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Physiological Effects of Winter Rape (*Brassica napus* var. *oleifera*) Prehardening to Frost. II. Growth, Energy Partitioning and Water Status during Cold Acclimation

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With 4 figures and 2 tables

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Abstract

Prehardening of winter rape, i.e. its early growth in a reduced day temperature (+12°C) from emergence to the beginning of cold acclimation at chilling temperatures (the 1st stage of cold acclimation), has a beneficial influence on frost resistance. In earlier studies it has been demonstrated that during prehardening, plants formed leaf rosettes and increased the photosynthetic efficiency at chilling temperatures. In the present study investigations were carried out on the effect of prehardening on the growth rate of the plants during the 1st stage of cold acclimation, and the progress of selected physiological processes occurring during this stage and resulting in increased frost resistance. It has been demonstrated that a greater inflow of photoassimilates during the 1st stage of cold acclimation results in greater increments of the dry mass of prehardened plants. These plants show also a distinct inhibition both of the elongation growth and the rate of expanding new leaves. The acquired energy is thus spent to a greater extent on the processes associated with increasing frost resistance, and not on growth. In prehardened seedlings, during the 1st stage of cold acclimation, there have been observed in leaves a higher rate of both water content decrease and drop in the osmotic potential of the cell sap and water potential in the tissues. Also an increased accumulation of soluble sugars and free proline was noticed. However, the beginning of these processes was not observed during the prehardening period. Prehardening stimulated the effectiveness of the 1st stage indirectly through changes leading to the increased amount of available energy and enabling the utilisation of the greater part of acquired energy in the cold acclimation process.

Key words: Cold acclimation — frost resistance — growth rate — proline — soluble sugars — water management

Abbreviations:

CA — 1st stage of cold acclimation (frost hardening) at +2°C; LT₅₀ — temperature at which 50 % of the

plants had been frost-killed; NPH — non-pre-hardened seedlings, seedlings grown from sprouting till the beginning of CA at 20/12°C (day/night); PH seedlings — prehardened seedlings, seedlings grown from sprouting till the beginning of CA at 12/20°C (day/night); ψ_{π} — osmotic potential; ψ_p — pressure potential (turgor); ψ_w — water potential.

Introduction

In earlier studies (Rapacz 1998a,b, Rapacz and Janowiak 1998) some physiological changes induced in winter rape by the process termed 'prehardening' were described. This process observed under field conditions in autumn may be performed under controlled conditions if the plant's growth is already from the sprouting phase at day temperatures, in the range of 10–15°C. Prehardened winter rape formed leaf rosettes, which may slightly increase its frost tolerance in a non-hardened and hardened state (Rapacz 1998a). This effect is similar to the observation that in winter rape, growth retardants, which inhibit hypocotyl elongation and make the seedlings form leaf rosettes, enhance also their frost tolerance (Kacperska-Palacz and Długołęcka 1971, 1972, Morrison and Andrews 1992).

On the other hand, the effectiveness of the 1st stage of cold acclimation (CA) (Kacperska-Palacz 1978) is greatly increased mainly by higher effectiveness of the photosynthetic apparatus during CA (Rapacz 1998a,b, Rapacz and Janowiak 1998). High photosynthesis thus increases the inflow of energy; under conditions of CA this is necessary, but is not sufficient for good hardening of the plants. The acquired energy, unlike under the spring conditions, must not be spent on the plants' growth, but, first

of all, on several intracellular changes connected with the increase of frost resistance. It is suggested that the first stage of frost hardening of herbaceous plants is related to the cessation of growth (Kacperska-Palacz 1978, Levitt 1980). Inhibition of cell expansion brings about decreased tissue hydration (Levitt 1980). This may, in turn, limit the probability of intracellular freezing and increased frost tolerance even in the absence of other hardening factors (Kacperska-Palacz 1978). On the other hand, the cessation of growth in terms of dry matter accumulation may reflect the worst energy supply caused by low net photosynthesis. Thus, plants, which grow faster, are more resistant (Hurry and Huner 1991). This was considered for spring and winter forms of wheat. Nevertheless, no relationship was found between frost resistance degree of spring or winter cultivars of grains and dry matter increase under field conditions (Fowler and Carles 1979). Tumanov (1960) wrote that low temperatures during cold acclimation were necessary to inhibit the growth rate, which is needed for accumulation of soluble sugars. Likewise Griffith and McIntyre (1993) concluded that the interrelationship between growth and frost tolerance is a quantitative one. Frost tolerance is induced only by low temperature, but the development of frost tolerance is dependent upon both irradiance, which affects the amount of photoassimilates available, and the day length, which may affect the partitioning of photoassimilates between growth and frost tolerance. The aim of the present study was to demonstrate the effect of prehardening on energy partitioning between growth and development of frost resistance.

