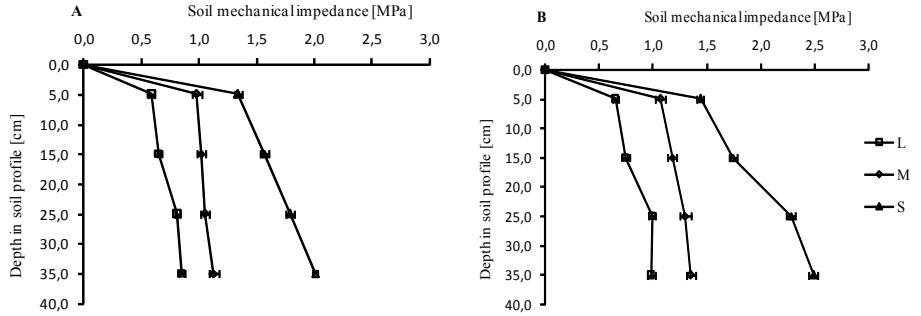
Statistical analysis

The experiments were performed according to a completely randomized design. All the results were analyzed by ANOVA (Statistics 5) using Duncan's multiple range tests ($P<0,05$). Angular transformations (arc sin $\sqrt{x}$) were performed when the variable involved was expressed in relative terms.

Results

Effect of soil compaction on growth traits. The growth under conditions of moderate (M) or severe (S) soil compaction, in comparison to treatment with low soil compaction (L) caused a decreased in dry matter of a stem and leaves (S) and roots (R), and an increase in shoot to root (S/R) dry matter ratio. Between 21 and 49 days of plant growth in M and S treatments, the dry matter of shoot decreased in comparison with the L treatment in maize to about 81 and 70% respectively, and in triticale to about 86 and 79% respectively. Similarly, in this period DM_R decreased in the M and S treatments in comparison with the L treatment in maize to about 70 and 62% respectively, and in triticale to about 71% and 59% respectively. Both maize seedlings and triticale seedlings grown under different levels of soil compaction (treatment M and S), showed slight and a statistically significant increase in the shoot to root dry mass ratio (DM_S / DM_R). For both moderate and severe soil compaction levels, an increase in the DM_S/DM_R ratio was observed in comparison to the low soil compaction treatment (Table 1. Fig. 2).

Distinct differences in dry matter distribution of the roots in the soil profile of root boxes were observed. In the M and S treatments compared to L, a slight increase of dry matter in root in the top level of the soil profile (0-15 cm) was observed. While a drastic decrease (from 50 to 10%) of R dry matter in treatments M and S in soil profile from 15 to 30 cm and from 30 to 40 cm, was observed (Table 2. Fig. 3).



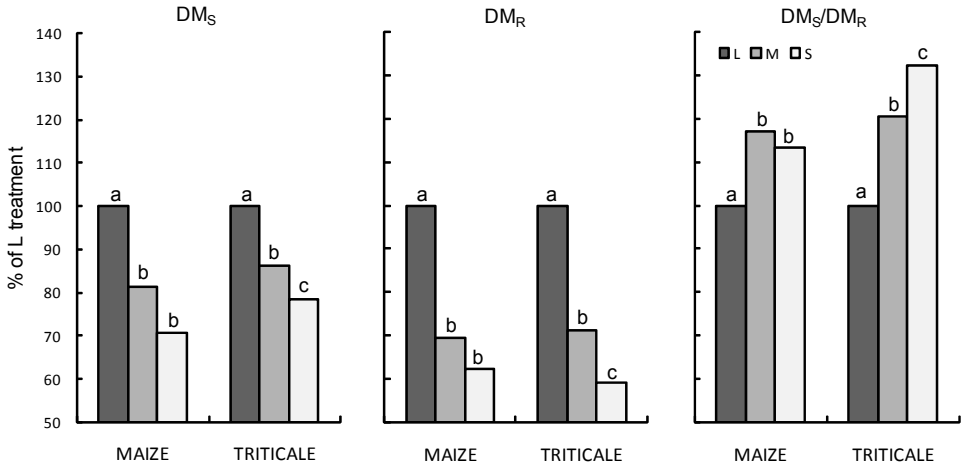
Mean values of 30 replications ± standard error of mean.

Fig. 1 Changes of soil mechanical impedance (MPa) in low (L), moderate (M) and severe (S) levels of soil compaction of the (A) air - dried soil and the (B) wet soil after watering to 65-70% of FWC soil through the depth in root-box

Table 1. Impact of soil compaction on shoot dry matter (DM_S) and roots (DM_R) in maize and triticale in 21 and 49 days after sowing

Treatment	DM_S	DM_R	$DM_S + DM_R$	DM_S/DM_R
Maize				
Days after sowing - 21				
L	0.509 a	0.260 a	0.769 a	1.96 b
M	0.430 b	0.185 b	0.615 b	2.33 a
S	0.380 b	0.164 c	0.544 c	2.32 a
Days after sowing - 49				
L	1.610 a	0.809 a	2.419 a	1.99 a
M	1.310 b	0.563 b	1.873 b	2.33 b
S	1.139 b	0.505 b	1.644 b	2.26 b
Triticale				
Days after sowing - 21				
L	0.350 a	0.211 a	0.561 a	1.66 a
M	0.319 ab	0.183 a	0.502 b	1.74 a
S	0.299 b	0.146 b	0.445 c	2.05 a
Days after sowing - 49				
L	0.789 a	0.461 a	1.250 a	1.71 a
M	0.681 b	0.329 b	1.008 b	2.06 b
S	0.619 c	0.274 c	0.893 c	2.26 b

Means within columns followed by the same letter do not differ significantly according to Duncan`s multiple range test ($\alpha = 0.05$). L – low , M – medium and S – severe soil compaction, respectively.



Bars marked with the same letter are not significantly different.

Fig. 2 Shoot dry matter (DM_S), root (DM_R) and shoot to root ratio (DM_S/DM_R) in seedlings of maize and triticale grown for 49 days in soil with different soil compaction levels (L- low, M- moderate, S- severe). Results are presented as a percent of treatment L.

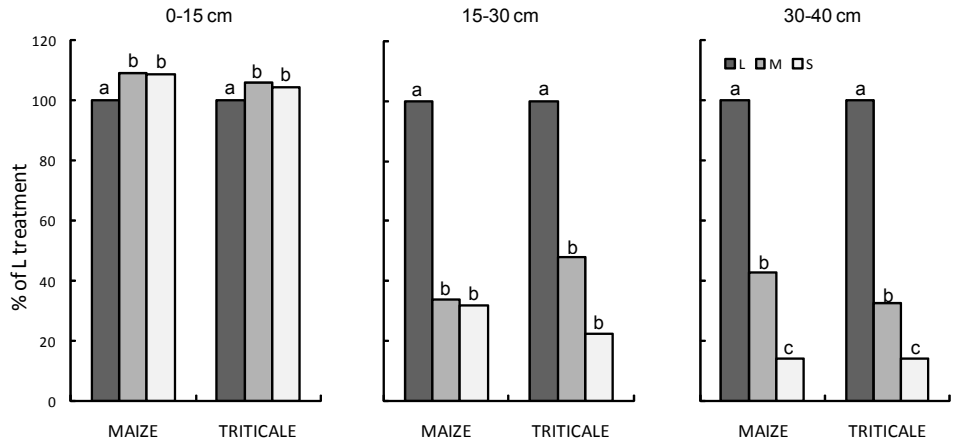
Index of relative growth rate (RGR) for seedlings of maize were higher in comparison to the values observed in triticale for all three treatments (Figure 3). The average values of relative growth rate for shoots and roots for three treatments of soil compaction was about 4.0 for maize and about 3.0 in triticale. The statistically significant decrease of RGR in treatments M and S was observed in comparison with treatment L.

Effect of soil compaction on root system structure. Different soil compaction levels did not influence the number of seminal and seminal adventitious roots but decrease their length, whilst in nodal roots a decrease in both their number and total length was observed (Table 3). After 7 weeks of seedlings growth a decrease in seminal root length in the M and S treatments in maize were about 20 and 27% respectively, and in triticale about 13 and 15% respectively. The decrease in the total length of seminal adventitious roots in maize was about 20 and 29%, respectively and in triticale about 12 and 18%, respectively. The total length of nodal roots in maize decreased by about 12 and 19% and in triticale by 19 and 31%, respectively. Observed changes of measured growth traits in M and S treatments in comparison with the L treatment, were greater for maize than for triticale. In triticale, the statistical differences between M and S in most cases were significant and in maize insignificant.

Table 2. Roots dry matter DM_R [g] developed in three layers of root box in seedlings of maize and triticale after 21 and 49 days growth in different soil compaction levels

Treatment	Soil layer in root-box profile [cm]		
	0 - 15	15 -30	30-40
Maize			
Days after sowing - 21			
L	0.102 b	0.091 a	0.067 a
M	0.121 a	0.043 b	0.021 b
S	0.118 a	0.029 b	0.017 b
Days after sowing - 49			
L	0.364 b	0.267a	0.178 a
M	0.397 a	0.090 b	0.076 b
S	0.395 a	0.085 b	0.025 c
Triticale			
Days after sowing - 21			
L	0.104 b	0.061 a	0.046 a
M	0.115 a	0.036 b	0.032 b
S	0.110 a	0.021 c	0.015 c
Days after sowing - 49			
L	0.221 b	0.115 a	0.126 a
M	0.233 a	0.055 b	0.041 b
S	0.230 a	0.026 c	0.018 c

Means within columns followed by the same letter do not differ significantly according to Duncan`s multiple range test ($\alpha = 0.05$). L – low , M – medium and S – severe soil compaction, respectively.



Bars marked with the same letter are not significantly different
L – low , M – medium and S – severe soil compaction, respectively.

Fig. 3 Roots dry matter (DM_R) developed in three layers of root-box in seedlings of maize and triticale grown for 49 days in soil with different soil compaction levels. Results are presented as a percent of treatment L.

Table 3. Relative growth rate (RGR- g $g^{-1}day^{-1}$) of seedlings of maize and triticale grown from 21 to 49 day in soil with different soil compaction levels. L – low , M – medium and S – severe soil compaction

Treatment	DM_S	% L	DM_R	% L	DM_S+ DM_R	% L
Maize						
L	4.11 a	100.0	4.05 a	100,0	4.09 a	100.0
M	3.98 b	96.8	3.97 b	98.1	3.98 b	97.3
S	3.92 b	95.3	4.02 ab	99.1	3.95 b	96.6
Triticale						
L	2.94 a	100.0	2.79 a	100.0	2.86 a	100.0
M	2.71 b	92.0	2.09 c	75.1	2.49 b	87.1
S	2.60 c	95.3	2.25 b	80.1	2.49 b	87.1

Means within columns followed by the same letter do not differ significantly according to respectively. Duncan`s multiple range test ($\alpha = 0.05$).

Discussion

Similarly as in case of responses of plants to other abiotic stresses, their responses to soil compaction are different among crop species and among cultivars within species (Iijima et al. 1994, Dennowisky 1992). In studies Iijima et al. (1994) specific response of four cereal species to the soil compaction was clearly noticeable in root distribution, nodal root number, leaf number and grain yield. Those authors reported that the responses in root growth for maize and rice were different in the downward penetration of the main axis and in higher order laterals. Species with "concentrated" type of a root system showed less restriction of root and shoot growth compared to species with a "scattered" type.

Used in this experiment, where the worst condition of soil strength was 1.68 MPa does not seem to be a critical value for root growth in maize (Imhoff et al. 2010). According to Dennovisky (1992) the root growth in triticale is limited at higher soil bulk density (1.65 g cm^{-3}) than in maize (1.46 g cm^{-3}). Obtained in this study results confirm these observations because observed changes of growth traits in soil compaction condition 1.34 and 1.58 g cm^{-3} in comparison with the 1.10 g cm^{-3} soil compaction, were greater for maize than for triticale.

The results obtained in this study indicate that the dry matter of maize and triticale is sensitive to soil compaction. Also our results indicated that root dry matter was inhibited by soil compaction, and that the shoot to root ratios (S/R) was significantly higher in compacted treatments. Changes in the S/R ratio have previously been reported in our earliest experiments (Grzesiak 2009). As shoot growth is checked by compaction, more assimilates may become available for root growth. Active substances can accumulate in root apices contacting compacted soil, coinciding with radial swelling and lateral proliferation of roots (Atwell 1988), and changes in the S/R have been frequently observed in response to different abiotic stresses. Masle and Passioura (1987) proposed that reduced leaf expansion of com-

pacted wheat plants is primarily a response to a hormonal message induced in the roots when they experience high soil strength. Clearly, more research on hormonal and source/sink mechanisms involved in controlling the root and shoot growth of plants grown on compacted soils is needed. A study by Hoffman and Jungk (1995) suggested that under soil compaction, shoot growth decreased when root growth was restricted and that both these traits were closely related, irrespective of the cause of root growth limitation by either compaction or water saturation. The reduction in dry mater of maize shoot and leaves with soil compaction was mostly due to decreased leaf area, stem diameter and plant height (Lipiec et al. 1996). In sunflower the reduction in whole plant leaf area development on compacted soils, is due not only to slower expansion rates, but also the smaller maximum size of individual leaves, and their thinness, despite they tended to have a higher specific leaf weight - SLW (Andrade et al. 1993).

Conclusion

The growth of maize and triticale was sensitive to different levels of soil compaction and the root dry matter was more suppressed than the dry matter of aboveground parts. Future studies of the response of plants to different soil compaction should examine the following in more detail i.e. chlorophyll fluorescence, hormonal signals from the roots and the relation between the sink-source and its water and carbon supply.

References

Andrade A, Wolfe DW, Fereres E (1993) Leaf expansion, photosynthesis, and water relations of sunflower plants grown on compact soil. Plant and Soil 149, 175-184

Atwell BJ (1988) Physiological responses of lupine roots to soil compaction Plant Soil 111,277-281

Bingham U, Bengough AG (2003) Morphological plasticity of wheat and barley roots in response to spatial variation in soil strength. Plant Soil 250, 273-282

Castillo SR, Dowdy RH, Bradford JM, Larson WE (1982) Effects of applied mechanical stress on plant growth and nutrient uptake. Agron. J 74, 526-530

Clark LJ, Whalley WR, Barraclough PB (2003) How do roots penetrate strong soil. Plant Soil 255, 93-104

Else MA, Janowiak F, Atkinson ChJ, Jackson MB (2009) Root signals and stomata closure in relation to photosynthesis, chlorophyll *a* fluorescence and adventitious rooting of flooded tomato plants. Annals of Botany 103, 313-323

Fageria NK, Balingar VC, Clark RB (2006) Physiology of crop production. pp. 23-60 The Haworth Press Inc. New York, London, Oxford

Grzesiak S, Grzesiak MT, Filek W, Hura T, Stabryła J (2002) The impact of different soil moisture and soil compaction on the growth of triticale root system. Acta Physiol. Plantarum 24, 331-342

Grzesiak MT (2009) Impact of soil compaction on root architecture, leaf water status, gas exchange and growth of maize and triticale seedlings, Plant Root 3, 10-16

Henderson CWL (1991) Sensitivity of eight cereal and legume species to the compaction status of deep, sand soils. Aust. J. Exp. Agric. 31, 347-355

Hillel D, van Bavel CHM (1976) Simulation of profile water storage as related to soil hydraulic properties. Soil Sci. Soc. of America J 40, 807-815
Hoffmann C, Jungk A (1995) Growth and phosphorus supply of sugar beet as affected by soil compaction and water tension. Plan and Soil 176, 15-25
Iijima M, Kono Y (1991) Interspecific differences of the root system structures of four cereal species as affected by soil compaction. Jpn. J. Crop Sci 60, 130-138
Iijima M, Kono Y, Yamauchi A, Pardalesjr JR (1994) Effects of soil compaction on the development of rice and maize root system. Envir. and Experimental Botany 30, 333-342
Iijima M, Higuchi T, Barlow PW (2004) Contribution of root cap mucilage and presence of root cap in maize Zea mays to the reduction of soil mechanical impedance. Ann. Bot 94-473
Ishaq M, Ibrahim M, Hassan A, Saaeed M, Lal R (2001) Subsoil compaction effects on crops in Punjab. II Root growth and nutrient uptake of wheat and sorghum. Soil and Tillage Res 60, 153-161
Jackson MB (2002) Long distance signaling from roots to shoots assessed the flooding story. J. of Experimental Botany 53, 175-181
Kono Y, Yamauchi A, Nonoyama T, Tatsumi T, Kawamura N (1987) A revised system of root-soil interaction for laboratory work. Environ. Control in Biol 25, 141-151
Lipiec J, Ishioka T, Hatano R, Sakuma T (1993) Effects of soil structural discontinuity on root and shoot growth in maize. Plant and Soil 157, 65-74
Lipiec J, Ishioka T, Szustak A, Pietrusiewicz J, Stępniewski W (1996) Effects of soil compaction and transient oxygen deficiency on growth, water use and stomata resistance in maize. ActaAgric.Scand. Sect. Soil and Plant Sci 46, 186-191
Masle J, Passioura JB (1987) The effect of soil strength on the growth of young wheat plants. Aust. J. Plant Physiol 14, 643-656
Masle J, Faraquhar GD, Gifford RM (1990) Growth and carbon economy of wheat seedlings as affected by the soil compaction to penetration and ambient partial of CO_2. Austr. J. Plant Physiol 17, 465-487
Masle J (2002) High soil strength: mechanical forces al play on root morphogenesis and in root:shoot signaling. *In*: Plant Roots the Hidden Half. Waisel Y, Eshel A, Kafkafi U, (Eds). pp. 807-819, Marcel Dekker Inc, New York, Basel
Materechera SA, Alston AM, Kirby JM, Dexter AR (1992) Influence of root penetration of seminal roots into a compacted soil. Plant and Soil 144, 297-303
O`Toole JC, DeDattaSK (1983) Genotypic variation in epicuticular wax of rice. Crop Sci 23, 392-394
Oussible M, Allmaras RR, Wych RD, Crookston RK (1993) Subsurface compaction effects on tillering and nitrogen accumulation in wheat. Agron. J 85, 619-625
Thangraj M, O`Toole JC, DeDatta SK (1990) Root response to water stress in rainfed lowland rice. Exp. Agric 28, 287-296
Tu JC, Tan CS (1991) Effect on soil compaction on growth, yield and root rots of white beans in clay loam and sand loam soil. Soil Biol. Biochem 23, 233-238
Vocanson A, Roger-Estrade J, Boizard H, Jeuffroy MH (2006) Effects of soil structure on pea (*Pisum sativum* L) root development according to sowing date and cultivar. Plant and Soil 281, 121-135
Williams SM, Weil RR (2004) Crop cover root channels may alleviate soil compaction effects on soybean crop. Soil Sci. Soc. Am. J 68, 1403-1409
Yamauchi A, Kono Y, Tatsumi J (1987) Comparison of root system structure of 13 species of cereals. Jap. J. Crop Sci 56, 618-631
Yamauchi A (1993) Significance of root system structure in relation to stress tolerance in cereal crop. Low-Input Sustainable Crop Production System in Asia. pp 347-360, Korean Society of Crop Science
Yu LX, Ray JD, O`Toole JC, Nguyen HT (1995) Use of wax-petrolum layer for screening rice root penetration. Crop Sci 35, 684-687

PLANT FUNCTIONING UNDER ENVIRONMENTAL STRESS P. 165-174

Cracow, 12th to the 15th September, 2012

HARMFULNESS OF *TETRANYCHUS URTICAE* KOCH AND *FRANKLINIELLA OCCIDENTALIS* PERGANDE FOR GREENHOUSE CUCUMBER TREATED WITH BIOSTIMULANTS

Anna Tomczyk, Dorota Rudzińska, Marcin Kowara

Department of Applied Entomology, Warsaw University of Life Sciences, Nowoursynowska 166, 02-786 Warsaw, Poland

Corresponding author: anna_tomczyk@sggw.pl

Abstract. The influence of *F. occidentalis* and *T. urticae* on net photosynthesis (P_N) of the plants treated and not treated with biostimulants: Asahi SL and Siapton 10 L was studied. The experiment was conducted under greenhouse conditions, on cucumber cv. Aramis. The plants were infested with spider mites and thrips (in separate greenhouse chambers), soon after first of 2 treatments with biostimulants. The plants with and without pests as well as treated and not treated with biostimulants were compared. The pest populations were monitored 6 weeks. The rate of photosynthesis of leaves was measured after 4, and 6 weeks from the beginning of pest infestation, using CO2 Analyzer, Li-COR 6400 XT. Negative influence of both pests on cucumber plants was observed, on the plants, treated and not treated with biostimulants after 6 weeks of pest feeding. It was in the contrast to the results obtained after 4 weeks of thrips feeding on these plants, where the stimulation of the photosynthesis was noticed. The rate of photosynthesis of damage plants was less affected after biostimulator application, as compared to the plants cultivated without biostimulants.

Key words: Cucumber, *Tetranychus urticae*, *Frankliniella occidentalis*, Plant damages, Asahi SL, Siapton 10L, Photosynthesis

Introduction

The most dangerous pests for greenhouse cucumber are two-spotted spider mite (*Tetranychus urticae* Koch) and western flower thrips (*Frankliniella occidentalis* Pergande) (Hao *et al.* 2002). Both pests feed on lower surface of the leaf blade, sacking the content of mesophyll cells. As a result of injuries visible white spots

are occurring, regular and small in the case of mite feeding and bigger, irregular, in the case of thrips injury. Spider mites as well as different species of thrips affect the different metabolic processes in the injured plants as they induce a biotic stress. The data of earlier studies showed influence of these pests on the activity of photosynthesis and the growth of their host plants (Tomczyk 1989, 1996; Shipp *et al.*1998; Hao *et al.* 2002; Dai *et al.* 2009). The strong decrease of photosynthesis intensity was often observed, in the plants injured by spider mites (Ferree and Hall 1980; De Angelis *et al.* 1983; Brito *et al.*1986; Tomczyk 1989; Park and Lee 2002; Haile and Higley 2003; Warabieda and Borkowska 2004; Bueno *et al.* 2009 as well as by thrips (Shipp *et al.* 1998; Hao *et al.* 2002; Deligeorgidis *et al.* 2006; Dai *et al.* 2009).

The main reason of lower photosynthesis intensity of plants injured by arthropods such as thrips or spider mites was a reduction of total chlorophyll content and chlorophyll fluorescence (Tomczyk 1989; Iatrou *et al.*1995; Haile *et al.* 1999; Bounfour *et al.* 2002; Warabieda and Borkowska 2004; Dai *et al.* 2009). However, some experiments have shown a stimulate effect of these pests on the process of photosynthesis (Tomczyk and Kropczyńska 1985; Tomczyk 1989; Trumble *et al.*1993; Reddall *et al.* 2007; Kou *et al.* 2011). In the opinion of some researchers, studies on plant physiology is essential for understanding the plant stress and for explaining the relationship between plants and stressors (Peterson and Higley 2001). Bilgin *et al.* (2010) have shown that biotic stress globally down regulates photosynthesis genes and suggest that this down regulation is an adaptive response to biotic attack.

The protection of plants against pests without chemical control can go in two directions: a decrease of the herbivore development or an increase of plant tolerance to the biotic stress. The plant treatment with some biostimulants can increase the plant tolerance to unfavorable conditions (including biotic factors such as patogens and pests), which induce a plant stress (Dąbrowski 2008).

The aim of the studies was to determine the influence of biostimulants Asahi SL and Siapton 10L on the intensity of photosynthesis in the leaves of cucumber plants injured by two-spotted spider mite (*Tetranychus urticae* Koch) and Western flower thrips (*Frankliniella occidentalis* Pergande).

Material and Methods:

The experiment was conducted under greenhouse conditions, on cucumber cv. Aramis. The plants were planted in two-leaf stage in the rings, filled with soil substrate. The infestation with pests was done soon after first treatment with biostimulants. There were applied twice during plant vegetation: 3 days before infestation with pests and 3 weeks after infestation. For the treatments 0.1% Asahi

SL (mixture of sodium o-nitrophenolan, sodium 5-nitroguaiacolan) and 0.25% Siapton 10L (mixture of amino acids and oligopeptides) were used.

The infestations with *T. urticae* and *F. occidentalis* were done in separate greenhouse chambers. However, part of the plants, treated and not treated with biostimulants, in the "thrips" chamber, was infested with *T. urticae,* to have plants injured by both pests. Initial pest populations were:

for *T. urticae* – 15 females per plant (5 per leaf)

for *F. occidentalis* – about 300 adult specimens per greenhouse (about 6 per plant)

The pest populations were monitored during 4 - 6 weeks, in 7 – 10 days intervals.Twelve groups of plants were tested, with 8 plants in each one:

1. Control plants (without pest and without biostimulants)
2. Plants treated with Asahi SL
3. Plants treated with Siapton 10L
4. Plants infested with *T. urticae*
5. Plants infested with *T. urticae* and treated with Asahi SL
6. Plants infested with *T. urticae* and treated with *Siapton 10L*
7. Plants infested with *F. occidentalis*
8. Plants infested with *F. occidentalis* and treated with Asahi SL
9. Plants infested with *F. occidentalis* and treated with Siapton 10L
10. Plants infested with *T. urticae* and *F. occidentalis*
11. Plants infested with *T. urticae* and *F. occidentalis* treated with Asahi SL
12. Plants infested with *T. urticae* and *F. occidentalis* treated with Siapton 10L

Net photosynthetic rate (P_N) of damaged and not damaged leaves was measured after 4 and 6 weeks from the beginning of pest infestation, using CO_2 Analyzer Li-COR 6400 XT. The measurements were done, in 7 replicates, on freshly detached leaves, after protection of petioles with wet cotton wool. The leaves were collected from the same part of the each plant (leaves no. 11 – 13). The average injury level after 4 weeks of mite feeding was about 35 - 40 % of the total leaf area, and 60 %, after 6 weeks. The injured leaf area caused by thrips feeding was about 25 - 30 %, after 4 weeks, and 55 – 60%, after 6 weeks. For the measurements of photosynthesis the leaf chamber fluorometer model 6400 - 40 with RedBlue LED light source was used. Light intensity was 800 μmol m^{-2} and the reference CO_2 concentration 400 μmol mol^{-1} .

Statistical methods

One-way ANOVA was used for pest populations and multifactor ANOVA for photosynthesis. Significance of differences was determined by Fisher's test with a significance threshold of 0.05.

Results

The development of *T. urticae* populations, during 6 weeks of feeding on cucumber plants, treated and not treated with biostimulants, is presented in Fig 1. During first 4 weeks of infestation the numbers of mobile stages of *T. urticae,* feeding on the plants was below 40 per leaf and they strongly increased during next 2 weeks. Higher number of mites was found on untreated plants as compared to treated with biostimulants. The difference between plants treated with Asahi and untreated was significant ($F_{2, 21} = 4{,}80$; $p = 0{,}0214$).

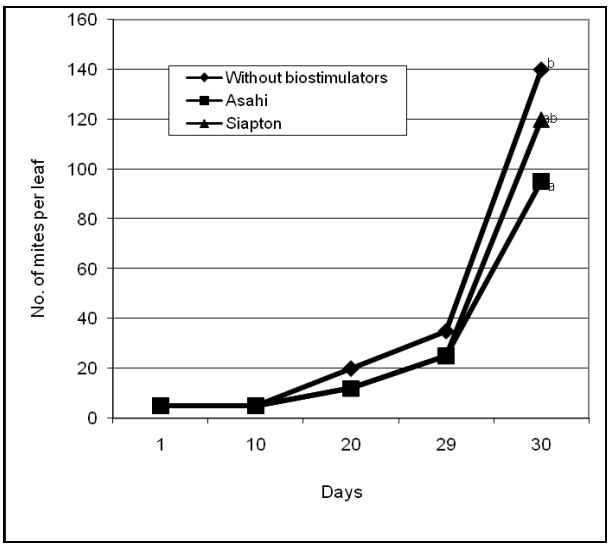


Fig 1. The development of *T. urticae* population on cucumber plants treated and untreated with biostimulants. The different letters above the lines indicate significant differences at p=0,05

The numbers of *F. occidentalis* (larvae and adults), during 6 weeks of experiment, are presented in Fig 2. The numbers of thrips increased, from initial 6 per plant, to 35 – 45, during 4 weeks and to 55 – 100, during 6 weeks of their feeding on cucumber. At that time more thrips were found on the plants treated with biostimulants as compared to untreated ones. The difference between Asahi treated and untreated plants was significant ($F_{2, 21} = 7{,}83$; $p = 0{,}005$).

The rates of net photosynthesis (P_N) of the plants with and without pests, as well as treated and not treated with biostimulants, 4 and 6 weeks after plant infestation, are presented in Fig.3 and Fig 4, respectively. The application of biostimulants on uninfested plants caused significant increase in the rate of photosynthesis, as com-

pared to the control plants (after 4 weeks: $F_{11, 72} = 8{,}71$ $p = 0{,}00$; after 6 weeks: $F_{11, 72} = 10{,}8$ $p = 0{,}00$). It was observed after 4 as well as after 6 weeks from the beginning of the experiment. However, all older plants (after 6 weeks from the beginning of infestation) had a lower level of photosynthesis than younger (after 4 weeks). The stimulate effect of biostimulant application on photosynthesis of not injured leaves was more evident in the case of Siapton 10L than Asahi SL treatment.

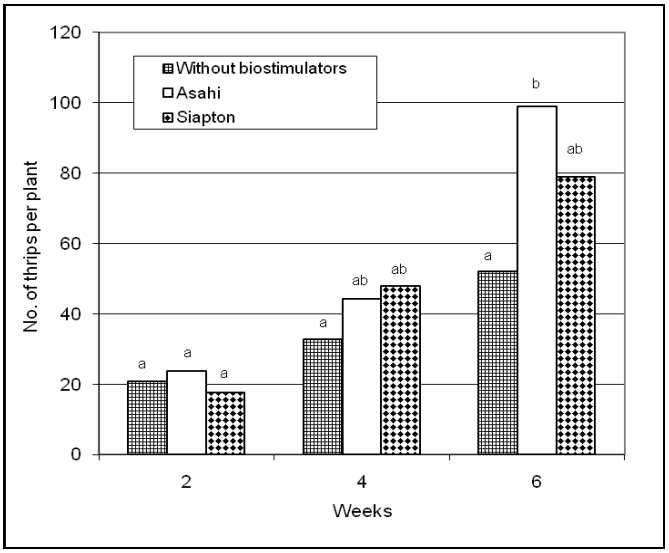


Fig 2. The development of *F. occidentalis* population on cucumber plants treated and untreated with biostimulants. The different letters above the bars indicate significant differences at p=0,05

The plants injured by *T. urticae*, not treated with biosimulants, had significantly lower photosynthesis rate, as compared to the control plants: 16 % after 4 weeks of infestation, and 29 % after 6 weeks of mite feeding. The application of biostimulants did not decrease the negative influence of spider mites on the net photosynthesis rate of cucumber plants after 4 weeks. However, it had a place after 6 weeks of infestation, when the leaves were 2 weeks older. The leaves of mite infested plants treated with Asahi SL had only 16 % lower rate of photosynthesis as compared to the uninfested leaves. The photosynthesis rate of the mite injured leaves of plants treated with Siapton 10 L was similar as compared to uninfested leaves.

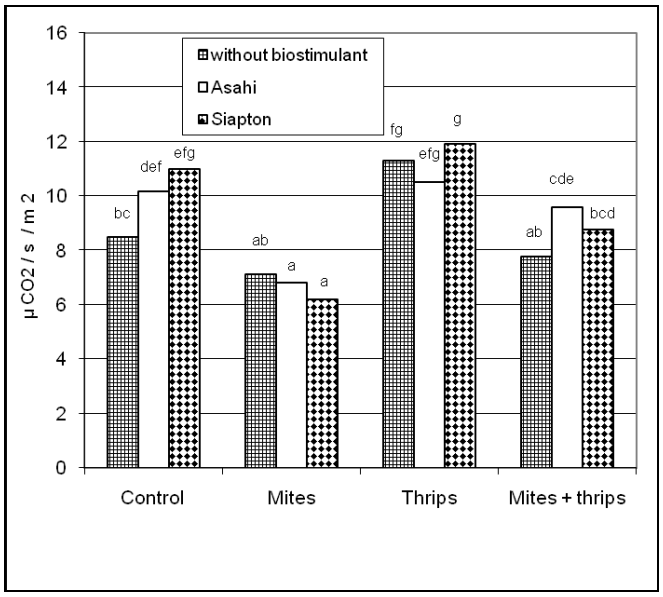


Fig 3. The rate of net photosynthesis (P_N) of the cucumber plants treated and untreated with biostimulants after 4 week infestation with *T. urticae* and *F. occidentalis.*
The different letters above the bars indicate significant differences at p=0,05

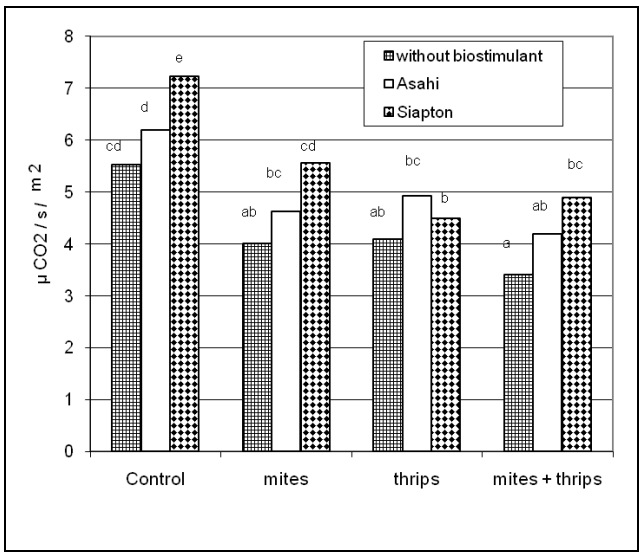


Fig 4 . The rate of net photosynthesis (P_N) of the cucumber plants treated and untreated with biostimulants after 6 week infestation with *T. urticae* and *F. occidentalis.*
The different letters above the bars indicate significant differences at p=0,05

Four weeks of *F. occidentalis* feeding on the plants caused a significant, more than 30 %, increase in the rate of photosynthesis of injured leaves, as compared to not injured ones. The application of biostimulants did not change this phenomenon. However, two weeks later (after 6 weeks of infestation – Fig. 4.) the rate of photosynthesis of the leaves injured by thrips decreased by 26 % in relation to not injured. The application of biostimulants did not significantly change this plant reaction. However, a small tendency for decrease of this negative influence of thrips on photosynthesis was observed.

Discussion

Greenhouse cucumber is a very good host plant for both *T. urticae* and *F. occidentalis*. The populations of these pests can reach high levels in short time as they have rapid reproductive rates. The feeding of high density populations of both pests result in heavy plant damages caused by removing mesophyll cell contents. Such injuries of plants create plant stress and induce reactions affecting a rate of photosynthesis, plant growth and yielding. In this studies possibility for the decrease of negative influence of *F. occidentalis and T. urticae* on the photosynthetic rates of infested cucumber plants, by applying biostimulants in their cultivation, was tested.

First measurements of the photosynthesis, after 4 weeks of pest feeding, showed a big difference in the rate of this process, between plants infested by mites and thrips. The photosynthetic rates of all plants injured by *T. urticae*, treated and not treated with biostimulants, were much lower as compared to uninfested plants. In the contrast, feeding of *F. occidentalis*, at the same time, resulted in the stimulation of photosynthetic process. The decline in the activity of photosynthesis in the leaves of different host plants attacked by high populations of spider mites was often observed (Ferree and Hall, 1980; De Angelis *et al.* 1983; Brito *et al.*, 1986; Tomczyk, 1989; Park and Lee, 2002; Haile and Higley, 2003; Warabieda and Borkowska, 2004, Bueno et al, 2009. However increase in the rate of photosynthesis, of the plants infested by spider mites was also observed, when density of their populations was low, or they were feeding by short time (Tomczyk and Kropczyńska 1985, Tomczyk 1989). In the current studies, probably the favorable conditions for the development of *T. urticae* population caused a rapid increase in the number of mites, resulted in serious destruction of plant tissues, including chlorophyll losses, and restricted possibility for CO_2 assimilation. The lower rate of photosynthesis in the leaves of plants strongly injured by spider mites was very often related to the decrease in the chlorophyll concentration in plant tissues (Tomczyk and Kropczyńska 1985, Tomczyk, 1989; Warabieda and Borkowska, 2004, Bueno *et al.* 2009). The application of biostimulants did not increase the tolerance of cucumber plants for stress caused by spider mite feeding.

The population of *F. occidentalis* developed slower than the population of *T. urticae*, in the same greenhouse conditions and probably induced defense reaction of injured plants. A strong increase in CO_2 assimilation was then observed in damaged leaves, even though the injured leaf area was in that time about 25–30 %. Almost the same, increased photosynthetic rate was observed also in plants treated with biostimulants. Many earlier studies demonstrated that plants can tolerate certain densities of thrips without any resulted damage such as growth inhibition or lower yield (Molema *et al.* 1955, Shipp 1995). However, high population of thrips can strongly damage plant tissues causing a reduction in photosynthesis, growth rate and yield (Shipp et all, 1998; Hao *et al.* 2002; Deligeorgidis *et al.* 2006; Dai *et al.* 2009).

The data of current experiment have shown that the combine feeding of *T. urticae* and *F. occidentalis* affected photosynthesis less than *T. urticae* alone, probably because the stimulative effect of thrips infestation reduced the negative influence of spider mite damage. The application of biostimulants enhanced this process, and specially the treatment with Asahi SL.

A longer feeding of both pests (6 weeks) increased a negative effect of damage on the process of photosynthesis, probably related to the increased level of biotic as well as abiotic stress. Probably a high temperature in the greenhouse, at that time, caused a rapid increase in pest populations, resulting in decline in the rate of photosynthesis. On the other hand the high temperature in the greenhouse affected also photosynthesis in undamaged leaves, as compared to the 4 week period of measurements. The application of biostimulants increased the level of plant tolerance to the injuries caused by both pests. This phenomenon was more evident after treatment with Siapton 10L. The increase in plant tolerance for biotic stress after treatment with biostimulants was also observed by other authors (Dąbrowski, 2008).

From the data presented in this paper can be concluded, that a harmfulness of *T. urticae* is higher as compared to *F. occidentalis* because it occurs earlier after the moment of pest attack and it is connected with strong inhibition of photosynthesis rate. An application of biostimulants on cucumber plants infested with *T. urticae* and *F. occidentalis* can increase the plant tolerance to this biotic stress.

Acknowledgements:

The studies were supported by the Polish Ministry of Science and Higher Education (grant no. N N310 119437)

References

Bilgin DD, Zavala JA *et al.* (2010) Biotic stress globally downregulates photosynthesis genes. Plant, Cell and Environment 33: 1597-1613

Bounfour M, Tanigoshi LK *et al.* (2002) Chlorophyll content and chlorophyll fluorescence in red raspberry leaves infested with *Tetranychus urticae* and *Eotetranychus carpini borealis* (Acari: Tetranychidae). Environ Entomol 31 (2): 215-220

Brito R, Stern VM, Sances FV (1986) Physiological response of cotton plants to feeding of three Tetranychus spider mite species (Acari: Tetranychidae). J Econ Entomol 79:1217-1220

Bueno AF, Bueno RC *et al.* (2009) Photosynthetic response of soybean to twospotted spider mite (Acari: Tetranychidae) injury. Braz Arch Biol Technol 52, 4: 825-834

Dai Y, Shao M *et al.* (2009) Effect of *Thrips tabaci* on anatomical features, photosynthetic characteristics and chlorophyll fluorescence of *Hypericum sampsonii* leaves. Crop Protection 28: 327-332

Deligeorgidis PN, Ipsilandis CG *et al.* (2006) Evaluation of the damage caused by *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae) on cucumber leaves (*Cucumis sativus* L., F_1 Kamaron). J Entomol 3 (1): 1-8

Dąbrowski Z. (2008). Biostimulators in modern agriculture: Field Crops, Vegetable Crops, Solanaceous Crops. Dąbrowski Z. (ed.). Editorial House Wieś Jutra, Warszawa.

De Angelis JR, Berry E, Krantz GW (1983) Photosynthesis, leaf conductance and leaf chlorophyll content in spider mite (Acari: Tetranychidae)-injured peppermint leaves. Environ Entomol 12: 345-348

Ferree DC, Hall FR (1980) Effects of soil water stress and two-spotted spider mites on net photosynthesis and transpiration of apple leaves. Photosynthesis Research 1: 189-197

Haile FJ, Higley LG (2003) Changes in soybean gas-exchange after moisture stress and spider mite injury. Environ Entomol 32: 433-440

Haile FJ, Higley LG, Ni X, Quisenberry SS (1999) Physiological and growth tolerance in wheat to Russian wheat aphid (Homoptera: Aphididae) injury. Environ Entomol 28: 787-794

Hao X, Shipp JL, Wang K., Papadopoulos AP, Binns MR (2002) Impact of western flower thrips on growth, photosynthesis and productivity of greenhouse cucumber Scientia Horticulture 92: 187-203

Iatrou G, Cook CM, Stamou G, Lanaras T (1995) Chlorophyll fluorescence and leaf chlorophyll content of bean leaves injured by spider mites (Acari: Tetranychidae). Exp. Appl. Acarol 19: 581-591

Kou JT, Shi SL, HU GX, Iing KK (2011) Comparison of compensatory photosynthesis between resistant and susceptible alfalfa clones as physiological response to damage by thrips. Acta Entomologica Sinica 54 (8): 910-917

Molema C, Steenhuis G, Ingamer H (1995) Genotypic effects of cucumber responses to infestation by western flower thrips. In: Parker BL, Skinner M, Lewis T (eds) Thrips biology and Management, Plenum Press, New York, pp 397-402

Park Y, Lee J (2002) Leaf cell and tissue damage of cucumber caused by twospotted spider mite (Acari: Tetranychidae). J. Econ Entomol 95: 952-957

Peterson RKD (2001) Photosynthesis, yield loss and injury guilds. In: Peterson RKD, Higley LG (eds) Biotic stress and yield loss,CRS press, New York, pp 83-97

Reddall A, Sadras VO, Wilson LJ, Gregg PC (2004). Physiological responses of cotton to two-spotted spider mite damage. Crop Sci 44: 835-846

Shipp JL (1995) Monitoring of western flower thrips on glasshouse vegetable crops. In: Parker BL, Skinner M, Lewis T (eds) Thrips biology and Management, Plenum Press, New York, pp 547-556

Shipp JL, Hao X, Papadopoulos AP, Binns M (1998) Impact of western flower thrips (Thysanopter: Thripidae) on growth, photosynthesis and productivity of greenhouse sweet pepper. Scientia Horticulturae 72: 87-102

Tomczyk A (1989) Physiological and biochemical responses of different host plants to infestation by spider mites (Acarina: Tetranychidae). Treatisis and Monographs, Warsaw Agricultural University, Poland, 112 p

Tomczyk A (1996) Mechanisms of physiological and biochemical interactions between spider mites (Tetranychidae) and their host plants. Acarology IX Proceedings, pp 25-28.

