Salt tolerance of barley induced by the root endophyte *Piriformospora indica* is associated with a strong increase in antioxidants

Helmut Baltruschat[1]*, József Fodor[2]*, Borbála D. Harrach[2], Elżbieta Niemczyk[3], Balázs Barna[2], Gábor Gullner[2], Anna Janeczko[3], Karl-Heinz Kogel[1], Patrick Schäfer[1], Ildikó Schwarczinger[2], Alga Zuccaro[1] and Andrzej Skoczowski[3]

[1]Institute of Phytopathology and Applied Zoology, Justus Liebig University Giessen, Heinrich-Buff-Ring 26-32, D-35392 Giessen, Germany; [2]Plant Protection Institute, Hungarian Academy of Sciences, Herman Ottó út 15, H-1022, Budapest, Hungary; [3]The Franciszek Górski Institute of Plant Physiology, Polish Academy of Sciences, Niezapominajek 21, 30-239 Cracow, Poland

Author for correspondence:
J. Fodor
Tel: +36 1487 7520
Fax: +36 1487 7555
Email: jfod@nki.hu

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Summary

• The root endophytic basidiomycete *Piriformospora indica* has been shown to increase resistance against biotic stress and tolerance to abiotic stress in many plants.
• Biochemical mechanisms underlying *P. indica*-mediated salt tolerance were studied in barley (*Hordeum vulgare*) with special focus on antioxidants. Physiological markers for salt stress, such as metabolic activity, fatty acid composition, lipid peroxidation, ascorbate concentration and activities of catalase, ascorbate peroxidase, dehydroascorbate reductase, monodehydroascorbate reductase and glutathione reductase enzymes were assessed.
• Root colonization by *P. indica* increased plant growth and attenuated the NaCl-induced lipid peroxidation, metabolic heat efflux and fatty acid desaturation in leaves of the salt-sensitive barley cultivar Ingrid. The endophyte significantly elevated the amount of ascorbic acid and increased the activities of antioxidant enzymes in barley roots under salt stress conditions. Likewise, a sustained up-regulation of the antioxidative system was demonstrated in NaCl-treated roots of the salt-tolerant barley cultivar California Mariout, irrespective of plant colonization by *P. indica*.
• These findings suggest that antioxidants might play a role in both inherited and endophyte-mediated plant tolerance to salinity.

Key words: antioxidant enzymes, ascorbic acid, calorimetry, ethane release, fatty acid unsaturation, *Hordeum vulgare* (barley), *Piriformospora indica*, salt stress.

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Introduction

High salt concentrations in soil and irrigation water are a major threat to agricultural production in arid and semiarid regions. The presence of excess ions in the rhizosphere causes injury to plant roots, followed by their gradual accumulation in the aerial parts with heavy damage to plant metabolism, which leads to stunted growth and reduced yield (Shannon, 1997). Plants have evolved complex mechanisms to counter NaCl toxicity and low water potential in soil caused by salinity as well as drought (reviewed by Munns & Tester, 2008). Furthermore, mutualistic symbiosis with mycorrhizal and endophytic fungi can confer salt tolerance to plants and decrease yield losses in cultivated crops grown in saline soils (Rodriguez

*These authors contributed equally to this work.

et al., 2004). Recently, a root-endophytic basidiomycete, *Piriformospora indica*, has been shown to improve plant resistance against root and leaf diseases and alleviate salt stress in barley (Waller *et al.*, 2005).

Piriformospora indica was isolated from the rhizosphere of *Prosopis juliflora* and *Zizyphus nummularia* in the Thar Desert in Rajasthan, India (Verma *et al.*, 1998). This fungus colonizes roots and increases the biomass of both monocot and eudicot plants (Varma *et al.*, 1999). In contrast to arbuscular mycorrhizal fungi, *P. indica* can be easily grown on synthetic media allowing for large-scale propagation and a possible use in plant production.

The aim of this study was to investigate the *P. indica*-mediated protective plant responses to moderate (100 mM NaCl) and high (300 mM NaCl) salt stress in barley. In order to elucidate physiological responses of *P. indica*-colonized barley plants to salinization, we measured important indicators of salt stress, such as metabolic heat production, lipid peroxidation and fatty acid composition; furthermore, we analysed antioxidant activities.

Earlier studies have demonstrated that salt-treated barley shows reduced metabolic activity and respiration rates (Criddle *et al.*, 1989; Jolivet *et al.*, 1990). Thus, calorimetrical determination of heat output can serve as a valuable tool for screening plants for salt tolerance (Criddle *et al.*, 1989; Schabes & Sigstad, 2004).

Lipid peroxidation is associated with cellular membrane damage elicited by salinity stress (Fadzilla *et al.*, 1997). NaCl treatment resulted in higher rates of lipid peroxidation in salt-sensitive plants than in salt-tolerant cultivars (Hernández *et al.*, 1995; Yang *et al.*, 2004). These observations suggest that the rate of lipid peroxidation can also be used to characterize how effectively *P. indica*-treated plants cope with salt stress.

Fatty acid desaturation is associated with salt stress in plants as well (Elkahoui *et al.*, 2004; Liang *et al.*, 2005). Previously, Berberich *et al.* (1998) have found that ω-3 desaturase genes are induced in roots of maize under high salt conditions. In agreement with this result, it has been shown that linolenic acid plays a pivotal role in the tolerance of tobacco plants to salt stress (Im *et al.*, 2002). Therefore, composition of fatty acids was analysed in leaves of uncolonized and *P. indica*-colonized salt-sensitive barley plants under salt stress conditions to characterize fatty acid desaturation.