Material and Methods

Plant material

Winter rape (cv. 'Górczański') plants were prehardened and hardened in the conditions described earlier (Rapacz and Janowiak 1998).

Growth analysis

Relative Growth Rate (RGR) was calculated according to Kvet et al. (1971):

$$\text{RGR} = \frac{\ln W_2 - \ln W_1}{t_2 - t_1} \cdot 1000 \text{ [mg g}^{-1} \text{ day}^{-1}\text{]}$$

where W_1 is shoot dry mass on the first day of investigated period, W_2 is shoot dry mass on the last day of investigated period, and $t_2 - t_1$ is length of investigated period (days). In the calculations the dry mass of fallen leaves was also taken into account.

Water balance analysis

The water content in the seedlings was estimated after drying them to a constant mass at $+70^\circ\text{C}$. For water potential (ψ_w) and osmotic potential (ψ_π) determinations, an HR 33T dewpoint microvoltmeter (VESCOR) connected with C-52 Sample Chambers was used. For determination of ψ_w , leaf discs were collected from the youngest fully expanded leaf and perforated, always in the same way, before measurement. ψ_π was measured in cell sap obtained from the remaining part of the leaf, squeezed after freezing in liquid nitrogen. ψ_p was calculated by subtraction of ψ_π from ψ_w .

Soluble sugars and free proline determination

Soluble sugars were determined by the anthrone method (Ashwell 1975, with modifications). The plant material (always from the youngest, fully expanded leaf) was homogenised in liquid nitrogen and the soluble sugars were extracted for 15 min with boiling distilled water. After centrifugation at $2000 \times g$, 0.2% anthrone (Sigma) in sulphuric acid was added to the supernatant and the sugars were determined spectrophotometrically ($\lambda = 620 \text{ nm}$) after heating at $+90^\circ\text{C}$ for 15 min. Sugar concentration was calculated from linear regression equation obtained from the calibration curves (based on known concentration of glucose, Sigma).

Free proline was determined by the colorimetric method according to Bates et al. (1973). The plant material (always from the youngest, fully expanded leaf) was homogenised in liquid nitrogen and the free proline was extracted with 3% sulphosalicylic acid. After centrifugation at $2000 \times g$, free proline concentration was determined as described in the original paper.

Statistical analysis of results

Statistical significance of differences between treatments was evaluated by variance analysis in a completely randomised design using the Duncan Multiple Range Test.

Results

Growth

Prehardened seedlings have shown a distinct inhibition of growth during CA. No elongation of their stems was observed (Fig. 1) and the rate of expanding new leaves as well as the rate of falling of the oldest leaves was much slower than in NPH seedlings (Fig. 2). However, the recorded increments of their dry mass were higher and relatively stable during successive 2-week long periods of CA (Table 1). In non-hardened seedlings, on the other hand, the RGR values during CA were systematically reduced.

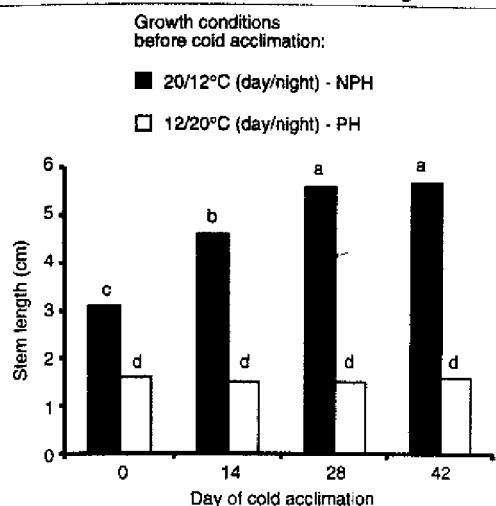


Fig. 1: Changes in stem length (sum of hypocotyl and epicotyl) of winter rape cv. 'Górczański' during cold acclimation at +2°C according to the preceding growth conditions. Values marked with the same letter are not significantly different ($P = 0.05$) based on the Duncan Multiple Range Test, $n = 7$ replications (plants)