Tomczyk A, Kropczyńska D (1985) Effects on the host plants. In: Helle W, Sabelis MW (eds) Spider mites, their biology, natural enemies and control. Elsevier, 1A pp 317-327.

Trumble JT, Kolodny-Hirsh DM, Ting IP (1993) Plant compensation for arthropod herbivory. Annu Rev Entomol 38: 93-119

Warabieda W, Borkowska B (2004) Chlorophyll fluorescence as a diagnostic tool for assessment of apple resistance against two-spotted spider mite (*Tetranychus urticae* Koch.). Electronic Journal of Polish Agricultural Universities, Horticulture, Volume 7, Issue 1

PLANT FUNCTIONING UNDER ENVIRONMENTAL STRESS P. 175-183

Cracow, 12th to the 15th September, 2012

RESPONSES OF PHOTOSYNTHESIS AND PHOTOSYSTEM II TO SALT STRESS IN TWO OAT CULTIVARS, *(AVENA SATIVA)* AND *(AVENA NUDA)*

Ewelina Janowska-Miąsik, Renata Tobiasz-Salach, Dorota Bobrecka-Jamro

University of Rzeszow, 35-601 Rzeszów, ul. Zelwerowicza 4, Poland.

Corresponding author: e_janowska@poczta.onet.pl

Abstract To investigate the changes occurring in PSII, chlorophyll (Chl) a fluorescence transient (OJIP) was measured in oat leaves after salt treatment. The plants were grown in modified Hoagland nutrient solution throughout the study period. Seven-day old seedlings were subjected to salinity stress 120 mM NaCl. Salt treatment of oat seedlings decreased the time at which the maximum fluorescence value was reached (Tfm) and the area above the fluorescence curve between Fo and Fm (Area) parameters, which is the confirmation of the fact that cereals have grown under stress condition. On the other hand salt stress did not induced any effects on the maximal efficiency of PSII photochemistry measured (Fv/Fm). This result suggests that analyzed oats showed resistance to salinity stress, and indicate that tolerance of PSII to salinity stress can be viewed as an important strategy for these cereal to grow in saline soil.

Key words: Chlorophyll a fluorescence; Oat; OJIP transient; Photosystem II; Salinity

Introduction

Plants like cereals which grown under field conditions are often exposed to various different stress agents concurrently. However, this study focuses only on a salt stress. On a global note, no other toxic substance restrict plant growth more than salt does. (Zhu, 2007). Increasing salinization poses a real threat to cultivatable land, since it may probably led to the loss of 30% of land within the next 25 years and even up to 50% by the year 2050.(Basu, 2009) Among the various sources of soil salinity, irrigation combined with poor drainage has turned out the most serious, since it represents losses of once productive agricultural land.(Zhu, 2007).

Munns (1995) has suggested that plant under salinity is inhibited through two phases. Initially, growth is affected because of cellular responses to the osmotic effect. In the following phase, growth is reduced due to the toxic effects of accumulated salts. Salt stress leads to a decreased efficiency of photosynthesis [Sayed, 2003], which is one of the most important metabolic process in plants and its performance is greatly affected under stress conditions. The four protein components of the photosynthetic electron transport chain responsible for the electron transfer from water to $NADP^+$ are Photosystem II (PSII), Photosystem I (PS I), cytochrome ($Cytb_6f$) complex, and ATP synthase. (Wydrzyński, 2008) PS II is more sensitive to all types of stresses as compared to PS I. (Apostolova, 2006). Since PSII is believed to play a key role in the response of photosynthesis to environmental perturbations (Baker, 1991), the effects of salinity stress on PSII have been investigated extensively. A useful and rapid measure of photosynthesis is the induction of Chlorophyll a fluorescence (Kautsy effect), which is a sensitive indicator of photosynthetic energy conversion that occurs during the light reaction stage (Schreiber *et al.,* 1995). Chl fluorescence can be modified by any factor affecting the light reaction pathway of photosynthesis, including many environmental stress agents (Lichtenthaler 1996). Recently, a dual-wavelength pulse-amplitude modulated fluorescence monitoring system was allowed to apply the Saturation Pulse method with the same ease and reliability for assessment of energy conversion efficiencies in PS I and PS II (Schreiber 2004, Schreiber and Klughammer, 2008). The objective of the study was to evaluate the effect of salt stress on Chlorophyl a fluorescence induction kinetics in a plant popularly referred to as a phytosanitary plant, with high resistance to many diseases, i.e., oats. On the basis of this, various parameters of fluorescence induction curves like Fv/Fm ratio, performance index (PI), area over the curve, etc. were measured.

Material and methods

Two oat cultivars, Bingo *(*Avena sativa*)* and Maczo *(*Avena nuda*)* were examined under greenhouse conditions. The plants were grown in modified Hoagland nutrient solution throughout the study period. The average temperature for day/night was 27/18 ^{0}C, relative humidity was 50-60%, the photoperiod for the day/night cycle was 16/8 h, and the maximum photosynthetically active radiation was about 1400 photons. Seven - day old seedlings were subjected to salinity stress 120 mM NaCl. First Chlorophyll *a* fluorescence measurements were performed first 7 days (18 BBCH) after stress application to observe reactions of photosynthetic apparatus. The next 22 days (28 BBCH) after stress application were devoted to the monitoring future stress application effects. Chlorophyll fluorescence parameters were measured with a direct fluorimeter (Pocket PEA, Han-

satech Instruments Ltd., King's Lynn, UK) Measurements of chlorophyll fluorescence were done on 25 plants from each treatment and had 4 repetitions for each plant. Chlorophyll fluorescence measurements were conducted on the first, second and third leaves of all plants, but only the average values are presented. Leaf samples were illuminated with strong light pulse (3500 photons) after 45 min in dark-adaptation. Upon irradiation, dark-adapted photosynthetic samples exhibit a fast fluorescence rise from the initial fluorescence intensity (Fo) to a maximal intensity (Fm) (Laz*á*r, 2006). The fluorescence intensity at 50µs was considered to be Fo, minimal fluorescence. The JIP test (Strasser B.J., 1995; Strasser R.J., 2000; 2004: 2010) was used to analyze each Chl *a* fluorescence transient. OJIP transient is non-destructive, easy, and allows rapid testing of any type of chlorophyll-containing sample in any forms.(Strasser R.J., 2004: 2010). Several researchers have reported that the measurement of OIJP transients is a dependable and sensitive method for the detection and quantification of salt induced changes in PS II and PSI of plants (Zribi 2009, Zushi 2012, Zhang 2008) The following parameters were also measured: The Performance Index (PI) which provided useful and quantitative information about the state of plants and their vitality (Strasser B.J., 2000). Maximal efficiency of PSII photochemistry (Fv/Fm), a parameter which is widely considered to be a sensitive indication of plant photosynthetic performance (Kalaji H.M., 2008). (Fv/Fo), value that is proportional to the activity of the water-splitting complex on the donor side of the PSII and could provide an estimation of leaf photosynthetic capacity. The time over which the maximum fluorescence value (Fm) was reached (Tfm), can be a useful indicator of sample stress which causes the (Fm) to be reached much earlier than expected. The area above the fluorescence curve between Fo and Fm (Area) is proportional to the pool size of the electron acceptors Qa on the reducing side of Photosystem II. If electron transfer from the reaction centres to the quinon pool is blocked , this area will be dramatically reduced.

The analysis of the variance (ANOVA) and the separation of the means (with the LSD test, $p<0,05$) were performed by using the software package Statistic ver. 8 (StatSoft Inc.,)

Result and discussion

Analyzing plants under the salinities used in this study, some important indicators regarding physiological changes were found, which could be useful in verifying and examining difference in tolerance to salt stress. Chlorophyll *a* fluorescence transient was measured to evaluate the effects of salt stress on the photochemical efficiency of PS II. The OJIP transient represents the successive reduction of elec-

tron transport pool of PS II. The intensity of fluorescence in the OJIP transient decreased with the plant growth. The OJIP chlorophyll fluorescence curves obtained from the 120mM NaCl treated Maczo cultivar showed slower fluorescence rise, and reached a lower P level after both (168h and 528h) of salt application (cf. green curve with the red curve) (Fig.1, Fig.2).

After 168 hour of salt treatment, the value of most parameters characterizing PS II functioning, were similar to those of control plants. Only PI values increased significantly as compared to control plants. (Table 1) Similar results were obtained Bacarin (2011) who studied salt stress on *Brassica napus L.*

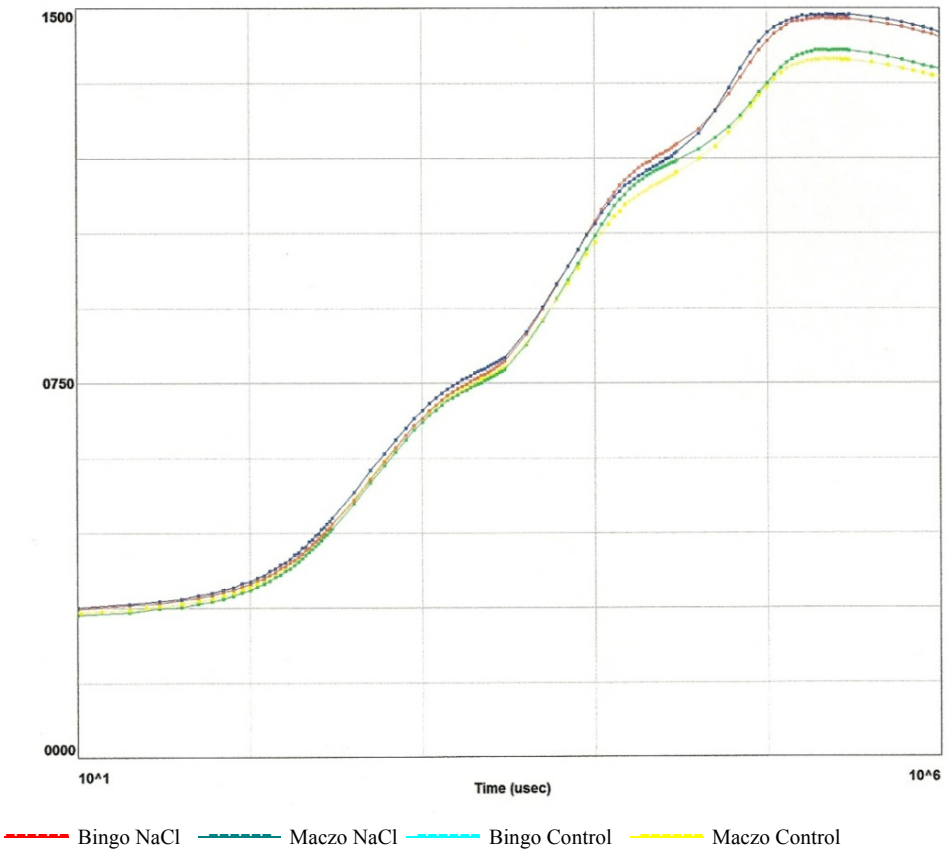


Figure 1. Chlorophyll *a* fluorescence induction curve of two oat cultivars Maczo and Bingo after 7 days of NaCl treatment

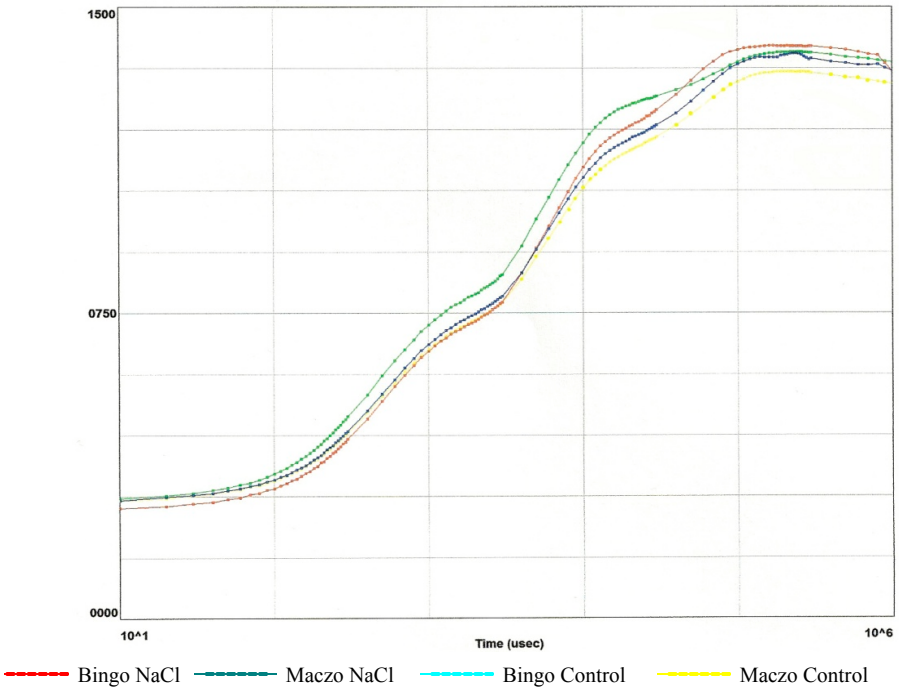


Figure 2. Chlorophyll *a* fluorescence induction curve of two oat cultivars Maczo and Bingo after 22 days of NaCl treatment

Larger changes in Chlorophyll *a* fluorescence parameters were observed 528 hours after the salinity application. Naked Maczo cultivar showed significantly higher Fo rate (average of 8.6%), and lower by 11, 9% Fv /Fo. The high Fo values can have several reasons, one of which may be increased number of inactive reaction centers where electron can not be transferred out of reduced primary quinine acceptor of PSII, second possibility is low energy transfer from LHC II to PS II reaction center, this may have been caused by the dissociation of LHC II from the PS II core (Havaux 1993, Murkowski 2002). Bingo cultivar received a higher PI rate, which probably suggest the stimulating effect of NaCl on this oat cultivar. Analyzing Fv/Fm parameter, which remain unchanged as duration of treatment of NaCl increase, we can draw a following conclusion that salinity had no effects on PSII photochemistry in a dark-adapted state. Very similar results in their experiments received (Brugnoli and Bjorkman, 1992; Morales et al., 1992; Abadia *et al.,* 1999). These results are contrary to many researchers, which reported that salinity reduces the Fv /Fm and Fv/Fo parameters. Their studies have shown that salt stress inhibits PSII activity. (Mistra 2006, Netondo 2004, Pereira 2000, Fricke and Peters

2002) The study also showed that salinity causes a significant decrease of Area and Tfm rates (tab. 2) Tfm decreased significantly (ca.7,2%), relative to control plants.

Tabela 1. Chlorophyll *a* fluorescence of two oat cultivars 168 hours after 120 mM NaCl application

Duration of treatment in [h] (I)	Cultivar (II)	Fo	Fm	Fv	Fv/Fm	Fv/Fo	Area	PI	Tfm
Control 0	Maczo*	281,5	1398,3	1116,8	0,799	3,969	21940,5	2,739	235,0
	Bingo	293,0	1486,8	1193,8	0,803	4,076	23118,8	3,131	265,0
168	Maczo*	276,8	1415,8	1139,0	0,804	4,117	22674,0	3,059	230,0
168	Bingo	290,8	1480,0	1189,3	0,804	4,093	22992,3	3,348	232,5
LSD α=00,5	IxII	**n.s.**	**n.s.**	**n.s.**	**n.s.**	**n.s.**	**n.s.**	**n.s.**	**n.s.**
Mean									
0		287,3	1442,5	1155,3	0,801	4,023	22529,6	2,935	250,0
168		283,8	1447,9	1164,1	0,804	4,105	22833,1	3,203	231,3
LSD α=00,5		**n.s.**	**n.s.**	**n.s.**	**n.s.**	**n.s.**	**n.s.**	**0,161**	**n.s.**
	Maczo*	279,1	1407,0	1127,9	0,802	4,043	22307,3	2,899	232,5
	Bingo	291,9	1483,4	1191,5	0,803	4,085	23055,5	3,239	248,8
LSD α=00,5		**6,6**	**35,7**	**37,5**	**n.s.**	**n.s.**	**n.s.**	**0,15**	**n.s.**
Total Mean		285,5	1445,19	1159,69	0,8	4,064	22681,4	3,069	240,625
* nacked									

Tabela 2. Chlorophyll *a* fluorescence of two oat cultivars 528h after 120 mM NaCl application

Duration of treatment in [h] (I)	Cultivar (II)	Fo	Fm	Fv	Fv/Fm	Fv/Fo	Area	PI	Tfm
Control 0	Maczo*	313,0	1348,5	1028,5	0,77	3,29	15169,3	2,69	240,0
	Bingo	314,5	1387,8	1073,3	0,77	3,42	17105,0	2,94	222,5
528	Maczo*	321,5	1390,0	1068,5	0,77	3,33	12462,8	2,71	222,5
528	Bingo	294,0	1406,0	1112,0	0,79	3,78	14117,0	4,32	225,0
LSD α=00,5	**IxII**	**6,73**	**n.s**	**n.s**	**n.s**	**0,13**	**n.s**	**0,39**	**n.s**
Mean									
0		313,8	13642,6	1050,9	0,77	3,353	16137,1	2,811	231,3
528		307,8	13998,0	1090,3	0,78	3,554	13289,9	3,519	213,8
LSD α=00,5		**n.s**	**n.s**	**31,7**	**n.s**	**0,125**	**1898,3**	**0,32**	**12,1**
	Maczo*	317,3	1365,8	1048,5	0,768	3,303	13816	2,700	231,3
	Bingo	304,3	1396,9	1092,6	0,782	3,601	15611	3,629	223,8
LSD α=00,5		**11,8**	**n.s**	**31,4**	**0,06**	**0,13**	**n.s**	**0,45**	**n.s**
Total mean		310,8	1381,3	1070,56	0,775	3,45	14718,5	3,165	227,5
* nacked									

The area over the fluorescence curve as compared to control leaves was decreased by 17,7% under 120 mM NaCl treatment. This decrease in area parameter with increasing duration of treatment of NaCl concentration suggests that the electron transfer rates at the donor side of PS II was inhibited.

The experiment has shown cultivar differences between the hulled and naked form of oats in respect of chlorophyll fluorescence regardless of salinity. Differences were seen at every stage of measurement (tab. 1, 2). Higher values indicating a better performance of light phase photosynthesis were obtained for the Bingo cultivar (hulled oat) compared to the Maczo cultivar (naked).

Conclusions

These studies under controlled conditions of two oat cultivars (Avena nuda*)* naked form of oat, and (Avena sativa) hulled form showed, that reaction to salinity of these two oat cultivars were varied. Both the measured and the calculated values of the analyzed fluorescence parameters indicate that the photosynthetic apparatus of Bingo cultivar of oat is more tolerant to salinity, compared with Maczo cultivar, which was characterized by a lower photosynthetic apparatus performance compared to a Bingo cultivar. Maczo obtain the lower values of the following parameters Fv (variable chlorophyll fluorescence), Fv/Fo (maximal efficiency of PSII photochemistry) and Area. These results, need to be supplemented by the analysis of the dark phase of photosynthesis, and the gas exchange in both forms of oats, because the provided information will give the opportunity to fully evaluate the photosynthetic performance in studied forms of oat.

References

Abadia A, Belkohodja R, Morales F, Abadiaa J. (1999). Effects of salinity on the photosynthetic pigment composition of barley (*Hordeum vulgare L.*) growth under a triple-line-source sprinkler system in the feld. Plant Physil. 154, 392-400.

Apostolova E.L., Dobrokova A.G., Ivanova P.I., Petkanchin I.B., Taneva S.G., (2006), Relationship between the organization of the supercomplex and the functions of the photosynthetic apparatus, J. Photochem. Photobiol. B: Biol.83 114-122

Bacarin M.A., Deuner S., Silva F.S.P., Cassol D., Silva D.M., (2011), Chlorophyll a fluorescence as indicative of the salt stress on *Brassica napus L.*Braz. J. Plant Physiol., 23(4) 245-253

Baker NR. (1991). Possible role of photosystem II in environmental perturbations of photosynthesis. Physiologia Plantarum 81, 563-570.

Basu S., Roychowdhury A.,Saha P., Sengupta DN (2009), differential antioxidative responses of indica rice cultivars to drought stress. Plant Growth regular10,219-225

Brugnoli E, Bjorkman O. (1992). Growth of cotton under continuous salinity stress: influence on allocation pattern, stomatal and non-stomatal components of photosynthesis and dissipation of excess light energy. Planta 187, 335-345.

Fricke W., Peters W.S., (2002) The biophysics of leaf growth in salt- stressed barley. A study at the cell level. Plant Physiol.129, 374-388

Govindjee,(1995), Sixty-three years since Kautsky: chlorophyll a fluorescence. Plant Physiol 22,131-160

Havaux M., (1993), Rapid photosynthetic adaptation to heat stress triggered in potato leaves by moderately elevated temperatures. Plant Cell Environ.16, 461-467

Kalaji H.M., Guo P., (2008), Chlorophyll fluorescence: A Useful tool in barley plant breeding progress, Photochemistry research Progress, Chapter12, 447-471

Lazár D., (2006) The polophasic chlorophyll a fluorescence rise measured under high intensity of exciting light. Funct. Plant Biol. 33, 9-30

Lichtenthaler H.K., (1996), Vegetation Stress: an introduction to the stress concept in plants. Plant Physiol.148, 4-14

Misra A.N., LatowskiD., Strzalka K., (2006) The xanthophyll cycle activityin Sidney Bean and cabbage leaves under salinity stress Plant Physiol.53,102-109

Morales F, Abadia A, Gomez-Aparis J, Abadia J. (1992). Effects of combined NaCl and CaCl2 salinity on photosynthetic parameters of barley grown in nutrient solution. Physiologia Plantarum 86, 419-426.

Munns R., Schachtman D.P., Condon A.G., (1995), The significance of two phase growth response to salinity in wheat and barley, Plant Physiol. 22, 561-569

Murkowski A., (2002), Oddziaływanie czynników stresowych na luminescencję chlorofilu w aparacie fotosyntetycznym roślin uprawnych .Monografia 61, Instytut Agrofizyki PAN, Lublin

Netondo G.W., Onyango J.C., Beck E., (2004) Sorghum and salinity: II Gas exchange and chlorophyll fluorescence of sorghum under salt stress Crop.Sci. 44, 806-811

Pereira W.E., de Siqueira D.L., Martinez C.A., Puiatti M., (2000) Gad exchange and chlorophyll fluorescence in four citrus rootstocks under aluminium stress Plant Physiol. 157, 513-520

Sayed O.H. (2003), Chlorophyll fluorescence as a tool in cereal crop research. Photosynthetica 41

Schreiber U., Bilger W., Neubauer C., (1995),Chlorophyll fluorescence as a non-instructive indicator for rapid assessment of in vivo photosynthesis. Ecophysiology of photosynthesis. Springer-Verlag, Berlin, 49-70.

Schreiber U., Klughammer C.,(2008), Saturation pulse method for assessment of energy conversion in PS I. PAM Appl. Notes 1, 11-14

Schreiber U.,(2004), Pulse-amplitude-modulation (PAM) fluorometry and saturation pulse method, Chlorophyll a fluorescence , a Signature of Photosynthesis. Springer Press, 280-312

Strasser B.J., Strasser R.J., (1995), Measuring fast fluorescence transient to address environmental questions, The JI –test, Photosynthesis, from light to biosphere, vol.5, 977-980

Strasser R.J., Srivastava A., Tsimilli-Michael M., (2000), The fluorescence transient as a tool to characterize and screen photosynthetic samples, Probing photosynthesis; Mechanism, regulation and adaptation, 443-480

Strasser R.J., Tsimilli-Michael M., Srivastava A.,(2004), Analysis of the Chlorophyll a fluorescence transien,: Chlorophyll fluorescence: A Signature of Photosynthesis, Advances in Photosynthesis and Respiration Series, vol 19, 321-362.

Strasser R.J.,Tsimilli-Michael M., Qiang S., Goltsev V., (2010), Simultaneous in vivo recording of prompt and delayed fluorescence and 820-nm reflection changes during drying and after rehydration of the resurrection plant *Haberlea rhodopensis*. Biochim., Biophys. Acta 1797, 1313-1326

Wydrzyński T.J.,(2008),Water splitting of Photosystem II: where do we go from here?, Photosynth. Res. 98 43-51, 321-330

Zhang L., Xing D., Rapid determination of damage to photosynthesis caused by salt and osmotic stress using delayed fluorescence of chloroplast. Photochem. Photobiol. Sci.7 ,(2008) 352-360

Zhu JK (2007), Plant salt stress, Encyclopedia of life science, John Wiley and sons, Ltd.

Zribi L., Fatma G., Fatma R., Salwa R., Hassen N., Nejib R.M.,(2009), Application of chlorophyll fluorescence for the diagnosis of salt stress in tomato *Solanum lycopersicum* Sci. Hortic.120, 367-372.

Zushi K., Kajiwara S., Matsuzoe N., (2012),Chlorophyll a fluorescence OJIP transient as a tool to characterize and evaluate response to heat and chilling stress in tomato leaf and fruit.Science Horticulturae 148, 39-46

PLANT FUNCTIONING UNDER ENVIRONMENTAL STRESS P. 185-192

Cracow, 12th to the 15th September, 2012

ER-BODIES IN *ARABIDOPSIS THALIANA* ROOT APICES UNDER CLINOROTATION AND AFTER X-RAY IRRADIATION

Svitlana Romanchuk

Cell biology and Anatomy Department, Institute of Botany of National Academy of Sciences of Ukraine 2, Thereschenkivska str., 01601, Kyiv – 1, Ukraine

Corresponding author: svet-romanchuk@yandex.ru

Abstract We studied the effect of clinorotation and X-Ray irradiation on ultrastructure of *Arabidopsis thaliana* endoplasmic bodies (ER-bodies) of root cells, which are granular endoplasmic reticulum derived organelles containing β-glucosidase. For the first time it was shown that ER-bodies in statocytes and cells of distal elongation zone with sensitivity to both studied stressors, as a result, an increase in the number and area of ER-bodies per section of a cell and in the variability of their forms was detected of these factors compared with controls. On the basis of obtained data, a role of ER-bodies in the stress reactions of plant cells is discussed in the up-to-date ideas on plant cell stress-sensitivity.

Key words: ER-bodies; β-glucosidase; clinorotation; X-ray irradiation; electron microscopy; root apices; *Arabidopsis thaliana*

Introduction

The study of plant cell reactions to various stress factors is a paramount task of plant biology. Such studies cover a range of biotic and abiotic effects on plants on earth. With the development of science and technology it has become possible to explore space by mankind. But realization of long-term space flight requires the life support bioregenerative systems, an indispensable component of which are plants as a source of oxygen, water and food. Although it is well known now that plants adapt to spaceflight factors, in particular to microgravity, by changing some their patterns at the cellular, physiological, biochemical and molecular levels, many questions on cause and effect of these changes are still open. In addition, it is nec-

essary to find the plant species which will be the most suited to the conditions in a space craft cabin (Kordyum 2002). Plants of the family *Brassicaceae* are known to be resistant to a variety of abiotic stresses, including irradiation (Saton et. al. 2001, Ogasawara 2001**,** Popova, Golldack 2007, Kuhlmann, Müller 2009). Among them there are many cultivated plants with which we encounter every day: cabbage, radish, mustard, rapeseed, etc., and *Arabidopsis thaliana* - a convenient model object. Clinorotation enables to reproduce partially the biological effects of microgravity in the laboratory. There are relatively much data on the changes in the ultrastructure of plastids and mitochondria in *A. thaliana* root and leaf cells under the influence of real and simulated microgravity (Guisinger, Kiss 1999, Kraft et. al. 2000). But information on the structure of endoplasmic reticulum (ER) in this species in altered gravity is absent. The family *Brassicaceae* is known to be characterized by the presence of ER-bodies in plant cells, which are derivative of granular endoplasmic reticulum (GER). ER-bodies have been in the first time described in radish cells using the electron microscopic method (Bonnett, Newcomb 1965). Later, these bodies were found in *A. thaliana* epidermis and cotyledon cells and it was shown that an enzyme β-glucosidase is its main component using the method of immunocytochemistry (Matsushima et. al. 2003). β-glucosidase (β-D-glucoside glucohydrolase, EC 3.2.1.21) catalyze the hydrolysis of aryl- and alkyl-β-glucosides, releasing glucose and an aglycone. β-glucosidase is appeared to perform the protective function (Xu et. al. 2004).

Earlier, an increase in the ER-bodies partial volume in cells under clinorotation has been reported (Kalinina 2007, Romanchuk 2010). So for us it became interesting to study the reaction of ER-bodies to other types of stress associated with both conditions on Earth and in space flight, in particular irradiation, since its contribution to genetic damages and cell physiological state changes may be significant (Kutsokon' et. al. 2003).

Materials and methods

Plant material. A. thaliana (L) Heynh. (line Columbia) seedlings were chosen for the study. Seeds were rolled up in the filter paper and treated with 70% alcohol for 30 second and washed with distilled water for 2 minutes; and clorox for 6 minutes and washed with distilled water 5 times for 5 minutes. Seeds planted of metal rod on mineral medium with an agarose additive (Murashige, Skoog 1962) in a Petri dish.

Clinorotation. Part of Petri dishes with seeds were placed in containers on a slow horizontal clinostat (2rpm). Other part of Petri dishes with seeds was in the stationary growth conditions. All plants grew in darkness at temperature 23°±1°C and humidity 67±1% during 3- and 7-days.

X-ray irradiation. 3-day-old seedlings were treated with X-ray radiation with a dose of 0.5 Gray and 8.0 Gray on the unit RUM-17 (Russia) (dose rate 0.43 cGr/sec). After irradiation and control seedlings grew for 10 days in light 12.000 lux (with 16/8 hours photoperiod) under the following conditions: temperature 23 ± 1°C, humidity 67 ± 1%.

Electron microscopy. The main root apices were fixed with glutar-aldehyde (2.5%) in a 0.1 M cacodylate buffer (pH 7.3) and postfixed with a 1% OsO4 solution in the same buffer. The specimens dehydration in a series of alcohols at increasing concentrations (15%, 30%, 50%, 70%, 80%, 90%, 96%, 100%) and propylene oxide as well as the specimen saturation with a mixture of epoxide resins were conducted according to the generally accepted method (Weigel, Glazebrook 2002). Ultrathin sections (50–70 nm) were prepared with the use of a RMC MT–XL microtome (USA). The sections were stained with uranyl acetate and lead citrate (Reynolds 1963) and studied with a JEM–1230 EX transmission electron microscope (Japan); the voltage was 60 kV. For the morphometric analysis, we used the negative photographs of cells taken with magnification of 3000x, 5000x, 8000x, 15000x, 25000x and 40000x. Negatives were scanned using the program HP Presicionscan Pro 3.1. Digital photographs were analyzed using the program UTHSCSA ImageTool, version 3.00. We measured the ER-body area and calculated its mean value and a number of ER-bodies per cell section.

Statistical analysis

All experiments were conducted three times. The obtained data were statistically evaluated using the program Statistica 6.0. Data are presented in the form of $M \pm m$, where M is the arithmetical mean and m is the standard deviation.

Results and discussion

We have shown that ultrastructure of both root statocytes and cells of distal elongation zone (DEZ) in 3- and 7-day old seedlings grown in the stationary conditions in darkness was typical for such cells described earlier (Tarasenko 1985, Dolan et. al. 1993, Atsushi et. al. 2009). On the sections of statocytes and DEZ cells, ER-bodies are the local enlargements of GER cisterns. Naturally, rounded or oval ER-bodies are surrounded with a single membrane with ribosomes, explaining their origin from GER, and they contain the thin fibrillar contents (fig. 1). ER-bodies are mainly localized in the central part of statocytes and DEZ cells. An average size of ER-bodies varied in seedlings of different age: in statocytes, it was 0,12±0,04 μ² in 3-day-old seedlings and 0,14±0,03 μ² in 7-day-old ones; in DEZ cells, it was 0,24±0,08 μ² and 0,25±0,011 μ², respectively. An average number of

ER-bodies was per statocyte section: 1,40±0,12 in 3-day-old seedlings and 1,50±0,05 in 7-day-old ones; it was per DEZ cell section 2,37±0,77 and 2,62±0,80, respectively. A total area of ER-bodies was per statocyte section: 0,16±0,06 μ² in 3-day old seedlings and 0,19±0,05 μ² in 7-day old ones, and it was per DEZ cell section 0,54±0,18 μ² and 0,60±0,20 μ², respectively (fig. 2). There were some differences in the ultrastructure and topography of ER-bodies under clinorotation. A shape and size of ER-bodies also changed in comparison with control. Especially, the changes were clearly observed in 7-day-old seedlings. Round, oval or elongated ER-bodies are mainly localized nearly the tonoplast and cytoplasmic membrane. A size of ER-bodies markedly increased per cell section, especially in DEZ cells*:* it was 0,16±0,02 μ² in 3-day old seedlings and 0,15±0,02 μ² in 7-day old ones in statocytes; and 0,29±0,07 μ² and 0,35±0,14 μ², respectively, in DEZ cells. Under clinorotation, a number of ER-bodies also increased per cell section: it was 2,20±0,61 in 3-day old seedlings and 2,87±0,13 in 7-day-old ones in statocytes, and it was 3,45±0,84 and 3,88±0,87, respectively, in DEZ cells. A total area of ER-bodies was per statocyte section 0,29±0,08 μ² in 3-day old seedlings and 0,40±0,13 μ² in 7-day old ones; it was per DEZ cell section 1,14±0,30 μ² and 1,32±0,25 μ², respectively (fig. 2).

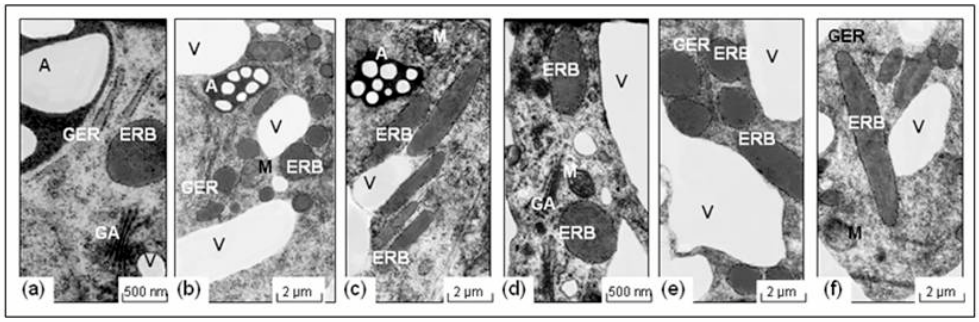


Fig. 1. Fragments of the root statocytes ((a), (b), (c)) and DEZ cells ((d), (e), (f)) of *A. thaliana*: ERB – ER-body, GER - granular endoplasmic reticulum, A - amyloplast, GA - Golgi apparatus, V - vacuole, M – mitochondria; (a), (d) - control, (b), (c), (e), (f) – X-Ray irradiation; (b), (e) - dose of 0.5 Gray, (c), (f) - dose of 8.0 Gray.

Plant radioresistance is known to be determined by meristems resistance (Mikheev et. al. 1998). ER-bodies are absent in root apical meristem cells, as we already reported, they are in statocytes and DEZ cells. According to Matushima (2002), in *A. thaliana* dry seeds, ER-bodies are absent and they appear in cotyledons during seed germination. ER-bodies formation is slow and varies from 48 to 66 hours after sprouting. Seedlings are known to be the most sensitive to radiation. Therefore, 3-day-old seedlings were chosen for irradiation in our

experiments. Cells perceive a dose in 0,5 Gray, under that DNA damage reaches a certain threshold, as a signal for induction and implementation of adaptive responses, including the activation of reparative systems. A dose in 8,0 Gray is not critical for *A. thaliana,* and seedlings grew normally during 8-10 days after irradiation by this dose (Danilchenko 2005). Our investigations of the ER-bodies ultrastructure in statocytes and DEZ cells of 13-day-old seedlings grown by the light showed that ER-bodies mainly localized in the central part of a cell, nearly GER profiles. An average size of ER-bodies per cell section was: 0,096±0,013 μ² in statocytes and 0,140±0,061 μ² in DEZ cells. An average number of ER-bodies per cell section was: 3,3±0,68 pieces in statocytes and 4,8±0,22 pieces in DEZ cells. An total area of ER-bodies per cell section was: 0,41±0,09 μ² in statocytes and 0,59±0,07 μ² in DEZ cells (fig. 3). However, after X-ray irradiation observed some differences in the ultrastructure and topography ER-bodies. ER-bodies are mainly localized nearly the tonoplast and cytoplasmic membrane (fig. 1).

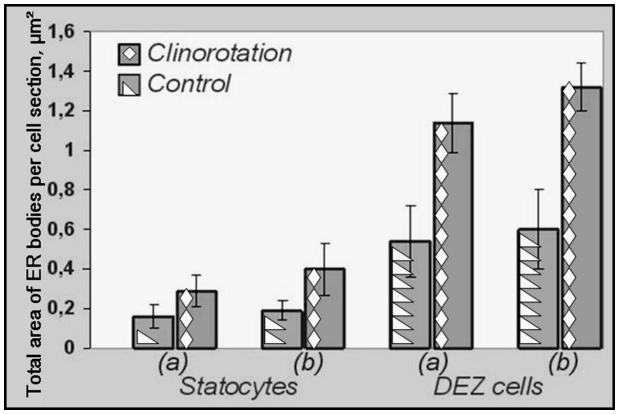


Fig. 2. Total area of ER-bodies (vertically, μm²) per cell section of the roots of seedlings of *A. thaliana*: (*a*) - 3-dayold roots; (*b*) - 7-dayold roots. Significant changes between control and experiment (<0.05).

The observed differences in the total area of ER-bodies per cell in both the root cap central statenchyma and DEZ may be explained with various functions of these tissues in root development. Statocytes are highly specialized cells for gravity perception. Cells in the DEZ distinguished themselves with special physiological properties and, therefore, respond to the action of exogenic signals, including gravity, and endogenic signals in a different way than other root cells (Baluska et. al. 2001) DEZ cells provide preparation to cell fast growth in the central elongation zone. As highly metabolizing cells, they are the most sensitive to altered gravity (Ishikawa, Evans 1993, Kordyum et. al., 2008). The results obtained by us showed that DEZ cells are sensitive to irradiation.

After X-ray radiation with a dose of 0,5 Gray, ER-bodies had a rounded shape and a small size. After X-ray radiation with a dose of 8,0 Gray, ER-bodies had an elongated shape, its length reached sometimes the half of cell. An average size of ER-bodies per cell section was: 0,17±0,07 μ² after X-ray radiation dose of 0,5 Gray and 0,280±0,04 μ² dose of 8,0 Gray in statocytes; and 0,240±0,017 μ² and 0,360±0,08 μ², respectively, in DEZ cells. An average number of ER-bodies per cell section was: 8,8±0,79 pieces after X-ray radiation dose of 0,5 Gray and 6,7±0,92 pieces dose of 8,0 Gray in statocytes; and 8,6±0,86 pieces and 7,5±0,50 pieces, respectively, in DEZ cells. A total area of ER-bodies per cell section was: 1,48±0,18 μ² after X-ray radiation dose of 0,5 Gray and 1,23±0,11 μ² dose of 8,0 Gray in statocytes; and 0,91±0,08 μ² and 1,71±0,03 μ², respectively, in DEZ cells (fig. 3).

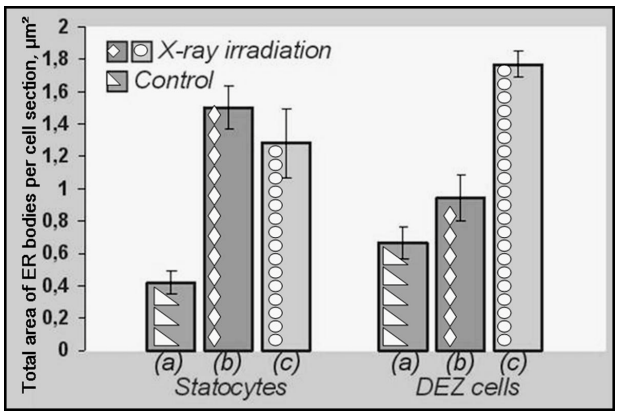


Fig. 3. Total area of ER-bodies (vertically, μm²) per cell section of the roots of seedlings of *A. thaliana*: (*a*) - 0 Gray; (*b*) - 0.5 Gray; (*c*) – 8,0 Gray. Significant changes between control and experiment(<0.05).

Thus, we in the first time described the influence of irradiation on the formation dynamics of ER-bodies, which are derivative of GER and contain an enzyme β-glucosidase, in root cap statocytes and in cells of the root distal elongation zone of *A. thaliana* seedlings.