Drought, salt and temperature extremes all induce the accumulation of reactive oxygen species (ROS), such as superoxide, hydrogen peroxide and hydroxyl radicals (Apel & Hirt, 2004). Plants are endowed with an array of radical scavengers and antioxidant enzymes that act in concert to alleviate oxidative stress. An imbalance between antioxidant defences and the amount of ROS results in cellular injury (Foyer & Noctor, 2000). An increasing body of evidence suggests that high salinity induces oxidative stress in plants that is at least partly responsible for tissue damage (Hernández *et al.*, 2000; Mittova *et al.*, 2004). Several studies have demonstrated that salinity increases antioxidant activities in salt-tolerant plants above the levels found in salt-sensitive plants (Gossett *et al.*, 1994; Gueta-Dahan *et al.*, 1997; Mittova *et al.*, 2004).

It has been previously shown that *P. indica* also induces antioxidants: the amount of ascorbic acid, the ratio of reduced to oxidized ascorbate and the activity of dehydroascorbate reductase were elevated in barley roots (Waller *et al.*, 2005). We addressed the question of whether antioxidants play a role in *P. indica*-mediated protection of barley against salt stress. Cultivated barley is a relatively salt-tolerant crop but there is a rather high variability among barley cultivars in this trait (Epstein *et al.*, 1980). Two contrasting genotypes, the salt-tolerant cultivar California Mariout and the salt-sensitive cultivar Ingrid, were chosen for this study to define antioxidant responses.

Materials and Methods

Plant inoculation and NaCl treatment

Seeds of salt-sensitive barley (*Hordeum vulgare* L.) cv. Ingrid and salt-tolerant cultivar California Mariout (Epstein *et al.*, 1980) were surface-sterilized for 10 min in 0.25% sodium hypochlorite, rinsed with water and germinated at 22°C on sheets of Whatman No. 1 filter paper in Petri dishes. After 2 d, one part of the germinating seeds was transferred to pots and grown in a 2 : 1 mixture of expanded clay (Seramis, Masterfoods, Verden, Germany) and Oil-Dri (equivalent to Terra Green, Damolin, Mettmann, Germany) in a growth chamber at 22 : 18°C day : night cycle, 60% relative humidity and a photoperiod of 16 h (200 μmol m^{-2} s^{-1} photon flux density), and fertilized weekly with 0.1% Wuxal top N solution (Schering, Düsseldorf, Germany, N : P : K, 12 : 4 : 6). The other part of the seeds was inoculated with *P. indica*: developing roots of 2-d-old germinating seeds were immersed in *P. indica* homogenate before transferring to pots and grown under the same conditions.

Piriformospora indica was propagated in liquid *Aspergillus* minimal medium (Peškan-Berghöfer *et al.*, 2004). Fungal mycelium was prepared for root inoculation as described by Druege *et al.* (2007). Root colonization was determined in 1-wk-old plants by the magnified intersections method (McGonigle *et al.*, 1990) after staining root fragments with 0.01% (w/v) acid fuchsin in lactoglycerol (Kormanik & McGraw, 1982). Fungal structures were visualized in the roots with a Zeiss Axioplan 2 microscope.

Salt-treated sets of uncolonized and *P. indica*-infected plants were exposed to salt from the age of 3 wk, continuously bottom-watered with sterile water containing 100 or 300 mM NaCl. Leaf and root samples were harvested after 1, 2, 3 and 4 wk periods of salt treatment. Control sets of barley plants were irrigated with sterile water.

Isothermal microcalorimetry

Four-centimetre-long apical leaf tips were excised from the youngest fully expanded leaves of 5-wk-old plants. Two leaf cuttings from different barley plants were placed into a sample

ampoule and heat production was recorded by a Thermal Activity Monitor LKB-2277 (Thermometric, Järfälla, Sweden) as described by Fodor *et al.* (2007).

Lipid extraction and separation

Leaf tissue (1.5 g) was ground in 7 ml of methanol–chloroform (2 : 1) with a mortar and pestle at 0–4°C, and vortexed thoroughly. The homogenate was centrifuged at 2000 ***g*** for 20 min at 7°C and the supernatant fluid was transferred to a clean tube. The residual pellet was extracted a second time with 2 ml of the same extraction mixture, vortexed and centrifuged as before. Subsequently, the supernatants were combined. Phase separation and isolation of particular lipid fractions was performed according to Żur *et al.* (2002).

Analysis of fatty acids

Fatty acid composition of phospholipids was analysed by a gas chromatograph (Hewlett Packard 5890 Series II) using capillary column GS-Alumina (30 m length, 0.542 mm in diameter purchased from J&W Scientific, Folsom, CA, USA) as described previously (Żur *et al.*, 2002). The relative amount of particular fatty acids was compared with internal standards (C17:0, Sigma-Aldrich, Munich, Germany). Double bond index was calculated by dividing by 100 the sum of the percentages of the unsaturated fatty acids, each multiplied by the number of its double bonds.

Ethane assay

Lipid peroxidation was monitored by detection of thermally produced ethane. Leaf samples from the youngest fully developed leaves of 5-wk-old plants (*c.* 400 mg) were placed into a 16 ml flask and sealed under nitrogen atmosphere. *In situ* decomposition of ω-3 unsaturated hydroperoxy fatty acids into ethane was accelerated by a brief heat treatment of the samples using a microwave oven according to Degousée *et al.* (1995). Gas chromatographic measurements were carried out as described by Fodor *et al.* (2007). Ethane was quantified by comparison to an authentic standard (Sigma-Aldrich).