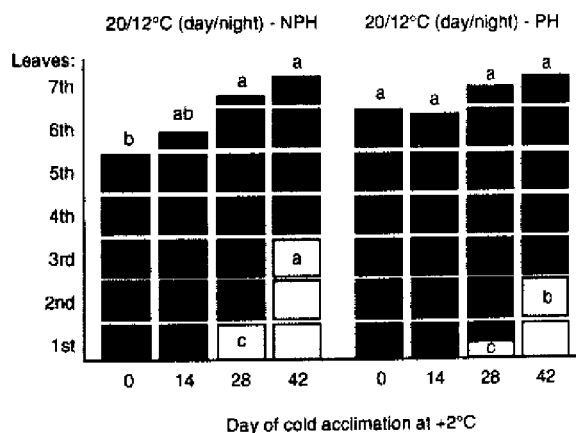


Fig. 2: The effect of growth conditions before cold acclimation on the development of winter rape leaves during cold acclimation at +2°C. The area of single rectangle represents the percentage of plants with the leaf fully developed (filled rectangles) or fallen (open rectangles). Values marked with the same letter are not significantly different ($P = 0.05$) based on the Duncan Multiple Range Test, $n = 30$ replications (plants)

Soluble sugars and free proline level

During the first 14 days of CA, in both PH and NPH seedlings, an increase in the content of soluble sugars and free proline was observed (Table 2). In PH seedlings this increase was markedly faster than in NPH ones. As a result, on the 14th day of CA, the content of soluble sugars in the seedlings was 50% higher and that of free proline 25% higher, while

Table 1: Relative Growth Rate (RGR)* of dry mass accumulation in shoots of winter rape cv. 'Górczański' seedlings during cold acclimation at +2°C, according to the preceding growth conditions

Successive days of cold	RGR [$\text{mg g}^{-1} \text{day}^{-1}$]	
	Thermal conditions before cold acclimation (day/night)	
	20/12°C (NPH)	12/20°C (PH)
1–14	21.3 ^b	27.2 ^a
14–28	16.0 ^c	23.2 ^b
28–42	13.0 ^d	27.0 ^a

* With regard to the mass of fallen leaves

Values marked with the same letter are not significantly different ($P = 0.05$) based on the Duncan Multiple Range Test, $n = 7$ replications (pots)

before CA the content of these substances was independent of the conditions of growth (Table 2).

Water status

Prehardening contributes very clearly to the changes in the water balance parameters during CA. A faster accumulation of osmotically active substances by PH seedlings during CA is manifested by a faster reduction of ψ_x (Fig. 3a). During CA no differences or significant changes in ψ_p in either PH or NPH seedlings were observed (Fig. 3c). As a result, the changes in ψ_w values (Fig. 3b) coincided closely with the changes in the values of ψ_x . Thus the reduction of the tissue hydration, observed during CA (Fig. 4) was not the result of changes in the value of water deficit, but was due to the accumulation of dry mass. The water content in the stem was more greatly reduced in PH seedlings (Fig. 4d), the difference being caused by variations in water content in leaves, both in blades (Fig. 4a) and petioles (Fig. 4b). Irrespective of the conditions of the initial growth, the stems contained similar amounts of water, undergoing a large progressive reduction during CA (Fig. 4c).

Discussion

It has been demonstrated so far that prehardening increases the resistance of winter rape plants through: (1) influence on the plant morphology (Rapacz 1998a) and (2) stimulation of the photosynthetic efficiency at CA temperatures (Rapacz 1998b, Rapacz and Janowiak 1998). The results

Table 2: Accumulation of separate soluble compounds in winter rape cv. 'Górczański' leaves during cold acclimation at +2°C according to the preceding growth conditions

Growth conditions before cold acclimation	Soluble sugars (mg glucose in 1 g of leaf fresh weight)		Free proline (µg in 1 g of leaf fresh weight)	
	Before CA	In 14 th day of CA	Before CA	In 14 th day of CA
20/12°C (day/night) — NPH	9.1 ^c	21.0 ^b	77.8 ^c	364.1 ^b
12/20°C (night/day) — PH	7.0 ^e	33.9 ^a	90.6 ^e	484.6 ^a

Values marked with the same letter are not significantly different ($P = 0.05$) based on the Duncan Multiple Range Test, $n = 7$ replications (pots)