Conclusions

The obtained data showed that production of ER-bodies increased under both clinorotation and X-ray irradiation. Based on the idea, that protective functions are intrinsic of ER-bodies an increase in the ER-bodies volume in cells may be considered as an adaptive cell response to the influence of these exogenic factors.

References

Atsushi J, Nagano AJ, Maekawa A, Nakano RT, Miyahara M, Higaki T, Kutsuna N, Hasezawa S, Hara-Nishimura I (2009) Quantitative analysis of ER body morphology in an *Arabidops*is mutant. Plant Cell Physiol 50(12): 2015–2022

Baluska F, Volkmann D, Barlow PW (2001) A polarity crossroad in the transition growth zone of maize root apices: cytoskeletal and developmental implications. J Plant Growth Regul 20: 170–181

Bonnett HTJ, Newcomb EH (1965) Polyribosomes and cister nal accumulations in root cells of radish. J Cell Biol 27: 423–432

Danilchenko OO (2005) Radioadaptive response induced by ultraviolet radiation in plants. Dissertation, Institute of Ukraine 123 pp

Dolan L, Janmaat K, Willemsen V, Linstead P, Poethig S, Roberts K, Scheres B (1993) Cellular organization of the *Arabidopsis thaliana* root. Development 119(1): 71–84

Guisinger MM, Kiss JZ (1999) The influence of microgravity and spaceflight on columella cell ultrastructure in starch-deficient mutants of *Arabidopsis.* American Jornual of Botany 86(10): 1357–1366

Ishikawa H, Evans ML (1993) The role of the distal elongation zone in the response of maize roots to auxin and gravity. Plant Physiol 102: 1203–1210

Kalinina IM (2007) Growth and differentiation of *Brassica rapa* L. root cells in microgravity and clinorotation. Dissertation, Institute of Ukraine 179 pp

Kordyum E (2002) Gravisensitivity of plant cells: status and prospects. J of Grav Physiol 9(1): 219- 220

Kordyum EL, Martyn GI, Ovcharenko YuV (2008) Growth and differentiation of root cap columella cells and a root proper in the stationary conditions and under clinorotation. Tsitol Genet 42(1): 3–12

Kraft TF, wan Loon JJ, Kiss JZ (2000) Plastid position in *Arabidopsis* columella cells is simular in microgravity and on a random-positioning machine. Planta 211: 415–422

Kuhlmann F, Müller C (2009) Independent responses to ultraviolet radiation and herbivore attack in broccoli. J Exp Bot 60(12): 3467–3475

Kutsokon' NK, Bezrukov VF, Lazarenko LM, Rashydov NM, Hrodzyns'kyĭ DM (2003) The number of aberrations in aberrant cells as a parameter of chromosomal instability. 1. Characterization of dose dependency. Tsitol Genet 37(4): 20–25

Matsushima R, Hayashi Y, Kondo M, Shimada T, Nishimura M, Hara-Nishimura I (2002) An endoplasmic reticulum derived structure that is induced under stress conditions in *Arabidopsis.* Plant Physiol 130:1807–1814

Matsushima R., Kondo M., Nishimura M., Hara–Nishimura I. (2003) A novel ER- derived compartment, the ER body, selectively accumulates a β-glucosidase with an ER retention signal in *Arabidopsis*. Plant J 33: 493–502

Mikheev AN, Guscha NI , Malinowski YY, Grodzinskiy DM (1998) The role of the proliferative activity of merisistematic cells in ensuring radioadaptive plant response. Dokladi NANU 10: 174–178

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15(13): 473–497

Ogasawara K, Yamada K, Christeller JT, Kondo M, Hatsugai N, Hara_Nishimura I, Hayashi Y, Yamada K, Shimada T, Matsushima R, Nishizawa NK, Nishimura M, Hara-Nishimura I (2001) A proteinase-storing body that prepares for cell death or stresses in the epidermal cells of *Arabidopsis*. Plant Cell Physiol 42: 894–899

Popova OV, Golldack D (2007) In the halotolerant *Lobularia maritima* (*Brassicaceae*) salt adaptation correlates with activation of the vacuolar H(+)-ATPase and the vacuolar Na+/H+ antiporter. J Plant Physiol 164(10): 1278–1288

Reynolds ES (1963) The use of lead citrate at high pH as an electron opaque stain in electron microscopy. J Cell Biol 17(1): 169–170

Romanchuk SM (2010) Ultrastructure of the statocytes and cells of the distal elongation zone of *Arabidopsis thaliana* under the conditions of clinorotation. Tsitol Genet 44 (6): 329–333

Saton H, Uchida A, Nakayama K, Okada M (2001) Water-soluble chlorophyll protein in *Brassicaceae* plants is a stress-induced chlorophyll-binding protein. Plant Cell Physiol 42(9): 906–1112

Tarasenko VA (1985) Ultrastructure of columella cells in the root cap of *Arabidopsis* under conditions of clinostating and microgravity. Extended abstract of cand. sci. (Biol.) dissertation, Institute of Leningrad" 35pp

Weigel D, Glazebrook J (2002) *Arabidopsis*: a laboratory manual. New York Gold Spring Harbor Laboratory Press: 354 pp

Xu Z, Escamilla-Trevino L, Zeng L, Lalgondar M, Bevan D, Winkel B, Mohamed A, Cheng CL, Shih MC, Poulton J, Esen A (2004) Functional genomic analysis of *Arabidopsis thaliana* glycoside hydrolase family 1. Plant Mol. Biol. 55: 343–367

PLANT FUNCTIONING UNDER ENVIRONMENTAL STRESS P. 193-206

Cracow, 12th to the 15th September, 2012

LEAD PREINCUBATION CONFERS NO RESISTANCE AGAINST *FUSARIUM OXYSPORUM* IN PEA PLANT

Arleta Malecka, Aneta Piechalak, Agnieszka Kutrowska, Barbara Tomaszewska

Department of Biochemistry, Institute of Molecular Biology and Biotechnology, Adam Mickiewicz University, Collegium Biologicum, Umultowska 89, 61-614 Poznań, Poland.

Corresponding autor: arletam@amu.edu.pl

Abstract Plants have evolved a wide range of mechanisms to cope with biotic and abiotic stresses. Both types of stressed impact the normal development of the plant. Emerging evidence suggest that hormones, such as abscisic acid or salicylic acid, proteins (e.g. transcription factors, kinase cascades) and ROS (reactive oxygen species) play important role in cross talk between different stresses. ROS have a main role in signal transduction in both abiotic and biotic stress. Different data allow to assume that plants in presence of an abiotic stress may present reduced or enhanced susceptibility to a biotic stress, and vice versa. This study was to determine ROS level in *Pisum sativum* which was pre-incubated with lead ions and after 24 hours inoculated with necrotrophic fungus *Fusarium oxysporum.* Main objective of this study was to confer or negate the role of inorganic element pre-incubation in elevating the resistance to biotic stress via cross talk. Our results shown rapid increase in level of superoxide anion and hydrogen peroxide in pea roots exposed to biotic and abiotic stress factors, especially to lead ions. Plant possess very efficient enzymatic antioxidative defense system: superoxide dismutase-SOD, catalase-CAT, and ascorbic peroxidase, which controls the cascades of uncontrolled oxidation and protect plant cells against oxidative damage. We have examined antioxidant system protection against oxidative stress damages: superoxide dismutase (SOD), catalase (CAT) and ascorbic peroxidase (APOX).

Key words: abiotic stress; antioxidative enzymes; biotic stress; lead; oxidative stress

Introduction

Plants are exposed to numerous biotic and abiotic stresses in their natural environment. These factors may affect their growth, development and reproduction. Abiotic stress can be caused by presence of trace metals, cold, salt or drought con-

ditions whereas biotic stress is caused by living parasitic organisms, for example: pathogens, bacteria, virus, fungi and oomycetes. Plants have evolved to live in environments where they are almost never exposed to one kind of stress, but often different abiotic and biotic factors work in combination. ROS have a central role in mediation between biotic and abiotic stress responses and are critical to both types of stress responses, although function differently in each (Atkinson and Urwin, 2012). In biotic stress the pathogen attack will trigger the production of ROS, mostly superoxide anion and hydrogen peroxide, in process known as the oxidative burst. This outburst limits pathogen spread by contributing to the hypersensitive response and cell death, which is assured by the coordinated down-regulation of ROS-scavenging mechanisms (Apel and Hirt, 2004; Torres, 2010). The role of ROS during this stress is to induce damage to the pathogen, to reinforce the plant cell wall and to act as a secondary messenger to prime the neighboring cells (Apel and Hirt, 2004; Torres et al., 2006). In abiotic stress the situation differs: down-regulation of ROS-scavenging mechanisms is not crucial, most important are even small perturbations in the balance between ROS generation and detoxication. Influence of abiotic factors also leads to increased ROS level in living cells, likewise observed in biotic stress response, but the consequence of ROS production is different and depends on the type of stress. In abiotic stress ROS damage to plant cells is undesirable and their removal is essential for the survival of the plant. To minimize damage caused by these potentially harmful molecules, plants produce antioxidants and ROS-scavening enzymes (Apel and Hirt, 2004). In biotic stress ROS damage dealt to both plant cells and pathogen is essential for the response to hostile organism. As stresses often occur in combination, the relationship between ROS signaling mechanism in different stress responses is complex. The presence of an abiotic factors can have the effect of reducing or enhancing susceptibility of plants to a biotic factors, and vice versa.

Interesting is question, whether pea plants grown for 24 hours in presence of trace metal such as lead ions are more or less susceptibile to necrotrophic fungus: *Fusarium oxysporum*. The aim of the present study was to answer this question. To find the answer we have shown changes in generation of superoxide anion and hydrogen peroxide in pea plants. Moreover, we have examined changes in enzymatic profiles of antioxidative enzymes: SOD, APOX and CAT from *Pisum sativum* roots. We shown the morphological changes in plant roots after pre-incubation of lead ions and inoculation with fungi.

Our results suggest that pre-incubation of *Pisum sativum* plants with lead ions confers no resistance in this plant to inoculation with spores of *Fusarium oxysporum*.

Materials and Methods

Plant material. Pea seedlings (*Pisum sativum* L., cv. Kwestor) were grown hydroponically on the Hoagland medium for 96 h in a growth chamber with 16/8 h photoperiod, day/night at room temperature and light intensity of 82 μmol m^2 s^{-1}. Then, the medium was changed into 100 x-diluted Hoagland medium supplemented with lead ions at the concentration of 0.1 mM. Lead ions originated from the solution of $(PbNO_3)_2$. After 24 hours medium was changed into 100 x-diluted Hoagland lead-free medium and the plant roots were inoculated with necrotrophic fungus *Fusarium oxysporum*. The roots were cut off after 0, 24, 48, 72, 96 and 168 hours of cultivation, respectively. Lead accumulated on the surface of roots was rinsed with 10 mM of $CaCl_2$.

Preparation of spore suspension and inoculation. *Fusarium oxysporum* f. sp. pisi strain number 1183 was obtained from Collection of Plant Pathogenic Fungi, the Institute of Plant Protection in Poznan. The pathogen was incubated in the darkness at 25 °C in Petri dishes (diameter 9 cm) on the potato dextrose agar (PDA) medium (Difco, pH 5,5). After 3 weeks of growth, the *Fusarium oxysporum* spore suspension was prepared. The spore suspension was obtained by washing the mycelium with sterile water and shaking with glass pearls. The number of spores was then determined using a Bűrker hemocytometr chamber. Seedlings were inoculated with the spore suspension at the concentration of 5 x 10^6 of spores per 1 ml. Inoculation was performed by injecting 10 μl of spore suspension into the roots, below shoot.

Index of tolerance. The index of tolerance (IT) was calculated according to Wilkins (1957):

$$IT = \frac{\text{average lenght of roots in tested solution}}{\text{average lenght of roots in control}} \times 100\%$$

Superoxide anion determination. Superoxide anion content was determined according to Doke (1983). The pea roots (0.5 g) were placed in the test tubes and filled with 7 mL of mixture containing 50 mM phosphate buffer (pH 7.8), 0.05 % NBT (nitro blue tetrazolium) and 10 mM of NaN_3. Next, the test tubes were incubated in dark for 5 min, and then 2 mL of the solution were taken from the tubes heated at 85 °C for 10-15 min, cooled in ice for 5 min and the absorbance was measured at 580 nm against the control.

Hydrogen peroxide determination. Hydrogen peroxide content was determined using the method described by Becana et al. (1986). The decrease of absorbance was measured at 508 nm. The reaction mixture contained: 50 mM phosphate buffer (pH 8.4), reagent containing 0.6 mM 4-(-2 pyridylazo) resorcinol, 0.6 mM potassium-titanium oxalate in (1:1). The corresponding concentration of H_2O_2 was determined against the standard curve of H_2O_2 (0.5-25 μM).

*In vivo detection of superoxide anion and hydrogen peroxi*de. After 48 and 72-hours cultivation with abiotic and biotic stress factors pea roots and shoots were submerged for 12 h in 100 μM of $CaCl_2$ containing 20 μM of dihydroethidium (DHE) adopting the method of Yamamoto et al., (2002) and in 4 μM dichlorodihydrofluorescein diacetate (DCFH-DA) in 5 mM dimethyl sulfoxide (DMSO) for 4 hours using method modified according to Afzal et al., (2003). After rinsing with 100 μM of $CaCl_2$ or 50 mM phosphate buffer (pH 7.4) the roots and shoots were observed with a confocal microscope (the model Zeiss LSM 510, Axioverd 200 M, Jena, Germany) equipped with a filter set no. 10, excitation 450-490 nm, emission 520 nm or more.

PAGE electrophoresis of antioxidant enzymes. Extract from pea roots plants were electrophoresed in 10 % (SOD, APOX) or 8 % (CAT) (w/v) polyacrylamide slab gel at pH 8.9 under nondenaturating conditions, according to Davis (1964). The activity of SOD was assayed according to Beauchamp and Fridovich (1971) in terms of its ability to inhibit the photochemical reduction of nitro blue tetrazolium (NBT). APOX activity was detected following the procedure of Mittler and Zilinskas (1993), and CAT activity was detected according to Woodbury and coauthors (1971). For APOX activity determination, 2 mM ascorbate was added to the buffer.

Protein quantification. Total soluble protein contents were determined according to the method of Bradford (1976), using the Bio-Rad assay kit with bovine serum albumin as a calibration standard.

Results

Pisum sativum seedlings were grown hydroponically with Hoagland medium in four variants: first -100 x diluted, clear Hoagland medium, second-supplemented with 0,1 mM $Pb(NO_3)_2$, third-inoculated by *Fusarium oxysporum* and, fourth- preincubated with 0,1 mM $Pb(NO_3)_2$ for 24 hours and inoculated by fungi. We observed morphologically changes in pea roots, the biggest in plants treated with lead. The color of roots changed from creamy white to light brown after 96 and 168 hours of cultivation time. We noticed in the roots of plants from second and fourth variants reduction of the total amount of lateral roots (Phot. 1).

Differences in IT values (Fig. 1) indicate that the highest sensitivity exhibits pea plants submitted to both stress factors: preincubation with lead and later inoculation with fungi (over 60%) and the highest resistance treated only with *Fusarium oxysporum* (over 80 %).

Phot. 1. Morphologically changes of pea roots grown hydroponically in Hoagland medium in the presence to 0,1 mM $Pb(NO)_3$ and *Fusarium oxyspor*um for 72 hours

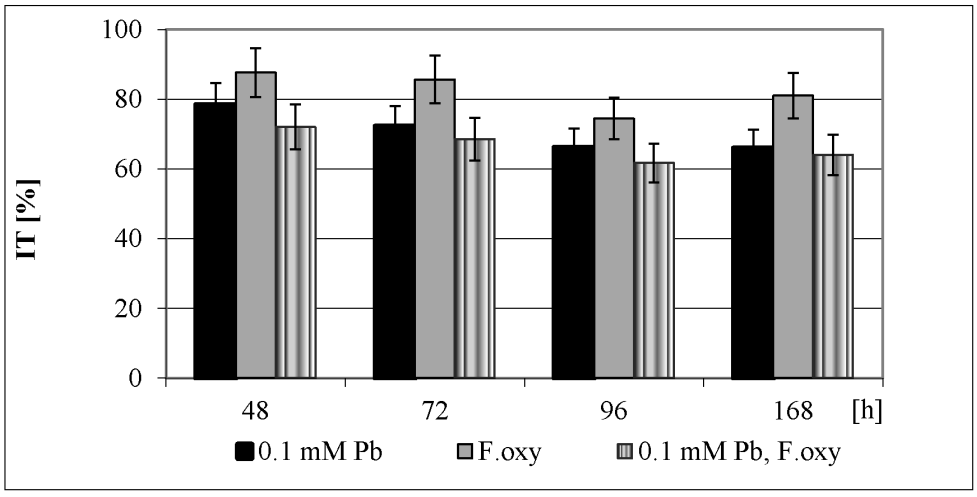


Fig. 1. Tolerance Index for *Pisum sativum* roots grown hydroponically in Hoagland medium in the presence to 0.1 mM $Pb(NO_3)_2$ and *Fusarium oxysporum* for 168 hours

Consistently, also the highest increase in roots biomass was observed after 168 hours in plants only inoculated and was similar to control plants (Fig. 2). In the same time the root mass of plants preincubated with Pb ions and inoculated by fungi was lowered for about 20% compared to control plants.

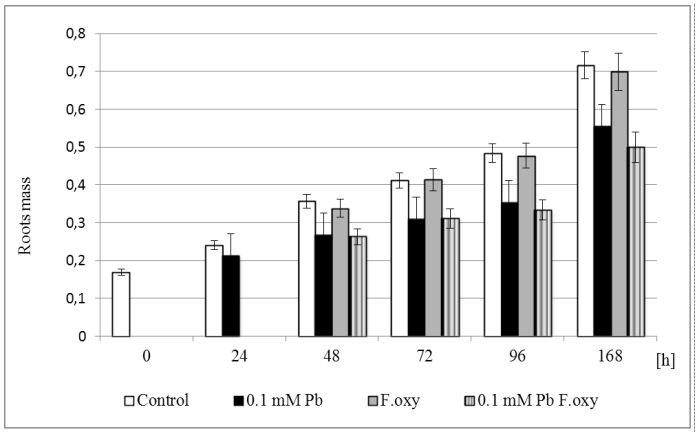


Fig. 2. Changes in roots mass of pea plants grown hydroponically in Hoagland medium in the presence to 0.1 $Pb(NO_3)_2$ and *Fusarium oxysporum* for 168 hours

In the presented study, we observed high concentration of superoxide anion (Fig. 3) in roots with the addition of Pb ions: after 24 hours of exposition to lead the level of $O_2^{-\bullet}$ increased by about three–fold, after removal of metal during remaining cultivation was observed gradual decrease of superoxide level. In plants treated with both factors (Pb and F. oxy) we observed similar level of $O_2^{-\bullet}$ like in plants exposed only to Pb ions. In both $O_2^{-\bullet}$ concentration were above two-fold higher in comparison to control plants and about 30% higher than in plants only inoculated with fungi. The level of superoxide anion in plants inoculated by fungi was maximal after 48 hours of cultivation (24h after inoculation) and was lower by 35 % and 25% than in plats treated only with Pb or fungi, respectively. We have noted a decrease in the $O_2^{-\bullet}$ level after 168 hours in all of the plants, which was probably due to either: activation of enzymatic (SOD) defense system or decline of ROS production rate.

The results of spectrophotometric studies on the level on $O_2^{-\bullet}$ in the pea roots were confirmed by using confocal microscopy technique (Phot. 2.) The most intensive fluorescence was observed in the pea roots treated for 48 hours with Pb ions and both stress factors, while in the pea roots inoculated by fungi the fluorescent signal was significantly lower. After 72 hours the fluorescence signal was less intense in all tested variants.

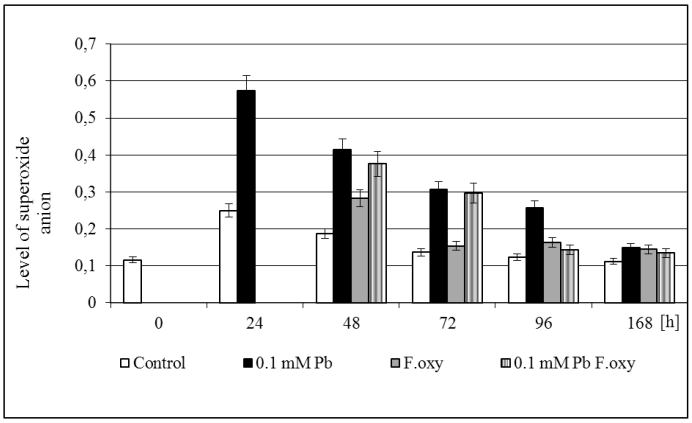
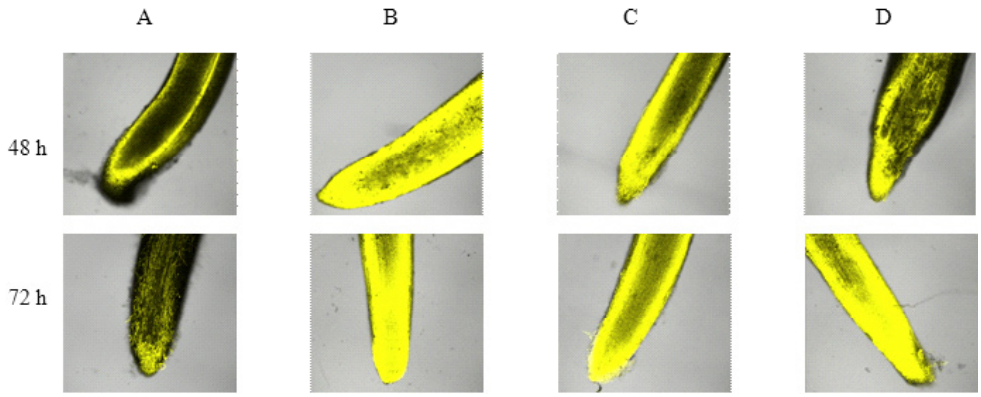


Fig. 3. Level of $O_2^{-\bullet}$ (A580 g^{-1} FW) in roots of *Pisum sativum* grown hydroponically in Hoagland medium in the presence to 0.1mM $Pb(NO_3)_2$ and *Fusarium oxysporum* for 168 hours



Phot. 2. Generation of $O_2^{-\cdot}$ in pea roots after 48 h and 72 h cultivation in abiotic and biotic stress. Fluorescent images of pea roots: (A) control roots (B) roots treating with 0.1 mM $Pb(NO_3)_2$ (C) roots treating with *Fusarium oxysporum* (D) roots treating with 0.1 mM $Pb(NO_3)_2$ and *Fusarium oxysporum.* The bar indicates 1 mm.

In all examined plants we observed changes in hydrogen peroxide concentration in roots (Fig. 4). After 48 hours of exposition to lead the level of H_2O_2 reached its maximum and increased six-fold in comparison to control plants. Also inoculation by fungi, with and in absence of metal, lead to increase of hydrogen peroxide concentration, its increased for about 70 % in comparison to control plants. The concentration of hydrogen peroxide in these plants was similar during the cultivation time.

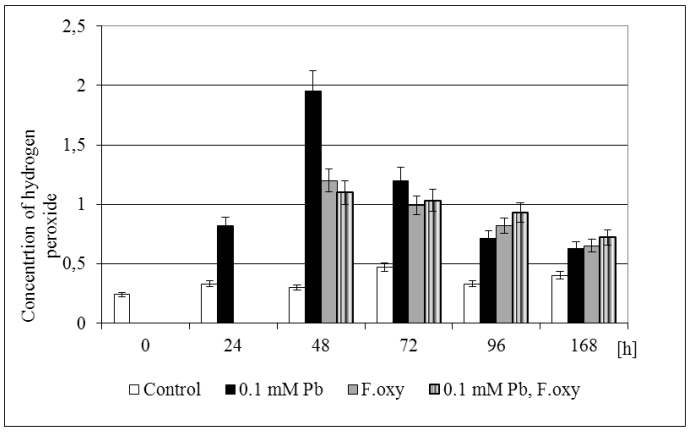
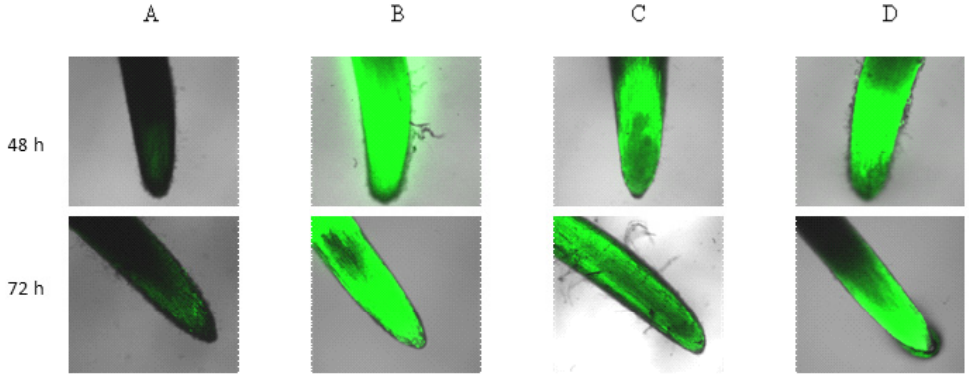


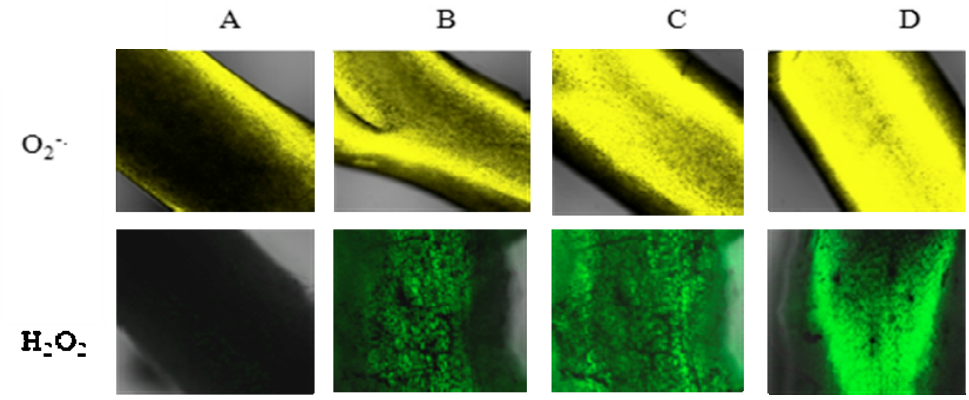
Fig. 4. Concentration of H_2O_2 (µmol x min^{-1}) in roots of *Pisum sativum* grown hydroponically in Hoagland medium in the presence to 0.1 $Pb(NO_3)_2$ and *Fusarium oxysporum* for 168 h.

Similarly, the studies on the level of H_2O_2 was also confirmed by applying confocal microscopy (Phot. 3). The DCFDA-derived fluorescence was higher in pea roots cultivated with Pb ions for 48 and 72 hours in comparison to plant from the remaining variants. The signal in plants inoculated by fungi and exposed to two stress factors was slightly lower after 72 hours cultivation.



Phot. 3. Generation of H_2O_2 in pea roots after 48 h and 72 h cultivation in abiotic and biotic stress. Fluorescent images of pea roots: (A) control roots (B) roots treating with 0.1 mM $Pb(NO_3)_2$ (C) roots treating with *Fusarium oxysporum* (D) roots treating with 0.1 mM $Pb(NO_3)_2$ and *Fusarium oxysporum*. The bar indicates 1 mm

We studied the level of ROS production in injection site in shoot pea plants exposed to abiotic and biotic stress factors. The most intensive DHE-fluorescence we observed in shoot plants inoculated by fungi and exposed to both stress factors after 48 hours cultivation time in comparison to control. Generation of hydrogen peroxide was the highest in plants treated with both stress factors too. The high fluorescence intensity we noticed also in shoots infection by fungi plants.

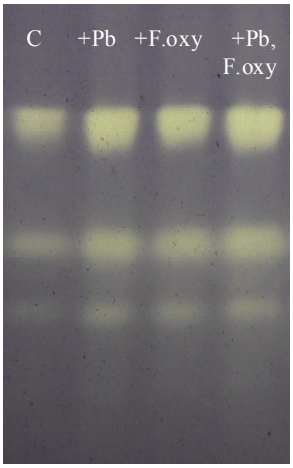


Phot. 4. Generation of $O_2^{\cdot-}$ and H_2O_2 in pea shoots after 48 h cultivation in abiotic and biotic stress. Fluorescent images of pea shoots: (A) control (B) shoots treating with 0.1 mM $Pb(NO_3)_2$ (C) shoots treating with *Fusarium oxysporum* (D) shoots treating with 0.1 mM $Pb(NO_3)_2$ and *Fusarium oxysporum*. The bar indicates 1 mm

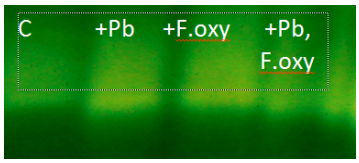
We studied the SOD isoenzymatic profiles (Phot. 5) in all variants: roots treated for 72 h with 0.1 mM Pb^{2+}, inoculated by fungi and inoculated by fungi after 24 hours preincubation with lead. After the gel analysis, we found the presence of 4 isoenzymatic forms of SOD. The greatest intensity was shown for I and III isoform in plants exposed to stress than in control plants. The isoforms of SOD remained at similar level for the plants treatment with stress factors and intensity were higher in comparison to control plants.

In catalase electrophoretic profiles one isoenzymatic form was found for all the options (Phot.6). The most intense stripe was observed for the plants treated with Pb ions and inoculated by fungi in comparison to control and treated with both stress factors plants.

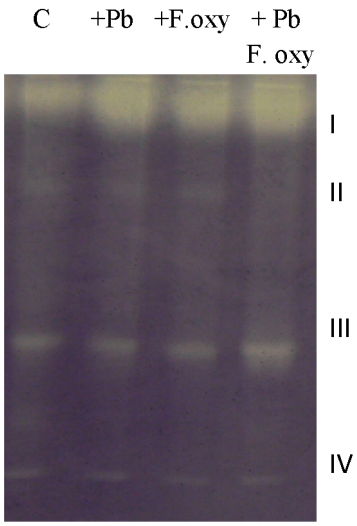
Furthermore, we have indicated the isoenzymatic profile of ascorbate peroxidase, we found four isoenzymatic forms (Phot.7). The most intense stripes were shown for the form I and III for all examined variants comparing to control plants. The isoforms II and IV remained stable and at similar level for all the studied plants.



Phot. 5. Effect of abiotic and biotic stress factors (72 h) on SOD isoenzymes from pea roots: native PAGE analysis 50 µg protein were put on 10 % gel. Staining was performed by the photochemical method, using NBT/ riboflavin



Phot. 6. Effect of abiotic and biotic stress factors (72 h) on CAT enzymatic profile from pea roots: native PAGE analysis 50 µg protein were put on 8 % gel



Phot 7. Effect of abiotic and biotic stress factors (72 h) on APOX isoenzymes from pea roots: native PAGE analysis 50 µg protein were put on 10 % gel

Disscusion

The interaction between abiotic and biotic stress is subject of intense research to expand our knowledge about plant adaptation. Early evidence for a cross-talk between abiotic and biotic stress responses was provided by Bressan et al. (1982). Authors demonstrated that pathogenesis-related (PR) proteins accumulate in tomato cells exposed to salt stress and that effector proteins involved in one of the tolerances have profitable characteristics for the other tolerances also. Other authors (Dombrowski, 2003) showed accumulation of insect resistance proteins in tomato plants grown in saline environment and wounded plants are more tolerant to salt stress (Capiati et al., 2006). It has been proposed that activation of broad salt tolerance responses in plants attacked by herbivorous insects would present an energetically expensive and probably unsustainable cost. For this reason, it not clear whether a wound mediated activation of salt tolerance is a functional redundancy in stress signaling pathways or it is specifically as a result of reactions that would protect the plant against defoliating insects (Dombrowski, 2003; Capiati et al. 2006; Orsini et al., 2010)

Several studies have indicated that stress responses as well as other fundamental physiological processes are controlled by a concerted action of different signaling pathways. The presence of an abiotic factors such as: metal ions can have the effect of reducing or enhancing sensitivity of plants to a biotic factors such as: fungi, and vice versa. The main role in mediation between biotic and abiotic stress responses have ROS and are essential to both types of stress responses (Atkinson and Urwin, 2012). Nevertheless, our current understanding of ROS participation in cross-talk between abiotic and biotic stress pathways is very limited.

The aim of the presented study was to examine the effects of cross-talk interactions of lead ions and *Fusarium oxysporum* infections on generation of ROS in plant organs and activity of enzymatic antioxidative enzymes such as: SOD, CAT and APOX.

We wanted to expand our knowledge about tolerance of pea plants pretreated for 24 hours with lead ions and after inoculated by fungi.

In our studies the highest sensitivity exhibits pea plants submitted to both stress factors: pre-incubation with lead and later inoculation with fungi, their IT values was over 60 % after 168 hours cultivation. In the same time IT level in plants infected by fungi was over 80 %. These results indicate, that pre-incubation pea plants of lead does not increase resistance to infection by *F. oxysporum*.

Similarly, differences in roots biomass and morphologically changes show, that pre-incubation with metal can have the effect of reducing sensitivity to biotic stress factors.

Plants exposed to lead ions and fungi had underdeveloped root systems, especially was seen reduction of the total amount of lateral roots. In these plants at the

same time we observed higher concentration of ROS in comparison to plants from other variants. The decreasing level of ROS after 96 or 168 hours in all studied plants was probably a result of increasing activity of antioxidative defense system. We observed the most intensive fluorescent at the site of fungi injection in pea plants shoot treated with biotic and both stress factors.

We determined the changes in electrophoresis profile of antioxidative enzymes in plants treated with abiotic and biotic stress factors. Catalase, ascorbic peroxidase and superoxide dismutase belong to the important enzymes removing reactive oxygen species like hydrogen peroxide and superoxide anion.

The intensity of SOD isoforms were higher in plants exposed to all stress factors in comparison to control plants, but on similar level. Similar results we obtained for APOX isoforms. In catalase electrophoretic profiles the most intense stripe was observed for the plants treated only with Pb ions or inoculated by fungi in comparison to control and treated with both stress factors plants.

Our results suggest that pre-incubation of *Pisum sativum* plants with lead ions confers no resistance in this plant to inoculation with spores of *Fussarium oxysporum.*

Unfortunately, there is not much papers which describe the cross-talk between trace metal and pathogens in plants, in addition sometimes there are conflicting data.

Some of the research suggest an antagonistic interaction between ABA-mediated abiotic stress signaling and disease resistance. This relationship may simply suggest that plants have developed strategies to avoid simultaneously producing proteins that are involved in abiotic and disease resistance responses. Moreover, the view that ABA-mediated abiotic stress signaling potentially takes precedence over biotic stress signaling, also supports the notion that water stress more significantly threatens plant survival than does pathogen infection (Fujita 2006). In *Nicotiana tabacum* and *Arabidopsis thaliana,* hypersensitive response and R-gene mediated defense responses to *Pseudomonas syringae* are compromised at high temperatures, allowing increased growth of these pathogens (Wang et al. 2008). *Also A. thaliana* exposed to drought stress allowed greater infection of an avirulent isolate *of P. syringae* (Mohr and Cahil, 2003). The antagonistic interaction of biotic and abiotic stress responses were shown by many authors (Xiong and Yang 2003; Koga and Mori 2003; Robert-Seilaniantz et al. 2007). An increase temperature can create a negative interactive effect by lowering resistance to bacteria, viral or fungi: in wheat higher mean temperatures observed over a 6 year experimental period correlated with heightened susceptibility to the fungus *Cochliobolus sativus* (Sharma et al, 2007). However, the development of transcriptomics and bioinformatics tools demonstrated that the interaction between these two stress responses is more complex than just antagonistic. One possible hypothesis to ex-

plain this kind of responses is that the induction of any particular pathway is the consequence of the integration of different signals (Robert-Seilaniantz et al., 2010).

In contrast, abiotic stress may also interact positively with pathogen stress. Drought stress enhanced resistance to the fungus *Botrytis cinerea* in tomato (Aucho, et al. 2006), increasing salt-induced osmotic stress was directly correlated with resistance to powdery mildew (Wiese et al. 2004).

Nemhauser and co-authors (2006) suggested that stress response seem to involve induction of common set of genes by both types stresses. In addition, specific genes to each stress are induced either by the same signal or other signal.

According to Atkinson and Urwin (2012) when examining the effects of an abiotic stress with simultaneous impact of a pathogen or herbivore, both positive and negative interactions have been observed depending on the timing, nature and severity of each stress.

Our results suggest that pre-incubation of pea plants with lead ions does not increase resistance in this plant to inoculation only with spores of fungi.

Perhaps, we used too high concentration of lead in our study, which not activate plants defense system, but cause oxidative damages by high level of ROS.

On the other hand, maybe our results are caused by negative interaction between lead and fungi pathogen.

There is the need for further research of large-scale transciptome, proteome and metabolome analyses in plant explanatory pathways in cross talk.

Acknowledgements

This work was partially supported by the (NCN) no N N305 381138.

References

Aebi HE (1983) Catalase in vitro. Methods of Enzymatic Analyses (Bergmeyer, H.U., ed.) Verlag Chemie, Weinheim 3: 273-282

Afzal M, Matsugo S., Sasai M, Xu B, Aoyama K, Takeuchi T. Method to overcome photoreaction, a serious drawback to the use of dichlorofluorescin in evaluation of reactive oxygen species. Biochem. Biophys. Res. Commun. 304, 619, 2003.

Apel K, Hirt H (2004). Reactive oxygen species: metabolism, oxidative stress and signal transduction. Ann Rev plant Biol 55: 373-399

Atkinson NJ, Urwin PE (2012) The reaction of plant biotic and abiotic stresses: from genes to the field. J Exp Bot 63, 3523-3544

Achuo EA, Prinsen E, Hofte M (2006) Influence of drought, salt stress and abscisic acid on the resistance of tomato to *Botrytis cinerea* and *Oidium neolycopersici.* Plant Pathology 55, 178-186

Becana M, Aparicio- Tejo P, Irigoyen JJ, Sanchez- Diaz M (1986) Some enzymes of hydrogen peroxide metabolism in leaves and root nodules of Medicago sativa. Plant Physiol 82: 1169-1171

Beauchamp C, Fridovich I (1971) Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. Anal Biochem 44: 276-287

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein- dye binding. Anal Biochem 72: 248-254

Bressan RA, Handa AK, Handa S, Hasegawa PM (1982) Growth and water relations of cultured tomato cells after adjustment to low external water potentials. Plant Physiol 70: 1303-1309

Capiati DA, Pais SM, Telles-Inon MT (2006) Wounding increase salt tolerance in tomato plants: evidence on participation of calmodulin-like activities in cross-tolerance signaling.J Exp Bot 57: 2391-2400

Davis BJ (1964) Disc electrophoresis II. Methods and application to human serum proteins. Ann NY Acad Sci 121: 404-427

Doke N. 1983. Involvement of superoxide anion generation in the hypersensitive response of potato tuber tissues to infection with an incompatible race of Phytophthora infestans and to the hyphal wall components. Physiological Plant Pathology 23(3): 345-357

Dombrowski JE (2003) Salt stress activation of wound-related genes in tomato plants. Plant Physiol 132: 2098-3107

Fujita M, Fujita Y, Noutoshi Y, Takahashi F, Narusaka Y, Yamaguchi-Shinozaki K, Shinozaki K (2006) Cross-talk between abiotic and biotic stress responses: acurrent view from the points of convergence in the stress signalling networks. Curr Opi Plant Biol 9: 436-442

Mittler R, Zilinskas BA (1993) Detection of ascorbate peroxidase activity in native gels by inhibition of the ascorbate- dependent reduction of nitroblue tetrazolium. Anal Biochem 212: 540-546.

Mohr PG, Cahil DM (2003) Abscisic acid influences the susceptibility of *Arabidopsis thaliana* to *Pseudomonas syringae* pv. tomato and *Peronospora parasitica*. Funct Plant Biol 30: 461-469

Nemhauser JL, Hong F, Chory J (2006) Different plant hormones regulate similar processes trough largely nonoverlapping transcriptional responses. Cell 126: 467-475

Orsini F, Cascone P, de Pascale S, Barbieri G, Corrado G, Rao R, Maggio A (2010) Systemin-dependent salinity tolerance in tomato:evidence of specific convergence of abiotic and biotic stress responses. Physiol Plant 138: 10-21

Robert-Seilaniantz A, Navarro L, Bari R, Jones JD (2007) Pathological hormone imbalances. Curr Opin Plant Biol 10: 372-379

Robert-Seilaniantz A, Bari R, Jones JDG (2010) A biotic or abiotic stress? In: Abiotic stress Adaptation in plants : physiolocical, molecular and genomic foundation, ed. by Pareek A, Sopory S.K, Bohnert H.J ang Govindjee. Springer Science + Business Media B.V, Chapter 6, pp 103-122

Sharma RC, Duveiller E, Ortiz-Ferrara G (2007) Progress and challenge towards reducing wheat spot blotch threat in the Eastern Gangetic Plains of South Asia: is climate change already taking its toll? Field crops Research 103, 109-118.

Torres MA (2010) ROS in biotic interactions. Physiol Plant 138, 414-429

Wang Y, Bao ZL, Zhu Y, Hua J (2009) Analysis of temperature modulation of plant defense against biotrophic microbes. Mol Plant-Microbe Interacton 22, 498-506

Wiese J, Kranz T, Schubert S (2004) Induction of pathogen resistance in barley by abiotic stress. Plant Biology 6: 529-536

Willkins, D,A.: A technique for the measurement of lead tolerance in plants. Nature 180: 37-38, 1957.