Antioxidant assays

Activities of ascorbate peroxidase (APX), catalase (CAT), dehydroascorbate reductase (DHAR) and glutathione reductase (GR), and the concentration of reduced and oxidized forms of ascorbic acid were detected in root extracts spectrophotometrically as described earlier (Harrach *et al.*, 2008).

Monodehydroascorbate reductase (MDHAR) activity was determined in 50 mM Tris-HCl buffer (pH 7.8) containing 1 mM ascorbate, 0.1 mM NADH and 0.2 U ml^{-1} ascorbate oxidase (Hossain *et al.*, 1984). The reaction was started by the addition of ascorbate peroxidase and followed by monitoring the consumption of NADH at 340 nm.

Statistical analysis

At least three independent experiments were carried out in each case. Statistical analysis was performed using Student's *t*-test and MANOVA. Differences were considered to be significant at $P < 0.05$.

Results

Piriformospora indica enhances shoot biomass under salt stress

Hyphal colonization of 1-cm-long root segments was estimated to be 50–60% in Ingrid barley and only the colonized plants were used in each experiment. The rate of colonization was not affected significantly by 3 wk exposure to salt stress (data not shown).

Barley plants irrigated with saline water for 2 wk showed stunted growth and underwent early senescence. The biomass of the youngest developed leaves slightly decreased under saline conditions, while older leaves exhibited chlorosis and subsequent necrosis. Mild salt stress (100 mM NaCl) caused a slight, but not significant, reduction in shoot fresh weight of barley plants. However, high-salt (300 mM NaCl) treatment caused substantial biomass reduction in uncolonized and *P. indica*-colonized cv. Ingrid and cv. California Mariout plants as well (Fig. 1).

Compared with uncolonized plants, shoot fresh weight of *P. indica*-colonized barley cv. Ingrid was enhanced about twofold under both control and saline conditions (Fig. 1). Even after exposure to 300 mM NaCl, *P. indica*-colonized plants produced shoot biomass comparable to uncolonized Ingrid barley grown under nonsaline conditions. Among plants grown in a highly saline environment, shoot fresh weight of salt-tolerant cv. California Mariout was significantly higher compared with the uncolonized cv. Ingrid, but the highest shoot biomass production was detected in *P. indica*-colonized Ingrid plants.

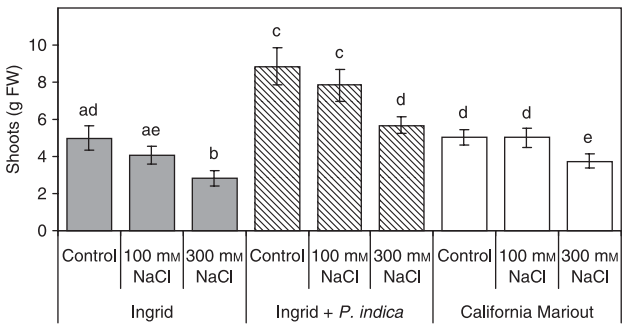

Fig. 1 Shoot fresh weight of 5-wk-old barley (*Hordeum vulgare*) plants, untreated (control) or treated with NaCl from 3 to 5 wk after germination. Ingrid is a salt-sensitive cultivar, California Mariout is a salt-tolerant cultivar, and plants of cv. Ingrid were uncolonized or *Piriformospora indica*-colonized. Letters indicate significant differences among treatments ($P < 0.05$).

Piriformospora indica counteracts the salt-induced decrease in heat efflux

The metabolic heat rates of leaf samples were reduced by *c.* 30% when Ingrid plants were exposed to 300 mM NaCl for 2 wk (Fig. 2a). Infection of roots with *P. indica* did not cause significant changes in heat production of leaves under nonsaline conditions.

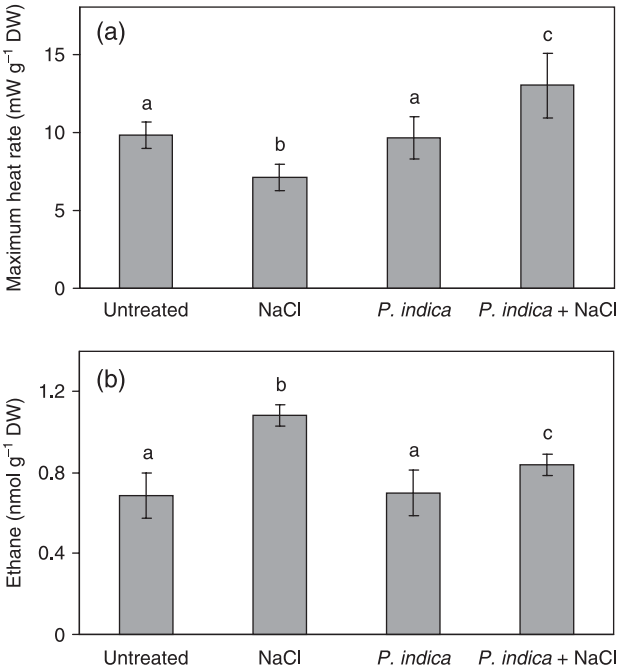

Fig. 2 Effects of salt treatment on metabolic heat efflux detected by isothermal calorimetry (a) and on lipid peroxidation estimated by thermally produced ethane (b) in leaves of 5-wk-old barley (*Hordeum vulgare*) cv. Ingrid plants. Control, untreated 5-wk-old barley; *P. indica*, *Piriformospora indica*-colonized plants; NaCl, plants treated with 300 mM NaCl from 3 to 5 wk after germination; DW, dry weight. Letters indicate significant differences among treatments ($P < 0.05$).