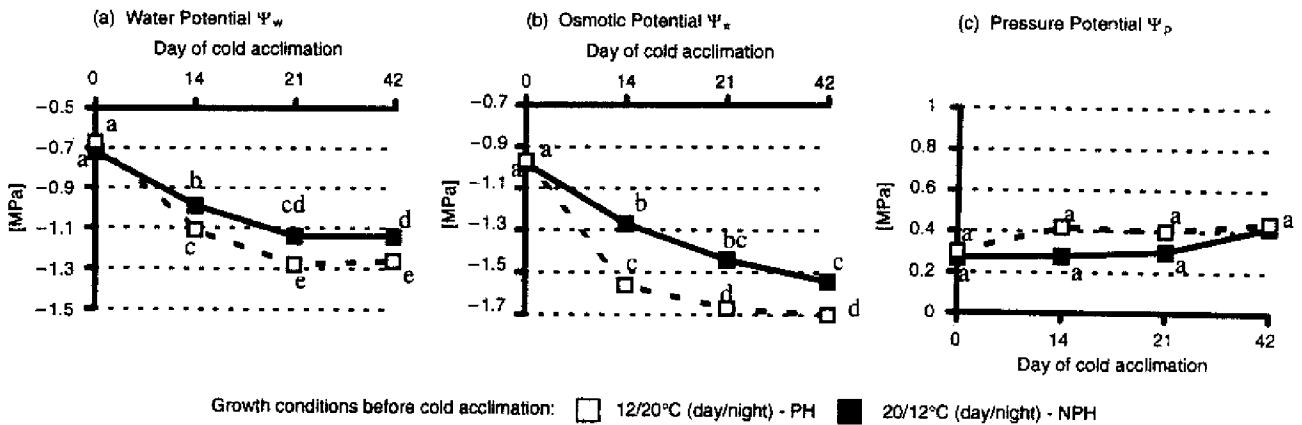


Fig. 3: Changes in water potential ψ_w , osmotic potential ψ_π and pressure potential (turgor) ψ_p in leaves of winter rape cv. 'Górczański' during cold acclimation at +2°C according to the preceding growth conditions. Values marked with the same letter are not significantly different ($P = 0.05$) based on the Duncan Multiple Range Test, $n = 7$ replications

obtained in the present study indicate, moreover, that prehardened plants are characterised not only by a better energy supply during the CA process, but also by a more rational utilisation of the acquired energy which is spent in greater part on the development of frost hardiness, and not on growth. Evidently the latter become inhibited. The stem shows no elongation (Fig. 1), and new leaves expand more slowly (Fig. 2). However, the RGR for the increment of dry mass observed during a 42-day-long CA was in these plants nearly 50% higher than in NPH seedlings (Table 1). The differences in RGR became greater in the successive, 2-week-long CA periods, as with differences in the efficiency of the photosynthetic apparatus and those in the degree of frost resistance (Rapacz and Janowiak 1998). This may be an indirect indication of a relation between intense photosynthesis during CA, increased dry mass accumulation and a greater increase in frost resistance. The same relation had been reported earlier by Hurry and Huner (1991). Considering the

inhibited elongating growth and expanding of new leaves in PH seedlings, the higher RGR values were associated with increased accumulation of dry mass in the earlier developed organs of the seedling. Consequently, during 42 days of hardening, in PH seedlings a much greater decrease in the water content in leaves could be observed (Fig. 4a,b). On the other hand, the stems of both PH and NPH seedlings accumulate dry mass to a similar degree (Fig. 4c). It is commonly regarded that the higher rate of water content decrease during CA indicates a higher degree of hardiness (Chen and Gusta 1978, Fowler and Carles 1979, Fowler et al. 1981). Reduced water content is connected with increased accumulation of osmotically active substances. In PH seedlings, during their CA, the values of ψ_π (Fig. 3a), and ψ_w (Fig. 3c) decreased faster and to the lower values. CA is always accompanied by a reduction of ψ_π in the cell sap, which is evidence of the reduction of ice formation (Levitt 1980). Chill induced changes in the water management may also activate mech-