Woodbury W, Spencer AK, Stahmann MA (1971) An improved procedure using ferricyanide for detecting catalase isozymes. Ann Biochem 44: 301-305

Xiong LZ, Yang YN (2003) Disease resistance and abiotic stress tolerance in rice are inversely modulated by an abscisic acid-inducible mitogen-activated protein kinase. The Plant Cell 15: 745-759

Yamamoto Y, Kobayashi Y, Rama Devi S, Rikiishi S, Matsumoto H (2002) Aluminum toxicity is associated with mitochondrial dysfunction and the production of reactive oxygen species in plant cells. Plant Physiol. 128: 63-72

PLANT FUNCTIONING UNDER ENVIRONMENTAL STRESS P. 207-221

Cracow, 12th to the 15th September, 2012

THE INFLUENCE OF SALINITY ON SOME BIOCHEMICAL PARAMETERS OF SELECTED CROP LEGUMES

Maciej Maciejewski*, Maria Tatrzańska**, Franciszek Dubet*, Agnieszka Płażek**

*The Franciszek Górski Institute of Plant Physiology, Polish Academy of Sciences in Krakow, Niezapominajek 21, 30-239 Krakow, Poland

**Department of Plant Physiology, University of Agriculture, Podłużna 3, 30-239 Krakow, Poland

Corresponding author: maciejmaciejewski@onet.pl

Abstract The salinity of agricultural land is not only limited to the areas with restricted precipitation (dry and semi-dry climate), but which, is more often, in areas where the developed system of agriculture requires intensive fertilization and irrigation. These lead to secondary saltiness and effectively prevent the growth of crop plants, also those which are a source of human food. The presence of excess of soluble salts in the soil leads in the plants to various biochemical and physiological changes. The studies aimed to show the changes in the biochemical processes that occur during excessive salinity of the ground. Experiments performed by the quasi-field conditions can be concluded that salinity results in the inhibition of growth of legume plants and that the changes of antioxidant enzymes activity are specific for each species of legumes under study. Moreover, salt stress forces the plants to change the mechanism of photosynthetic absorption of CO_2, leading to a stronger carbon reassimilation, for reasons of stomata closed.

Key words: salinity; ROS; carbon isotope ratio $^{13}C/^{12}C$; antioxidants; legumes

Introduction

Soil salinity. The world in its development, actually meets with two opposing phenomena which affect each other. On one side it is a continuous increase in population, which in 2011 exceeded 7 billion, while on the other there is a decrease in food productivity due to the influence of abiotic and biotic stresses limiting plant crops. It is therefore important to seek to increase the yield per unit area but not only to increase the area under cultivation. Limiting plant growth and produc-

tivity seems to be the result of some stresses - mostly low temperature (cold and frost), drought (water deficit) and salinity. Global climate's changes, make drought and salinity of more and more important. According to Ghassemi et al. (1995) salinity can be divided into primary and secondary stage. First one is the result of long-term salinity build-up of salt in the soil because of two natural processes: the first is the weathering of rocks containing various types of soluble salts especially sodium, magnesium and calcium chlorides and to a lesser extent, sulfates, and carbonates, while the second is the deposition in soil of a natural ocean salt (composed mainly of NaCl) carried by wind and rain. It is said that the second type of salinity is "transient" because of the changes in the location in the soil profile and thus in the root zone. Contrary, so called secondary salinity is caused by human activities in an environment that changes the balance between the incoming water and plants and soil transpiration. The most important in this case is a watering of a field by irrigation systems, using water usually containing small amounts of soluble salts. Contrary to pure rain or snow water supplied to plants in naturally conditions, the artificial field watering leads to accumulation of these salts being non-utilized by plants. Subsequently, it leads to an increase of amounts of salt accumulated in groundwater. When water evaporate salts are leaving on the surface to form a "salt burn". All kind of water used to water plants contains dissolved salts, both the low and of good quality. In general, salts are common and essential component of the soil, while at the same time plant are fertilized (Kotuby-Amacher *et al*. 2000). Excessive build-up of ions of soluble salts in water leads to a phenomenon called soil salinity, which is considered as the electrical conductivity of the solution at 4 dS m^{-1} ≈ 40 mM NaCl or greater [Chinnusamy et al. 2005]. Special parts of the environment are agricultural areas where the biggest problem for humans is a direct effect of salinity on crop plant production. Around the world, there is no climate zone, which would be free from saline land. Part of the problem is resulted from watering of plants, which makes the soil in large quantities new salts, which are not there before (Munns *et al*. 2008). The problem of saline soils of varying degrees, nature and properties meets more than 100 countries all over the world, it is estimated that 20% of irrigated agricultural land is damaged by salt (Lauchli *et al*. 2008).

In temperate conditions, the causes of excess salts in the soil include mainly anthropogenic factors: inadequate (excessive and unbalanced) mineral fertilization, the use of salt to counter the effects of winter, water leakage from landfills steel industry, mining or sodium factory, emissions from chemical factory (production of potassium fertilizers) and brown coal power station. The sources include natural origin, which once regularly frequent or prolonged droughts, especially in areas where water is running low. These phenomena lead to a situation in which the accumulation of ions Na^+, K^+, Ca^{2+} or Cl^- (Rengasamy *et al*. 2006, Mahajan and Tuteja 2005).

Glycophytes and halophytes. Plant growth and development in a variety of environmental conditions led to the division in terms of sensitivity to salinity. Glycophytes are called plant adapted to grow in a low salt concentration in the substrate. It is assumed that the content does not exceed 0.5%. Increasing salinity in the soil causes a rapid abnormal life processes. The group glycophytes include crop and weeds for which the concentration between 100-200 mM NaCl results in growth inhibition and eventually death (Munns and Termaat 1986).The second group of plants known as halophytes for the optimum salt concentration is between 300-400 mM NaCl. Formation tolerance mechanisms during adaptation enables phylogenetic allows halophytes growth, further measurement of ions in plants treated salinity stress showed that ions are accumulated. While in glycophytes there is a tendency to remove salts (Zhu 2007).

Effect of salinity on the plants is based on several actions: it reduces the potential of the water, leading to the presence of physiological drought. Under such conditions, the plants may not get out of the water due to the increased osmotic potential in the soil, in spite of its sufficient availability. Another effect of soil salinity is supplying the ionic imbalance and interfering with the ability to download ions. In this case, transport phloem-xylem is slowed due to the weak flow of water within the plant. Such processes in disorders of water leads to loss of cell turgor, stomata closure, a significant decrease in transpiration, photosynthesis and efficiency of other metabolic processes (protein synthesis, the release of energy from fat). Visible consequence of these processes is to limit the growth and yield (Nawaz *et al.* 2010).

Reactive Oxygen Species (ROS). Reactive oxygen species such as superoxide anion - O_2- , hydrogen peroxide - H_2O_2, hydroxyl radical - ·OH and singlet oxygen - $^1O_2$ in homeostasis near conditions are produced during normal cell metabolic processes taking place during the growth and development of plants (synthesis of some metabolites, lignification processes, plants aging) (Kacperska 2007). Plants subjected to various environmental stresses, such as drought, high temperature, high light intensity, salinity, and the impact of herbicides and other chemicals. As the effect the disorder in the balance between the production of active forms of oxygen and their decomposition by antioxidant compounds, takes place. Often the result of such action is the oxidative damage (Parida and Das 2005) of cell structures. Next, there is the formation of an excess of reactive oxygen species. Simultaneously with the formation of ROS plants have evolved defenses against excess oxidants. ROS action seriously disrupts the functioning of the normal metabolism of nucleic acids, proteins and lipids. From the other side antioxidants, having a high level in plants, show a larger-induced plant resistance to oxidative damage (Spychalla and Desborough 1990). Salt stress induces a particular activity of antioxidant enzymes such as catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR), guaiacol peroxidase (GP) and, especially, superoxide dismutase

(SOD) [Mittova et al. 2003], which catalyzes the decomposition oxidants (ROS). Subsequently, hydrogen peroxide is eliminated by ascorbate peroxidase localized in the thylakoid membranes.

Discrimination of carbon isotope ratio $^{13}C/^{12}C$. On Earth, there are two stable isotopes of carbon, ^{12}C and ^{13}C. The most common is the isotope ^{12}C, the amount of which is almost 99%. C3 plants use little more readily CO_2 with a lighter isotope of carbon, as a result we have a differentiation in value of ^{13}C to ^{12}C ratio in plant tissues, which is less than in air. As a result it appears the variation the content of these isotopes in the various compounds involved in the processes of physical and biochemical conversion of carbon in plant cells which takes place during photosynthesis (Farquhar *et al.* 1989). The parameter called discrimination coefficient informs about proportion between amount of assimilates containing carbon uptake from atmospheric CO_2 or CO_2 originating from internal tissue respiration. Variation of the ratio values indicates the difference between objects under study with respect to the duration of stomata closing (enabling reassimilation of CO_2).

Discrimination coefficient is calculated according to the formula (O'Leary 1993):

$$\Delta = \frac{(^{13}C/^{12}C)\ \text{plant}}{(^{13}C/^{12}C)\ \text{air}} - 1$$

Material and methods

Plant material. The study was conducted during the growing season from August to September 2012, the plant material consisted of 4 species of Polish grain legumes: pea (*Pisum sativum* L.) cultivar 'Wenus', yellow lupine (*Lupinus luteus* L.) cultivar 'Mister', white lupine (*Lupinus albus* L.) cultivar 'Butan', blue lupine (*Lupinus angustifolius* L.) cultivar 'Sonet' and soybean (*Glycine* Willd.) cultivar 'Augusta'. Species were selected in terms of their sensitivity to salt stress: 'Wenus' is a cultivar relatively resistant, while 'Augusta' is sensitive one. In the study there was also used two species of intermediate resistance to stress ('Mister', 'Butan'). Containers were filled with perlite substrate and placed outdoors under plastic tunnel and then were divided into plots with an area of 0.7 m^2. Next containers were equilibrated with 220 l of NaCl solution of concentration of (0, 20, 70 mM). After sowing the seeds were watered with three NaCl concentrations 0, 20 and 70 mM and contained commercial fertilizers Agrofoska and Florovit. After eight weeks of plant growth the test material was collected. Samples for the deter-

mination of antioxidant enzymes (superoxide dismutase, catalase, peroxidase non-specific) were taken from fully developed leaves at 8 week-old plants. Antioxidant activity calculated per 1 g fresh weight or per 1 mg of protein estimated according to (Bradford 1976). In addition fresh and dry weight of plants were measured. Statistical analysis was performed using STATISTICA 10 using Student's t test.

SOD activity. SOD activity was measured according to Droillard *et al*. (1987). The frozen tissue (about 0,5g) was homogenized using Tissue Lyser with 1,3 ml of extraction buffer (50 mM potassium phosphate, pH 7,8 and 1% PVPP) and centrifuged (16000 x g) at 4^{o}C for 10 min. A sample of tissue extract (20µl) was added to 1 ml of assay buffer containing nitrobluetetrazolium (NBT) (56 mM), xanthine (0,1 mM) and potassium phosphate buffer (50 mM pH 7,8, 1mM EDTA). The reaction was started by adding 10 µl of xanthine oxidase (0,03 U). The absorbance at 560 nm was been recorded for 120 s. The SOD activity was calculated as the percentage of inhibition on NBT reduction. One unit of SOD was the amount of extract causing 50% inhibition of reduction of NBT to NBT-diformazan. The determination of SOD activity was done in 5 replicates (five independent samples collected from different plants).

CAT activity. Catalase activity was estimated according to Aebi (1984). Samples of leaves were homogenized at 4^{o}C with a 50 mM phosphate buffer (pH7,5) and 1mM EDTA and centrifuged at 16000 x g for 10 min. CAT activity was assayed in a reaction mixture composed of a 50 mM phosphate buffer (pH 7,5) to which 30 % H_2O_2 was added to reach an absorbance value in the range of 0,520-0,550 (λ= 240 nm). The reaction was started after adding 200 µl of crued extracts to the reaction mixture. CAT activity measured as the decrease in absorbance at 240 nm. As a consequence of H_2O_2 consumption. The decrease in absorbance of 0,0145 responded with 1 µmol H_2O_2 decomposed per minute per mg of protein. The determination of CAT activity was done in 5 replicates (five independent samples collected from different plants).

POX activity. Peroxidase activity was measured according to the method described by Bergmeyer (1965). Leaf discs were ground to a fine powder with liquid nitrogen and extracted with 50 mM of phosphate buffer (pH 7.0) and 1 mM EDTA (SIGMA-ALDRICH). The extract was centrifuged (14 000 rpm) at 4°C for 10 min and the supernatant was used as the crude extract. 2 cm^3 of 50 mM phosphate buffer (pH 7.0) was mixed with 12 µl of 0.5% p-phenylenediamine and 12 µl of crude extract. The oxidation of p-phenylenediamine was initiated by the addition of 12 µl of buffered H_2O_2 (0.15 cm3 of 30% H_2O_2 (w/v) mixed with 50 cm3 of extract buffer) to a prepared mixture. The absorbance was measured at 460 nm. The total peroxidase activity was expressed as an increase in absorbance of the sample after 1 min and expressed as per 1 mg of FW. The determination of POX activity was completed in 5 replicates.

Results

Increasing the salt concentration in the medium resulted in a reduction of plant growth. For most species concentration limit was about 70 mM NaCl, with the only surviving plants peas. Fig. 1 shows the fresh weight of the plants. The largest decrease in the fresh weight of pea plants was observed between the control objects, and the concentration of 70 mM NaCl. On the other hand, 20 mM salt found to stimulate the growth of fresh weight. The decrease in fresh weight significantly reacted white lupine plants (down 50%) and soybeans (70% decrease). The least fresh weight decrease as influenced by salinity was observed in the yellow lupine.

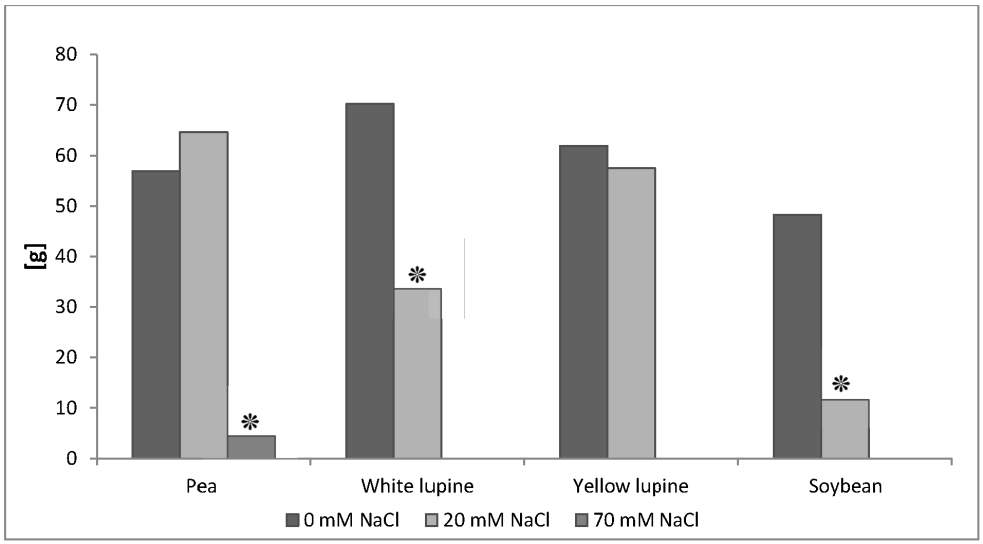


Fig. 1. The influence of salinity on the fresh weight of studied legume plants

Fig. 2 shows the dry weight of legumes. As in the previous graph greatest decrease dry matter was characterized by a soybean (about 80% reduction in dry matter). At the same time these plants reached the highest dry matter value in the control. Other species responded also significantly for salinity were pea and white lupine, however pea showed a significant increase in dry weight between the control object and the concentration of 20 mM NaCl (5g). Yellow lupine was the least sensitive specie for the influence of the salinity.

Fig. 2a shows a value of water [g] which is need to hydration of 1 g DW plant tissue (so called relative water content RWC). The most susceptible species was a Pea because increasing of salinity make hydration of leaves to decrease, starting to 20 mM NaCl. It is correlated with the results of FW and DW because it was

a normal reaction to physiological changes in plants The highest RWC value was observed in plants of both lupine, but in plants of White lupine despite of decrease of FW and DW, increase hydration was noted. The lowest level of hydration showed plants of soybean, due to the low content of water in tissues generally but the differences were not significant.

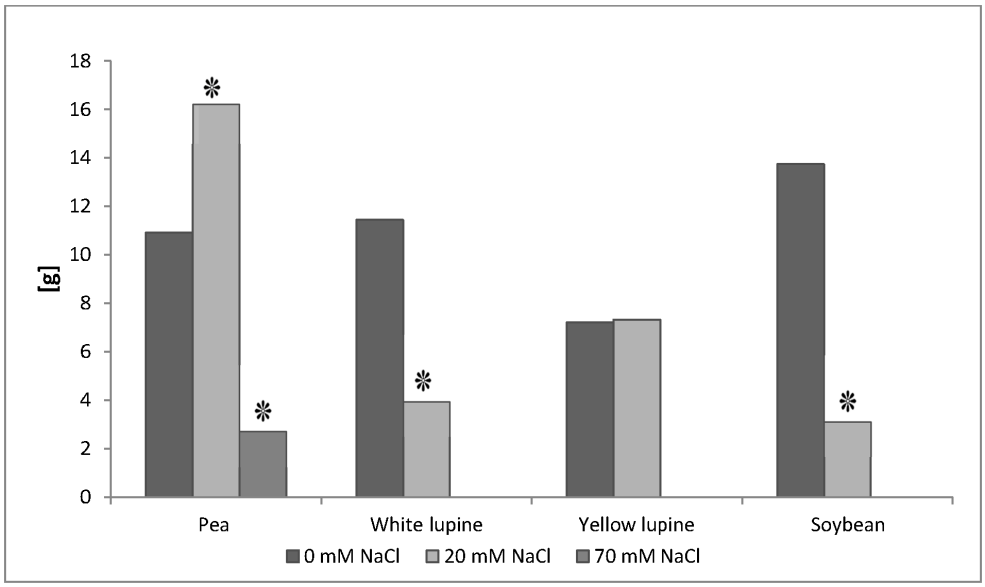


Fig. 2. The influence of salinity on the dry weight of studied legume plants

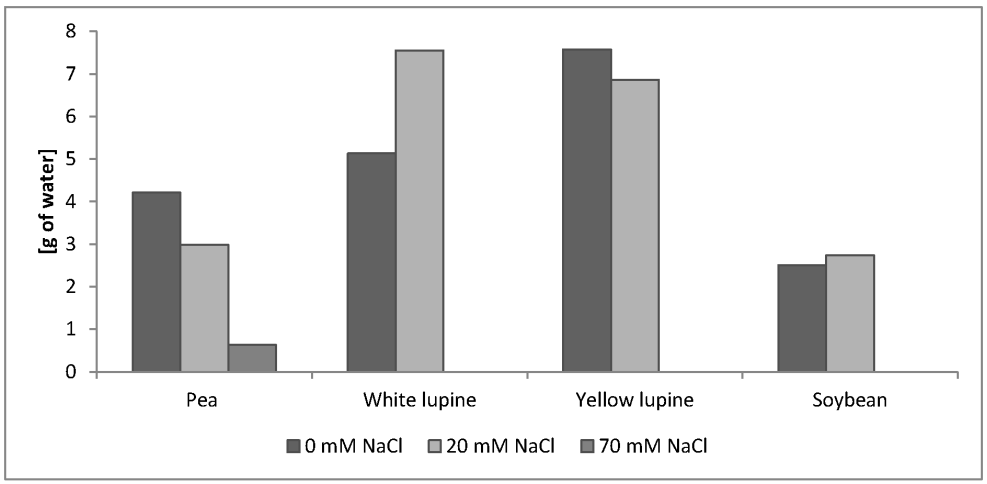


Fig. 2a. The relative water content (RWC) in plants tissue of studied legumes

The protein content was presented in Fig. 3. In all species of legume plants protein levels remained at the same level. With an equally high protein content in plants, two species of lupine can note a downward trend with increasing concentration of salt, however, there are no significant differences. Compared to the control, only of pea plants reacted significantly with respect to the change in the protein content. 20 mM concentration of salt stimulated a significant increase in the protein content of the plants (about 0.1 mg), while 70 mM NaCl resulted in a significant decrease with this respect.

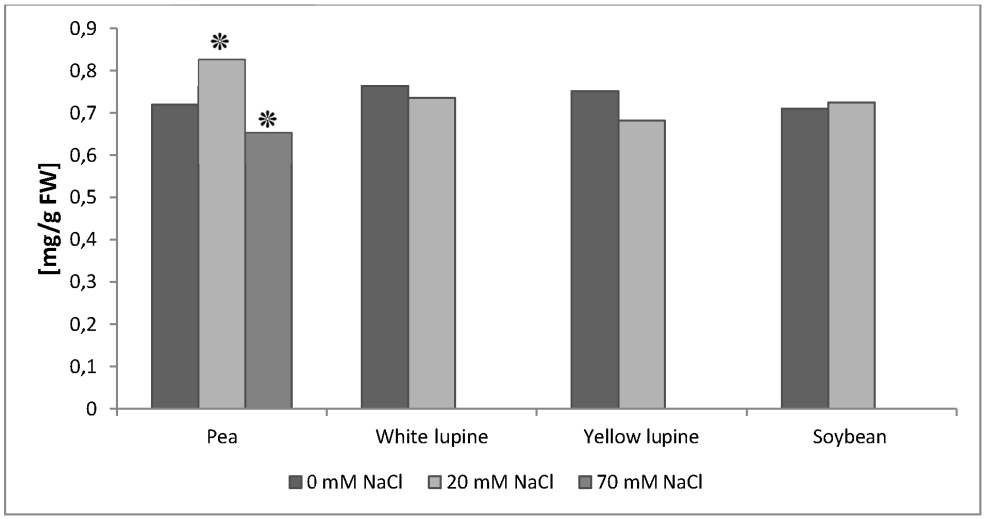


Fig. 3. The influence of salinity on the protein content in leaves of studied legume plants

The activity of antioxidant enzymes were converted into fresh weight and protein.

Fig. 4 shows the activity of superoxide dismutase per g fresh weight. Lupines demonstrate downward trend in the levels of SOD, however, compared to the control level is greater in lupine crops. Compared to the control significant increase in activity was observed in pea plants grown in 70 mM NaCl, with a simultaneous decrease in the 20 mM NaCl. The increase in SOD activity was also observed in soybean plants, however, the increase was not statistically significant. The calculation of the activity per mg of protein (Fig. 5) gave similar results as the SOD activity in the case of fresh weight.

Catalase activity are presented on figures 6 and 7. A significant increase in activity compared to control, both in terms of fresh weight, and the protein was observed in plants of pea, and amounted about 30%. Noting the increasing trend with increasing salt concentration in the soil. In plants of white lupine activity decreased with increasing salinity but it was not statistically significant. However, the level of this enzyme was the highest in the control objects of all plant species. In other species the level of activity at a similar level fluctuated.

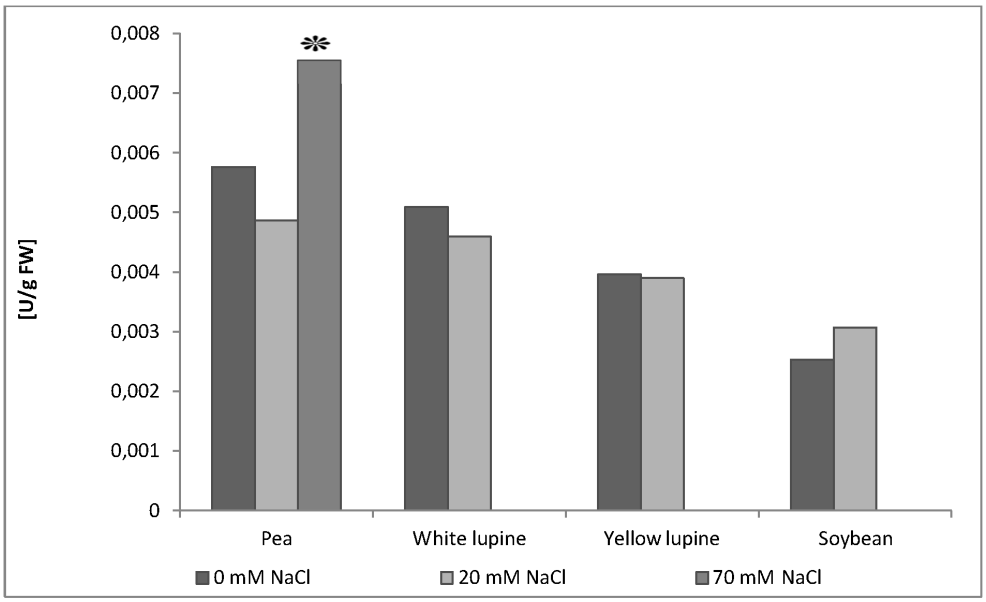


Fig. 4. Activity of SOD per 1g of FW

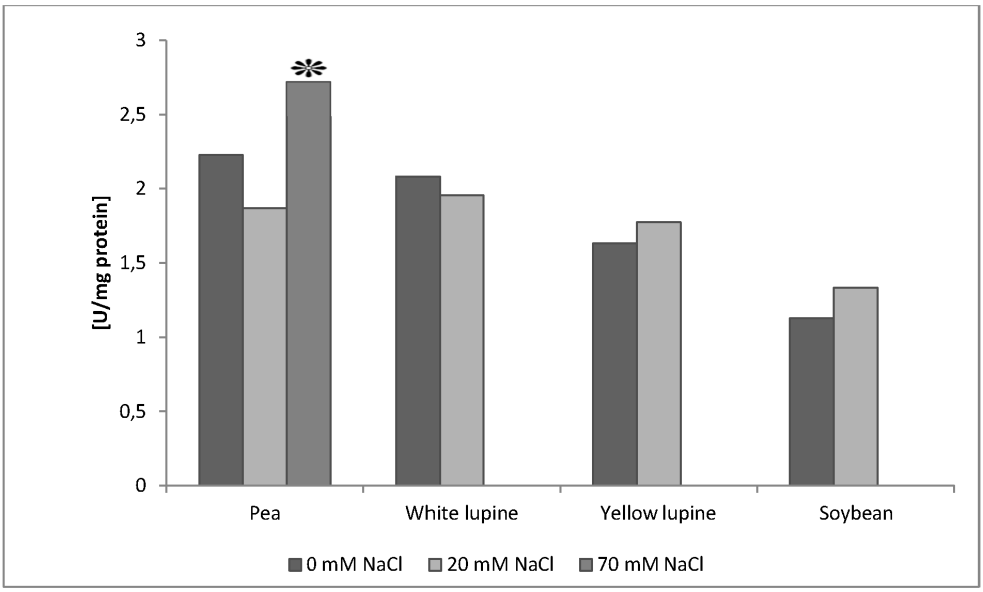


Fig. 5. Activity of SOD per 1mg of protein

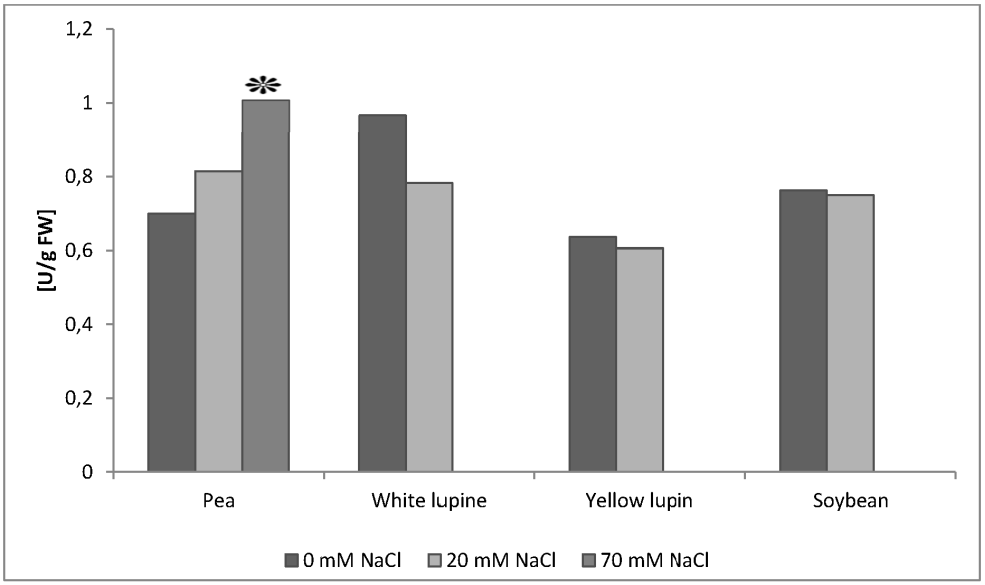


Fig 6. Activity of CAT per 1g of FW

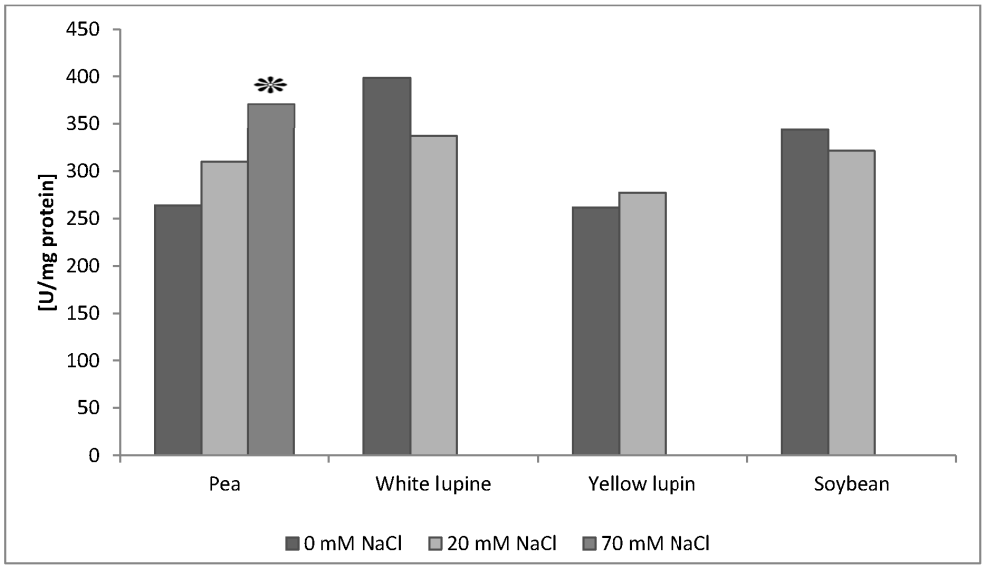


Fig. 7. Activity of CAT per 1 mg of protein

Figure 8 and 9 shows the changes in POX activity. Soybean showed a significant increase in the peroxidase activity (50%), both in terms of fresh weight and protein. In yellow lupine POX despite the high activity in the control plants showed a decrease in the level of activity, however, by a statistically significant only in terms of fresh weight. The level of peroxidase activity in pea plants remained at a similar level, only slightly reducing its activity. The lowest level of POX activity was observed in white lupine plants.

Table 1 shows the results of $^{13}C/^{12}C$ carbon isotope discrimination. Discrimination of carbon in plants that grow without the addition of NaCl is varied depending on the species. The lowest (most negative) showed discrimination blue lupine plants in all organs, while the highest (least negative) soybean plants (in small pods almost 4 units). Increasing the salt concentration increases discrimination in every organs. Responded most poorly ventilated organs such as pods and seeds (yellow lupine, pea) in which CO2 reassimilate was the least negative. Organs well ventilated (leaves) strongly discriminate isotopes of carbon.

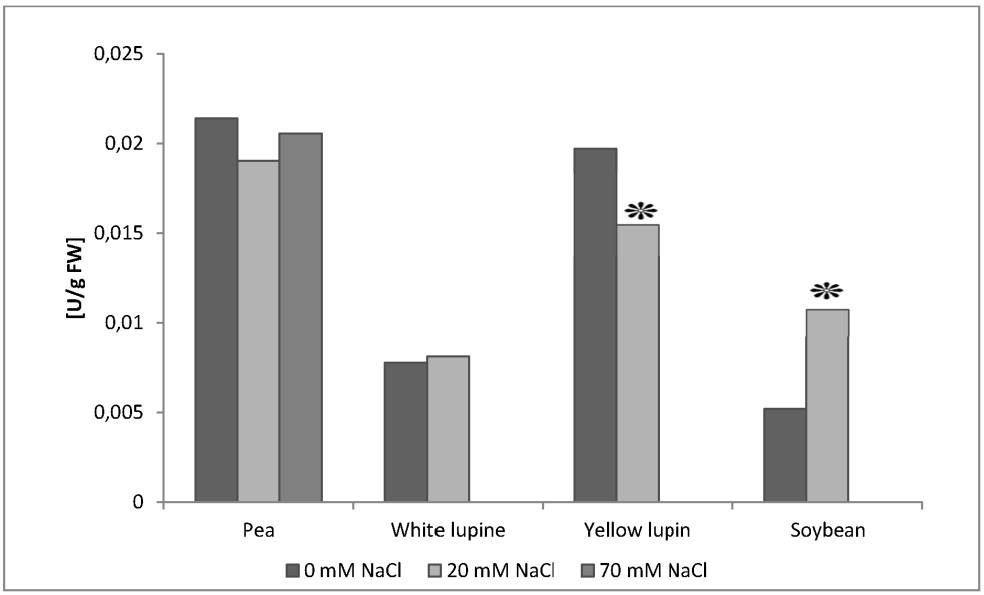


Fig. 8. Activity of POX per 1g of FW

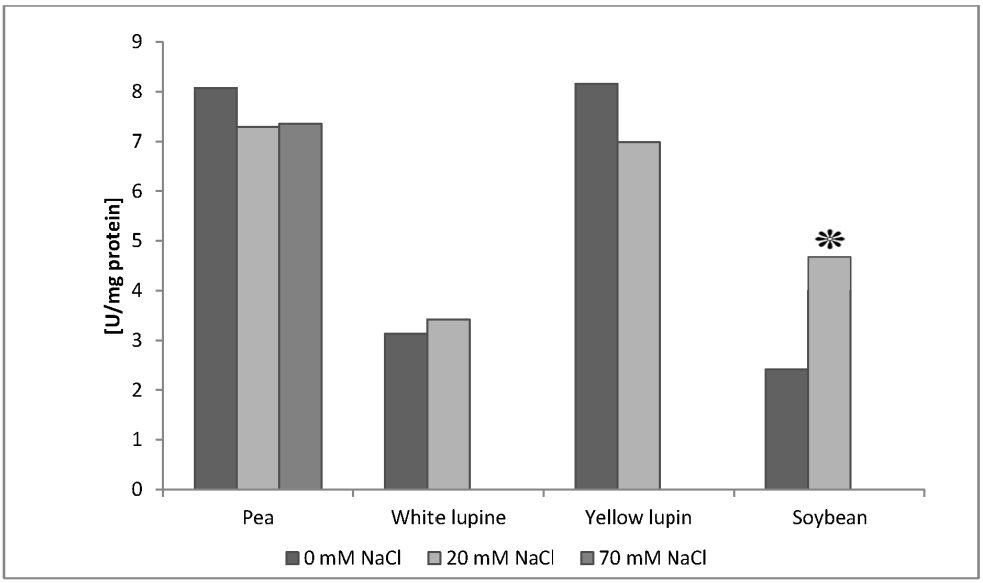


Fig. 9. Activity of POX per 1mg of protein

Table 1. Discrimination of carbon isotope ratio $^{13}C/ ^{12}C$

	Lupinus luteus L. cv. Mister	*Lupinus angustifo-lius* L. cv. Sonet	*Pisum sativum* L. cv. Wenus	*Glycine* Willd. cv. Augusta
0 mmol/l NaCl				
leaves	-31.20	-32.70	-30.13	-28.14
stem	-30.37	-30.96	-29.27	-28.18
tendrils			-30.04	
small pods		-30.32	-28.44	-26.49
pods without seeds	-28.25		-28.65	
seeds	-27.89		-27.47	
40 mmol/l NaCl				
leaves	-30.71	-31.40	-29,87	-28. 02
stem	-28.97	-29.64	-28.50	-27.94
tendrils			-29.14	
small pods		-29.68	-27.71	-26.17
pods without seeds	-27.30		-27.76	
seeds	-26.73			
70 mmol/l NaCl				
leaves			-29.00	
stem			-27.60	
tendrils			-28.96	

Discussion

No doubt, salt stress, which is one of the most important abiotic stress causes in legumes a number of changes in physiological and biochemical parameters. The first obvious features is the inhibition of growth and thus decrease in fresh and dry weight of the aboveground part and roots (Parida and Das 2005). Each of the species tested, reacted to the change in this parameter, although the species were more resistant to salinity. Similar results of FW and DW were achieved in the study of *Pisum sativum* by Borucki and Sujkowska (2008). Biochemical parameter that reacts strongly to salinity is increased production of ROS by simultaneous changes in the activity of antioxidant enzymes, especially SOD, CAT, POX. In this study, the plants can be identified in whom antioxidant system work in a way specific to the species, causing them more resistant (pea) or more sensitive to salinity (soybeans). Several authors (Hernandez *et al.* 2000, Sreenivasulu *et al.* 2000, Sairam *et al.* 2000) suggests that higher activity of antioxidant enzymes plays an important in conferring tolerance to environmental stresses different cultivars.

The study of Dionisio-Sese and Tobita (1998) compared the activity of antioxidant enzymes in plants sensitive and tolerant to salinity. Two sensitive rice varieties showed a decrease in SOD activity and an increase in peroxidase activity in response to stress. At the same time varieties showed an increase in lipid peroxidation, electrolyte flow and accumulation of Na^+. Varieties less sensitive to salinity were two different mechanisms of protection against ROS. One of them showed a slight increase in SOD activity declined slightly, while addition of peroxidase activity without altering the activity level of lipid peroxidation, electrolyte flow and accumulation of Na^+. In the second embodiment of the Na^+ accumulation was recorded with simultaneous lesions similar to those in sensitive cultivars.

An interesting phenomenon is the result of the level of activity of antioxidant enzymes in plants soybean (*Glycine*). Comparing them with research Comba *et al.* (1998) observed that 50 mM NaCl increases the activity of SOD and CAT, whereas in our study 70 mM NaCl resulted in a complete inhibit growth of plants. Simultaneously, in a concentration of 20 mM NaCl, there was no change in activity.

Discrimination $^{13}C/^{12}C$ isotope carbon showed that the treated plants contained more heavier isotope of carbon, compared to control plants. The most different of the carbon isotope discrimination between leaves, seeds and tendrils for *Pisum sativum* were observed. However, the analyzes were carried out at a concentration of 70 mM NaCl due to the inhibition of the generative form. Differences between Δ leaves, tendrils and seed increased with increasing concentration. Similar results were shown in the experience of Brugnoli and Lauteri [1991] where the biggest differences between the results achieved and the seed leaves. In addition, values between leaves and stems were very close.

Conclusions

- Salinity (0-70mM) inhibits the growth of studied legumes
- Salinity influence activity of SOD, CAT and POX specifically for each plant species
- Pea is the most resistant species to salinity, while soybean is the most susceptible one
- The decrease of level of discrimination ratio ^{13}C to ^{12}C in organs less ventilated (seeds) is observed

Acknowledgement. This research was supported by Action COST FA 0901: Putting Halophytes to work- from Genes to Ecosystem made in The *Franciszek Górski* Institute of Plant Physiology, Polish Academy of Sciences in Krakow.

References

Aebi, H. 1984. Catalase in vitro. Methods in Enzymol. 105, p.121-126

Bergmeyer H.U. 1965. Methods in Enzymatic Analysis, 2nd edn.,. Academic Press, New York. p. 990

Borucki W., Sujkowska M. 2007. The effects of sodium chloride-salinity upon growth, nodulation and root nodule structure of pea (*Pisum sativum* L.) plants. Acta Physiol. Plant. 30, p.293-301

Bradford M.M. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, p.248-254.

Brugnoli E., Lauteri M. 1990. Effects of salinity on stomatal conductance, photosynthetic capacity and carbon isotope discrimination os salt-tolerant (*Gossypium hirustum* L.) and salt-sensitive (Phasoleus vulgaris L.) C3 non-halophytes. Plant Physiol. 95, p.628-635

Chinnusamy V., Jagendorf A., Zhu J-K. 2005. Understanding and improving salt tolerance in plants. Crop Sci 45, p.437-448.