When *P. indica*-colonized Ingrid plants were grown in a high-saline environment, the amount of heat production was significantly ($P < 0.05$) above that observed in uninfected plants.

Changes in fatty acid composition

Fatty acid composition of phospholipid fractions prepared from leaves of salt-sensitive Ingrid barley is listed in Table 1. The major fatty acid species were palmitic (C16:0), palmitooleic (C16:1), stearic (C18:0), oleic (C18:1), linoleic (C18:2) and linolenic (C18:3) acids. Analysis of fatty acid composition in barley leaves indicated that the fully saturated C16:0 palmitic acid was the predominant C16 fatty acid, whereas C18 fatty acids mostly consisted of unsaturated species (Table 1). We found a slight salt-induced shift from C16 fatty acids to C18:3 fatty acid upon high-salt treatment. This increase was accompanied by a small but significant rise in the overall proportion of unsaturated fatty acids, in the ratio of C18:3 to C18:2 fatty acids and in the double bond index, which is a more precise indicator of fatty acid desaturation (Table 1).

In leaves of *P. indica*-colonized plants, the proportion of C16:1 fatty acid increased, whereas the molar percentage of C18:1 fatty acid significantly decreased compared with the uninfected plants (Table 1). The proportion of linolenic acid and the derived values for indicators of fatty acid desaturation were slightly elevated upon inoculation with the endophyte. Interestingly, when *P. indica*-inoculated Ingrid plants were subjected to salt, we could not find further changes in the molar percentages of C16 or C18 fatty acids, except for C16:1, which was again down-regulated to the concentration detected in leaves of salt-treated uninfected plants (Table 1).

Piriformospora indica reduces lipid peroxidation in leaves of salt-treated barley

High salinity stress induced the peroxidation of membrane lipids as demonstrated by the emission of thermally produced

Table 1 Fatty acid composition in phospholipids isolated from leaves of barley (*Hordeum vulgare*) cv. Ingrid

Fatty acid	Untreated	NaCl	*Piriformospora indica*	*P. indica* + NaCl
16:0	16.9 ± 0.7	15.4 ± 0.7*	15.8 ± 1.0	16.3 ± 1.1
16:1	2.3 ± 0.2	1.8 ± 0.4	2.8 ± 0.2*	1.9 ± 0.4
18:0	2.4 ± 0.5	2.3 ± 0.3	2.2 ± 0.5	2.3 ± 0.6
18:1	2.7 ± 0.2	2.5 ± 0.2	1.9 ± 0.3*	2.2 ± 0.1*
18:2	25.9 ± 2.2	23.0 ± 1.2	22.6 ± 4.1	23.1 ± 0.9
18:3	49.9 ± 2.5	55.0 ± 1.9*	54.7 ± 5.6	54.1 ± 2.5*
18:3:18:2	1.95 ± 0.25	2.39 ± 0.19*	2.47 ± 0.70	2.32 ± 0.19*
U:S	4.17 ± 0.29	4.65 ± 0.28*	4.54 ± 0.39	4.33 ± 0.46
DBI	2.06 ± 0.04	2.15 ± 0.04*	2.13 ± 0.08	2.12 ± 0.06

Molar percentages of fatty acids ± SD are shown. NaCl, treatment with 300 mM NaCl from 3 to 5 wk after germination; 18:3 : 18:2, ratio of linolenic to linoleic acid; U : S, ratio of unsaturated to saturated fatty acids; DBI, double bond index = Σ(mol % fatty acid × number of double bonds)/100.

*Significant difference between treated and control plants at $P < 0.05$ level.

Table 2 Statistical analysis (MANOVA) for testing the effect of salt concentration, time-point of sampling and root colonization by *Piriformospora indica* on activities of APX, CAT, GR, DHAR and MDHAR antioxidant enzymes in roots of barley (*Hordeum vulgare*) plants

	F					
Factors	APX	CAT	GR	DHAR	MDHAR	df
Salt	**79.15**	**639.74**	**324.52**	**1042.74**	**527.26**	2.69
Time	**22.04**	**58.15**	**54.16**	**93.84**	**12.46**	3.69
P. indica	**144.55**	**335.00**	**30.12**	**279.90**	**124.46**	1.69
Salt × time	**24.95**	**81.22**	**26.78**	**87.67**	**24.78**	6.69
P. indica × salt	**77.40**	**48.39**	**61.41**	**26.41**	**93.78**	2.69
P. indica × time	**38.93**	**174.46**	2.29	**6.80**	**5.09**	3.69

Plants were treated with NaCl between the ages of 3 and 7 wk after germination. The salt factor has three concentrations: 0, 100 and 300 mM NaCl, the time factor has four levels: 1, 2, 3, 4 wk after NaCl treatment; the *P. indica* factor has two levels: uncolonized and *P. indica*-colonized cv. Ingrid; df, degrees of freedom; APX, ascorbate peroxidase; CAT, catalase; GR, glutathione reductase; DHAR, dehydroascorbate reductase; MDHAR, monodehydroascorbate reductase. Significant ($P < 0.05$) *F*-values are indicated by bold characters.

ethane derived from the decomposition of the 16-hydroperoxide of linolenic acid. *P. indica* by itself did not affect the emission of ethane from leaves of cv. Ingrid (Fig. 2b). The rate of ethane release from the leaves of salt-treated Ingrid plants increased by 60% compared with the unsalinized control. However, high salt exposure accelerated the rate of lipid peroxidation by only 20% in leaves of *P. indica*-colonized plants (Fig. 2b).