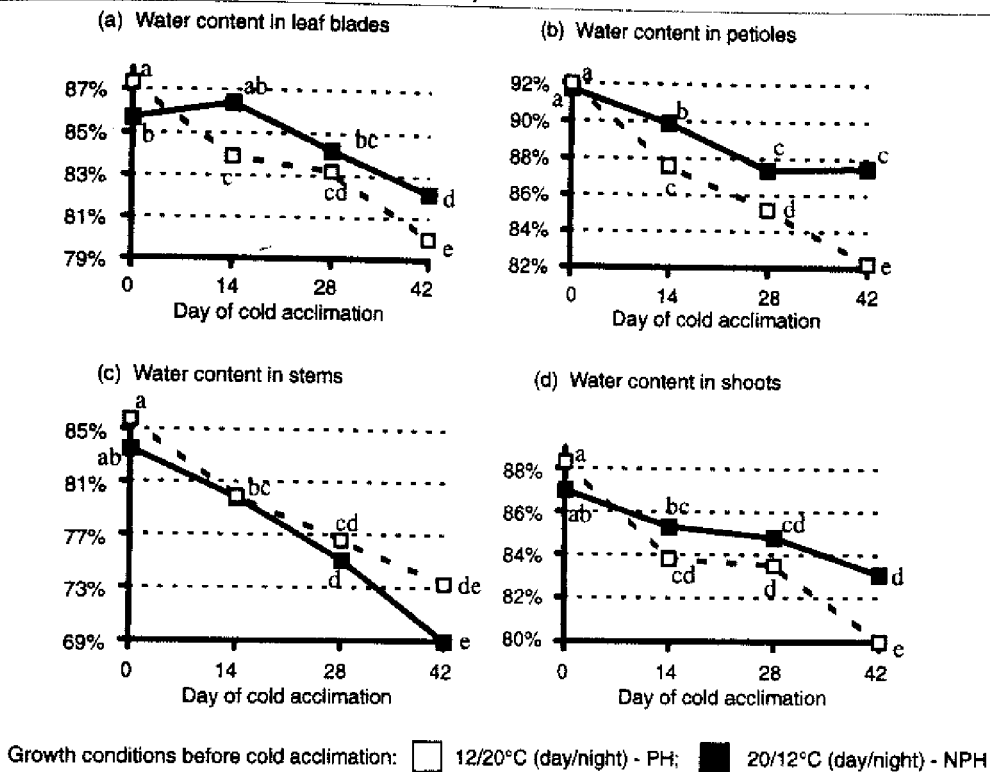


Fig. 4: Changes in water content in various parts of winter rape cv. 'Górczański' leaves during cold acclimation at +2°C according to the preceding growth conditions. Values marked with the same letter are not significantly different ($P = 0.05$) based on the Duncan Multiple Range Test, $n = 7$ replications

anisms responsible for the metabolic adjustment of the cells to functioning under reduced water potential (Kacperska 1993), thus protecting the cells against frost induced desiccation. The rate of ψ_{π} reduction during CA may be regarded as a specific marker of the degree of frost hardening (Dörffling et al. 1990, Tantau and Dörffling 1991).

PH seedlings accumulated more soluble sugars and free proline during CA (Table 2). The content of soluble sugars is directly associated with photosynthetic ability during CA (Tumanov 1940, Hurry et al. 1995) and it may be the physiological marker of the acclimation degree. Their level in the state of optimal acclimation is a good indicator of cultivar resistance (Akerman 1927, Fowler et al. 1981, Rybka 1993, Collins and Rhodes 1995, Hurry et al. 1995). The content of free proline may be another such marker (Paquin 1984, Dörffling et al. 1990, Alberdi et al. 1993). The aforementioned fine-particle substances, besides their non-specific functions connected with the reduction of the ψ_{π} , may perform also protective functions. Sugars may stabilise the cellular membranes during freezing (Rudolph and Crowe 1985), and protect them against the detrimental effects of dehydration

(Crowe et al. 1984). They may also (together with thermostable proteins) protect the enzymes against dehydration (Robertson et al. 1994). Proline may stabilise cellular membranes during freezing (Rudolph and Crowe 1985). It cannot be excluded, however, that during CA the increasing proline level is the result of decreasing ψ_{π} (Trotel et al. 1996).

So far, cold acclimation has been investigated on plants not subjected to prehardening. Prehardening of winter rape allows for on the other hand, a much higher frost resistance during CA than that observed in previous reports (Kacperska and Kulesza 1987, Andrews and Morrison 1992, Solecka and Kacperska 1993, Laroche et al. 1992). With similar duration of CA, LT_{50} of PH seedlings is more than 5°C lower than in the above quoted studies or in the NPH controls (Rapacz and Janowiak 1998). Hence, in PH seedlings it has been observed that the examined processes connected with CA were much more intense than it has been reported so far (Kacperska 1993).