Comba M.E., Benevides M.P., Tomaro M.L. 1998. Effects of salt stress on antioxidant defence system in soybean root nodules. Aust. J Plant Physiol. 25(6), p.665-671

Dionisio-Sese M.L., Tobita S. 1998. Antioxidant responses of rice seedlings to salinity stress. Plant Sci. Limerick 135, p.1-9

Droillard, M.J., Paulin A.,. Massot J.C. 1987. Free radical production, catalase, and superoxide dismutase activities and membrane integrity during senescence of petals of cut carnation (*Dianthus caryophyllus*). Physiol. Plant. 71:197–202

Farquhar G.D., Ehleringer J.R., Hubick K.T. 1989. Carbon isotope discrimination and photosynthesis. Annu. Rev. Plant Physiol. Plant Mol. Biol 40, p. 503-537

Ghassemi F., Jakeman A.J., Nix H.A. 1995. Salinization of land and water resources. Human causes, extent management and case studies. University of New South Wales Press Ltd, Sydney

Hernandez J.A., Olmos E., Corpas F.J., Sevilla F., 2000. Tolerance of pea (pisum sativum L.) to long term salt stress in associated with induction of antioxidant defences. Plant Cell Environ. 23, p.853-862

Kacperska A. 2007. Stres spowodowany zasoleniem- stres solny. In: Fizjologia roślin. J. Kopcewicz, S. Lewak. 2007. PWN, W-wa, p.655-659

Kotuby-Amacher J., Koenig R., Kitchen B. 2000 Salinity and plant tolerance. Electronic Publishing, Utah State University extension

Lauchli A., James R., Huang C.X., McCully M., Munns R. 2008 Cell-specific localization of Na+ in roots of durum wheat and possible control points for salt exclusion. Plant Cell Environ. 31, p. 1565–1574

Mahajan S., Tuteja N. 2005. Cold, salinity and drought stressed: an overview. Arch. Biochem. Biophys. 444, p.139-158

Mittova V., Tal M., Volokita M., Guy M. 2003. Up-regulation of the leaf mitochondrial and peroxisomal antioxidative systems in response to salt-induced oxidative stress in the wild salt-tolerant tomato species *Lycopersicon penneli*. Plant Cell Environ. 26, p. 845-856

Munns R., Termaat A. 1986. Whole plant responses to salinity. Aust. J Plant Physiol. 13, p.143-160

Munns R., Tester M. 2008. Mechanism of salinity tolerance. Annual Review of Plant biology 59, p.651-681

Nawaz K., Hussain K., Majeed A., Khan F., Afghan S., Ali K. 2010. Fatality of salt stress to plants: Morphological, physiological and biochemical aspects. African Journal of Biotechnology vol. 9(34), p. 5475-5480

O'Leary, M.H. 1993. Biochemical basis of carbon isotope fractionation. In R. Ehleringer et al. stable isotopes and plant carbon-water relations. Academic Press, New York, p. 19-28

Parida A.K., Das A.B. 2005. Salt tolerance and salinity effects on plants: a review. Ecotox. And Environ. Safety 60, p.324-349

Rengasamy P. 2006. World salinization with emphasis on Australia. Journal of Experimental Botany 57(5), p. 1017-1023

Sairam R.K., Srivastava G.C., Saxena D.C. 2000. Increased antioxidant activity under elevated temperatures: a mechanism of heat stress tolerance in wheat genotypes. Biol. Plant 43, p.245-251

Spychalla J.P., Desborough S.L. 1990. Superoxidase dismutase, catalase and alpha-tocopherol content of stored potato tubers. Plant Physiol. 94, p. 1214-1218

Sreenivasulu N., Grimm R., Wobus U., Weachke W. Differential response of antioxidant compounds to salinity stress in salt tolerant and salt sensitive seedling of foxtail millet (Setaria italic) Physiol. Plant. 109, p.435-442

Zhu J.K. 2007. Plant salt stress. In: Encyclopedia of life sciences. Doi: 10.1002/ 9780470015902. a0001300.pub2

PLANT FUNCTIONING UNDER ENVIRONMENTAL STRESS P. 223-231

Cracow, 12th to the 15th September, 2012

MORPHOLOGY, ANATOMY AND SURFACE ULTRASTRUCTURE OF *NUPHAR LUTEA* (L.) SMITH. TERRESTRIAL, FLOATING AND SUBMERGED LEAVES

Elena Klimenko

Institute of Botany, National Academy of Sciences of Ukraine, Tereschenkivska St. 2, 01601, Kyiv, Ukraine

Corresponding author. li_grey@mail.ru

Abstract Anatomy and the surface ultrastructure of floating, submerged and terrestrial leaves of the heterophyllous aquatic plant *Nuphar lutea* (L.) Smith. were investigated. Submerged leaves differed from floating and terrestrial leaves by the: absence of a cuticle, stomata, asterosclereids, differentiated parenchyma, as well as the presence of small intercellular spaces. A significant reduction in the thickness of submerged leaf blades with increasing the water depth was demonstrated: a number of mesophyll layers decreased. Such anatomical patterns of submerged leaves are considered as phenotypic plasticity that makes it possible plant adaptation to light absorption in water column. The hydropotes structure was similar to that of salt glands, so they can release certain substances outside. It is supposed that described features of anatomy and the surface ultrastructure of floating, terrestrial and submerged leaves from the different water depth are conditioned by their existence in the different physicochemical environments.

Key words: *Nuphar lutea*; leaf; heterophylly; anatomy; hydropote

Introduction

Variations in leaf traits such as size, shape, thickness, and pigmentation are widespread (Titus and Sullivan 2001). Heterophylly, the production of two or more leaf forms within the same individual, is a common characteristic of aquatic and amphibious plants (Hutchinson 1967; Deschamp and Cooke 1985). These plants are typically confronted with abruptly different microenvironments – air and water – that contrast strikingly as media for plant life (Maberly and Spence 1989; Titus and Sullivan 2001). Controlled by the developmental program or environmental

factors, or both, plants produce distinct types of leaves corresponding to changes in the water level and the seasons (Lin et all 2005). Heterophylly provides aquatic plants an advantage in adapting to the environment.

Nuphar lutea (L.) Smith. is known to be a heterophyllous aquatic plant with floating and submerged near-bottom leaves. Floating leaves has long petioles, emerge in May and exist during summer. Submerged leaves have short petioles, exist over an all year and can grow in different depth, up to 3 m. After decrease in water level in river, plants grow at the riverside, so that their leaves with short petioles are in air.

The primary goal of this research was to study heterophylly in *N. lutea* by describing the surface and internal anatomy of terrestrial, floating and submerged leaves.

Material and methods

Plant material. Mature, without any visible damage terrestrial, floating and submerged *Nuphar lutea* (L.) Smith leaves were collected from plants grown in the basin of the Psyol river in Ukraine during 2009–2011 years. Floating and submerged leaves were collected from the same plant. Submerged near-bottom leaves were collected at the depth of 0.5 and 1.5 m. 3 leaves of each type were collected.

Preparation of leaf tissue. To examine internal leaf structure, 0.5x1 cm pieces from the centre of the leaf lamina were fixed in 2.5% glutaraldehyde (0.1 M cacodylate buffer, pH 7.3) for 12 h at ambient temperature and then in 1% OsO_4 in the same buffer for 12 h at 4 C°. Samples were dehydrated through a graded alcohol series and embedded in epon-araldit resins. Ultrathin sections were obtained on an ultramicrotome RMC MT-XL (USA). Semithin sections (1 μm) were stained with 0.12% toluidine blue and examined with a light microscope NF (Carl Zeiss, Germany). Ultrathin sections (about 55 nm) were stained with uranyl acetate and lead citrate and examined with a transmission electron microscopes JEM 1200EX and JEM 1230EX (JEOL, Japan). Tissue for scanning electron microscopy was fixed in 1% paraformaldehyde (0.1 M cacodylate buffer, pH 7.3), dehydrated in an ethanol series. Specimens were then sputter coated with gold and observed using a JSM-35 scanning electron microscope (Japan).

Quantification of leaf structure and surface ultrastructure. Total leaf thickness, leaf palisade mesophyll thickness, spongy mesophyll thickness, height and width of epidermis and palisade cells, fractional area of intercellular air space were measured from digital photo with program Image Tool for Windows. Photos were made with using a Leitz light microscope (Leitz Dialux 20 EB, Leitz, Wetzlar, Germany) attached to a PC-based image processing system. Leaf histological analysis was performed on ten transverse sections of each leaf type. 100 epidermis and palisade cells from 3 leaves of each type of leaves was taken for the measurement. The

amount of hydropotes was counted on the digital photo of abaxial epidermis and recalculate to the 1 mm^2.

Statistical analysis

Differences in anatomical parameters were tested by applying Student t-test. The level of significance was accepted at $p < 0.05$ (Lakin 1990).

Results

Morphology and anatomy of terrestrial leaves. Terrestrial leaves form rosette on the ground surface. They are large, green, simple, smooth-edged, circular, peltate, without stipules. Petioles are short and triangular. Stomata placed on the leaf adaxial side and are absent on its abaxial side. On the abaxial side, there are the great quantity of hydropotes (on average 211 on 1 mm^2), placed diffusely. A cuticle covers both leaf sides. Cells of the adaxial epidermis have an oval shape. Cells of abaxial epidermis are elongate and significantly larger than previous (Table 1). Mesophyll is differentiated on palisade and spongy parenchyma (Fig. 1). Palisade parenchyma consists of 3-5 layers of cylindrical cells, which sizes are 29.9±1.1 (height) x13.7±0.3 (width) μm (Table 1). Intercellular spaces vary by size. There are asterosclereids and latex vessels in mesophyll. Chloroplasts located along anticlinal cell walls. Spongy mesophyll consists of thin-wall oval cells and large intercellular spaces (aerenchyma).

Morphology and anatomy of floating leaves. Floating leaves are large, green, simple, smooth-edged, circular, peltate, without stipules. Petioles are triangular. A petiole length corresponds to the length of the pond. Stomata placed on the leaf adaxial side, and they are absent on its abaxial side, where the great quantity of hydropotes placed diffusely (on average 118 on 1 mm^2). A cuticle covers both leaf sides. Cells of the adaxial epidermis have an oval shape. Abaxial epidermal cells are more elongate and larger than those of the adaxial epidermis (Table 1). Mesophyll is differentiated on palisade and spongy parenchyma (Fig. 1). Palisade parenchyma consists of 4-5 layers of cylindrical cells, which sizes are 40.2±0.8 (height) x11.4±0.2 (width) μm (Table 1). Intercellular spaces vary by size. There are asterosclereids and latex vessels in mesophyll. Chloroplasts located along anticlinal cell walls. Spongy mesophyll consists of thin-wall oval cells and large intercellular spaces (aerenchyma).

Morphology and anatomy of submerged leaves from 0.5 m. Submerged leaves are green, simple, undulate, and circular, without stipule, with short triangular petioles. They formed rosette on the bottom of pond. Stomata and cuticle are absent. Hydropotes appears on the abaxial side of leaf. They amount are significantly low (on average 21 on 1 mm^2). Cells of the adaxial and abaxial epidermis have an oval

shape and elongate. They high are almost similar (Table1). Mesophyll is homogenous; number of its layers is 7 (Fig. 1). Heights of mesophyll cells vary from 8.3 to 33.2 µm. Mesophyll cells are oval, with big vacuole, chloroplasts located along tangential cell walls.

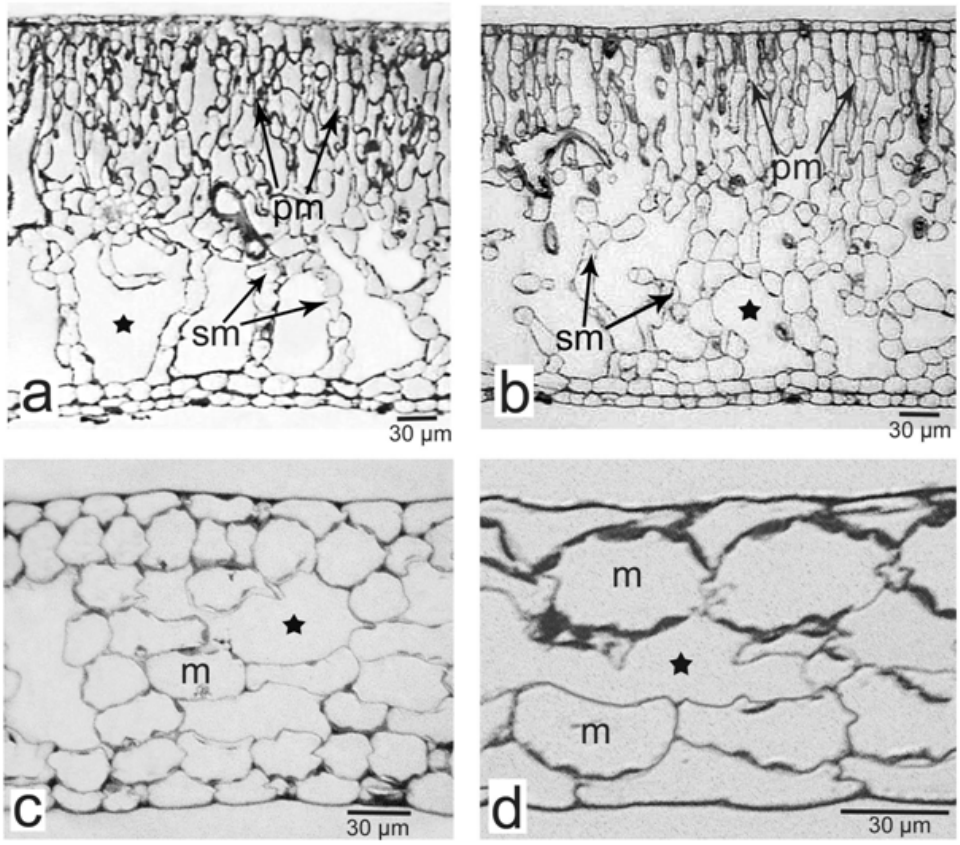


Fig. 1 Comparison of internal anatomy of N. lutea terrestrial (a), floating (b) and submerged leaves from the depth of 0.5 m (c) and 1.5 m (d). Mesophyll tissue of terrestrial and floating leaves has palisade (pm) and spongy layers (sm), chloroplasts located along anticlinal cell walls and large intercellular space (*). In contrast, leaves formed under water have a homogeneous mesophyll (m) with chloroplasts that expose lengthwise the tangential cell walls and little intercellular space (*). Bars: a, b, c, d – 30 µm

Table 1 Anatomy features of *N. lutea* terrestrial, floating and submerged leaves, n=100, P=0.05

Leaves	Leaf thickness µm, *M±m*	Height of epidermis cells		Fractional area of intercellular air space %, *M±m*	Amount of hydropotes on 1 mm^2
		adaxial µm, *M±m*	abaxial µm, *M±m*		
Terrestrial	588.0±2.8	11.2±0.2	18.3±0.2	26.13±0.6	211±13.5
Floating	549.8±0.7	12.9±0.3	15.3±0.3	43.6±0.8	118±3.06
Submerged 0.5 m	162.2±4.4	12.3±0.5	13.6±0.4	39.1±0.8	21±1.2
Submerged 1.5 m	70.8±0.3	8.7±0.9	11.6±0.9	12.5±0.7	8.5±0.6

Morphology and anatomy of submerged leaves from 1.5 m. Leaves are green, simple, undulate, and circular, without stipule, with short triangular petioles. They formed rosette on the bottom of pond. Stomata and cuticle are absent. Hydropotes appears on the abaxial side of leaf. They amount are on average 8.5 on 1 mm^2. Adaxial and abaxial epidermis cells have irregular form and considerably elongate. Mesophyll is not differentiated. They are 2-3 homogeneous mesophyll cell layers with small intercellular space area (Fig. 1). Heights of mesophyll cells vary from 7.7 to 27.4 µm. Mesophyll cells are oval, with big vacuole, chloroplasts located along tangential cell walls. They are 2-3 homogeneous mesophyll cell layers with small intercellular space area.

Ultrastructure of hydropotes. Hydropotes of terrestrial, floating and submerged leaves are cupped and raised above the epidermis. They represent three-cells glands, a basal cell of which is rectangular, it is named a «foot cell» (Lüttge 1971). Its wall is cellulose, twisting, and form protuberances at the anticlinal wall that bordering with a mesophyll layer. Cell wall protuberances are coated by the plasmalemma. «Foot cells» are rich with the cytoplasm, have large nuclei, numerous condensed mitochondria, numerous free ribosomes and small vacuoles, dictyosomes are rare. Cells of a hydropote are connected between themselves by numerous plasmodesmas. The middle and upper cells are filled with the dense homogeneous osmiophil content (Fig. 2).

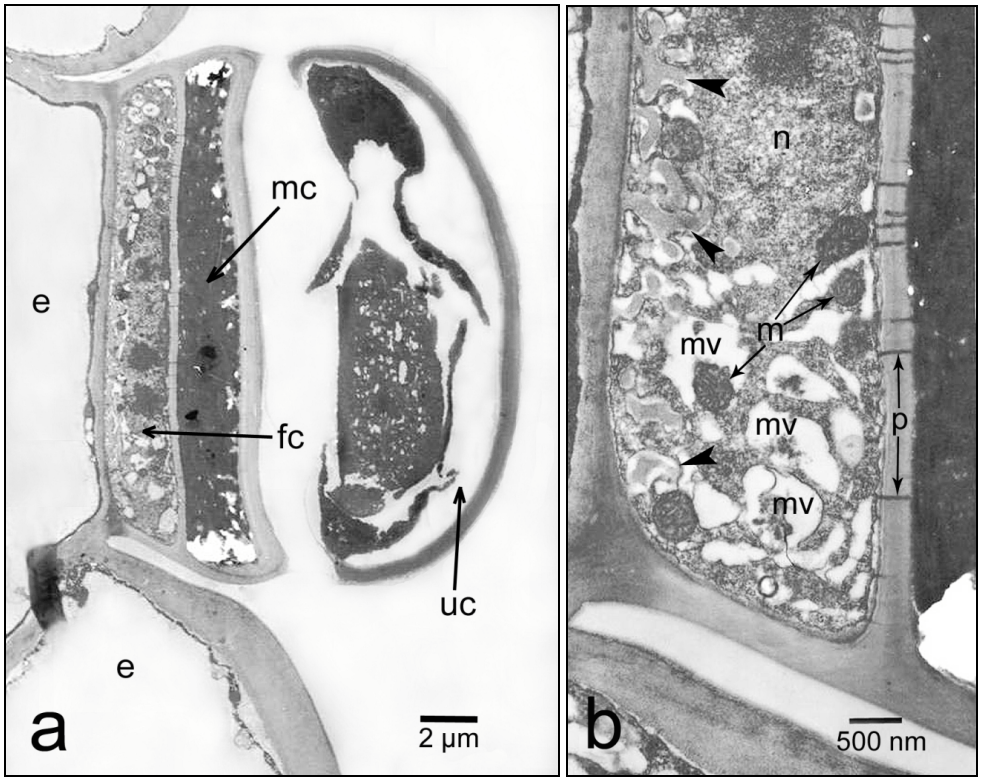


Fig. 2 TEM micrographs illustrating a hydropote. a – TEM micrograph of a hydropote. b – TEM micrograph taking at the «foot cell» of hydropote. Hydropote consist of three cells: fc – basal «foot cell», mc – medium cell, uc – upper cell. «Foot cell» connecting with medium cell by numerous plasmodesma (p). Upper cell are filled with dense homogeneous osmiophil content. The basal walls of «foot cell» bear towards the cytoplasm many protuberances (arrow), big nuclei (n), numerous mitochondrion (m) and microvacuoles (mv). Bars: a – 2 µm, b – 500 nm

Discussion

There are no significant differences in a shape of *N. lutea* terrestrial, floating and submerged leaves, but their internal anatomical patterns are strongly notable, especially of submerged leaves.

Anatomical patterns of floating and aerial leaves are similar, but there are some differences. A blade thickness of aerial leaves and a size of their abaxial epidermal cells are larger than those of floating leaves. However, a size of both palisade parenchyma cells and intercellular spaces are smaller than those in floating leaves

(Table 1). Increasing of the leaf blade thickness may be explained by the enlargement of abaxial epidermal cells and aerenchyma volume that correlate with a decrease in palisade parenchyma cell size. A reduced partial volume of intercellular spaces may be explained by more tightly spaced palisade parenchyma cells, as well as by a corresponding decrease in cell number and volume of this tissue. As was found in the most aquatic species, terrestrial leaves are generally thicker than underwater. In *Ranunculus flabellaris* this feature correlates with thicker epidermal cells, higher length/width ratios for mesophyll cells, and more intercellular space area (Young et all 1987). In a heterophyllous aquatic plant *Batrachium peltatum*, terrestrial and floating leaves are significantly different in their thickness. The thickness of terrestrial leaf blades was 400±36 μm, and it of floating leaves was 272±17 μm. It is assumed that increasing of the terrestrial leaf thickness is caused by their existence in the more arid conditions compared with floating leaves (Nielsen and Sand-Jensen 1992).

Submerged leaves of *N. lutea* are differing significantly in the anatomical structure from terrestrial and floating leaves. They lack stomata, cuticle, asterosclereids, differentiated parenchyma, the volume of intercellular spaces and the leaf thickness reduced. In the subepidermal parenchyma cells are formed two loop-shaped curves of the cell wall in each cell. Bends formed between two adjacent parenchyma cells of each layer in the direction of the long axis of the underwater leaf (Nedycha 2011). A significant difference in the anatomical structure of terrestrial and submerged leaves was shown by the example of heterophyllous aquatic plant *R. flabellaris*. It has no cuticle, stomata, differentiated mesophyll, its leaf blade thickness and a cell size decreased. Authors believe these changes are caused by the cumulative effect of environmental factors (Young et all 1987).

In the submerged leaves of *N. lutea* that grow at different depths, there are differences in a shape of epidermal cells, the thickness of a leaf blade, a number of mesophyll layers, a size of mesophyll cells and a volume of intercellular spaces. These changes may be associated with the influence of abiotic factors (temperature, flow rate, depth of water, light intensity). It is known that *Veronica anagallis-aquatica* plants decrease a cell size and increase a cell density with increasing flow velocity (Boeger and Poulson 2003). But, in our opinion, the anatomical parameters of *N. lutea* submerged leaves are the most depend on the depth of the plants growth. Submerged leaves from the depth of 1.5 m have only 2–3 mesophyll layers and small intercellular spaces (12.5±0.65). Leaves collected at the depth of 0.5 m have 7 mesophyll layers and a greater volume of intercellular spaces (39.1±0.81). With increasing depth the intensity of light decreases. A reduced number of parenchyma layers may be considered as adaptation necessary for light adequate absorption by all leaf layers. A dependence of the leaf blade thickness and plant biomass from the light intensity was described in *Rumex crispus*: a number of green leaves and a dry mass (weight) of plants decreased in plants growing in a blackout (Laan and Blom 1990).

There is information concerning the formation of specialized cells – hydropotes in the leaf epidermis of aquatic plants. Kaul (1976) and Wilkinson (1979) pointed that hydropotes occur in many different, widely separated groups of aquatic angiosperms and in aquatic ferns (Carpenter 2006). Grüss (1926) illustrated the abaxial hydropotes with nuclei in *Nuphar, Nymphaea*, and *Victoria* species. A size and density of hydropotes in the epidermis depends from its type. It is suggested that hydropotes are available only on the lower epidermis of floating leaves, or on leaf both surfaces in a wide range of aquatic angiosperms and aquatic ferns (Kaul 1976; Wilkinson 1979; Carpenter 2006). These structures assign various functions: glands that secrete mucus (Juniper and Jeffree 1983), structures that perform the function of absorption (Lüttge et all 1971; Wilkinson 1979).

However, according to the information obtained by us for *N. lutea*, hydropotes have the structure similar to that of salt glands: the formation of cell wall protuberances, large nuclei, and a significant amount of free ribosomes, small vacuoles, mitochondria with developed cristae, and the absence of reserve substances (materials). Gunning and Pate (1969) have termed cells with wall protuberances "transfer cells". Cell wall protuberances are always coated by the plasmalemma. Thus, an increase in the cell wall surface is equivalent to an increase of the plasmalemma surface. The presence of large nuclei in a transfer hydropote cell "foot-cell" may be related to the secretory function (Luttge and Kraft 1969). However, the absolute volume of gland nuclei is usually not larger than that of nuclei in usual parenchyma cells. Hydropotes on the lower surface of *Nymphaea* floating leaves are salt-transporting gland cells. In hydropotes cells nearly all the total space, not occurred by the cell wall protuberances and the large nuclei, is occupied by mitochondria (Lüttge and Higinbotham 1979). Lüttge and co-workers have shown that *Nymphaea* hydropotes can absorb sulphate, and they are effective about twice than surrounding epidermal cells (Lüttge 1971). Thus, it may be assumed that hydropotes of *Nymphaeales* have secretory and absorptive functions (Lüttge and Kraft 1969; Wilkinson 1979) highly specialized to their aquatic habitats (Carpenter 2006).

Conclusions

This study provides us with more evidence in favor of significant phenotypic plasticity of plant leaves as an important structure providing a successful course of photosynthesis. Leaf anatomy of terrestrial, floating and submerged leaves is shown to be strongly different and conform to their environmental conditions. A significant reduction in the thickness of the submerged leaf blade with increasing depth of the water by reducing the number in mesophyll layers can be seen as an adaptation necessary for the absorption of light in all mesophyll layers. Hydropotes have the structure similar to that of salt glands. According to the hydropote ultrastructure, it may suppose that they can release certain substances outside. The further study to find out what kind of substances they release is required.

References

Boeger MRT, Poulson ME (2003) Morphological adaptations and photosynthetic rates of amphibious *Veronica anagallis-aquatica L.* (*Scrophulariaceae*) under different flow regimes. Aquat Bot 75:123–135

Carpenter KJ (2006) Specialized structures in the leaf epidermis of basal Angiosperms: morphology, distribution, and homology. Am J Bot 93:665–681

Deschamp PA, Cooke TJ (1985) Leaf dimorphism in the aquatic angiosperm *Callitriche heterophylla.* Am J Bot 72:1377–1387

Grüss J (1926) Die Haustoren der *Nymphaeaceen*. Berichte der Deutschen Botanischen Gesellschaft 45:454–458

Gunning BSE, Pate JS (1969) "Transfer cells" plant cells with wall ingrowths, specialized in relation to short distance transport of solutes – their occurrence, structure, and development. Protoplasma 68:107-133

Hutchinson GE (1967) A treatise on limnology. Wiley, New York

Juniper BE, Jeffree CE (1983) Plant surfaces. London, Arnold

Kaul RB (1976) Anatomical observations on floating leaves. Aquat Bot 2:215–234

Laan P, Blom PM (1990) Growth and survival responses of *Rumex* species to flooded and submerged conditions: the importance of shoot elongation, underwater photosynthesis and reserve carbohydrates. J Exp Bot 228:775-783

Lakin GF (1990) Biometrics. High School, Moscow

Lin B-L, Wang H-J, Wang J-S, Zaharia LI, Abrams SR (2005) Abscisic acid regulation of heterophylly in *Marsilea quadrifolia L.*: effects of R-(□) and S-(+) isomers. J Exp Bot 56:2935-2948

Lüttge U (1971) Structure and function of plant glands. Annu Rev Plant Physiol 22:23-44

Lüttge U, Higinbotham N (1979) Transport in plants. Springer-Verlag, New-York

Lüttge U, Pallaghy CK, von Willert K (1971) Uptake by glands and epidermal cells of water lily (*Nymphaea*) leaves with special reference to the effect of Poly-L-Lysine. J Membrane Biol 4:395-407

Maberly SC, Spence DHN (1989) Photosynthesis and photorespiration in freshwater organisms: amphibious plants. Aquat Bot 34:267–286

Nielsen SL, Sand-Jensen K (1993) Photosynthetic implications of heterophylly in *Batrachium peltatum* (Schrank) Presl. Aquat Bot 44:361-371

Nedycha OM (2011) Heterophylly in plants. Alterpres, Kyiv (in Russian, with English abstract)

Titus J, Sullivan PG (2001) Heterophylly in the yellow waterlily, *Nuphar variegata* (*Nymphaeaceae*): effects of [CO_2], natural sediment type, and water depth. Amer J Bot 88:1469−1478

Wilkinson HP (1979) The plant surface (mainly leaf). In: Metcalfe CR, Chalk L (ed) Anatomy of the dicotyledons, 2nd ed., vol. 1. Clarendon Press, Oxford, UK, pp. 97–165

Young JP, Horton RF (1987) Heterophylly in *Ranunculus flabellaris*: the effect of abscisic acid on leaf anatomy Ann Bot 60:117–125

PLANT FUNCTIONING UNDER ENVIRONMENTAL STRESS P. 233-239

Cracow, 12th to the 15th September, 2012

THE IMPACT OF PLANT-DERIVED SMOKE ON SEED GERMINATION IN THE CONTEXT OF SWAILING

Renata Bączek-Kwinta, Marcin Markowicz

University of Agriculture in Krakow, Faculty of Agriculture and Economics, Department of Plant Physiology, ul. Podłuzna 3, 30-239 Kraków, Poland

Corresponding author: rrbaczek@cyf-kr.edu.pl

Abstract Smoke generated by wildfires have special ecological impact in various fire-prone environment in Africa, Australia, and both Americas. However, in the countries of moderate climate zones, swailing (controlled burning of meadows) is often, although illegally, practised (BrE) by farmers. Hence, the influence of smoke generated from plant debris on seed germination and radicle growth of 4 popularly grown crops, and 3 weed species, occurring in Central Europe, was studied. Smoke had stimulative effect on seed germination of 6 from 7 studied species. There was no impact on germination of meadow cress seeds, but in this case strong stimulation of radicle growth was noticed. The influence of smoke on germination was the strongest in case of red cabbage, as the difference between smoke-treated and control seeds was 60%, and the stimulation of growth of radicles was obtained. Summing up, plant-derived smoke can stimulate seed germination and seedling vigour of some crop and weed plants of moderate climate countries. Therefore, smoke generated by swailing can cause local changes in both the natural and agricultural environments.

Key words: germination; seed; smoke; weed

Introduction

The role of fire-related cues (smoke and heat) in alterations of ecosystem properties in fire-prone areas such as chapparal, fynbos, kwongan or matorral, in Australia, both Americas and Africa is obvious (Roche *et al*. 1997; Keeley and Fotheringham 1998, Brown *et al*. 2003; Crosti *et al*. 2006). Since the 1990s it has been known that not only heat, but also smoke itself can affect seed germination (De Lange and Boucher 1990), having both positive and negative impact (Drewes *et al*. 1995; Light *et al*. 2002; Daws *et al*. 2007). Physiologically active substances pres-

ent in smoke are mainly nitric oxides and butenolide derivatives. The latter have wider physiological and ecological impact than nitric oxides (Flematti *et al.* 2004; 2005; Van Staden *et al.* 2005). They have been termed karrikinolides or karrikins (KAR). The compound 3-methyl-2H-furo[2,3-c]pyran-2-one, obtained from plant-derived smoke, has been referred to KAR_1 (Chiwocha *et al.* 2009). Karrikins are water soluble, thermostable, long lasting in solution and highly active at very low concentration of 1^{-9} M in darkness (Flematti *et al.* 2004; Light et al 2005). Physiologically active butenolide derivatives can be even synthesized in laboratory conditions (Flematti *et al*. 2005; Light *et al*. 2005; Nagase *et al* 2008; Sun *et al.* 2008).

Physiological impact of smoke compounds on seeds of approx. 1200 species proves their physiological and ecological role in various ecosystems. However, one must remember that the number of 1200 is only 0.38% all known plant species according to the *International Union for Conservation of Nature and Natural Resources* (total 315 000, www.iucnredlist.org/documents/summarystatistics/2010_1RL_Stats_Table_1.pdf]. Moreover, most of species can form various ecotypes, and cultivars of crops are bred in the majority of the countries. Hence, the studies on the impact of smoke and its compounds should not be restricted to fire-prone environments. It was borne in mind that swailing (burning of meadows), often practised by farmers in the areas of moderate climate, may also have an impact on the local vegetation. Hence, the purpose of this work was to examine the influence of smoke on seed germination and radicle growth of selected popularly grown crops and some weed species occurring in Central Europe.

Materials and methods

Seed material. Seeds of seven species were used. Four crops: lettuce (*Lactuca sativa* L.*,* two cultivars having seeds insensitive to light: 'Królowa Majowych' and 'Rozalka'), rapeseed (*Brassica napus* L., cv. 'Mlochowski'), red cabbage (*B. oleracea* L. *var. capitata* f. rubra, cv. 'Langedijker Polana') and white cabbage (*B. oleracea* L. *var. capitata* f. alba cv. 'Ditmarska') were examined, as well as three weed species: coltsfoot (*Tussilago farfara* L.), meadow cress (*Cardamine pratensis* L.) and sorrel (*Rumex acetosa* L.). Lettuce and coltsfoot belong to the *Asteraceae*, whereas cabbages and meadow cress to *Brassicaceae*, and sorrel belongs to *Polygonaceae* family.

The source of smoke and smoke treatment. Two sets of seeds of each species (and cultivar in case of lettuce) were placed in sealed boxes, in Petri dishes of a diameter 4 cm, on a moistened blotting paper. Semi-dried plant debris (leaves and stems of various grass species, 0.5 g FW), collected from a local meadow, was burned in a glass dish, and smoke was directed to one of the boxes by a fan from a distance approx. 1 m, for 3 min. Burned and cooled ash was left in the box, to-

gether with the seeds, in an open dish, for 24 h. The treatment simulated the impact of swailing, when not only smoke affects the environment, but also the volatiles emitted from the burned debris. Control seeds were kept with similar dish filled with unburned debris. Germination was performed in darkness for 5 days, the temperature was 20/17ºC (day/night), relative humidity ca. 90%. Germination counts were performed daily. Germination was considered when the radicle protruded 1 mm.

Statistical analysis

Each treatment consisted of four replicates of 10 seeds. The percentage of germinated seeds of individual species and cultivar was arcsine transformed and the data were compared among the species (also cultivar) and the treatment using one-way analysis of variance (ANOVA at $p<0.05$). In case of the length of the radicles, Student t-test was used to compare two means among the species, at $p=0.05$.

Results and discussion

The volatile compounds contained in smoke and/or burned plant debris had stimulative effect on seed germination of all the studied species but meadow cress (Table 1, Fig.1). Germination stimulation was noticed just on the 1st day in case of lettuce, rapeseed, sorrel and coltsfoot (Table 1). Lettuce seeds are often used as a germination model, also in smoke experiments (Jager *at al*. 1996), but the response of seeds may greatly vary upon the cultivar due to its differentiated light-sensitivity. In this work, performed on two cultivars having seeds insensitive to light (unpublished results), genotypic differentiation was shown as different germination pattern of seed of both used cultivars (‘Rozalka’ seeds germinated faster, Table 1), but their positive response to smoke was distinct (Table 1). Moreover, growth of ‘Rozalka’ radicles was improved, as on the 4th day they were approx. 4 mm (20%) longer than in the control ones (Table 2). Sparg *et al*. (2005) discussed the role of smoke at the post-germination level, based on their results as well as these obtained by Brown *et al*. (2003), and the improvement of seedling vigour was raised.

Germination of seeds of rapeseed (Table 1) and both cabbage species was stimulated by the treatment (Table 1, Fig. 1). The impact of smoke was stronger in the case of red than white cabbage seeds, as the differences in percentages were 60 and 25%, respectively. Additionally, the effect of smoke on growth of red cabbage radicles was distinct (Table 2), whereas there was no such response in case of white cabbage (data not shown). Interestingly, smoke had no effect on germination of seeds of meadow cress, which belongs to the same family as cabbage and rapeseed (*Brassicaceae*), but triggered strong positive growth response in meadow cress

radicles, because their length was 160% of control upon the smoke treatment (Table 2).

Table 1. The effect of smoke on seed germination during first 3 days. Mean percentage of seeds germinated in four Petri dishes (number of seeds in each dish = 10) ± standard deviation (SD) were given

Classification	Species, cultivar	Treatment: control (C), smoke (S)	Day of germination, % of germinated seeds		
			1.	**2.**	**3.**
Crops	Lettuce, 'Królowa Majowych'	C	5 ±7.07 a	90 ±0.00 b	100 ±0.00 b
		S	15 ±7.07 a	95 ±7.07 b	100 ±0.00 b
	Lettuce 'Rozalka'	C	50 ±7.07 a	80 ±7.07 b	90 ±7.07 b
		S	70 ±14.2 a	95 ±7.07 b	95 ±7.07 b
	Red cabbage	C	0.0 ±0.00 a	25 ±14.1 b	50 ±0.00 c
		S	0.0 ±0.00 a	85 ±7.07 b	85 ±7.07 b
	White cabbage	C	0.0 ±0.00 a	65±7.07 b	80 ±0.00 c
		S	0.0 ±0.00 a	90 ±14.1 b	95 ±7.07 b
	Rapeseed	C	25 ±35.4 a	95 ±0.00 b	100 ±0.00 b
		S	50 ±28.3 a	95 ±14.1 b	100 ±7.07 b
Weeds	Coltsfoot	C	48 ±3.18 a	48 ±3.18 a	48 ±3.18 a
		S	55 ±7.07 a	55 ±7.07 a	55 ±7.07 a
	Meadowcress	C	100 ±0.00 a	100 ±0.00 a	100 ±0.00 a
		S	100 ±0.00 a	100 ±0.00 a	100 ±0.00 a
	Sorrel	C	0 ±0.00 a	85 ±7.07 b	90 ±0.00 b
		S	10 ±0.00 a	90 ±14.1 b	95 ±7.07 b

Mean values with different letters (a–c) are significantly different (P=0.05)

Physiological basis for the mode of action of smoke compounds has not been unraveled yet. Egerton-Warburton (1998) indicated that smoke acts as a scarifying agent to the seed surface, but in the paper of Briggs and Morris (2008) it was shown that the mechanism is not universal. On the other hand, most researchers agree that smoke and its main physiologically active component, butenolide, interacts with gibberellin, ABA and auxin pathways in seeds (Light *et al*. 2005; Chiwocha *et al*. 2009). It can probably result in up-regulation of expansins, the proteins disrupting the hydrogen bonds within the cell wall (Jain *et al.* 2008). Smoke compounds, possessing their oxidative role, may also interfere with the cell redox status (Light *et al.* 2009). All these circumstances lead to the supposition that the key smoke compounds, butenolides, act as a new class of plant growth regulators (Light *et al.* 2009).

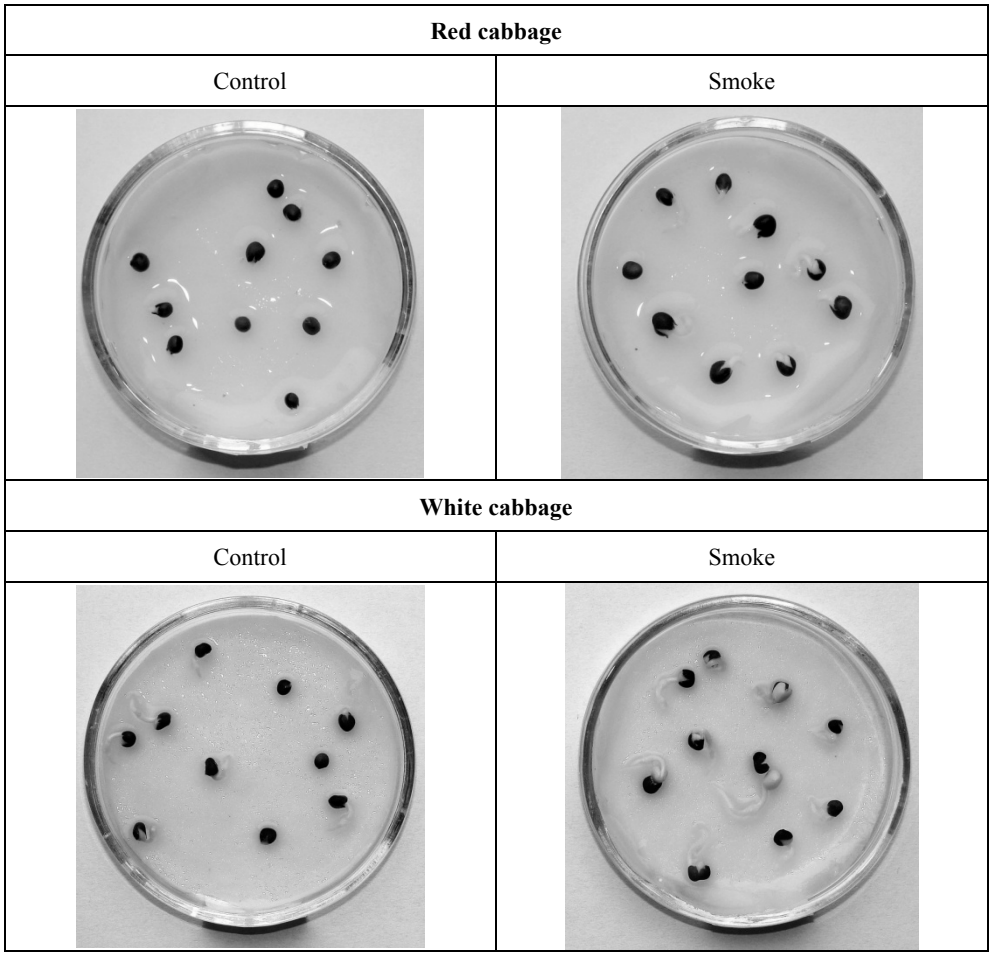


Fig. 1. Germinated cabbage seeds, day 2. Samples representative for 4 replicates (Petri dishes)

The results of the studies on the impact of smoke on germination can be applicable in some cases, as the smoke extract can be used in case of late sowing to accelerate germination (Light and Van Staden 2004), to overcome the light requirement for germination (Merrit *et al*. 2006) and to improve the vigour of some indigenous plants (Sparg *et al*. 2005; Emery and Lacey 2010). Some papers also emphasized the role of smoke compounds as conditioning agents in unfavourable conditions (Jain *et al*. 2008). In case of seeds of persistent weed or invasive species, such as *Avena fatua* L. (Kępczyński *et al*. 2010, 2012), the experiments can

explain the mechanism of germination stimulation and may improve new methods for prevention. Other valuable application is the re-vegetation of degraded areas (Light and Van Staden 2004).

Table 2. The effect of smoke on length of the radicles on the 4th day of germination (only the significant differences were shown). Means of 4 Petri dishes ± SD were given. The significance of differences between means was evaluated by Student's *t*-test: * - differentiation at P=0.05, ** - P=0.01, *** - P=0.001.

Species, cultivar	Treatment: control (C), smoke (S)	The length of the radicles [mm] on the 4th day of germination
Lettuce 'Rozalka'	C	22.7 ± 0.90 **
	S	27.2 ± 2.01 (120%)
Red cabbage	C	11.5 ± 6.60*
	S	18.5 ± 6.60 (161%)
Meadowcress	C	24.4 ± 5.70***
	S	38.9 ± 6.20 (159%)

References

Briggs CL, Morris EC. (2008) Seed-coat dormancy in *Grevillea linearifolia*: little change in permeability to an apoplastic tracer after treatment with smoke and heat. Ann Bot 101:623–632.