Piriformospora indica further increases antioxidant enzyme activities induced by salt treatment in barley roots

Statistical analysis revealed significant ($P < 0.05$) effects of salt concentration, duration of salt treatment and root colonization by *P. indica* on the activities of antioxidant enzymes (Table 2). Enzyme activities were affected in barley roots by NaCl in the following order (from highest to lowest effect): DHAR, CAT, MDHAR, GR and APX. MDHAR activity was the least affected by the time points. On the other hand, GR activity was the least affected by *P. indica*, which exerted a very high effect on CAT and DHAR activities. Changes in salt concentration significantly affected the time-dependent responses of plants, as evaluated by enzyme activities. Furthermore, root colonization by the endophyte also had significant time-dependent effects on enzyme activities, particularly on CAT and APX, and to a lesser extent on DHAR and MDHAR. Its effect on GR activity was not significant.

In roots of uncolonized Ingrid plants, enzyme activities were markedly increased after salt treatment, peaked at 1 wk after salt exposure and then gradually returned to the corresponding basal levels over the next 3 wk. Only MDHAR activity was found to be enhanced by salt throughout the experiment (Fig. 3). Both the increase and then the decline of enzyme activities were modest when the plants were exposed to 100 mM NaCl compared with the plants subjected to high salt.

In *P. indica*-colonized Ingrid and in California Mariout plants, the salinity-induced changes in enzyme activities were different from those associated with salt stress in uninfected Ingrid barley. First, the time for antioxidant enzymes to reach the peak activities was longer: 3 wk after salt exposure. Second, the ceiling rates of the enzyme activities were significantly higher. Third, a less pronounced decrease was observed in enzyme activities at 4 wk after salt treatment (Fig. 3).

Piriformospora indica enables barley roots to maintain ascorbate in its reduced state under salt stress

Colonization of barley by *P. indica* enhanced both ascorbic acid concentration and the ratio of reduced to oxidized ascorbate about twofold in plant roots after saline exposure (Fig. 4). We could not detect ascorbate in *P. indica* grown axenically in liquid medium.

Strikingly, salt treatment had the opposite effect on ascorbic acid concentrations in uncolonized than in *P. indica*-colonized Ingrid plants (Fig. 4), and therefore salt did not affect significantly the amount of ascorbate (Table 3). However, the *P. indica*-dependent response of ascorbate to salinization was highly significant. The amount of reduced ascorbate strongly declined in uninfected roots after 1 wk of high-salt treatment. By contrast, salinization further increased the ascorbate concentration in the colonized plants at the first time-point of sampling (Fig. 4a). The amount of ascorbate then gradually decreased but still remained above the values recorded for the control plants grown under nonsaline conditions. Furthermore, *P. indica*-colonized plants maintained efficient redox balance of ascorbate even after 3 wk of salt treatment (Fig. 4b). Statistically significant ($P < 0.05$) time-dependent or endophyte-dependent effect of salinization was not observed for the ascorbate : DHA ratio (Table 3). Nevertheless, both *P. indica* and salinity exerted significant effect on ascorbate redox state (Table 3). Remarkably, in uninfected plants, a strong decrease in the ratio of reduced to oxidized form of ascorbate was already detectable 1 wk after salinization: the ascorbate : DHA ratio decreased by *c.* 80% (Fig. 4b).