All the biochemical and physiological changes connected with the frost hardening process, reported in the present study, were more intense in PH seedlings. This observation refers also to other

investigated changes, such as the ratio of fatty acid unsaturation or the synthesis of thermostable proteins (unpublished data). All those changes began to appear no earlier than $+2^{\circ}\text{C}$ during CA. This observation indicates that during prehardening the 1st stage of frost hardening has not yet begun, and the influence of prehardening on the development of frost hardiness is rather indirect — through changes leading as a consequence to the increase of the energy available for the hardening process. This event should be associated with the increased inflow of photoassimilates during the 1st stage of frost hardening (stimulation of the effectiveness of photosynthesis at hardening temperatures, Rapacz 1998b, Rapacz and Janowiak 1998) and with the inhibition of the elongation growth and expansion of new leaves during this period, thanks to which the greater part of the acquired photoassimilates may be utilised in the processes connected with the development of frost resistance. The observed inhibition of elongation growth may be the result of changes in the hormonal balance, which, already during prehardening, make the plants form rosettes. This phenomenon would be analogous to the observation that the application of some growth retardants not only promotes the formation of rosettes before CA, but slows down the growth and increases the accumulation of sugars or reduces water content already during CA (Tumanov 1960, Dolnicki 1969, Kacperska-Palacz and Długolecka 1971).

Based on the results of the present study as well as of earlier ones (Rapacz 1998a,b, Rapacz and Janowiak 1998) it can be established that under natural conditions the preparation of winter rape plants for winter begins already at the moment of emergence, and comprises prehardening and then the 1st, 2nd and 3rd stages of frost hardening (Kacperska-Palacz 1978). At present, it is difficult to decide whether, or rather at what rate, prehardening affects the development of frost resistance in other species of herbaceous plants, especially the monocotyledons.

Zusammenfassung

Physiologische Wirkungen in der Winterraps (*Brassica napus* var. *oleifera*) Frosthärtung. II. Wachstum, Energieaufteilung und Wasserstatus während der Kälteakklimatisation

Vorhärtungen von Winterraps, die im frühen Wachstum bei reduzierten Tagstemperaturen ($+12^{\circ}\text{C}$) vom Keimen bis zum Beginn der Kälteanpassung bei Kühlttemperaturen (1. Stadium der Kälteakklimatisation) auftraten, haben einen günstigen Einfluß auf die

Frostresistenz. In früheren Untersuchungen wurde gezeigt, daß während der Vorhärtung im Blattrosettenstadium die Photosyntheseeffizienz bei Kühlttemperaturen zunahm. In der vorliegenden Untersuchung wurden Experimente durchgeführt, um den Einfluß der Vorhärtung auf die Wachstumsrate der Pflanzen während des ersten Stadiums der Kälteanpassung und des Fortschrittes ausgewählter physiologischer Prozesse, die während dieses Stadiums auftreten und zu einer Verbesserung der Frostresistenz führen, zu untersuchen. Es wurde gezeigt, daß eine größere Zufuhr von Photoassimilaten während des ersten Stadiums der Kälteanpassung zu einer größeren Zunahme der Trockenmasse vorgehärteter Pflanzen führt. Diese Pflanzen zeigen eine deutliche Inhibierung des Längenwachstums und der Ausbreitungsrate neuer Blätter. Auf diese Weise wird die Energie in einem größeren Ausmaß Prozessen zugeführt, die mit der Erhöhung der Frostresistenz in Verbindung stehen. In vorgehärteten Sämlingen während des ersten Stadiums der Kälteanpassung wurden in den Blättern eine hohe Rate der Wassergehaltsabnahme und des Rückgangs im osmotischen Potential des Zellsaftes und des Wasserpotentials der Gewebe beobachtet. Ferner erhöhte sich die Akkumulation löslicher Zucker und freien Prolins. Allerdings konnte der Beginn dieser Vorgänge nicht während der Vorhärtungsphase beobachtet werden. Vorhärtung stimulierte die Effizienz im ersten Stadium indirekt über Änderungen, die zu einer Erhöhung der verfügbaren Energie führten und damit zur Nutzung eines größeren Teils der Energie, die für Abhärtungsprozesse gegenüber Kälte zur Verfügung gestellt wurde.

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