Brown NAC, Van Staden J, Daws MJ, Johnson T (2003) Patterns in the seed germination response to smoke in plants from the Cape Floristic Region, South Africa. S Afric J Bot 69:514-525

Chiwocha SDS, Dixon KW, Flematti GR, Ghisalberti EL, Merritt DJ, Nelson DC, Riseborough J-AM, Smith SM (2009) Karrikins: A new family of plant growth regulators in smoke. Plant Sci 177:252–256

Crosti R, Ladd PG, Dixon KW, Piotto B (2006) Post-fire germination: the effect of smoke on seeds of selected species from the central Mediterranean basin. Forest Ecol Management 221:306-312

Daws MI, Davies J, Pritchard HW, Brown NAC, Van Staden J (2007) Butenolide from plant-derived smoke enhances germination and seedling growth of arable weed species. Plant Growth Regul 51:73-82

De Lange JH, Boucher C (1990) Autecological studies on *Audouinia capitata* (Bruniaceae). 1. Plant-derived smoke as a seed germination cue, S Afr J Bot 56:700-703

Drewes FE, Smith MT, Van Staden J (1995) The effect of a plant-derived smoke extract on the germination of light-sensitive lettuce seed. Plant Growth Regul 16:205–209

Egerton-Warburton IM (1998) A smoke-induced alteration of the sub-testa cuticle in seeds of the post-fire recruiter, *Emmenanthe penduliflora* Benth. (Hydrophyllaceae). J Exp Bot 49:1317-1327

Emery NJ, Lacey E. (2010) Optimizing the application of smoke water to maximize germination of the flannel flower, *Actinotus helianthi*. Seed Sci Technol 38:797-801

Flematti GR, Ghisalberti EL, Dixon KW, Trengove RD (2004) A compound from smoke that promotes seed germination. Science 305: 977

Flematti GR, Ghisalberti EL, Dixon KW, Trengove RD (2005) Synthesis of the seed germination stimulant 3-methyl-2H-furo[2,3-c]pyran-2-one. Tetrahedron Lett 46:5719-5721

International Union for Conservation of Nature and Natural Resources:www.Iucnredlist.org/ documents /summary statistics/2010_1RL_Stats_Table_1.pdf. Accessed 7 of January 2013

Jäger AK, Light ME, Van Staden J (1996) Effects of source of plant material and temperature on the production of smoke extracts that promote germination of light-sensitive lettuce seeds. Env Exp Bot 36:421-429

Jain N, Soos V, Balazs E, Van Staden J (2008) Changes intercellular macromolecules (DNA, RNA and protein) during seed germination in tomato, following the use of a butenolide, isolated from plant-derived smoke. Plant Growth Regul 54:105-113

Jain N., Ascough GD, Van Staden J. (2008) A smoke-derived butenolide alleviates $HgCl_2$ and $ZnCl_2$ inhibition of water uptake during germination and subsequent growth of tomato – possible involvement of aquaporins. J Plant Physiol. 165:1422-1427

Keeley JE, Fotheringham CJ (1998) Smoke-induced seed germination in California chapparral, Ecology 79:2345-2371

Kępczyński J, Cembrowska D, Van Staden J (2010) Releasing primary dormancy in *Avena fatua L.* caryopses by smoke-derived butenolide. Plant Growth Regul 62:85–91

Kępczyński J, Cembrowska-Lech D, Van Staden J (2012) Necessity of gibberellin for stimulatory effect of KAR1 on germination of dormant *Avena fatua* L. caryopses. Acta Physiol Plant DOI 10.1007/s11738-012-1080-1

Light ME, Burger BV, Van Staden J (2005) Formation of a seed germination promoter from carbohydrates and amino acids. J Agric Food Chem 53:5936-5942

Light ME, Daws MI, Van Staden J (2009) Smoke-derived butenolide: Towards understanding its biological effects. S Afr J Bot 75:1-7

Light ME, Gardner MJ, Jäger AK, Van Staden J (2002) Dual regulation of seed germination by smoke solutions. Plant Growth Regul 37:135-141

Light ME, Van Staden J (2004) The potential for smoke in seed technology. S Afr J Bot. 70:97-101

Nagase R, Katayama M, Mura HH, Matsuo N, Tanabe Y (2008) Synthesis of the seed germination stimulant 3-methyl-2H-furo[2,3-c]pyran-2-one utilizing direct and regioselective Ti-crossed aldol addition. Tetrahedron Lett 49:4509-4512

Roche S, Dixon KW, Pate JS (1997) Seed ageing and smoke: partner cues in the amelioration of seed dormancy in selected Australian native species. Aust J Bot 45:783-815

Sun K, Chen Y, Wagerle T, David L, Currie M, Chmura P, Song Y, Xu M (2008) Synthesis of butenolides as seed germination stimulants. Tetrahedron Lett 49:2922-2925

Van Staden J, Jäger AK, Light ME, Burger BV (2004) Isolation of the major germination cue from plant-derived smoke. S Afr J Bot 70:654–659

PLANT FUNCTIONING UNDER ENVIRONMENTAL STRESS P. 241-253

Cracow, 12th to the 15th September, 2012

EFFECT OF SALT STRESS ON ANTIOXIDANT ACTIVITY IN LEAVES OF BARLEY, MAIZE, SORGHUM AND SPINACH

Maria Tatrzańska*, Maciej Maciejewski*, Agnieszka Płażek**, Franciszek Dubert*

*The Franciszek Górski Institute of Plant Physiology of Polish Academy of Sciences, Niezapominajek 21, 30-239 Kraków

**Department of Plant Physiology, University of Agriculture, Podłużna 3, 30-239 Kraków

Corresponding author; e-mail: marysia133@gmail.com

Abstract The aim of the experiments was to compare the salt tolerance of such plant species as barley, maize, sorghum and spinach. Plants were grown in perlite and were treated with the following NaCl concentrations: 0, 20 and 70 mM. Fresh weight (FW), dry weight (DW) and relative water content (RWC) of aboveground parts of plants were estimated. In addition, total protein content, the activities of superoxide dismutase (SOD), catalase (CAT) and non-specific peroxidase (POX) were measured in the leaves. The increasing NaCl concentration reduced or completely inhibited the plant growth of all species under study. Barley and spinach showed the least decrease of FW and DW under salt stress compared to the other studied plant species. The decrease of FW at 70 mM of NaCl was more visible than a decline of DW and amounted to 28.5%, 64.7%, 62.5% and 50% for barley, maize, sorghum and spinach respectively. The decrease of DW amounted to 54.5%, 33.3%, 19.2% and 50% for barley, maize, sorghum and spinach respectively. Barley and spinach plants grown at 70 mM of NaCl demonstrated an increase in RWC compared to the control, while values of this parameter in maize and sorghum plants grown at 70 mM were reduced significantly. Barley, sorghum and spinach plants grown at 20-70 mM of NaCl did not demonstrate any changes in SOD and CAT activities, that increased only in the leaves of maize plants grown at 70 mM of NaCl. The increasing salinity affected POX activity in all studied plant species, but these changes were specific for each species. Taking into consideration DW reduction, sorghum plants could be recognized as more salt tolerant than other species under study, however barley and spinach induce defence mechanism against salinity which is the uptake of greater amount of water to dilute ion concentration in cells.

Key words: Catalase; Fresh and dry weight; Relative Water Content; Salt stress; Peroxidase; Superoxide dismutase

Introduction

According to recent reports, more than 6% of the total land area is saline. The problem affects all continents. Particularly affected areas are semi-arid deserts (FAO 2008). The emergence of this problem is closely related to the increased demand for food. The growing number of people means that more and more scientists consider the possibility of using saline lands for agriculture.

The salinity of the soil solution occurs when the content of soluble salt is higher than 0.2% (Siyal et al. 2002). Excessive salt concentration in the soil is mainly found in arid and semi-arid areas (Kumar and Reddy et al. 2003) and irrigated with sea water (Zhu 2001). in most cases soil salinity is an effect of salt accumulation over long cultivation periods and deforestation (Brini et al. 2009). Salinity is an additional consequence of excessive and unbalanced fertilization in intensively cultivated areas and urban areas as a result of the use of salt for de-icing streets in winter (Zimny 2004).

The effect of high concentrations of salts in environment, are osmotic and ion toxicity stresses. A high concentration of sodium ions disturbs the osmotic balance and results in inhibition of plant water uptake. The toxic influence of Na^+ may be manifested by the premature death of leaves, degradation of the cell membranes, inhibition of many enzymes, as well as damages of photosynthetic apparatus (Mitsuya et al. 2003). Plants have different sensitive degree to salinity depending on species and plant development. In addition, the sensitivity of the plant can be confirmed by other adverse environmental conditions (Munns and Tester 2008). Most of the crops respond to salinity as typical glycophytes and demonstrate differentiated tolerance levels to this stress. According to Munns and Tester (2008) barley can be recognized as the most salt tolerant cereal and rice as the most sensitive one, however some authors have stated that, among many genotypes of barley, some of them demonstrated high salt sensitivity (Kalaji et al. 2011).

Salinity causes also oxidative stress, which damages the cell structure by oxidation of lipids, proteins and nucleic acids and interferes with metabolic processes, forming reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), superoxide ($O_2^{\bullet -}$), and hydroxyl ($OH^{\bullet}$) radicals (McKersie and Leshem, 1994; Pastori and Foyer, 2002). Damage of membrane structure as a result of salinity can be demonstrated by the toxic influence of Na^+ ions, which cause strong membrane depolarization and the consequent effect of lipid peroxidation (Yasar et al. 2006). In order to minimize the effects of oxidative stress, plants form the ROS defence system consisting of oxidizing molecules including glutathione, ascorbate, carotenoids and tocopherols. In addition, antioxidant system involves scavenger enzymes such as: superoxide dismutase (SOD), calatase (CAT), ascorbate peroxidase (APX), guaiacol peroxidase (GPX), and glutathione reductase (GR) (Alscher et al. 1997 Apel and Hirt 2004). Enzymes capture ROS under different cellular com-

partments in the form of isoenzymes (Asada 1992; Alscher et al. 2002; Shigeoka et al. 2002; Apel and Hirt 2004). Superoxide dismutase converts $O_2^{\cdot -}$ to H_2O_2, while catalase and ascorbate peroxidase convert H_2O_2 to H_2O and O_2 (Mehdy 1994). Thus, ROS concentration depends on the balance between production and scavenging, and the correct functioning of antioxidant system is clearly important requirement for plant survival (Scebba et al. 1999). Hydrogen peroxide is recognised as the signal factors initiating several processes initiated under various environmental stresses (Mehdy 1994; Bestwick et al. 1998). Taking into account so important role of H_2O_2 in plant defence response against various stresses, an inhibition of the hydrogen peroxide scavengers could characterize genotypes more tolerant to the stress. Arabidopsis mutants lacking cytosolic or chloroplastic ascorbate peroxidase were found to be more salt tolerant (Munns and Tester 2008).

Salinity induces oxidative stress due to increased stomatal closure and a decrease of NADPH consumption by the Calvin Cycle. When ferredoxine is over-reduced during photosynthetic electron transfer, electrons may be transferred from PSI to oxygen to form superoxide radicals by the process called Mehler Reaction. This reaction initiates production of more harmful hydroxyl radical (Türkan and Demiral, 2009).

The aim of this study was to compare the salt tolerance of such plant species as barley, maize, sorghum and spinach grown at 20 and 70 mM of NaCl. In the study fresh and dry weight as well as relative water content of aboveground parts of plants were estimated. Moreover, we investigated the antioxidative process occurring in the leaves of studied plant species under salt stress. The activities of superoxide dismutase, catalase and non-specific peroxidase were measured.

Material and methods

Plants material and growth conditions. In the experiment the seeds of the maize (*Zea mays* cv. Król), sorghum (*Sorghum bicolor* cv. Bicolor), spinach (*Spinacia oleracea* cv. Olbrzym zimowy) and barley (*Horedum vulgare* cv. Poldek) were used.

The experiment was conducted outdoors under an open foil tunnel in four plastic pools (4.5 m x 2.4 m x 0.8 m) filled with perlite. The pools were equipped with drains to remove an excess of salt solution. Before planting, the pools were irrigated up to full capacity with the NaCl solutions of 20 and 70 mM. The third control pool was irrigated with water without salt. All NaCl solutions, including pour water, were supplemented with Hoagland medium (1938). Particular solutions were prepared in plastic barrels (220 dm^3) equipped with pumps and water meters. Seeds were sown directly to saline perlite, finally obtaining 100 plants of each species for each salt concentration. Plants in the pools were watered each day with the same amount of solution depending on the requirements. Plants were grown for 7 weeks

(June – August). Plant samples for all analyses were collected at the end of the experiment.

Fresh and dry weight and relative water content. Fresh weight (FW) was estimated for aboveground plant parts, next their dry weight (DW) was evaluated after 48 h-drying at 70 °C. Relative water content (RWC) in the leaves was calculated according to the formula: (FW – DW)/DW. The RWC parameter means number of grams of water needed to hydrate 1 g of plant dry weigh. Analyses were done in 15 replicates.

Enzyme assays. For enzymatic assays, leaf samples (the 3rd leaf counting from the top of the plant) were collected and were frozen in liquid N_2 and stored in –80°C until homogenization.

Assay of superoxide dismutase (SOD) activity. SOD activity (EC: 1.15.1.1) was measured according to Droillard et al. (1987). The frozen tissue (about 0.5 g) was homogenized using Tissue Lyser with 1.3 cm^3 of extraction buffer (50 mM potassium phosphate, pH 7.8 and 1% PVPP) and centrifuged (16 000 x g) at 4°C. a sample of tissue extract (20 µl was added to 1 cm^3 of assay buffer containing nitrotobluetetrazolium (NBT) (56 mM), xanthine (0.1 mM) and potassium phosphate buffer (50 mM, pH 7.8, 1mM EDTA). The reaction was started by adding 10 µl of xanthine oxidase (0.003 U). The absorbance was measured using spectrophotometer LKB Ultrospec 2100 pro (Biosciences Amersham, Sweden) at 560 nm was recorded for 60 s. The SOD activity was calculated as the percentage of inhibition of NBT reduction. One unit of SOD was the amount of extract causing 50% inhibition of reduction of NBT to NBT-diformazan. The determination of SOD activity was done in 5 replicates.

Determination of catalases (CAT) activity. Catalase activity was determined according to the method described by Aebi (1984). Leaf tissue was homogenized at 4°C in phosphate buffer at a concentration of 50 mM (pH 7.5) supplemented with 1 mM EDTA, and then centrifuged for 10 min at 13000 rpm. Enzyme activity was measured spectrophotometrically (LKB Ultrospec 2100 pro Biosciences Amersham, Sweden) at λ = 240 nm, assuming the activity per unit amount of enzyme which decomposes 1µmol H_2O_2 and which corresponds to the absorbance decline of 0.0145 per minute. Assays were done in 5 replicates (in five leaves from different plants of each object)

Determination of non-specific peroxidase (POX) activity. Peroxidase activity was measured spectrophotometrically according to the method described by Bergmeyer (1965). Leaf samples were ground to a fine powder with liquid nitrogen and extracted with 50 mM phosphate buffer (pH 7.0) and 1 mM EDTA (SIGMA). The extracts were centrifuged (16 000 x g) at 4 ºC for 10 min and the resulting supernatants were used as the crude extracts. Two cm^3 of 50 mM phosphate buffer (pH 7.0) was mixed with 12 µl of 0.5% p-phenylenediamine and with 12 µl of crude extract. The oxidation of p-phenylenediamine was initiated by addition of 12 µl of

buffered H_2O_2 (0.15 cm^3 of H_2O_2 mixed with 50 cm^3 of extract buffer) to prepared mixture. The absorbance was measured at λ=460 nm (using the same spectrophotometer as above). The peroxidase activity was expressed as difference of absorbances of sample recorded at the beginning of measurement and after 1 min per g protein. Assays were done in 5 replicates (in five leaves from different plants of each object).

Protein determination. Protein concentration in the leaves was determined according to Bradford (1976) using the Bio-Rad (Munich, Germany) protein assayed with bovine serum albumin as a calibration standard.

Statistical analysis

All data were analysed with Statistica 9.0 software (Statsoft, Tulsa, OK, U.S.A.) using a one-way ANOVA. In the figures the means and standard error (SE) are presented.

Results

Fresh and dry weight and relative water content. The increase of NaCl concentration reduced or completely inhibit the growth of all plant species under study (Fig. 1 and 2). Plants of spring barley grown at 20 and 70 mM NaCl demonstrated a slight, but significant decrease in FW and DW in relation to the control plants, but no significant differences were observed between FW of plants treated with salt stress.

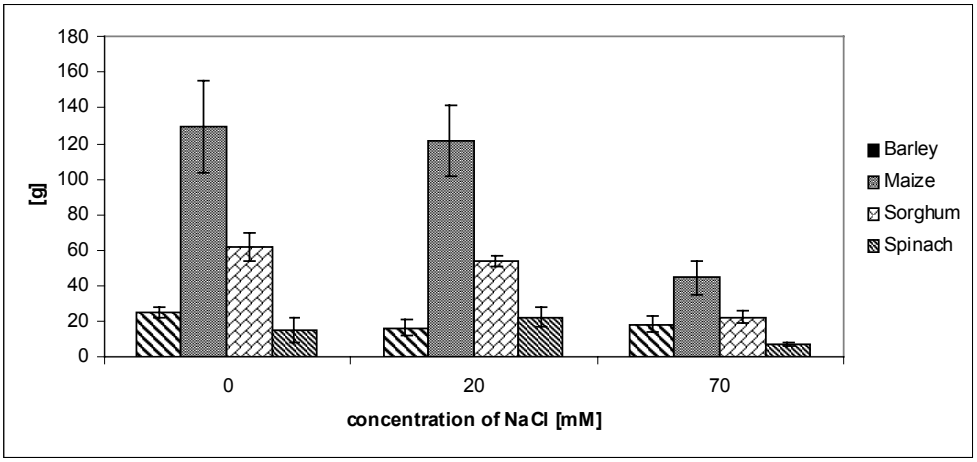


Fig. 1. Fresh weight [g] of barley, maize, sorghum and spinach plants grown at 20 and 70 mM of NaCl. Results shown as means ± SE (n=15)

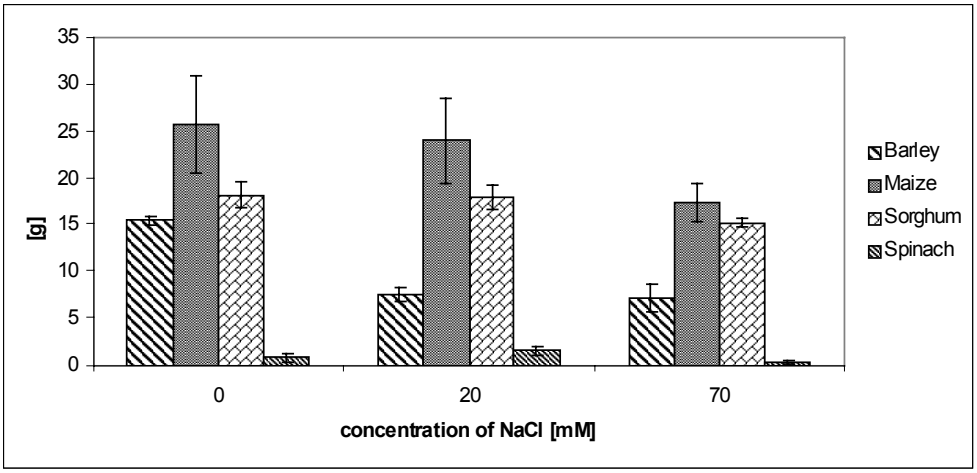


Fig. 2. Dry weight [g] of barley, maize, sorghum and spinach plants grown at 20 and 70 mM of NaCl. Results shown as means ± SE (n=15)

NaCl concentration of 70 mM decreased considerably FW and DW of maize, sorghum and spinach plants. The decrease of FW at 70 mM of NaCl was more visible than a decline of DW and amounted to 28.5%, 64.7%, 62.5% and 50% for barley, maize, sorghum and spinach respectively, while the decrease of DW amounted to 54.5%, 33.3%, 19.2% and 50% for barley, maize, sorghum and spinach respectively. Barley plants grown at 20 mM of NaCl demonstrated an increase in RWC, whereas in higher salt concentration of 70 mM it did not change (Fig. 3). Values of this parameter in maize plants grown at 20 mM did not differ from those of the control, but at 70 mM RWC was reduced significantly. The increasing NaCl level decreased gradually RWC in plants of sorghum. Most diverse species was the spinach, in which plants a significant decrease in the RWC at 20 mM NaCl relative to the control was observed, but at 70 mM NaCl the degree tissue hydration increased significantly. It is worth to add, that control spinach plants demonstrated 35-fold higher tissue hydration compared to barley, 4-fold higher than sorghum and 8 times higher than maize.

Protein content. Analysis of total protein content showed that the protein content in the leaves of barley, maize, sorghum and spinach did not change under the increasing salt concnetration (Fig. 4).

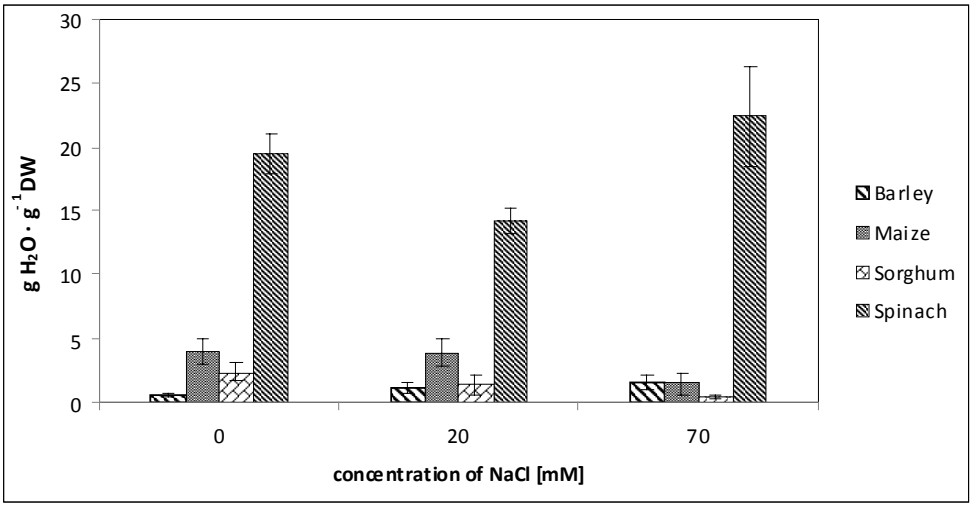


Fig. 3. Leaves relative water content [g of H_2O g^{-1} of DW] of barley, maize, sorghum and spinach plants grown at 20 and 70 mM of NaCl. Results shown as means ± SE (n=15)

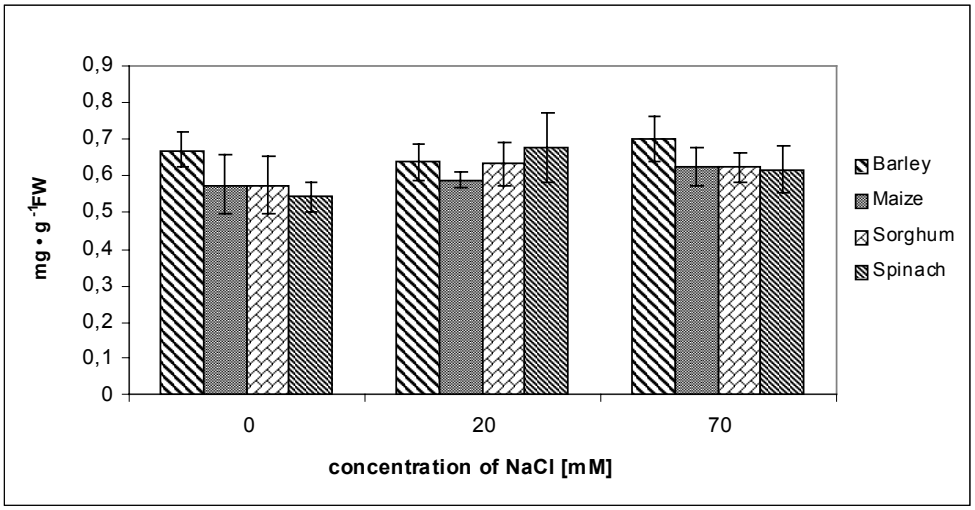


Fig. 4. Protein content in leaves of plants barley, maize, sorghum and spinach grown at 20 and 70 mM of NaCl. Results shown as means ± SE (n=5)

SOD activity. The increasing salinity did not influence SOD activity in the leaves of barley, sorghum and spinach plants (Fig. 5). In the case of maize, 20 mM of NaCl decreased SOD activity in relation to the control, but this change was not statistically proved. Salt concentration of 70 mM increased SOD activity in leaves of this plant species and it differed from that noted in the plants grown at 20 mM, but it was on the same level as the control plants.

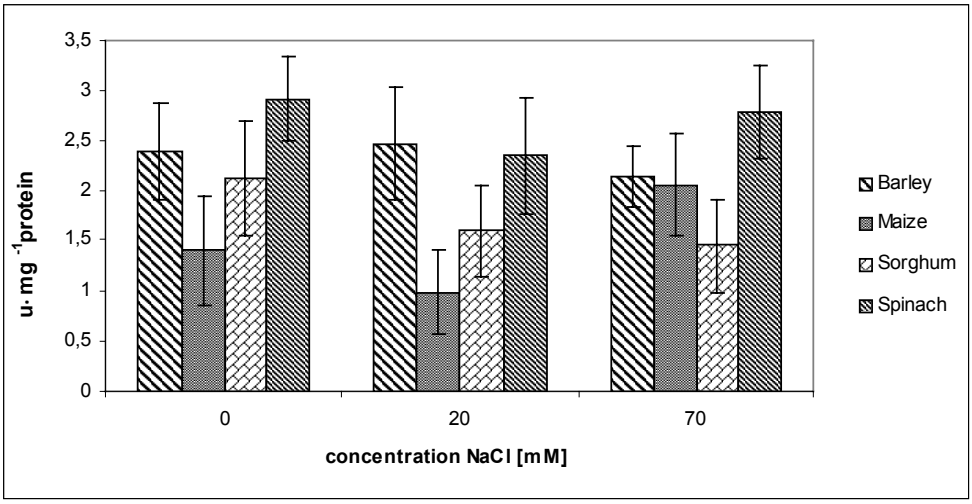


Fig. 5. Superoxide dismutase (SOD) activity in leaves of barley, maize, sorghum and spinach plants grown at 20 and 70 mM of NaCl. Results shown as means ± SE (n=5)

CAT activity. The increasing salt level caused similar changes in CAT activity as in the SOD one. In the case of barley, sorghum and spinach the activity of this enzyme noted in the leaves of control plants did not differ from the activity found in plants grown at 20 and 70 mM (Fig. 6). The significant changes of CAT activity were observed only in the leaves of maize. Plants grown at 20 mM demonstrated a decline of CAT activity, however this reduction was not statistically significant as compared to the control. Higher salt level – 70 mM increased significantly value of this parameter in relation to that noted in the plants treated with salt concentration of 20 mM, but it was not significantly higher than in the control.

POX activity. Plants of barley grown at 20 and 70 mM of NaCl showed significant decrease in non-specific peroxidase activity in relation to the control plants (Fig. 7). In maize plants at 20 mM a significant increase in the activity of this enzyme as compared to the control was found, while the higher salt content - 70 mM decreased it to the control level. In the case of other studied plant species the rising salt amount in the perlite did not influence activity of POX in the leaves.

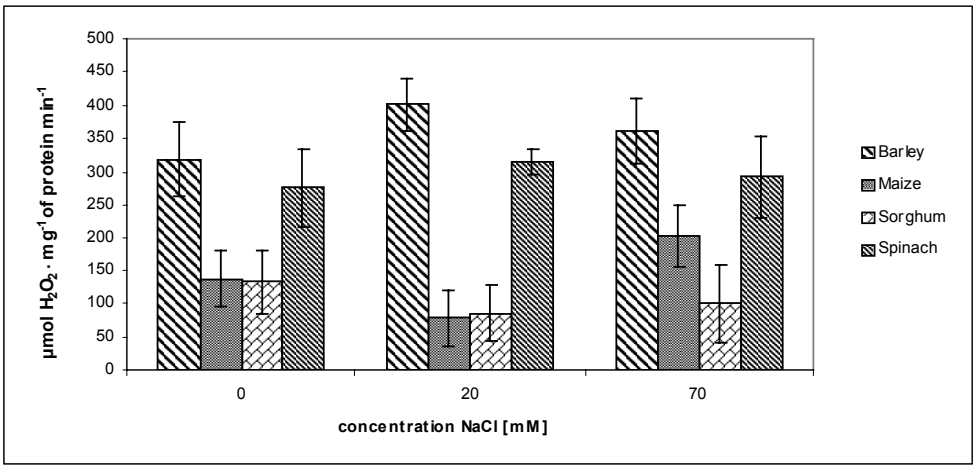


Fig. 6. Catalase (CAT) activity [µmol H_2O_2 g^{-1} of protein min^{-1}] in leaves of barley, maize, sorghum and spinach plants grown at 20 and70 mM of NaCl.
Results shown as means ± SE (n=5)

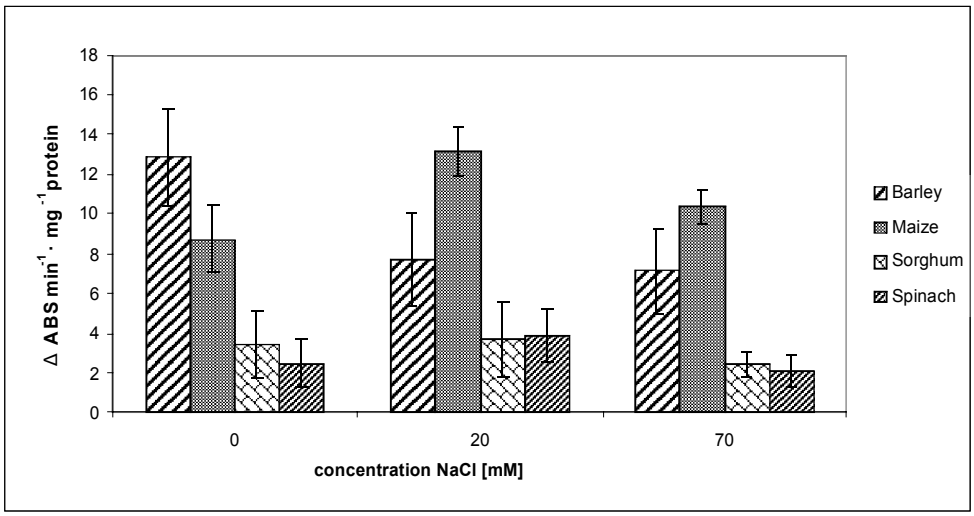


Fig. 7. Non-specific peroxidase (POX) activity [ΔABS g^{-1} of protein min^{-1}] in the leaves of barley, maize, sorghum and spinach plants grown at 20 and 70 mM of NaCl.
Results shown as means ± SE (n=5)

Discussion

Most of the crops respond to salinity as typical glycophytes and demonstrate differentiated tolerance levels to this stress. Munns and Tester (2008) stated that the variation in salinity tolerance in dicotyledonous species is greater than in monocotyledonous. These authors include barley to the most salt tolerant cereals, whereas rice to the most sensitive ones. According to these authors some legumes can be more sensitive to salt stress than rice. Our previous study confirm this statement. We found that yellow and narrow-leafed lupine as well as pea were very sensitive, even to 40 mM of NaCl (data unpublished).

The most considerable criterion of salt tolerance is a production of biomass, which is significantly declined due a decrease in net photosynthesis rate inhibited via stomata closure (Munns and Tester 2008; Pérez-López *et al*. 2009). In cereals, the major effect of salinity is a reduction of the number of tillers (Munns and James, 2003). The toxic effect of Na^+ ions is demonstrated by the premature senescence of older leaves and a decrease of chlorophyll amount (Khatkar and Kuhad, 2000). Under salinity, plant growth is reduced due to ion toxicity and ion imbalance as well as due to poor water relations. The plant response to a salinity is reduction of cell water potential by decreasing the osmotic potential and by reducing of turgor pressure. Reducing water use by a decline of transpiration rate is a positive mechanism reducing salt loading in the plant (Romero-Aranda *et al.* 2001).

In our investigation barley and sorghum demonstrated the lowest reduction of fresh weight among all studied plant species. Barley dry weight production was inhibited in higher degree than in the case of maize and sorghum and it was observed already at 20 mM, while in other plant species under study the significant reduction of DW was found at 70 mM of NaCl. Munns and Tester (2008) stated that barley is the most salt tolerant cereal, however Kalaji *et al.* (2011) demonstrated that among barley species occur both salt sensitive and tolerant cultivars. Results of many studies indicate that tolerance to salinity is associated with plant genotype rather and not with plant species.

Almodaves and Sharif (2005) showed that sorghum has a moderate tolerance to saline soil, whereas according to Krishnamurthy *et al.* (2007) it should be relatively more resistant to salinity than maize. Our results improved this statement. The DW reduction in the case of sorghum plants grown at 70 mM of NaCl was less than that observed in maize ones. Spinach plants showed FW production at 20 mM NaCl on the same level as the control plants, while a FW decline was observed at 70 mM NaCl. It could be due to the fact that low salinity may stimulate the growth and development of some plant species (Chartzoulakis *et al*. 2002; Slama *et al*. 2008). According to Basin *et al*. (2010) spinach is a halophyte. The authors demonstrated that range 50-100 mM NaCl slightly increased weight, while the loss of weight was observed at 200 mM.

It is worth to note that DW reduction in barley was not caused by lower tissue hydration. This plant species and spinach demonstrated a different defence mechanisms to salinity compared to the maize and sorghum. Especially spinach showed in the control plants significantly higher relative water content than in other plants under study. Values of RWC in barley and spinach increased under salt stress. The higher water uptake to plants is a resistance mechanism to salinity and it prevents the high concentration of ions in cytoplasm (Sairam and Tyagi 2004).

Many studies conducted on various genotypes showed differences in the expression or activity of antioxidant (Sairam and Tyagi 2004; Tounekti *et al*. 2011). Tester and Munns (2008) suggest that differences in the antioxidant activity may be related to the degree of closure of the stomata and therefore changing the intensity of gas exchange. Plants that exhibit tolerance to stress tend to minimize damage to their metabolism, for example, by regulating enzymatic activity, including enzymes that inactivate ROS, which show increased activity following stress. The increase can be attributed to a favorable adjustment of enzyme activity or new synthesis of these proteins that may be an indicator of salt tolerance (Jin et al., 2009).

According to the Saiva and Tyagi (2004) an increase in the activity of such enzymes as SOD, CAT, POX, APX is observed in many plant species under salinity. In the presented study, only maize plants showed an increase of SOD and CAT activities in plants grown at 70 mM. Analyzing pattern of changes of these both enzymes it could be supposed that H_2O_2 amount generated by SOD was reduced by activated CAT. It is worth to note that in plants grown at 20 mM a slight decrease in SOD and CAT activities with simultaneous increase of POX activity was observed. According to Mittler (2002) APX and CAT belong to different classes of scavengers. APX is activated by H_2O_2 in the μM range, while CAT activity by mM one and the latter is responsible for scavenging of ROS excess in the cells under stress. In our experiment SOD and CAT activities were induced at 70 mM, whereas the increase in POX activity was evident as early as at 20 mM NaCl.

Azevedo-Neto *et al*. (2006) reported increased activity of SOD and POX in maize plants grown at 100 mM NaCl, whereas there was no significant effect of salt on the growth of CAT activity. The results published by El-Sayed et al. (1994) indicated an increase of SOD and POX activities, and no increase in CAT activity in sorghum grown in salinity from 50 to 150 mM of NaCl.

Conclusions:

– Barley and spinach show the least decrease of fresh and dry weight under salt stress (0-70 mM NaCl) compared to the other studied plant species. These both species demonstrate defence mechanism against salinity which consists in the greater uptake of water to dilute the salt ions.

– SOD and CAT activities increase in the leaves of maize plants grown at 70 mM of NaCl.
– The increasing salinity affects POX activity in all studied plant species, but these changes are specific for each species.

Acknowledgement

The study was supported by the Polish Ministry of Science and Higher Education No. 813/N-COST/2010/0 (within COST Action FA0901)

References

Almodares A., Sharif, ME (2005) Effect of water quality on yield of sugar beet and sweet sorghum. J Environ Biol 26: 487-493.

Alscher RG, Donahue JL, Cramer CL (1997) Reactive oxygen species and antioxidants: relationship in green cells. Physiol. Plant. 100:224–233.

Alscher RG, Erturk N, Heath LS (2002) Role of superoxide dismutases (SODs) in controlling oxidative stress in plants. J. Exp. Bot. 53:1331–1341.

Apel K, Hirt H (2004) Reactive oxygen species: metabolism, oxidative stress, and signal transduction. Annu. Rev. Plant Biol. 55:373–399.

Asada K (1992) Ascorbate peroxidase – a hydrogen peroxide-scavenging enzyme in plants. Physiol. Plant. 85:235–241

Azevedo-Neto AD, Prisco, JT, Enéas-Filho J, Medeiros, JR, Gomes-Filho, E (2006) Hydrogen peroxide pre-treatment induces salt-stress acclimation in maize plants. J Plant Physiol 162:1114–1122.

Bestwick CHS, Brown IR, Mansfield JW (1998). Localised changes in peroxidase activity accompany hydrogen peroxide generation during the development of a non-host hypersensitive reaction in lettuce. Plant Physiol. 118:1067-1078

Brini F, Amara I, Feki K, Hanin M, Khoudi H, Masmoudi K (2009) Physiologcal and molecular analyses of seedling of two Tunisian durum wheat (Triticum turgidum L. subsp. Durum[Desf.) varieties showing contrasting tolerance to salt stress. Acta Physiol Plant 31: 145-154

Chartzoulakis K, Loupassaki M, Bertaki M, Androulakis I (2002) Effects of NaCl salinity on growth ion content and CO_2 assimilation rate of six olive cultivars, Scientia Horticulturae, 96: 235-–247

El-Sayed, H, El-Haddad, M, Oleary,JW (1994).Effect of salinity and K/Na ratio of irrigation water on growth and solute content of Atriplex amnicola and Sorghum bicolor. Irrig. Sci., 14: 127–133

FAO (Food and agriculture organization) (2008) FAO Land and Plant Nutrition Management Service:

Jin X, Huang Y, Zeng F, Zhou M, Zhang G (2009) Genotypic difference in response of peroxidase and superoxide dismutase isozymes and activities to salt stress in barley. Acta Physiol. Plant. 31:1103–1109

Khatkar D, Kuhad MS (2000) Short-term salinity induced changes in two wheat cultivars at different growth stages Biol. Plant. 43: 629–632.

Kalaji HM, Bosa K, Kościelniak J, Hossain Z (2011) Chlorophyll *a* fluorescence–A useful tool for the early detection of temperature stress in spring barley (*Hordeum vulgare L.*) J of Integrative Biology.12: 925–934

Krishnamurthy A, Moore JK, Zender CS, Luo C (2007) Effectsof atmospheric inorganic nitrogen deposition on ocean biogeochemistry.J. Geophys. Res., 112:

McKersie BD, Leshem YY (1994) Stress and stress coping in cultivated plants. Kluwer Academic Publishes, London.

Mehdy MC (1994) Active oxygen species in plant defense against pathogens. Plant Physiol 105: 467-472

Mittler R (2002) Oxidative stress, antioxidants and stress tolerance. Trends Plant Sci. 7: 405–410
Mitsuya S, Kawasaki M, Taniguchi M,, Miyake H (2003b) Light dependency of salinity-induced chloroplast degradation. *Plant Production Science ,* 6*:* 219–223.
Munns R, Tester M (2008) Mechanisms of salinity tolerance, Annu Rev Plant Biol 59: 651–81
Munns R, James RA (2003) Screening methods for salinity tolerance :a case study with tetraploid wheat. Plant and Soil 253: 201–218
Pastori GM, Foyer CH (2002) Common components, networks, and pathways of cross-tolerance to stress. The central role of 'redox' and abscisic acid-mediated controls. Plant Physiol. 129:7460–7468
Pèrez-Lòpez U, Robredo A, Lacuesta M, Mena-Petite A, Muñoz- Rueda A(2009) The impact of salt stress on the water status of barley plants is partially mitigated by elevatd CO_2. ? Environmental and Experimental Botany 66: 463–470
Ramadan T, Flowers TJ (2004) Effects of salinity and benzyl adenine on development and function of microhairs of Zea mays L, Planta, 219:639– 648
Romero-Arnada R, Soria T, Cuartero J (2001) Tomato plant-water uptake and plant-water relationships under saline growth conditions. Plant Sci 160: 265–272
Sairam RK, Tyagi A (2004) Physiology and molecular biology of salinity stress tolerance in plants, Current Science, 86/ 3
Scebba F, Sebastiani L, Vitagliano C, 1999. Protective enzymes against activated oxygen species in wheat (Triticum aestivum L.) seedlings: Responses to cold acclimation. Journal of Plant Physiology 155: 762–768
Shigeoka S, Ishikawa T, Tamoi M, Miyagawa Y, Takeda T, Yabuta Y, Yoshimura Y (2002) Regulation and function of ascorbate peroxidase isoenzymes. J. Exp. Bot. 53:1305-1319.
Siyal AA, Siyal AG, Abro ZA (2002) Salt affected soils their identification and reclamation, Pakistan J. Appl. Sci. 2: 537–540
Slama I, Ghnaya T, Savourè A, Abdelly C (2008), Combned effect of long-term salinity and soil drying on growth, water relation, nutrient status and proline accumulation *of Sesuvium portulcastrum,* C.K.Biologies, 331: 4424–51
Taunekti T, Vadel AM, Oñate M, Khemira H, Munnè- Bosch (2011) Saly-induced oxidative stress in rosemary plants: Damage or protection? Environmental and Experimental Botany. 71: 298–305
Türkan I, Demiral T (2009) Recent development in understanding sainity tolerance, Environmental and Experimental Botany, 67: 2–9
Yasar FO, Uzal S, Tuufenkci, Yidiz K (2006) Ion accumulation in different organs of green bean genotypes grown under salt stress. Plant Soil Environ 52: 476 – 480
Zhu JK (2001) Plant salt tolerance. Trend Plant Sci. 6: 66–71
Zhu H, Ding GH, Fang K, Zhao FG, Qin P(2006) New perspective on the mechanism of alleviating salt stress by spermidine in barley seedlings, Plant Growth Regul. 49:147–156

PLANT FUNCTIONING UNDER ENVIRONMENTAL STRESS P. 255-265

Cracow, 12th to the 15th September, 2012

EFFECTS OF SELECTED BIOSTIMULATORS ON THE COURSE OF GENERATIVE PHASE AND YIELD STRUCTURE PARAMETERS OF PEA AND LUPINE PLANTS

Agnieszka Kalandyk*, Franciszek Dubert*, Maciej Maciejewski*, Maria Tatrzańska*, Tomasz Krępski**, Agnieszka Płażek**

** The Franciszek Górski Institute of Plant Physiology Polish Academy of Sciences in Krakow*

*** Department of Plant Physiology, Faculty of Agriculture and Economics, The Hugo Kołłątaj University of Agriculture in Krakow*

Corresponding author: akalandyk@interia.pl

Abstract Arable crops during the growing season are subjected to climatic stress belonging to abiotic stresses such as excessive temperature fluctuations, spring frosts or prolonged summer heat, fluctuations in soil water content - periodic droughts or periodic excess leading to flooding rain, wind, causing lodging plants, factors which are cause of yield loss. The aim of this research was to determine the effectiveness of the selected six preparations of stimulants, plant hormones and substances with anti-stress, in mitigating the effects of abiotic stress associated with vegetation in field. The plant material were two varieties of pea: Bohun and Cysterski and two varieties of yellow lupine: Talar and Dukat. The active substances were administered in the form of spraying at the beginning of flowering. The effectiveness of individual biostimulators was determined by measuring parameters that characterize the generative phase (number of flowers, pods, and the degree of abortion of flowers) and the parameters of yield (number of seeds per plant, number of seeds per pod, seed weight per plant, weight of 1000 seeds) as compared to control plants. The strongest stimulators among the tested substances were zearalenone and Asahi, which showed activity against all tested variety.