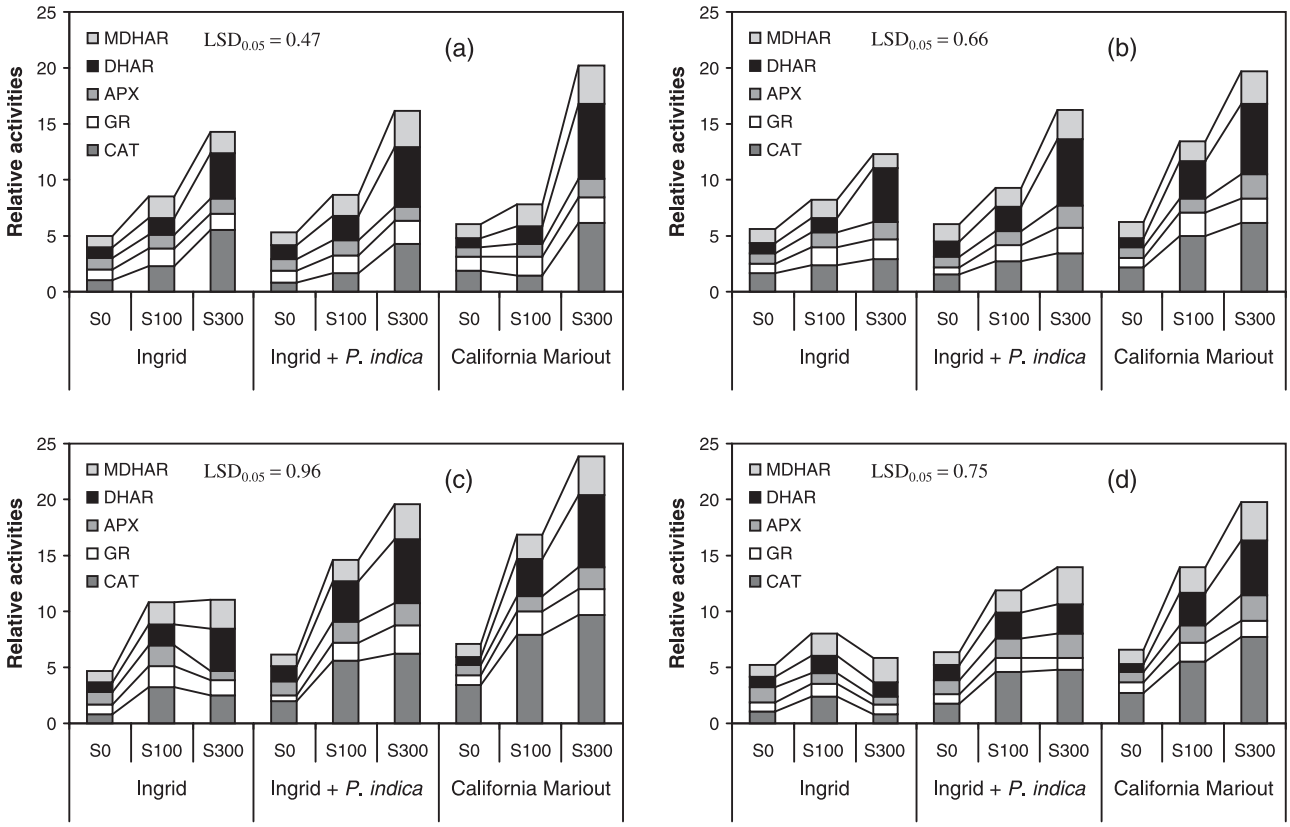

Fig. 3 Relative enzyme activities of catalase (CAT), glutathione reductase (GR), ascorbate peroxidase (APX), dehydroascorbate reductase (DHAR) and monodehydroascorbate reductase (MDHAR) in roots of salt-sensitive barley (*Hordeum vulgare*) cv. Ingrid, *Piriformospora indica*-colonized cv. Ingrid and salt-tolerant cv. California Mariout after 1 (a), 2 (b), 3 (c) and 4 wk (d) of salt exposure. Plants were treated with NaCl from 3 to 7 wk after germination. Enzyme activities were normalized to the activities of enzymes measured in roots of unsalinized (S0) Ingrid plants at 1 wk after treatment. Activity level of 1 represents 72.25, 0.34, 1.15, 0.83 and 0.60 mmol g^{-1} FW min^{-1} activities of CAT, GR, APX, DHAR and MDHAR, respectively. S0, S100, S300, treated with 0, 100 and 300 mM NaCl, respectively; $LSD_{0.05}$, least significant difference between means at $P = 0.05$.

Table 3 Statistical analysis (MANOVA) for testing the effect of salt concentration, time-point of sampling and root colonization by *Piriformospora indica* on ascorbic acid content and ratio of reduced ascorbate to oxidized ascorbate in roots of barley (*Hordeum vulgare*) cv. Ingrid

	F		
Factors	ASC	ASC : DHA	df
Salt	0.38	**194.78**	1.77
Time	**30.53**	**26.16**	2.77
P. indica	**454.30**	**208.49**	1.77
Salt × time	**11.88**	0.67	2.77
P. indica × salt	**105.53**	0.03	1.77
P. indica × time	**20.40**	**8.07**	2.77

Barley plants were treated with NaCl from 3 to 6 wk after germination. ASC, ascorbic acid; DHA, dehydroascorbic acid; df, degrees of freedom. The *P. indica* factor has two levels: uncolonized and *P. indica*-colonized cv. Ingrid; the time factor has three levels: 1, 2 and 3 wk after NaCl treatment; the salt factor has two levels: 0 and 300 mM NaCl. Significant ($P < 0.05$) *F*-values are indicated by bold characters.

Discussion

As a result of the symbiosis with *P. indica*, barley tolerates a moderate salt stress (100 mM NaCl) in hydroponic culture (Waller *et al.*, 2005). Here we could show that *P. indica* protects barley even from high salt stress (300 mM NaCl). However, the mechanism of *P. indica*-induced salt tolerance has not yet been investigated.

In order to get a better understanding of the impact of *P. indica* on the establishment of salt tolerance, we assessed biochemical markers for salt stress, such as metabolic activity, fatty acid composition and lipid peroxidation. Previous studies have demonstrated a salt-induced increase in lipid peroxidation (Hernández *et al.*, 1995; Yang *et al.*, 2004) and a marked reduction in metabolic heat production (Criddle *et al.*, 1989) in salt-sensitive plants, while these parameters were unaltered in salt-tolerant cultivars. We provide clear evidence that salt-induced responses indicated by heat emission and ethane production in the *P. indica*-infected salt-sensitive barley cv. Ingrid resemble those found in salinity-tolerant plants. Our calorimetric studies indicated that the rate of metabolic activity

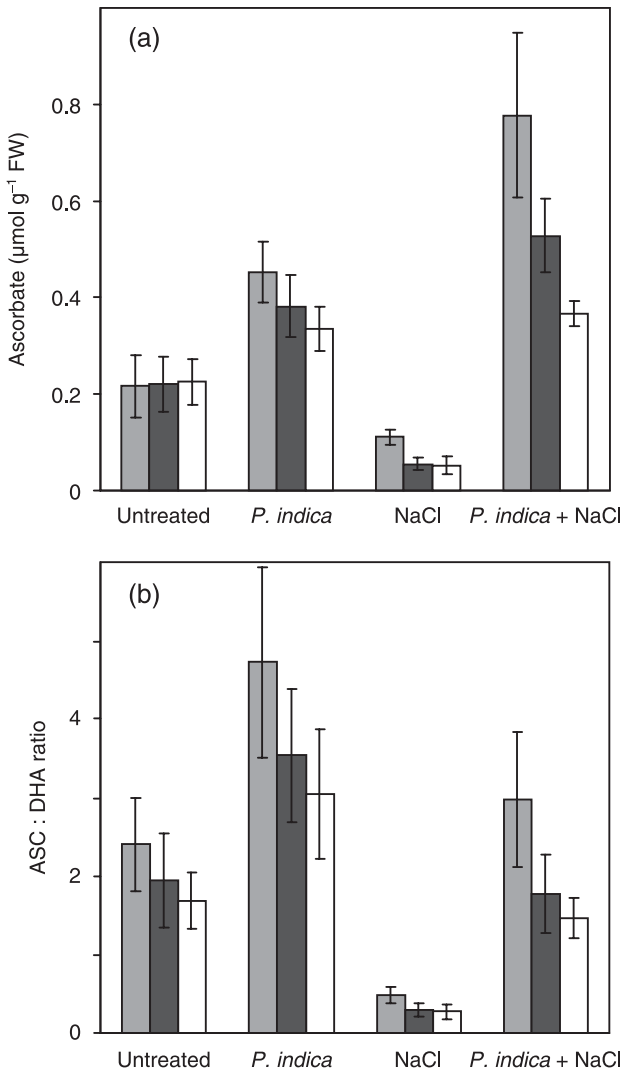

Fig. 4 Amount of reduced ascorbate (a) and ratio of reduced to oxidized ascorbate (b) in roots of salt-sensitive barley (*Hordeum vulgare*) cv. Ingrid plants after 1 (grey bars), 2 (black bars) and 3 (white bars) wk of salt exposure. The plants were untreated or treated with 300 mM NaCl from the age of 3 wk, and uncolonized or *Piriformospora indica*-colonized. ASC, reduced ascorbic acid; DHA, dehydroascorbic acid.

increased in leaves of *P. indica*-infected plants after salt treatment. Therefore, the endophyte seemed to overcompensate the salt-induced inhibition of leaf metabolic activity. Previous results have shown that the extent of natural herbicide resistance of wild oat biotypes is tightly correlated with the rate of heat production upon herbicide exposure, owing to the activation of metabolic pathways required for defence responses (Stokłosa *et al.*, 2006). This suggests that enhanced tolerance to salt stress can be associated with higher metabolic activity in *P. indica*-colonized barley.

Previous studies have shown that exogenously applied unsaturated fatty acids can protect barley during NaCl-induced stress (Zhao & Qin, 2005). Thus, lipid desaturation could be an important component of plant tolerance in response to salt stress. *P. indica* colonization leads to a significant reduction in the proportion of oleic acid in barley leaves, as was previously found in salt-treated barley roots (Zhang *et al.*, 2002; Liang *et al.*, 2005). Similar to salinity, *P. indica* slightly increased the proportion of C18:3 fatty acid in the phospholipid fraction isolated from barley leaves. With one exception (C16:1), *P. indica* induces changes in fatty acid compostion similar to those induced by salinity. Such effects on the fatty acid composition of host plants may display a symbiotic adaptive strategy mediated by the endophyte to cope with salt stress in hostile environments (Rodriguez *et al.*, 2008). We speculate that *P. indica* might induce similar effects on fatty acid composition of the host plants in its original habitat, the arid Thar desert.

Salt-induced lipid peroxidation was significantly attenuated in *P. indica*-treated plants. Cellular membrane damage as a result of salt stress is associated with an accumulation of ROS (Hernández *et al.*, 1995), which can be toxic to living cells causing oxidative damage to DNA, lipids and proteins. On the other hand, ROS can act as signalling molecules for stress responses (Apel & Hirt, 2004). According to a recent report, endophytic fungi characterized by their broad host ranges can confer effective tolerance to ROS under abiotic stress conditions such as salinity (Rodriguez *et al.*, 2008). Interestingly, the clavicipitaceous fungal endophyte, *Epichloë festucae*, which has a restricted host range, can generate superoxide by a NADPH oxidase to establish a mutualistic association with *Lolium perenne* (Tanaka *et al.*, 2006). In *P. indica*-colonized barley roots, we could not detect H_2O_2 accumulation at penetration sites or in the infected cells (data not shown).