Key words: yellow lupine *(Lupinus luteus)*; pea *(Pisum sativum)*; abiotic stresses; biostimulators

Introduction

Plants from the *Fabaceae* family have many advantages. They are used as forecrop for other plant species, because they are able to fix atmospheric nitrogen through a symbiosis with nodule bacteria. Lupine develops a tap root system so it possess the ability to transport water and minerals from the deeper layers of the soil which results in better availability of water and minerals for other plants. Legume seeds are used as a forage component, because they possess an optimal amino acid composition and are rich in proteins and fat. Some legumes are also used as source of human food.

Despite so many advantages, the acreage of crops in Poland (and also in Europe) is very small. The reason for this is the low stability of yielding, perhaps resulting from plant susceptibility to climatic stress, especially drought stress, which causes significant crop losses.

One of the options to mitigate the effects of stress is the use of biostimulators, which according to European Biostimulants Industry Consortium – EBIC (2011) are various formulations of compounds, substances and other products that are applied to plants or soils. Assumption is made that biostimulants would be helpful in facing to the most important challenges of global agriculture and environmental protection issues in coming years.

According to the newest definition (EBIC 2011) biostimulators do not act as trophic compounds and do not eliminate or reduce stress. Instead they increase plants ability to elicits and increase exploitation of their genetic potential for better performing under given conditions thus are complementary to fertilizers and plant protection (Gawronska *et al*. 2008, Przybysz *et al.* 2008).

Most biostimulants positively affect wide range of processes/parameters (Gawronska *et al.* 2008, Przybysz *et al.* 2008) but some preparations are strictly targeted to particular ones.

Among positive effects of biostimulators most often reported are: increased biomass accumulation and yield, higher yield quality, better efficiency of photosynthetic apparatus (manifested by increased leaf area, chlorophyll content, intensity of photosynthesis and parameters of chlorophyll a fluorescence), improved plant water status and nutrients uptake, lowered membrane injuries, hormonal changes. Mode of action of them is stimulatory or protective against stressors, often both, though common is opinion that positive effects of biostimulators are more evident or even only seen when plants are under stress both biotic and abiotic.

The aim of this research was to determine the effectiveness of the chosen stimulators, plant hormones and other substances active in mitigating effects of environmental stresses associated with plant vegetation in the field for example: high and low temperature, drought, periodic flooding and wind causing plant lodging.

Material and methods

Two cultivars of pea: Bohun and Cysterski and two cultivars of yellow lupine: Talar and Dukat were used as plant material. The experiment was conducted in 2011 in Krakow and consisted of seven treatments: control and six which were treated with particular stimulator. Each treatment consisted of two experimental plots with 50 plants on each.

At the stage of flowering plants were sprayed with solutions of stimulators (150 ml per plot). They were:

- Asahi (1 ml/l) – commercial product, it is a mixture of three nitrophenolic compounds, according to the producer Asahi stimulates practically all main physiological processes in plants
- Biochikol (100 ml/l) – commercial product, it contains chitosan, which demonstrates a fungicide-like properties and stimulates the plant defense mechanism
- Tytanit (0,3 ml/l) – commercial product, contains titanium salt, which typically improves pollen grain vitality
- Hydrogen peroxide – signaling substance, its activity is often similar to plants hormones
- 1-Naphthaleneacetic acid – NAA (2 mg/l) synthetic analogue of auxin. Auxin coordinate of many physiological and developmental processes in plants, it controls their life cycle and is essential for regulation of growth of root system as well as flowering and fruit formation.
- Zearalenone – ZEN (2mg/l) – it is a new plant growth regulator. It has strong toxic action to animal organisms. However Zen is present in the shoot apices of many plants, for instance in wheat, carrot, rape, celery, white been, and cotton. Its content increases with the advancement of the vernalization and photoinduction processes, indicating that this substance is related to flowering. Moreover ZEN improved the induction and differentiation of wheat and rape callus. During the production of haploid embryos obtained via pollination of wheat by maize, the effect of ZEN was similar to 2,4-D. These results suggest that ZEN may be treated as a substance regulating numerous physiological processes (Biesaga-Kościelniak 2001).

In the growing period, the following stress conditions: cold during the germination of plants, the initial drought, followed by two periods of heavy rain, strong winds cause temporary lodging partial canopy and finally excessive precipitation hindering seed maturation. The control plants were subjected to the same stress factors, but not treated with any of the active substances.

For individual plants following parameters were measured: number of flowers per plant, number of pods per plant, the degree of flower abortion and the parameters of yield: number of seeds per plant, number of seeds per pod, weight of seeds per plant and weight of 1000 seeds.

Statistical analysis of results was performed using StatSoft Statistica v. 10. The significance of differences between the objects was tested using t Test.

Results

Effects of biostimulators on number of flowers, pods and abortion of flowers. The obtained results presented in Table 1 indicated that for pea cultivar Cysterski the highest increase of pods number per plant resulted ZEN and it was 22% as compared to control. Asahi resulted in 15% increase. Other treatments did not show statistically significant differences as compared to control. In the case of flowers number per plant values in all objects did not significantly differs as compared to control. The highest degree of flowers abortion showed control plants (45.2%) and the lowest – object treated with ZEN (34.1%).

Table 1. Effects of biostimulators on number of flowers, number of pods per plant and percent of aborted flowers for two pea cultivars: Cysterski and Bohun. Stars indicate statistically significant differences compared to control, $p< 0.05$

Treatment	Number of flowers/plant	% of control	Number of pods/plant	% of control	Abortion of flowers [%]
Cysterski					
Control	**10.9**	**100.0**	**5.95**	**100.0**	**45.2**
Asahi	11.0	100.9	6.82	114.6	38.0
NAA	9.8	89.9	6.10	102.5	37.6
Chitosan	10.8	99.1	5.98	100.5	44.8
H_2O_2	10.2	93.6	6.09	102.3	40.1
Tytanit	10.3	94.5	6.08	102.2	41.0
ZEN	11.0	100.9	7.28 *	122.3	34.1
Bohun					
Control	**11.6**	**100.0**	**6.57**	**100.0**	**43.1**
Asahi	13.4 *	115.6	7.94 *	120.9	40.7
NAA	12.3	106.0	6.86	104.4	44.2
Chitosan	12.6	108.6	7.02	106.8	44.3
H_2O_2	12.6	108.6	6.97	106.1	44.5
Tytanit	11.7	100.9	6.71	102.1	42.4
ZEN	12.9	111.2	8.28 *	126.0	35.9

Results presented in Table 1 indicated that for Bohun cultivar Asahi resulted in 16% increase of flowers number per plant and 21% increase of pod numbers per plant as compared to control, while in the case of ZEN it was 11% and 26% - increase, respectively. Other objects did not show statistically significant differences as compared to control. The highest degree of flowers abortion showed object after treatment with H_2O_2 (44.5%) and the lowest – object treated with ZEN (35.9%).

The obtained results presented in Table 2 indicated that for lupine cultivar Talar Asahi resulted in 65% increase of flowers number per plant and 60% increase of pods number per plant as compared to control, while in the case of ZEN it was 51% and 45% - increase, respectively. The highest degree of flowers abortion showed object after treatment with Tytanit (80.0%) and the lowest – object treated with Chitosan (74.6%).

Table 2. Effects of biostimulators on number of flowers, number of pods per plant and percent of aborted flowers for two lupine cultivars: Talar and Dukat. Stars indicate statistically significant differences compared to control, $p< 0.05$.

Treatment	Number of flowers/plant	% of control	Number of pods/plant	% of control	abortion of flowers [%]
Talar					
Control	**21.1**	**100.0**	**5.34**	**100.0**	**74.7**
Asahi	34.8 *	164.9	8.55 *	160.1	75.4
NAA	18.7	88.7	4.08	76.4	78.2
Chitosan	13.3	63.0	3.38	63.3	74.6
H_2O_2	14.5	68.7	3.17 *	59.4	78.1
Tytanit	22.2	105.2	4.44	83.1	80.0
ZEN	31.9 *	151.2	7.75 *	145.1	75.7
Dukat					
Control	**29.3**	**100.0**	**8.64**	**100.0**	**70.5**
Asahi	43.8 *	149.6	12.01 *	139.0	72.6
NAA	26.4	90.1	7.66	88.7	71.0
Chitosan	25.2	86.0	6.99	80.9	72.2
H_2O_2	18.1 *	61.8	4.56 *	52.8	74.8
Tytanit	22.4	76.4	5.41	62.6	75.8
ZEN	37.4 *	127.7	11.65 *	134.9	68.9

Results presented in Table 2 indicated that for Dukat cultivar Asahi resulted in 50% increase of flowers number per plant and 39% increase of pods number per plant as compared to control, while in the case of ZEN it was 28% and 35% - increase, respectively. Other stimulators declined values of studied parameters. Similarly to Talar cultivar, also in this case the highest degree of flower abortion showed plants after treatment with Tytanit (75.8%) and the lowest – object treated with ZEN (68.9%).

Effects of biostimulators on yield structure parameters. For pea cultivar Cysterski increase of weight of seeds per plant was obtained only in case of the treatment with ZEN and it was 28% higher than control. In case of number of seeds per plant values in all objects did not show statistically significant differences as compared to control (Figure 1a).

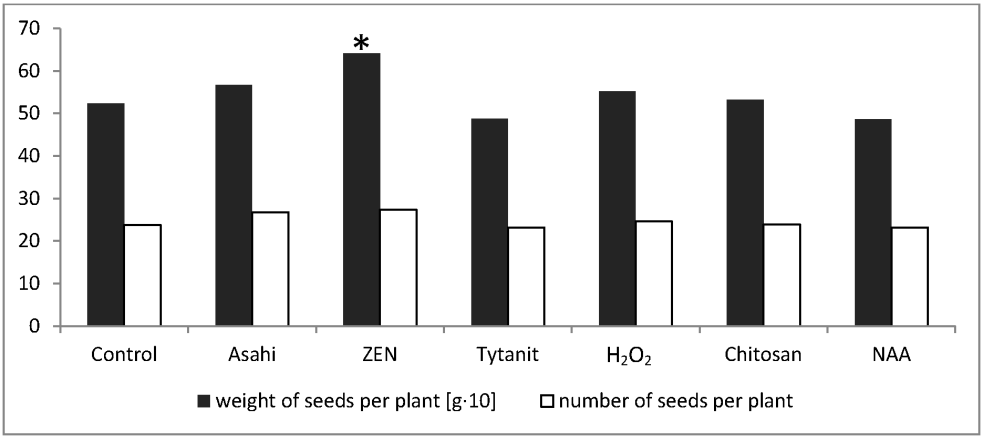


Figure 1a. Effect of biostimulators on the yield parameters of pea cultivar Cysterski. Stars indicate statistically significant differences compared to control, $p< 0.05$

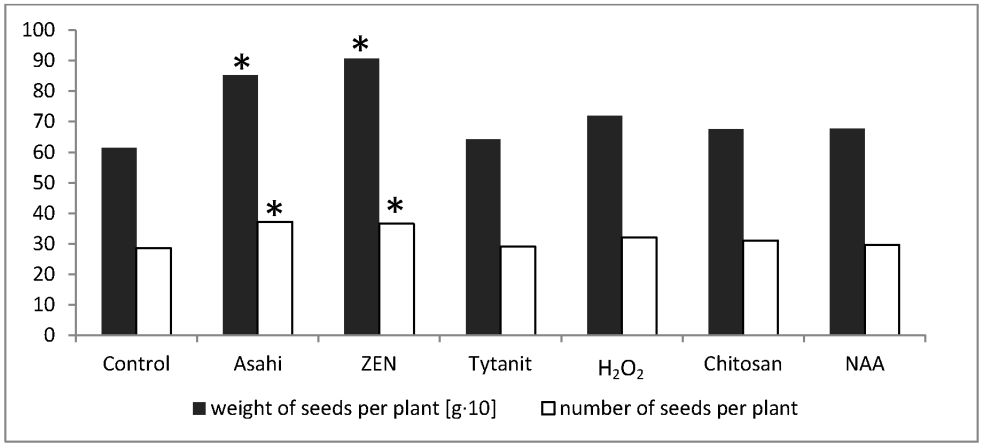


Figure 1b. Effect of biostimulators on the yield parameters of pea cultivar Bohun. Stars indicate statistically significant differences as compared to control, $p< 0.05$

Results presented in Table 3 indicated that in the case of the number of seeds per pod and weight of 1000 seeds, individual objects were not significantly different, but the highest weight of seeds per plant after treatment with ZEN results from the highest weight of 1000 seeds (237 g).

In case of Bohun cultivar the highest weight of seeds per plant was obtained after treatment with ZEN and it was 47% higher than control, next Asahi - 39%

increase compared to control. In case of number of seeds per plant increases of values were obtained after treatment with Asahi (30%) and ZEN (28%) as well (Figure 1b).

Table 3. Effect of biostimulators on selected parameters of yield structure in two pea cultivars. Stars indicate statistically significant differences compared to control, $p < 0.05$

Treatment	Number of seeds per pod	Mass of 1000 seeds [g]	Number of seeds per pod	Mass of 1000 seeds [g]
	Cysterski		Bohun	
Control	**4.09**	**231**	**4.33**	**215**
Asahi	4.14	212	4.67 *	229 *
NAA	4.01	209	4.31	228 *
Chitosan	3.98	230	4.42	216
H_2O_2	4.22	228	4.61 *	227 *
Tytanit	3.93	223	4.30	220
ZEN	3.99	237	4.35	247 *

Results presented in Table 3 indicated that Asahi and H_2O_2 resulted in formation the highest number of seeds per pods (4.67 and 4.61, respectively). Similarly to Cysterski cultivar, the highest weight of seeds per plant after treatment with ZEN results from the highest weight of 1000 seeds (247 g).

Similarly to pea, also in case of lupine the highest weight and number of seeds per plant were obtained after treatments with Asahi and ZEN. For Talar cultivar the highest weight of seeds per plant was obtained in case of treatment with Asahi and it was 93% higher than control. Next ZEN - 64% increase. Highest number of seeds was obtained in the object treated with Asahi and it was 70% higher than control, next ZEN - 53%. After treatment with H_2O_2 values of weight of seeds and number of seeds per plant declined significantly (Figure 2a).

The obtained results presented in Table 4 indicated that ZEN resulted in formation the highest number of seeds per pods (3.53) and H_2O_2 – the lowest (2.57). However, after treatment with hydrogen peroxide was observed highest weight of 1000 seeds (156 g). Significant increase in the weight of 1000 seeds was also observed after treatment with Asahi (146 g) and ZEN (141 g).

For Dukat cultivar the highest weight of seeds per plant was obtained in case of treatment with ZEN and it was 72% higher than control. Next Asahi - 61% increase (Figure 2b).

Highest numbers of seeds was obtained in the object treated with ZEN and it was 46% higher than control, next Asahi - 44%. Similarly to Talar cultivar, also for Dukat cultivar treatment with H_2O_2 resulted in significant decline of weight of seeds and number of seeds per plant (Figure 2b).

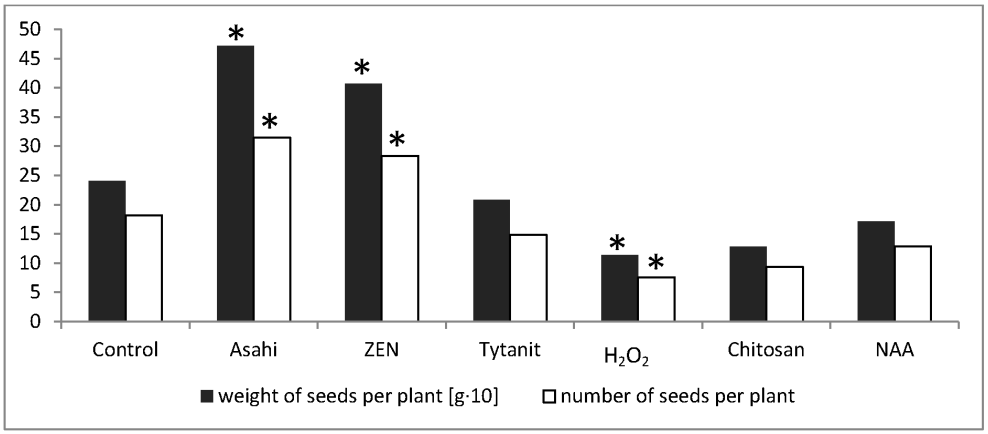


Figure 2a. Effect of biostimulators on the yield parameters of lupine cultivar Talar. Stars indicate statistically significant differences compared to control, $p< 0.05$

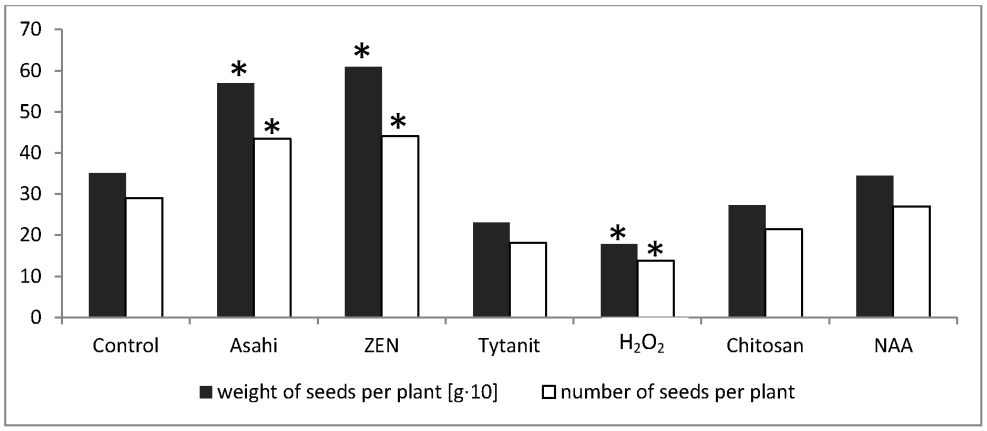


Figure 2b. Effect of biostimulators on the yield parameters of lupine cultivar Dukat. Stars indicate statistically significant differences compared to control, $p< 0.05$

Similarly to Talar cultivar, also in case of Dukat ZEN resulted in formation the highest number of seeds per pods (3.62). Significant increase in the weight of 1000 seeds compared to control was observed in almost all of investigated treatments (Table 4).

Table 4. Effect of biostimulators on selected parameters of yield structure in two lupine cultivars. Stars indicate statistically significant differences compared to control, $p< 0.05$

Treatment	Number of seeds per pod	Mass of 1000 seeds [g]	Number of seeds per pod	Mass of 1000 seeds [g]
	Talar		Dukat	
Control	**3.18**	**129**	**3.20**	**118**
Asahi	3.41	146 *	3.45	131 *
NAA	2.97	135	3.17	132 *
Chitosan	2.85 *	133	3.01	127 *
H_2O_2	2.57 *	156 *	3.16	130 *
Tytanit	3.35	140	3.27	135
ZEN	3.53 *	141 *	3.62 *	137 *

Discussion

The results of presented studies are similarly to the results of many experiments conducted in other plant species which indicate that spraying of plants with Asahi SL result in the increase of plant crops (Czeczko and Mikos-Bielak 2004; Maciejewski *et al*. 2007; Gawronska *et al.* 2008; Kwiatkowski and Juszczak 2011). However, it can be seen strong relationship between action of the stimulator and weather conditions (Budzynski *et al.* 2008; Malarz 2008; Michalski *et al.* 2008; Przybysz *et al.* 2008). According to Matysiak *et al.* (2011), one can obtain diverse reaction of crop plants to Asahi application related to the year of study.

Kozak *et al.* (2008) reported that Asahi SL applications in soybean cultivation significantly increased the height of plants and the first pod formation, the number of pods per plant, the number of seeds per plant and the yields of seed and straw.

Matysiak *et al.* (2011) similarly to Kositorna *et al.* (2008), haven't observed any changes in contents of sugars, potassium and sodium in sugar beet, which is contrary to results obtained by Cerny *et al.* (2002), who found near 14% increase of sugar content as well as remarkable increase of root mass.

According to Czeczko and Mikos-Bielak (2004) single spraying of potato plants with Asahi SL increases of tuber mass by 14%. Maciejewski *et al.* (2007, 2008) emphasized the differentiation in potato plant crops as related to particular cultivating season as well as the cultivar studied. Budzynski *et al.* (2008) stated that single application of Asahi SL increased yield of oil rape seeds by 3%, while double application resulted in 5 % increase. Significant increase of rape seeds mass obtained also Spychaj-Fabisiak *et al.* (2011) as well, Budzynski *et al.* (2008), Malarz *et al.* (2008) and Przybysz *et al.* (2010). Budzynski *et al.* (2008) and Malarz *et al.* (2008), proved that even greater seed number per pod (23%) is obtained after application of the biostymulator on plants with advanced developmental stages.

Biesaga – Kościelniak and Filek (2010) showed that exogenous application of zearalenone and its derivatives can stimulate generative development in winter

plants, which suggest its participation in the mechanism of flowering. Moreover, treatment with zearalenone had an effect on calli proliferation and cell differentiation. The effect of zearalenone was similar to the activity of auxins in in vitro cultures, which may confirm the hormonal properties of zearalenone in plants. Watering and soaking wheat and soybean grains with zearalenone solution resulted in higher yields of these plants.

Dziurka *et al.* (2012) reported that ZEN significantly increased yielding of yellow lupin. This higher productivity was due to the effect of ZEN on the increase in the value of the coefficient of the use of biological potential of the plants.

Conclusions

- The most active stimulators among all tested substances are ZEN and Asahi, which show activity in case of all investigated cultivars.
- In case of pea, Asahi causes the increase of 21% in seed number and 24% in seed weight, while ZEN affects the increase of 24% and 38%, respectively.
- In lupine Asahi causes the increase of 56% in seed number and 76% in seed weight, while ZEN increases studied parameters of 49% and 68%, respectively.
- Both substances are worthy to be studied in the further researches.

Acknowledgments. This research was supported by the Ministry of Agriculture and Rural Developmet, grant no. HOR hn-801-3/2011

References

Basak A. 2008. Biostimulators – definitions, classification and legislation. p. 7–17. In: „Biostimulators in Modern Agriculture. General Aspects" (H. Gawrońska, ed.). Editorial House Wieś Jutra, Warsaw, 89 pp.

Biesaga – Koscielniak J. 2001. Zearalenone as a new hypothetical regulator of the vegetative and generative plant development. (Zearalenon jako nowy, hipotetyczny regulator wzrostu i rozwoju roślin). The *F. Górski* Department of Plant Physiology PAS. Monografie. Rozprawa habilitacyjna.

Biesaga – Kościelniak J., Filek M. 2010. Occurrence and Physiology of Zearalenone as a New Plant Hormone. Sociology, Organic Farming, Climate Change and Soil Science. Sustainable Agriculture Reviews Volume 3, pp 419–435.

Budzyński W., Dubis W., Jankowski K. 2008. Response of winter oilseed rape to the biostymulator Asahi SL applied in spring. p. 18–25. In: „Biostimulators in Modern Agriculture. Field Crops" (Z.T. Dąbrowski, ed.). Editorial House Wieś Jutra, Warsaw, 119 pp.

Cerny I., Pacuta V., Feckova J., Golian J. 2002. Effect of year and Atonik application on the selected sugar beet production and quality parameters. J. Central Eur. Agric. 3 (1): 15–22.

Czeczko R., Mikos-Bielak M. 2004. Effects of Asahi bio-stimulator application in the cultivation of different vegetable species. Ann. UMCS, Agric. 59 (3): 1073–1079.

Dziurka M., Ostrowska A., Mirek M., Biesaga–Koscielniak J., Janeczko A. 2012. Improvement of growth and seed yield qualitty of Lupinus luteus L. plants as affected by aplication of zearalenone. Acta Physiol. Plant. 34 (Suppl 1):S1–S116

EBIC, European Biostimulants Industry Consortium, 2011 http://www.biostimulants.eu/.
Gawrońska H., Przybysz A., Szalacha E., Słowiński A. 2008. Physiological and molecular mode of action of Asahi SL biostymulator under optimal and stress conditions. p. 54–77. In: „Biostimulators in Modern Agriculture. General Aspects" (H. Gawrońska, ed.). Editorial House Wieś Jutra, Warsaw, 89 pp.
Gawronska H., Przybysz A., Wrochna M., Szalacha E., Slowinski A. 2008. Biological basis of mode of action of the Asahi SL biostimulator. Monograph series: Biostimulators in modern agriculture, General Aspects. Wies Jutra, Warsaw: 54-76.
Kositorna J., Smoliński J. 2008. Asahi SL biostimulator in protection of sugar beet from herbicide stress. p. 41–50. In: „Biostimulators in Modern Agriculture. Field Crops" (Z.T. Dąbrowski, ed.). Editorial House Wieś Jutra, Warsaw, 119 pp.
Kozak M., Malarz W., Serafin-Andrzejewska M., Kotecki A. 2008. The effects of sowing rate and Asahi sl biostimulator on soybean growth and yield. Monographs series: Biostimulators In modern agriculture, Field Crops. Wies Jutra, Warsaw: 77–84.
Kwiatkowski C.A., Juszczak J. 2011. The response of sweet basil (Ocimum bassilicum L.) to the application of growth stimulators and forecrops. Acta Agrobot. 64 (2): 69–76.
Maciejewski T., Michalski T., Bartos-Spychała M., Cieślicki W. 2008. Effect of the application of biostymulator Asahi SL on the yield of potato tubers and their quality. p. 52–60. In: „Biostimulators in Modern Agriculture. Solanaceous Crops" (Z.T. Dąbrowski, ed.). Editorial House Wieś Jutra, Warsaw, 97 pp.
Maciejewski T., Szukała J., Jarosz A. 2007. Wpływ biostymulatora Asahi SL na cechy jakościowe bulw ziemniaków. J. Res. Appl. Agric. Eng. 52 (3): 109–112.
Malarz W., Kozak M., Kotecki A. 2008. The use of Asahi SL biostimulator in spring rape growing. p. 25–33. In: „Biostimulators in Modern Agriculture. Field Crops" (Z.T. Dąbrowski, ed.). Editorial House Wieś Jutra, Warsaw, 119 pp.
Matysiak K., Adamczewski K., Kaczmarek S. 2011. Wpływ biostymulatora Asahi SL na plonowanie i wybrane cechy ilościowe i jakościowe niektórych roślin rolniczych uprawianych w warunkach Wielkopolski. Progress in Plant Protection/Postępy w Ochronie Roślin 51 (4)
Michalski T., Horoszkiewicz-Janka J., Bartos-Spychała M. 2008. Efficiency of Asahi SL in protection of barley and wheat mixture in comparison with pure sowing. p. 50–59. In: „Biostimulators in Modern Agriculture. Field Crops" (Z.T. Dąbrowski, ed.). Editorial House Wieś Jutra, Warsaw, 119 pp.
Przybysz A., Gawronska H., Slowinski A. 2008. The effect of Asahi SL on growth, efficiency of photosynthetic apparatus and yield of field grown oil seed rape. Monographs series: Biostimulators In modern agriculture, Field Crops. Wies Jutra, Warsaw: 7–17.
Przybysz A., Wrochna M., Słowiński A., Gawrońska H. 2010. Stymulatory effect of Asahi SL on selected plant species. Acta Sci. Pol., Hortorum Cultus 9 (2): 53–64.
Spychaj-Fabisiak E., Murawska B., Pacholczyk Ł. 2011. Values of quality traits of oilseed rape seeds depending on the fertilization and plant density. J. Elementol. 16 (10): 115–124.

PLANT FUNCTIONING UNDER ENVIRONMENTAL STRESS P. 267-274

Cracow, 12th to the 15th September, 2012

COMPERATIVE STUDY OF BIOSTIMULATOR AND A GROWTH RETARDANT EFFECTS ON SELECTED MORFOROGICAL TRAITS OF WINTER WHEAT RELATED TO LODGING RESISTANCE

Agata Kaczor, Jacek Żebrowski

Department of Plant Physiology, Institute of Applied Biotechnology and Basic Science, University of Rzeszow

Corresponding author: jazeb@univ.rzeszow.pl

Abstract Intensive management in majority of cereal crops requires chemical control of lodging to minimize possible yield losses due to losing by plants their vertical position. A variety of exogenous growth retardants, inhibiting culm elongation, is widely applied in farming to reduce the risk of lodging. An alternative approach based on application of biostimulators is also taken recently into consideration. Reports on use of biostimulators to improve wheat standability are so far lacking. This work presents preliminary comparative studies on the effects of a biostimulator and a commercial growth retardant on selected culm traits related to lodging resistance in winter wheat (*Triticum aestivum*).

Key words: winter wheat; biostimulators; lodging resistance

Introduction

Intensive crop production for high yield, based on high-input of nitrogen nutrition and watering bears the risk of permanent losing upright position of plants within canopy. This usually affects grain yield and grain quality in cereals, including wheat (Pinthus 1973, Rajkumara 2008). High level of nitrogen supply promotes plant height, leaf expansion and shoot total weight, that makes the plants more exposed to wind loads and thus more susceptible to falling down during heavy rains (Berry et al. 2004).

A common practice in high-input cereal management is use of plant regulators (PGRs), which through shortening the main culm reduce effectively the suscepti-

bility of plants to lodging (Berry et al. 2000, Rajala and Peltonen-Sainio 2001, Rahman et al. 2006, Ramburan and Greenfield 2007). Majority of so far used PGRs can be classified into two groups, as antigibberelins, i.e. containing compounds inhibiting gibberelin biosynthesis at various steps of the pathway, or releasing the ethylene within tissues (Rademacher 2000, Rajala 2004).

The plant growth regulators are well established to be effective in minimising lodging risk and thus increasing yield in the case when lodging occurs (Shekoofa and Eman 2008, Tripathi 2004). However, at seasons without lodging, or in stands suffering from drought or other abiotic stresses, the treatment may be neutral or even may have negative effect on yield formation (Ma and Smith 1991, Haskins and McMullen 2007, Ottman 2011, Pavlista et al. 2010).

Recently, a group of chemicals considered as biostimulators have been introduced to practice or are being tested for their protective activity under unfavourable environmental conditions in various crops (Djanaguiraman 2005, Gawrońska et al 2008, Łyszkowska et al. 2008, Masheva et al 2010, Przybysz et al 2010) .Their possible effect on lodging has not been evaluated so far. The aim of this paper was to assess the impact of ARY 107, a chemical classified as a biostimulator, on winter wheat shoot traits related to lodging. The action pattern is compared with that of a growth retardant Stabilan 750 SL.

Material and methods

The winter wheat cv. Turnia plants were grown in a mini-plot field experiment in 2011/2012. At the ZS 31 Zadoks stage (Zadoks et al. 1974), at the onset of shooting the plants were sprayed with Stabilan 750 SL, containing chlormequat chloride as an active ingredient, at the rate of 1.5 l/ha and with ARY 701 kindly provided by Arysta LifeScience, considered as a biostimulator, according to manufactures recommendation. The plants were analyses for the growth rate at the phase of pseudostem and culm development twice a week. At the late milk maturity plants were collected for measurements of culm length, internode length and internode diameter. The diameter was determined with a digital caliper as an average of the ellipse longer axis and the perpendicular to it shorter axis. Additionally the lower part of culm, 15 cm long, was tested for stiffness in bending using the Instron system 5542 (Instron Ltd., High Wycombe, England), driven with a computer and the Bluehill 2 software. This parameter was evaluated as the slope of the force to deflection curve recorded in the course of bending of the specimen tested as a cantilever beam.

Results and discussion

Stabilan 750 SL, a growth retardant containing chlormequat chloride as an active ingredient, reduced as expected the axial growth rate of shoot in winter wheat cv. Turnia, including both the phase of pseudostem (Fig. 1) and the culm formation (Fig. 2). In consequence, the final culm length of mature plants was decreased by 17 percent compared to non-treated plants (Fig. 3). The second lower internode, expected to contribute to plant lodging resistance (Pinthus 1973), was reduced by about percent (Fig. 4), that was accompanied by its thickening by about 2 percent (Fig. 5).

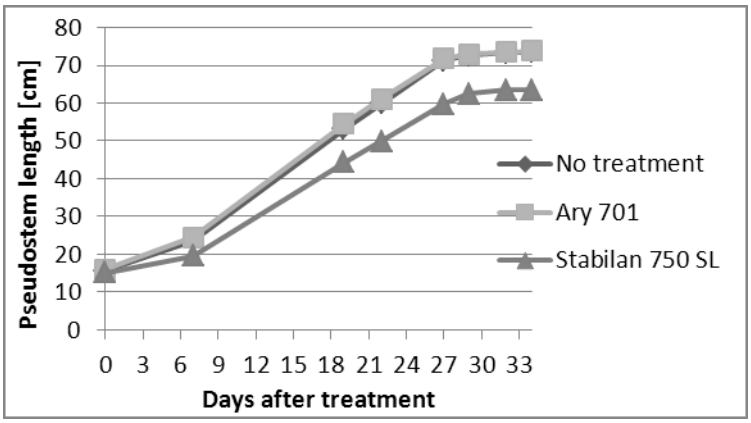


Figure 1. The influence of the growth retardant Stabilan 750 SL and a biostimulator ARY 701 on the psudostem elongation dynamics in winter wheat cv. Turnia

On the contrary, the treatment of plants with Ary 701, considered as a biostimulator, only slightly and statistically insignificantly stimulated the culm and lower internode elongation (Figs. 1, 3, 4). However, the thickening of the basal culm part was in this case much more pronounced (by 4 percent) relative to that caused by Stabilan 750 SL (Fig. 5). Therefore, the Stabilan 750 SL and the Ary 701 showed quite different patterns of culm morphology modification. In the case of the former the thickening of culm, a feature relevant for plant lodging resistance, occurred at the expense of the investment of assimilates into axial growth. In turn, the plants treated with Ary 701 showed culm thickening along with increase of internode length.

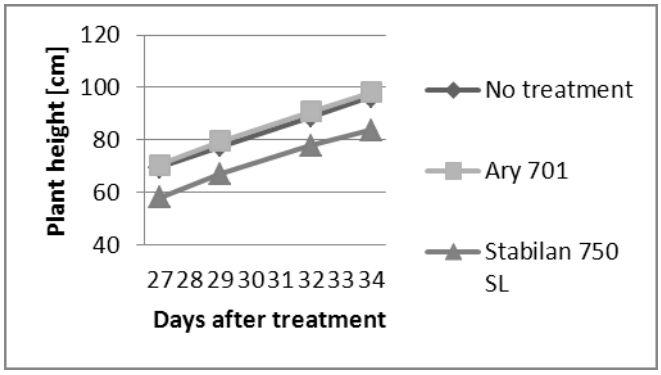
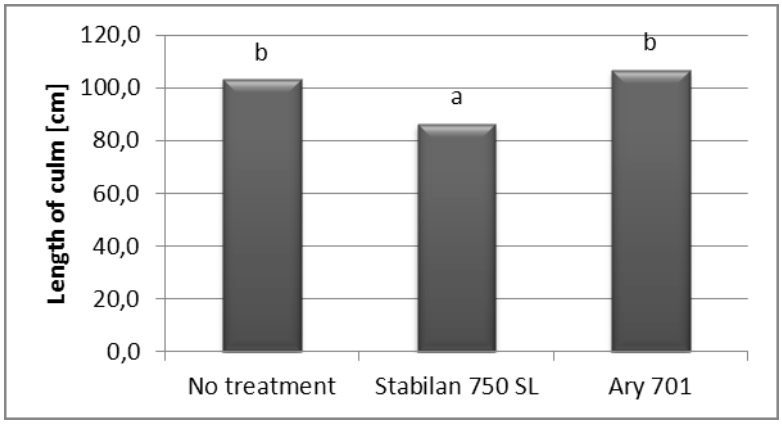


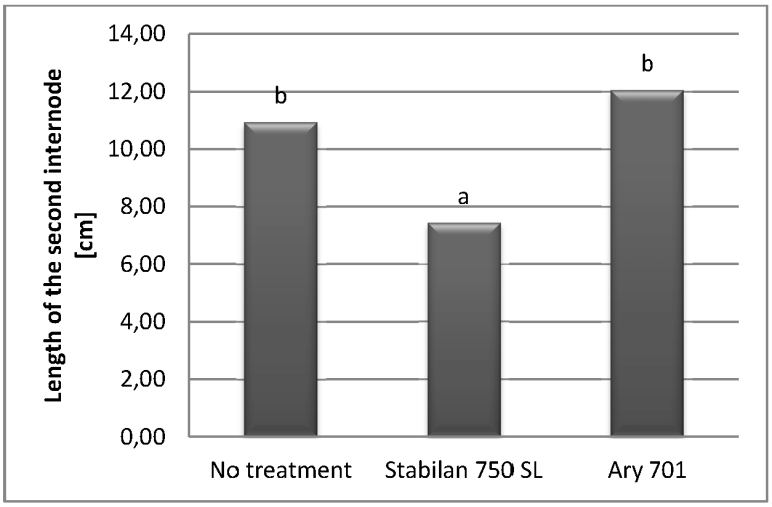
Figure 2. The effect of the growth retardant Stabilan 750 SL and a biostimulator ARY 701 on the culm elongation dynamics in winter wheat cv. Turnia

The manipulation of plant height is an effective tool for improving the lodging resistance of cereal crops produced in intensive management system (Berry et al 2000). Reduction of the culm length affects both culm resistance to bending as well as the drag force imposed by wind on a plant (Berry et al. 2004). Both impacts make the growth retardants effective in lodging control in cereals. Mechanisms of action of growth retardants differ in dependence on chemical nature of active ingredient, and may rely on regulation of gibberelin biosynthesis or ethylene content in culm tissues (Rajala 2004).



[a] different letters in the rows indicate significant differences among means for $p < 0.05$.

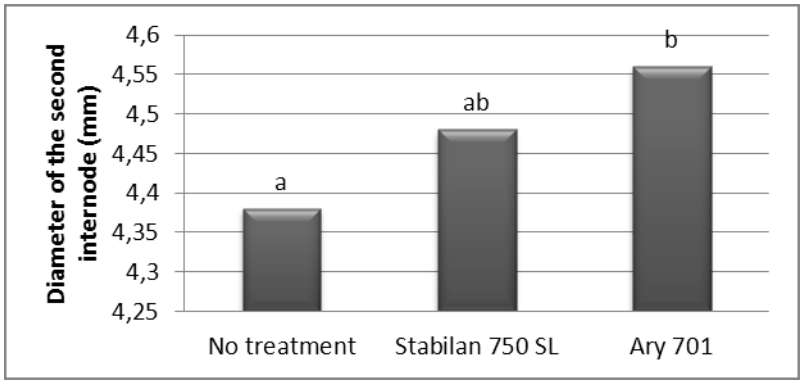
Figure 3. The final length of culm of plants treated with growth retardant Stabilan 750 SL and a biostimulator ARY 701 in winter wheat cv. Turnia.



[a] different letters in the rows indicate significant differences among means for $p < 0.05$.