Our previous report demonstrated that *P. indica* enhances the ratio of reduced to oxidized ascorbate and induces DHAR activity in colonized barley (Waller *et al.*, 2005). Since ascorbate was not found in *P. indica*, we can assume that the fungus induces the accumulation of ascorbate in plant root cells. Ascorbic acid acts as a primary substrate in the ascorbate-glutathione cycle for detoxification of hydrogen peroxide. In addition, it acts directly to neutralize oxygen free radicals (Foyer & Noctor, 2000). Under the high salt stress condition, *P. indica*-infected Ingrid plants maintained an efficient redox balance of ascorbate and contained higher ascorbate concentration than the unsalinized control, although the concentration of reduced ascorbate decreased over time in roots of salt-treated infected plants. Strikingly, ascorbate content and the ratio of reduced to oxidized ascorbate dramatically decreased in roots of salt-treated uninfected plants soon after 1 wk of salt exposure. These findings are consistent with those presented by Mittova *et al.* (2004), who found that the ratio of ascorbate to DHA decreased in the salt-sensitive *Lycopersicon esculentum* under salt stress, and increased in the salt-tolerant *Lycopersicon pennellii*. Other investigators have shown that ascorbate content decreased in salt-sensitive and salt-tolerant pea cultivars as well, but the

decline was greater in the NaCl-sensitive plants (Hernández *et al.*, 2000). The importance of ascorbate in cellular protection under salt stress has also been demonstrated on an ascorbate-deficient *Arabidopsis* mutant. Impaired in the ascorbate-glutathione-cycle, it accumulated high amounts of ROS and showed increased sensitivity to salt stress (Huang *et al.*, 2005). Consistently, exogenously applied ascorbate increased the resistance to salt stress and attenuated the salt-induced oxidative burst (Shalata & Neumann, 2001).

Alternatively, ascorbate can improve the tolerance of barley to high salinity via processes related to root growth. Ascorbic acid and high ratio of reduced to oxidized ascorbate accelerate root elongation and increase root biomass (Córdoba-Pedregosa *et al.*, 2005).

Earlier studies have suggested that tolerance of plants to salt stress is associated with the induction of antioxidant enzymes (Hernández *et al.*, 2000; Bor *et al.*, 2003, Sekmen *et al.*, 2007). We found that NaCl increased the activities of CAT, APX, DHAR, MDHAR and GR in roots of salt-stressed barley. Although enzyme activities decreased after an initial induction in both salt-sensitive and -tolerant plants, their decline was delayed and less pronounced in *P. indica*-colonized Ingrid barley and in the salt-tolerant cv. California Mariout. Our data highlight the importance of these enzymes in tolerance of barley to salinity. MDHAR activity remained elevated up to 4 wk under high saline conditions in roots of both salt-sensitive and -tolerant barley cultivars. CAT and APX showed a sustained increase in the activities in *P. indica*-infected Ingrid barley after long-term exposure to NaCl. By contrast, their activities decreased in uninfected Ingrid barley after 4 wk of salt exposure. In agreement with these data, overexpression of CAT, APX or DHAR in transgenic plants enhanced tolerance to salt stress (Badawi *et al.*, 2004; Ushimaru *et al.*, 2006; Nagamiya *et al.*, 2007). Surprisingly, *Arabidopsis* double mutant plants deficient in cytosolic and thylakoid APX also show enhanced tolerance to salinity, suggesting that ROS such as H_2O_2 could be responsible for activation of an abiotic stress signal that leads to enhanced stress tolerance (Miller *et al.*, 2007).

The mechanism responsible for *P. indica*-mediated up-regulation of the plant antioxidant system is not known. It has been shown recently that *P. indica* is able to produce auxin when associated with plant roots (Sirrenberg *et al.*, 2007). Exogenous auxin has been found to transiently increase the concentration of ROS and then prevent H_2O_2 release in response to oxidative stress (caused by paraquat) and enhance APX activity, while decreasing CAT activity (Joo *et al.*, 2001; Pasternak *et al.*, 2007). On the other hand, *P. indica* increased the amount of methionine synthase, which plays a crucial role in the biosynthesis of polyamines and ethylene (Peškan-Berghöfer *et al.*, 2004). Transgenic tobacco plants overproducing polyamines also have enhanced tolerance toward salt stress, and salt treatment induces antioxidant enzymes such as APX, superoxide dismutase and glutathione S-transferase more significantly in these transgenic plants than in wild-type controls (Wi *et al.*, 2006). *Sebacina vermifera*, an endophyte closely related to *P. indica*, down-regulates ethylene production in *Nicotiana attenuata* (Barazani *et al.*, 2007). Interestingly, our preliminary results suggest that *P. indica* induces ethylene biosynthesis in barley roots. Ethylene signalling may be required for plant salt tolerance (Cao *et al.*, 2006), and ethylene may induce some antioxidant enzymes when plants are exposed to heat stress (Larkindale & Huang, 2004). However, further experiments are necessary to clarify the function of phytohormones in *P. indica*-induced salt tolerance in barley.

In conclusion, our results demonstrated that a high-saline environment is well tolerated by salt-sensitive barley when previously inoculated with the mutualistic basidiomycete *P. indica*. This endophyte appears to confer tolerance to salt stress, at least partly, through the up-regulation of ascorbate and antioxidant enzymes. Our observations are only correlative but supported by the fact that elevated antioxidant activities are also demonstrated under saline conditions in barley cv. California Mariout, which is genetically tolerant to salt. However, several possible symbiotic mechanisms could account for salt tolerance. For example, root endophytes may act as a biological mediator allowing symbiotic plants to activate stress response systems more rapidly and strongly than non-symbiotic plants (Rodriguez *et al.*, 2004). Since *P. indica* has a broad host range and can easily be propagated in axenic culture on a large scale, we emphasize the high potential of the endophyte in protecting crops against salt stress in arid and semiarid agricultural regions.

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