Figure 4. The influence of Stabilan 750 SL and ARY 701 on the length of the second lower internode

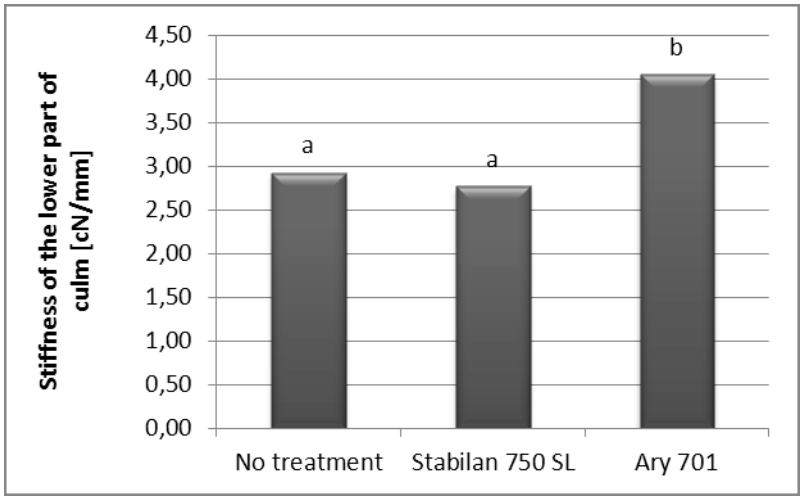
Inhibitors of gibberelin biosynthesis may disturb the chemical pathways at different control points, e.g through specific inhibition of *ent*-copalyl diphosphate synthase or inhibition of oxygenation of *ent*-kaurene to *ent*-kaurenoic acid. Stabilan 750 SL containing chlormequat as an active compound showing the former action mode.



[a] different letters in the rows indicate significant differences among means for $p < 0.05$.

Figure 5. The influence of Stabilan 750 SL and ARY 701 on the diameter of the second lower internode

Mechanical properties of basal culm part are another, apart from the culm length feature of plant relevant for resistance to lodging in cereals (Pinthus 1973) . Increase in stiffness of culm may result from increase of culm diameter and culm wall thickness, the geometrical component of stiffness, as well as from improvement of the effective culm Young's modulus, related to the contribution of strengthening tissues on cross – section and the cell wall mechanical properties. Although Stabilan 750 SL increased the culm diameter, no changes in culm stiffness were observed (Fig. 6), that may indicate some deterioration of mechanical properties of cell walls. In turn, Ary 701 significantly improved this parameter (Fig. 6), that may suggest occurrence of some advantageous changes in cell wall material properties along with alteration of the cross –section dimension.



[a] different letters in the rows indicate significant differences among means for $p < 0.05$.

Figure 6. The effect of Stabilan 750 SL and ARY 701 on stiffness of the lower part of culm

Conclusions

This preliminary study indicates, that effects of tested growth retardant and a biostimulator are to some extend complementary in controlling the lodging resistance in winter wheat.

References

Berry PM, Griffin JM, Sylvester-Bradley R, Scott RK, Spink JH, Baker CJ, Clare RW (2000). Controlling plant form through husbandry to minimize lodging in wheat. Field Crops Research, 67, pp 59-81.

Berry PM., Sterling M, Spink JH, Baker CJ, Sylvester-Bradley R, Mooney SJ, Tams AR, Ennos AR. Donald LS (2004). Understanding and reducing lodging in cereals. Advances in Agronomy. 84, pp 217-271.

Djanaguiraman M, Sheeba JA, Durga Devi D, Bangarusamy U (2005). Effect of Atonik seed treatment on seedling physiology of cotton and tomato. J. Biol. Sci., 5 (2), pp 163-169.

Gawrońska H, Przybysz A, Szalacha E, Slowiński A (2008). Physiological and molecular mode of action of Asahi SL biostimulator under optimal and stress conditions. Biostimulators in modern agriculture. General aspects (ed. Dabrowski Z). Wieś Jutra, Warszawa, pp 7-17.

Haskins B, McMullen G, (2007). Crop canopy management through nitrogen and plant growth regulators. IREC Farmers' Newsletter, 175, Autumn, pp 10-13.

Łyszkowska M, Gajc-Wolska J, Kubiś K (2008). The influence of biostimulators on field and quality of leaf and iceberg lettuce - grown under field condition. Biostimulators in Modern Agriculture. Vegetable Crops., (ed. Dabrowski Z). Wydawnictwo Wieś Jutra, Warszawa.

Ma B, Smith DL (1991). The effects of ethephon, chlormequat chloride and mixtures of ethephon and chlormequat chloride applied at the beginning of stem elongation on spike-bearing shoots and other yield components of spring barley (Hordeum-vulgare L). Journal of Agronomy and Crop Science-Zeitschrift Fur Acker Und Pflanzenbau, 166, pp 127-135.

Masheva S, Valchev N, Yankova V (2012). Effect of biostimulator Aveikan on growth manifestations yield and phytosanitary status in leek variety Starozagorski 72. Agricultural Science and Technology, 4, pp 256-259.

Ottman M (2011). Lodging Control for Wheat and Barley in Arizona. Western Farm Press, The University of Arizona, AZ1532, pp 1-2.

Pavlista AD, Hergert GW, Baltensperger D, Knox S (2010). Reducing Height and Lodging of Winter Wheat, Plant Management Network.

Pinthus MJ (1973) Lodging in wheat, barley, and oats: the phenomenon, its causes, and preventive measures. Advances in Agronomy, 25, pp 209-263.

Przybysz A, Wrochna M, Slowinski A, Gawronska H (2010). Stimulatory effect of Asahi SL on selected plant species. Acta Scientiarum Polonorum / Hortorum Cultus, 9, pp 53-64.

Rademacher W (2000). Growth retardants: Effects on gibberellin biosynthesis and other metabolic pathways. Annual Review of Plant Physiology and Plant Molecular Biology, 51, pp 501-531.

Rahman ATMF, Ajala BA, Azele ME (2006). Effect of Cycocel in the prevention of lodging in wheat (Triticum aestivum) with the application of nitrogen fertilization. Hamdard Medicus, 49, pp 53-58.

Rajala A (2004). Plant growth regulators to manipulate oat stands. Agricultural and Food Science, 13, pp 186-197

Rajala A, Peltonen-Sainio P (2001). Plant growth regulator effects on spring cereal root and shoot growth. Agronomy Journal, 93, pp 936-943.

Rajkumara S (2008). Lodging in cereals – a review. Agric. Rev., 29, pp 55 – 60.

Ramburan S, Greenfield PL (2007). The effects of chlormequat chloride and ethephon on agronomic and quality characteristics of South African irrigated wheat. S. Afr. J. Plant Soil, 24, pp 106-113.

Shekoofa A, Emam Y (2008). Effects of Nitrogen Fertilization and Plant Growth Regulators (PGRs) on Yield of Wheat (Triticum aestivum L.) cv. Shiraz. Journal of Agricultural Science and Technology, 10, pp 101-108.

Tripathi SC, Sayre KD, Kaul JN, Narang RS (2004). Lodging behavior and yield potential of spring wheat (Triticum aestivum L.): Effects of ethephon and genotypes. Field Crops Research, 87, pp 207-220.

Yamaguchi S (2008). Gibberellin metabolism and its regulation. Annual Review of Plant Biology, 59, pp 225-251.

Zadoks JC, Chang TT, Konzak CF (1974). A Decimal Code for the Growth Stages of Cereals. Weed Research, 14, pp 415-421.

PLANT FUNCTIONING UNDER ENVIRONMENTAL STRESS P. 275-287

Cracow, 12th to the 15th September, 2012

THE REDOX STATUS OF PLANT TISSUES INFLUENCES THE RESISTANCE OF WINTER RYE SEEDLINGS TO SNOW MOULD CAUSED BY *Microdochium nivale* (FR) Samuels & Hallett

Franciszek Dubert[1*], Agnieszka Płażek[2], Ewa Pociecha[2], Irena Kolasińska[3], Franciszek Janowiak[1]

[1]*Institute of Plant Physiology, Polish Academy of Sciences, Niezapominajek 21, 30-239 Kraków, Poland,*

[2]*Department of Plant Physiology, University of Agriculture, Podłużna 3, 30-239 Kraków, Poland,*

[3]*Department of Plant Genetics and Breeding, Institute of Plant Breeding and Acclimatization, Radzików, 05-870 Błonie, Poland,*

Corresponding author: dubert@ifr-pan.krakow.pl

Abstract The disease of grasses and cereals called snow mould is a serious problem in Polish agriculture as it results in a decrease of crop yield and its quality. One of the causes of snow mould is the fungus *Microdochium nivale*, which develops in conditions existing under snow cover – low temperature, very weak light, and high air humidity. The aim of the study was to verify whether the redox status of plant tissues plays an important role in plant resistance to this pathogen. The experiment was performed on 2-week-old seedlings of six breeding lines and cultivar 'Stach' of winter rye, which were inoculated with *Microdochium nivale* mycelium in controlled conditions. At various time points during the pathogenesis, analyses of the level of low molecular antioxidants (LMA) and the amount of phenolics – one of the most important members of LMA – were conducted. Sensitivity to the pathogen was evaluated on the basis of plant regrowth ability after infection and was expressed as Average Regrowth Index (ARI) coefficient. Phenolic level was 2-3 times higher in the leaves as compared to the crowns. In leaves of less susceptible plants phenolic content was greater as compared to more susceptible ones. In both more and less sensitive plants the infection with snow mould fungus resulted in a small, insignificant decrease of phenolic content. The above mean that genetically determined greater phenolic content in the leaves is decisive for a higher degree of resistance to snow mould. In both groups of genotypes studied, the longer the time of cultiva-

tion of control plants under conditions favorable for inoculation, the greater the increase of antioxidant level. This increase was higher in the more resistant lines. The snow mould fungus infection process, separated from the influence of growth conditions stress, resulted in a decrease in antioxidant content. This decrease was particularly marked in the more resistant lines. This can be due to the fact that the more resistant lines consumed more of their antioxidants to decompose (inactivate) molecules of ROS secreted by pathogenic fungi in the course of pathogenesis development, which supports the hypothesis about a protective role of LMA compounds against pathogen attack. Significant though not excessively high correlation was fund between LMA and phenolic content in the leaves. This correlation proves that phenolics belong to low molecular antioxidants. However, it was relatively low, which indicates that phenolics may react to infection by pathogenic fungi somewhat differently from other antioxidative molecules, and furthermore, the share of phenolics in total antioxidant pool may vary in particular winter rye lines studied.

Key words: low molecular antioxidants; phenolic compounds; snow mould, winter rye

Abbreviations: ARI – average regrowth index; LMA – low molecular antioxidants; R – plants resistant to snow mould; ROS – reactive oxygen species; S – plants susceptible to snow mould

Introduction

Rye (*Secale cereale* L.) is a cereal which plays a major role in the feeding of European populations, due to its winter hardiness and low environmental and nutritional demands (Schlegel 2010). Today rye is cultivated on 11 million ha worldwide. The main rye producers include Russia, Poland, and Northern European countries. It is used for food and forage. Food made from whole rye is valued for its health benefits such as well-balanced composition of macronutrients, high dietary fibre content, and high level of minerals, vitamins, sterols, and phenolics.

Rye is the most frost tolerant among all cereals, and thus it can be cultivated under conditions where other cereals fail. The greatest danger for winter survival of rye is its low resistance to fungi which can attack when the temperature at snow-plant interface remains around 0°C. In Poland and other cold and temperate regions, the most dangerous is pathogenic fungus *Microdochium nivale* (Fr) Samuels & Hallett. Pink snow mould disease caused by this fungus can inflict damages leading to serious yield losses and a decrease in yield quality (Smith 1981). Because of the harmful effects of fungicide use on the ecosystem and also on human health, a lot of interest has been shown in resistant genotype production. However, as yet no cultivars highly resistant to snow mould have been obtained. One of the reasons is that neither the mechanism of infection nor the physiological background for snow mould resistance has been fully explained (Bojarczuk *et al.* 1990).

One of the factors contributing to the mechanisms of frost and pathogen resistance is a change in phenolic content (Dixon and Paiva 1995). Phenolic synthesis is initiated quickly as a response to various biotic and abiotic stresses (Graham and

Graham 1996). Phenolic compounds comprise a diverse group of so called secondary metabolites. They have a large range of structures and functions. Phenolics can be classified into water-soluble phenolic acids, phenylpropanoids, flavonoids and quinones and water-insoluble condensed tannins, lignins and cell-wall bound hydroxycinammic acids. They are considered as the most important and numerous group of compounds in the plant kingdom (Naczk and Shahidi 2004).

Polyphenols possess the ideal structure for free radical scavenging activity. They have been shown to be more effective antioxidants than tocopherols or ascorbate. Antioxidative properties of polyphenols arise from their high activity as hydrogen or electron donors and the ability to chelate transition metal ions (Rice-Evans *et al.* 1997). Another mechanism underlying the antioxidative properties of phenolics is the ability of flavonoids to alter peroxidation kinetics by a modification of the lipid packing order and to decrease the fluidity of the membranes (Arora *et al.* 2000). Moreover it has recently been shown that phenolics can be involved in hydrogen peroxide scavenging cascade in plant cells (Takahama and Oniki 1997). Phenolics, together with other compounds possessing redox properties, belong to so called low molecular antioxidants (LMA). They accompany bigger molecules of enzymatic antioxidants like peroxidases, catalases, and superoxide dismutases. In higher plants the first group includes glutathione, ascorbate, tocopherol, proline, betaine, and others, which are also information-rich redox buffers and important redox signaling components, interacting with biomembrane-related compartments (Sanchez-Moreno 2002). An evolutionary consequence of aerobic life for higher plants, reactive oxygen species (ROS) are formed by partial reduction of molecular oxygen. The above enzymatic and non-enzymatic antioxidants in higher plants can protect their cells from oxidative damage by scavenging ROS. They provide essential information on cellular redox state and regulate gene expression associated with biotic and abiotic stress responses to optimize defense and survival. Special attention is given to ROS and ROS-antioxidant interaction as a metabolic interface for different types of signals derived from metabolism and from the changing environment, which regulates the appropriate induction of acclimation processes or execution of cell death programs, which are the two essential directions for higher plants (Hong-bo *et al.* 2008).

The aim of the study was to investigate whether redox status of leaves as well as phenolic content in leaves and crowns of winter rye plants determine their snow mould resistance. In the study six breeding lines and one cultivar ‘Stach’ of winter rye differing in the degree of snow mould resistance were used.

Material and Methods

Plant material and growth conditions. Winter rye genotypes (cv. 'Stach' and six inbred lines nos. 1, 2, 3 5, 22, 23) were received from the Institute of Plant Breeding and Acclimatization in Radzików (Poland). The seeds were sown to pots containing a mixture of soil and sand (3 : 1 v/v) and cultured for six weeks in a growth chamber at 18°C (day/night) at 10 h photoperiod with a light intensity of 250 μmol m^{-2} s^{-1} PPFD. Next the plants were pre-hardened for two weeks at 12ºC at 10 h photoperiod with a light intensity of 250 μmol m^{-2} s^{-1} PPFD. Subsequently, the plants were cold acclimated for three weeks at 2ºC at 8 h photoperiod with a light intensity of 150 μmol m^{-2} s^{-1} PPFD.

Microdochium nivale inoculation. Inoculum was prepared according to the method described by Prończuk and Prończuk (1987). Mycelium of the isolate of *M. nivale* no. 38z/5a/01 collected from rye in 2001 was kindly provided by Prof. Maria Prończuk from Plant Breeding and Acclimatization Institute, Radzików, Poland. Mycelium was grown on the Potato Dextrose Agar (PDA) medium (Sigma-Aldrich) for 7 days at 19°C in darkness. Then the mycelium was transferred to sterile mixture of soil, peat, and sand (3/1;v/v), containing 5% w/w of ground wheat seeds, and was cultured for 14 days under the same light/temperature conditions.

Half of the cold hardened seedlings were inoculated according to Prończuk *et al.* (2003). The mycelium was gently mixed and spread on the soil of each plant. Each pot was covered with moistened blotting paper and black plastic foil to imitate the conditions under the snow cover and kept for 5 weeks at 2°C in darkness.

Estimation of plant Average Regrowth Index (ARI) after M. nivale inoculation. At the end of 5 weeks of incubation at 2°C in darkness, the blotting paper and foil were removed and then leaves were cut and allowed to regrow for 3 days at 12°C in 100 μmol m^{-2} s^{-1} PPFD light and next for 7 days at 18°C in 250 μmol m^{-2} s^{-1} PPFD light. Plant regrowth was evaluated using the visual rating scale (0-5) where '0' denotes a plant without any visible symptoms of infection and '5' – a completely dead plant with no signs of leaf elongation.

The Average Regrowth Index (ARI) was calculated from these ratings according to the formula: $[(n \times 0) + (n \times 1) + \ldots\ldots + (n \times 5)] \times N^{-1}$, where: n – number of plants corresponding to each disease rating (0-5), N – total observations.

ARI was calculated in 5 replicates as an average from five pots (25 plants in a pot, each pot = one replicate). Decreased values of ARI mean an increase in plant resistance to the pathogen.

Phenolic estimation. Samples of leaves and crowns (250 mg) were collected from both infected and non-infected seedlings 1, 5, 9, and 13 days after inoculation. The samples were quickly frozen and kept at -80ºC until the analyses. They were boiled in 1 cm^3 of 80% ethanol, then homogenised in 2 cm^3 of 80% ethanol

and centrifuged at 2500 × *g* for 20 min. The supernatant was mixed with 25% Na_2CO_3 and Folin-Ciocalteau reagent (Singleton and Rossi 1965). The absorbance of samples was measured at λ=760 nm using spectrophotometer Ultrospec 2100 pro (Biosciences Amersham, Sweden). Total phenolic content was calculated as milligrams of chlorogenic acid per 1 g of fresh weight (FW). This procedure allows to estimate the level of water soluble phenolic compounds with aromatic ring and with at least one hydroxyl group.

Total antioxidant determination in leaf tissues. Samples of the leaves (250 mg) were collected from control and inoculated plants 1, 5, 9, and 13 days after inoculation. Plant material was freeze-dried and samples were ground with ball mill MM400 (Retsch, Germany) in Eppendorf vials, to which 1 cm^3 of 50% ethanol was then added and shaken for two hours at room temperature. The extracts were then centrifuged for 20 min in a refrigerated centrifuge at 18000 × g (MPW-350R, Poland) and the supernatant was used for the measurements. The total content of antioxidants (free radical-scavenging activity) in the leaf tissues was measured by DPPH method according to Brand-Williams *et al.* (1995) with some modifications adapting the protocol to 96-well microtitre plates and to the measurement of absorbance by microtitre plate reader**.** Next 0.5 mM solution of stable free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH, SIGMA) in methanol was used. The absorbance was determined after 30 min of reaction at 37°C at λ = 515 nm using reader Model 680 (Bio-Rad Laboratories, USA). The results were expressed as μmoles of Trolox equivalents per 1 g F.W. For each treatment ten measurements were made on five independent samples each collected from two different plants.

Statistical analysis

All effects of infection on the phenolic and low molecular antioxidant content in rye seedlings were tested with *F*-test (ANOVA/MANOVA). The results obtained were analysed using Duncan's multiple range test at $p<0.05$. The Shapiro-Wilk test was used to estimate if the resulting data of regrowth after *M. nivale* inoculation had parametric distribution. Differences in regrowth were estimated using the non-parametric Friedman test. All statistical analyses were conducted using STATISTICA 9.0 package.

Results and discussion

Susceptibility to snow mould

The values of ARI coefficient defining susceptibility level of breeding lines and cv. 'Stach' to pathogen *M. nivale* are presented in Table 1. Genotypes of winter rye under study can be classified into groups of more susceptible (lines 5, 22 and 23) and more resistant plants (lines 1, 2, 4 and cv. 'Stach'). Mean value of ARI for the susceptible group amounts to 3.86 ± 0.20, while for the resistant one it is equal to 2.21 ± 0.19.

Table 1. Values of the ARI (Average Regrowth Index) coefficient of resistance to *M. nivale* for winter rye breeding lines and cv. 'Stach'. R – lines more resistant to *M nivale* infection; S – lines more susceptible to *M. nivale* infection. Mean values ± SE

Breeding line or cultivar	ARI	Resistance label
1	2.18 ± 0.42	R
2	2.20 ± 0.44	R
4	2.06 ± 0.48	R
5	4.29 ± 0.32	S
22	3.15 ± 0.40	S
23	4.13 ± 0.30	S
'Stach'	2.42 ± 0.21	R

Changes in phenolic concentration

Analysis of variance showed significant influence of genotype and the stage of pathogenesis – from the moment of inoculation to the day of analysis – on phenolic level in leaves and crowns.

In the leaves of all breeding lines and cv. 'Stach' phenolic content was 2 to 3 times greater as compared to the crowns (Table 2). This result is surprising as crowns are the main gate for pathogenic fungus *M. nivale* attack. However, a reverse interpretation is also possible. Crowns contain more meristematic tissues, mainly lateral shoot buds, as compared to leaves, whose blades, with the exception of narrow strip in the lowest leaf part, are composed of stable, mature cells. Meristematic cells are characterized by a greater share of cytoplasm, nucleus, and cell organelle in whole cell volume and, moreover, their cell wall is thinner. Taking these facts into account, it could be assumed that meristematic tissues are of special importance for pathogenic fungus as the source of nutrients. These are synthesized in leaves but their distribution between leaves and crown could be an important element of plant defence strategy (Pociecha *et al.* 2008, 2010). Moreover, plant inoculation with snow mould takes place in the cold, which increases phenolic compounds and cell wall lignin synthesis. Thereby, they play an important role in the development of the plant's hypersensitive response (Lamb and Dixon 1997).

Table 3 presents mean phenolic content in leaves and crowns of both groups: susceptible and resistant to *M. nivale*. In the leaves of both control and infected seedlings belonging to the resistant group, phenolic content was greater as compared to the susceptible plants. Infection of seedlings did not significantly change phenolic content either in more or less susceptible plants. It means that genetically determined greater phenolic content in the leaves is decisive for resistance to the studied pathogen. No relationship between changes in phenolic content and the degree of resistance to *M. nivale* was found.

Table 2. Changes in phenolic content (mg g^{-1} FW) in the leaves and crowns of breeding lines and cv. 'Stach' of winter rye 1, 5, 9 and 13 days after seedling inoculation with *M. nivale*. Values marked with the same letter within each breeding line or cultivar do not differ statistically according to multiple range Duncan's test ($p<0.05$)

Line	Days	Leaves		Crowns	
		Control	Infected	Control	Infected
1	1	0.77 bc	0.75 bcd	0.3 f	0.27 f
	5	0.73 cd	0.7 d	0.32 f	0.32 f
	9	0.84 a	0.79 ab	0.38 e	0.4 e
	13	0.85 a	0.84 a	0.4 e	0.39 e
2	1	0.83 d	0.9 cd	0.25 g	0.17 h
	5	0.84 cd	0.88 cd	0.45 e	0.27 fg
	9	1.14 a	1.03 b	0.3 fg	0.26 g
	13	0.93 c	0.82 d	0.35 f	0.28 fg
4	1	0.71 cd	0.69 cd	0.23 g	0.26 g
	5	0.68 cd	0.6 d	0.24 g	0.3 fg
	9	0.7 cd	0.75 c	0.37 ef	0.4 ef
	13	0.95 b	1.13 a	0.43 e	0.41 e
5	1	0.86 bc	0.84 bcd	0.29 g	0.26 g
	5	0.88 ab	0.79 cd	0.29 g	0.34 efg
	9	0.96 a	0.77 d	0.34 fg	0.43 e
	13	0.9 ab	0.9 ab	0.35 efg	0.41 ef
22	1	0.73 ab	0.66 ab	0.35 de	0.32 de
	5	0.62 b	0.62 b	0.29 e	0.37 cde
	9	0.75 a	0.68 ab	0.4 cd	0.42 cd
	13	0.63 b	0.7 ab	0.46 c	0.42 cd
23	1	0.58 d	0.66 cd	0.3 ef	0.26 f
	5	0.59 d	0.58 d	0.31 ef	0.31 ef
	9	0.62 cd	0.74 ab	0.33 ef	0.37 e
	13	0.68 bc	0.77 a	0.37 e	0.37 e
'Stach'	1	0.79 bc	0.77 c	0.34 defg	0.32 efg
	5	0.76 bc	0.81 bc	0.27 g	0.31 fg
	9	0.89 b	0.85 bc	0.4 def	0.46 d
	13	0.91 ab	0.82 bc	0.43 de	0.4 def

Table 3. Mean phenolic content (mg g^{-1} FW) in leaves and crowns of two groups of winter rye lines susceptible (S) and resistant (R) to *M. nivale* 1, 5, 9 and 13 days after inoculation

Days after inoculation	Control (C)		Infected (I)	
	Group S	Group R	Group S	Group R
Leaves				
1	0.723	0.775	0.720	0.778
5	0.697	0.753	0.663	0.748
9	0.777	0.893	0.730	0.855
13	0.737	0.903	0.790	0.873
Crowns				
1	0.313	0.280	0.280	0.255
5	0.297	0.320	0.340	0.300
9	0.357	0.363	0.407	0.380
13	0.393	0.403	0.400	0.370

The results obtained in studies conducted on plants grown under abiotic and biotic stress are usually difficult to interpret due to combined plant reaction to both stresses. We attempted to separate the influence of darkness, high humidity, and cold from fungus infection. The results were obtained by subtracting the value of phenolic content estimated for the first day after the inoculation from the values for consecutive days (Table 4). This calculation was performed for leaves and crowns of both groups: resistant (R) and susceptible (S) to *M. nivale*. It was assumed that changes of mean values of phenolic content in susceptible breeding lines (lines 5, 22 and 23) as well as the resistant ones (cv. 'Stach' and lines 1, 2 and 4) which took place between the 1st and 9th day of pathogenesis (measured in control plants) illustrate their reaction to specific growth conditions, which are necessary to the development of the infection (darkness, cold, high air humidity). Furthermore, it was assumed that changes in phenolic content in seedlings of inoculated plants (measured in inoculated plants), demonstrated combined effects of the stress of growth conditions and stress resulting from pathogenesis. Thus the difference of these changes between control and inoculated plants describes the effects on phenolic content of the stress of snow mould pathogen infection itself without the effect of growth conditions stress.

In the leaves of control plants of R group, growth conditions induced an increase of phenolic content, while in S group nonspecific and significantly weaker changes of the compounds took place. The infection with snow mould fungus resulted in both more and less sensitive plants in a small decrease of phenolic content. In the crowns of control plants the abiotic stresses resulted in an increase of phenolic content, which was higher in more resistant lines than in the sensitive ones. Similarly, inoculation with the fungus followed by the development of pathogenesis resulted in a small increase of phenolic content.

Table 4. Fluctuations in phenolic content in the leaves and crowns of winter rye lines susceptible (S) and resistant (R) to *M. nivale*, influenced by plant growth conditions (in control plants) and by infection process (in inoculated plants)

Days after inoculation	The influence of growth conditions		The influence of *M. nivale* infection	
	Group S	Group R	Group S	Group R
Leaves				
5	↓ (0.026)	↓ (0.022)	↓ (0.031)	↓ (0.008)
9	↑ (0.054)	↑ (0.118)	↓ (0.044)	↓ (0.041)
13	↑ (0.014)	↑ (0.128)	↑ (0.056)	↓ (0.033)
Crowns				
5	(0.016)	↑ (0.040)	↑ (0.076)	↑ (0.005)
9	↑ (0.044)	↑ (0.083)	↑ (0.083)	↑ (0.042)
13	↑ (0.080)	↑ (0.123)	↑ (0.040)	↓ (0.008)

Calculations:

1. The influence of plant growth conditions (darkness, low temperature, high air humidity) on phenolic content in control plants was calculated through subtraction of phenolics content on the 1st day from phenolics content 5, 9 or 13 days after inoculation (data in Table 3), separately in S and R plant groups.
2. The influence solely of fungal infection was calculated separately for S and R plant groups according to the formula: $(I_t - I_1) - (C_t - C_1)$, where t = 5, 9 or 13 (data in Table 3).

Changes in low molecular antioxidants (LMA) content in leaves

Table 5 presents LMA content, while Table 6 shows mean values for both R and S groups of the breeding lines studied. Generally, growth conditions applied during inoculation procedure did not influence LMA content in the control seedlings. Only in plants of line 1, 2 and cv. 'Stach' LMA amount increased during 13 days of the inoculation (Table 5). In infected plants LMA level decreased during pathogenesis in lines 1, 2, 5 and 22, while it did not change in cv. 'Stach'. In both control and infected plants mean LMA content was lower in the lines belonging to R group compared to S group, though at the last day of the analyses (13th day of pathogenesis) a strong decrease of antioxidant content took place in both groups independently of their pathogen susceptibility level (Table 6).

Similarly to phenolic content analysis (Table 4), Table 7 presents an attempt to separate the influence of plant growth conditions from the influence of fungal infection on the dynamics in the changes of LMA. The specific stresses accompanying plant growth conditions necessary for successful fungus inoculation caused an increase in antioxidant content, though in the resistant group this increase remained till the last day of the analysis, while in more susceptible lines a marked decrease of LMA level was observed on the 13th day of the analysis.

The snow mould fungus infection process, separated from the influence of plant growth conditions stress, resulted in the seedlings of both more and less sensitive lines in a decrease of antioxidant content. The observed decline was particularly noticeable in the more resistant lines. This result can be connected with different susceptibility to infection of the breeding lines studied.

Table 5. The changes in low molecular antioxidant content expressed as Trolox equivalent [µmol Trolox g^{-1} DW] in leaves of winter rye breeding lines and cv. 'Stach' 1, 5, 9 and 13 days after seedling inoculation with *M. nivale*. Values marked with the same letter within each breeding line and cultivar do not differ statistically according to multiple range Duncan's test ($p<0.05$)

Line	Days after inoculation	Control	Infected
1	1	23.55 c	29.54 b
	5	24.54 c	28.40 b
	9	29.77 b	27.85 b
	13	34.17 a	24.13 c
2	1	31.88 b	31.30 b
	5	31.66 b	33.04 ab
	9	36.58 a	31.89 b
	13	23.93 c	21.15 c
4	1	30.33 ab	21.41 c
	5	31.14 a	27.49 b
	9	29.10 ab	29.60 ab
	13	31.91 a	23.36 c
5	1	33.98 cd	41.47 a
	5	38.67 abc	35.69 bc
	9	39.61 ab	35.49 bc
	13	33.72 cd	29.64 d
22	1	34.10 ab	32.07 bc
	5	31.33 bc	32.54 abc
	9	32.50 abc	35.84 a
	13	28.99 cd	27.16 d
23	1	24.68 cd	28.97 b
	5	26.77 bcd	27.53 bc
	9	29.76 b	35.47 a
	13	24.02 d	28.76 b
„Stach"	1	25.80 b	39.93 a
	5	32.84 a	33.47 a
	9	32.93 a	39.64 a
	13	34.17 a	31.42 a

Table 6. Mean content of low molecular antioxidant content expressed as Trolox equivalent [µmol Trolox g^{-1} DW] in leaves for two groups of winter rye lines susceptible (S) and resistant (R) to *M. nivale* 1, 5, 9 and 13 days after inoculation

Days after inoculation	Control (C)		Infected (I)	
	Group S	Group R	Group S	Group R
1	30.92	27.89	34.17	30.55
5	32.26	30.05	31.92	30.60
9	33.96	32.10	35.60	31.25
13	28.91	31.05	28.52	25.02

It is possible that the resistant lines utilized more of their antioxidants to decompose (inactivate) molecules of ROS secreted by pathogenic fungi in the course of the pathogenesis, which supports the hypothesis about the protective role of these compounds against pathogen attack. Our results related to LMA content in control plants grown only under abiotic stresses are similar to those presented by other authors who have shown an increase in LMA under abiotic stresses. An increase in antioxidant pools in maize seedlings under short-term osmotic stress was observed by Kolarovic *et al.* (2009). Kellos *et al.* (2008) found that not only osmotic stress but also treatment with salicylic acid or hydrogen peroxide increased the level of low molecular antioxidants, especially in the stress tolerant maize genotype. Guo *et al.* (2006) also reported that antioxidant content decreased greatly in drought-sensitive cultivars under drought stress and in chilling-sensitive cultivars under chilling stress in contrast to resistant cultivars.

Table 7. Fluctuations in phenolics content in the leaves of winter rye lines susceptible (S) and resistant (R) to *M. nivale*, influenced by plant growth conditions (in control plants) and by infection process (in inoculated plants)

Days after inoculation	The influence of growth conditions		The influence of *M. nivale* infection	
	Group S	Group R	Group S	Group R
Leaves				
5	↑ (1.34)	↑ (2.16)	↓ (3.59)	↓ (2.11)
9	↑ (3.04)	↑ (4.21)	↓ (1.61)	↓ (3.65)
13	↓ (2.10)	↑ (3.16)	↓ (3.63)	↓ (8.69)

Calculations as described in Table 4.

A small but statistically significant negative correlation ($r = -0.245$; $p<0.05$) between ARI index and phenolic content in the leaves was found. This fact indicates that greater phenolic amount is accompanied by higher resistance to the pathogen in breeding lines of rye. A correlation between ARI and leaf antioxidant content was found ($r = 0.306$; $p<0.05$). Taking in account the fact that a higher value of ARI indicates higher elevated susceptibility of seedlings to fungal infection, it could be assumed that greater antioxidant amount is accompanied by higher pathogen sensitivity. Significant though not excessively high correlation ($r = 0.264$; $p<0.05$) between antioxidant and phenolic content in leaves was also found. This correlation seems to arise from the fact that phenolics belong to low molecular antioxidants. This correlation coefficient is relatively small, which may indicate that phenolics may react to infection somewhat differently from other antioxidative molecules and furthermore, the share of phenolics in total antioxidant pool may vary in particular winter rye lines studied. It is worth mentioning that Javanmardia (2003) found a strong positive relationship between the antioxidant activity and total phenolic acids content in the tested basil accessions. However, this specific

plant material is characterized by the dominance of phenolics (concentration 40-times higher as compared to rye seedlings) and relatively small amount of other antioxidants (total antioxidants amount similar to those observed in rye seedlings). This proves that rye plant material is relatively more abundant in antioxidants other than phenolics. This is confirmed by the finding that in winter rye resistance is linked to defence response expressed by higher activity of such antioxidant enzymes as catalase, superoxide dismutase, and non-specific peroxidase (Pociecha *et al.* 2013).

References

Arora A, Byrem TM, Nair MG, Strasburg GM (2000) Modulation of liposomal membrane fluidity by flavonoids and isoflavonoids. Archives of Biochem and Biophis 373: 102–109

Bojarczuk M., Bojarczuk J., Krel E. 1990. Efficiency of breeding diploid winter rye population resistant to snow muld (*Fusarium nivale*/Fr./Ces.). Hd Rośl Aklim Nas 34: 37–46

Brand-Williams W, Cuvelier ME, Berset C (1995) Use of a free-radical method to evaluate antioxidant activity. Food Sci. Technol.-Lebensm.-Wiss. Technol. 28: 25–30

Dixon RA, Paiva NL (1995) Stress-induced phenylpropanoid metabolism. Plant Cell 7:1085– 1097

Guo Z, Ou W, Lu S, Zhong Q (2006) Differential responses of antioxidative system to chilling and drought in four rice cultivars differing in sensitivity. Plant Phys Bioch 44: 828–836

Hong-Bo S, Li-Ye C, Ming-An S, Jaleel CA, Hong-Mei M (2008) Higher plant antioxidants and redox signaling under environmental stresses. C R Biol 331 (6):433-441

Javanmardia J, Stushnoff C, Locke E, Vivanco JM (2003) Antioxidant activity and total phenolic content of Iranian Ocimum accessions. Food Chemistry 83: 547–550

Kellos T, Timar I, Szilagyi V, Szalai G, Galiba G, Kocsy G (2008) Stress hormones and abiotic stresses have different effects on antioxidants in maize lines with different sensitivity. Plant Biol 10 (5):563-572

Kolarovic L, Valentovic P, Luxova M, Gasparikova O (2009) Changes in antioxidants and cell damage in heterotrophic maize seedlings differing in drought sensitivity after exposure to short-term osmotic stress. Plant Growth Regulation 59 (1):21-26

Lamb C, Dixon RA (1997) The oxidative burst in plant disease resistance. Annu Rev Plant Physiol Plant Mol Biol 48: 251–75

Naczk M, Shahidi F (2004) Extraction and analysis of phenolics in food. J Chromat 1054: 95–111.

Pociecha E, Płażek A, Janowiak F, Dubert F, Kolasińska I, Irla M (2013) Factors contributing to enhanced pink snow mould resistance of winter rye (*Secale cereale* L.) - pivotal role of crowns. Physiol Molec Plant Pathol 81: 54–63

Pociecha E, Płażek A, Rapacz M, Niemczyk E, Zwierzykowski Z (2010) Photosynthetic activity and soluble carbohydrate content induced by the cold acclimation affect frost tolerance and resistance to *Microdochium nivale* of androgenic festulolium genotypes. J Agron & Crop Sci 196: 48–54.

Prończuk M, Madej L, Kolasińska I (2003) Research for resistance to *Microdochium nivale* among inbred lines of rye. Plant Breed Seed Sci 48:83–86 .

Prończuk M, Prończuk S (1987) Przydatność „metody chłodniowej" w ocenie odporności życicy trwałej na *Fusarium nivale* (Fr) Ces. Biul IHAR 162: 27–32

Rice-Evans CA, Miller NJ, Paganga G (1997) Antioxidant properties of phenolic compounds. Trends in Plant Sci 2: 152–159

Sanchez-Moreno C (2002) Review: Methods used to evaluate the free radical scavenging activity in foods and biological systems. Food Science and Technology International 8 (3):121-137

Schlegel R (2010) Plant Breeding. Updates Version 11.10. New Delhi, Mumbai, Chennai, Kolkata, Bangalore: BOD, Viva BVooks Private Limited, 2010

Singleton VS, Rossi JA Jr (1965) Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagent. Amer J Enol Viticult 16: 144-157

Smith JD (1981) Snow molds of winter cereals: guide for diagnosis, culture, and pathogenicity. Canad J Plant Pathol 3:15–25

Takahama U, Oniki T (1997) A peroxide/phenolics/ascorbate system can scavenge hydrogen peroxide in plant cells. Physiol Plant 101: 845–852

The Editors

Maciej T. Grzesiak is a Research Associate at the Department of Ecophysiology at the Institute of Plant Physiology, Polish Academy of Sciences in Cracow. He graduated in chemistry at the Jagiellonian University in Cracow in 1999. He received the Ph.D. degree in plant physiology from Agricultural University in Cracow in 2005. In the years 2007-2008 he worked as a post-doc at the Department of Renewable Resources, University of Alberta, Canada. His scientific interests focus on issues concerning different aspects of the influence of water stress (drought or waterlogging) and soil compaction on physiological and biochemical processes in different crop species, application of modern analytical techniques and their use in evaluating the origins of variation in response of crop plants to environmental stresses. His research also examines the significance of root system structure of maize and triticale in relation to abiotic stress tolerance.

Andrzej Rzepka is a Professor of Biology at the Pedagogical University of Cracow. He graduated in Biology at the College of Education in Cracow in 1980. He received his Ph.D. in 1989 and habilitation in 2009 in the biological sciences at the Pedagogical Academy in Cracow. Now he is the director of the Institute of Biology at the Pedagogical University of Cracow. He gives lectures in plant physiology for bachelor and undergraduate students and teaches seminars for graduate and Ph.D. students. His research focuses on the response of lower and higher plants to abiotic stresses. In the case of lower plants, he examines changes in metabolic activity in response to drought, light, elevation of carbon dioxide, and hypoxia stresses. In the case of higher plants, he investigates the effects of ozone, herbicides, allelopathic compounds, heavy metals, and salinity on plant growth and development He conducts research on the mechanisms of acclimation and adaptation of plants to stressful conditions.

Tomasz Hura is an Associate Professor of plant physiology at the Institute of Plant Physiology, Polish Academy of Sciences in Cracow. He graduated in chemistry at the Jagiellonian University in Cracow in 1997. He received his Ph.D. in 2002 and habilitation in 2012 in plant physiology from Agricultural University in Cracow. In the years 2004-05 he was holder of EU project CropStress fellowship at the Plant Breeding Institute, University of Hohenheim, Stuttgart, Germany. His research interests focus on molecular, biochemical, physiological, and morphological adaptation of plants to drought stress. His recent research examines the role of phenolic compounds in the mechanism of drought tolerance in triticale and the application of chlorophyll fluorescence parameters and chlorophyll emission and absorption spectra in the response of crop plants to soil drought.

Stanisław Grzesiak is a Professor at the Institute of Plant Physiology, Polish Academy of Sciences in Cracow. He graduated in biology at the Faculty of Biology and Earth Sciences, Jagiellonian University in Cracow in 1971. He received the Ph.D. degree in 1978 and habilitation in 1991 in plant physiology from Agricultural University in Cracow. In the year 2002 he received the title of Professor from the President of Poland. In the year 1979 he worked as a post-doc at the Agriculture Canada, Research Center, Lethbridge, Alberta, Canada. He also worked as a visiting professor at the Missouri State University, Columbia, MO, USA (1984-85) and Agricultural University, Nagoya, Japan (1994-95). Professor Grzesiak has worked on the responses of crop plants to air gaseous pollution (SO_2, O_3), water stress, and soil compaction. His research also focuses on physiological aspects of the variations in stress susceptibility indexes between and within plant species or varieties under drought, flooding, and soil compaction stresses.

Plant-stress conferences in Cracow aiming at the popularization of the knowledge of plant stress physiology, presenting state-of-the-art research, and providing the possibly to exchange opinions and ideas as well as initiate new scientific projects.

International Conferences „Plant Functioning Under Environmental Stress" are organized by the F. Górski Institute of Plant Physiology of the Polish Academy of Sciences in Cracow under auspices of the Polish Botanical Society and Committee of Physiology, Genetic and Breeding of Plant, Polish Academy of Sciences in co-operation with Slovak Agricultural University in Nitra, Plant Protection Institute, Hungarian Academy of Sciences, Pedagogical University in Cracow, University of Life Sciences (SGGW) in Warsaw, and Agricultural University in Cracow.

The conference in 2012 had 176 participants including 47 from abroad (Australia, Canada, Denmark, England, Germany, Hungary, Iran, Pakistan, Portugal, Russia, Slovakia, Turkey, Ukraine). Eight plenary lectures were delivered, and 35 oral and 134 poster presentations were given (abstracts are available at the APP website at www.springer.com).

ISBN 978-83-86878-30-7