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RESEARCH PAPER

Proline induces the expression of salt-stress-responsive proteins and may improve the adaptation of *Pancratium maritimum* L. to salt-stress

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Abstract

Proline is an important component of salt-stress responses of plants. In this study the role of proline as part of salt-stress signalling in the desert plant *Pancratium maritimum* L. was examined. The data showed that salt-stress brought about a reduction of the growth and protein content, particularly at 300 mM NaCl, that was significantly increased by exogenous proline. In the leaves, salt-stress up-regulated ubiquitin, a small protein targeting damaged proteins for degradation via the proteasome, up to 5-fold as detected by western blotting. This change was also affected by proline even in non-stressed leaves. However, salt-stress resulted in a decrease in the amount of ubiquitin-conjugates, particularly in the roots, and this effect was reversed by exogenous proline. Severe salt-stress resulted in an inhibition of the antioxidative enzymes catalase and peroxidase as revealed by spectrophotometric assays and activity gels, but the activity of these enzymes was also maintained significantly higher in the presence of proline. Salt-stress also up-regulated several dehydrin proteins, analysed by western blotting, even in non-stressed plants. It is concluded that proline improves the salt-tolerance of *Pancratium maritimum* L. by protecting the protein turnover machinery against stress-damage and up-regulating stress protective proteins.

Key words: Growth, *Pancratium maritimum* L., proline, salt-responsive genes.

Introduction

Although in the past most attention has been concerned with the role of proline as a compatible osmolyte (Yancey *et al*., 1982; Samaras *et al*., 1995) and osmoprotectant (Stewart and Lee, 1974; Csonka, 1981; Le-Rudulier *et al*., 1984; MacCue and Hanson, 1990; Serrano and Gaxiola, 1994), its further roles in stress tolerance have received far less attention. Working with *Arabidopsis* mutants, Werner and Finkelstein (1995) found that a proline-deficient mutant, selected for its ability to germinate on saline media, was unable to continue growth on saline media because it could not accumulate proline to the equivalent level of the wild type. Their conclusion was that the proline-deficient mutation disrupted the perception of both ionic and osmotic stresses, in addition to other aspects of stress-induced signals.

Exogenous proline has been reported to protect plants under stress. It improved the tolerance of somatic embryos of celery (*Apium graveolens* L. cv. SB 12) to partial dehydration (Saranga *et al*., 1992). Okuma *et al*. (2000) found that exogenous proline improved the growth of salt-stressed tobacco cell cultures and the improvement was attributed to the role of proline as an osmoprotectant for enzymes and membranes against salt inhibition rather than as a compatible solute. However, Nanjo *et al*. (1999) using transgenic *Arabidopsis* plants with reduced pyrroline-5-carboxylate synthase (P5CS) activity, which is the rate-limiting enzyme in proline synthesis from glutamate, demonstrated that these plants were unable to grow on saline media and that feeding with L-proline, but not D-proline, could increase their ability to withstand the salt.

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They came to the conclusion that, in addition to various known roles of proline, it was also involved in the synthesis of key proteins that are necessary for stress responses. However, Iyer and Caplan (1998) showed that a proline metabolite such as pyrroline-5-carboxylate, but not proline itself, at 1 mM concentrations is able to induce stress-regulated genes such as dehydrins and *sal*T.

In this work, the possibility was investigated that proline may confer a protective effect by protecting or inducing stress-protective proteins. In this context, the effect of exogenous proline and/or stress on the expression of ubiquitin, antioxidative enzymes and dehydrins is examined. If proline simulates the effect of water stress on these proteins in non-stressed plants and mitigates the negative effects in stressed plants, then it might be able to confer its effect at least partially by inducing the stress-adaptive responses.

Materials and methods

The desert plant *Pancratium maritimum* L. was used as an experimental plant. Seeds of *Pancratium* were collected from dehiscing capsules during September from the Baltim area of Egypt (Zahran and Willis, 1992). A large amount of seed was collected, mixed and used for all subsequent work. Seeds were stored in paper bags at room temperature until used.

Growth conditions and stress treatments

Seeds were germinated in 7 cm pots containing washed sand moistened with water until the first leaf emerged, then fed with Long Ashton nutrient solution (Hewitt, 1966) until the age of 28 d. Seedlings were incubated in a plant growth room at 27 °C and 16 h photoperiod (200 μmol $s^{-1}m^{-2}$). Stress treatments were carried out under the same conditions.

Seedlings were germinated as described above until the age of 28 d and then treated as follows. (1) For salt treatment, seedlings were fed with Long Ashton nutrient solution containing 0, 75, 150, 225 or 300 mM NaCl. (2) For salt treatment in the presence of proline, seedlings were fed with Long Ashton nutrient solution containing 5 mM proline combined with the above levels of NaCl.

Seedlings were watered every day with 200 ml of nutrient solution per pot. After 10 d of stress treatment, seedlings were removed from the sand, washed very briefly in distilled water, dried between two layers of filter paper, wrapped in tin foil and frozen directly in liquid nitrogen. Seedlings were then kept at –80 °C until used for further analysis.

Determination of the water contents and dry weights of seedlings

Seven seedlings from each treatment were weighed (shoot and root separately) and dried at 70 °C for 2 d. The dry weights were then recorded and water contents (%) were determined and expressed as means plus or minus standard error of the mean (SE). The fresh weight of the roots was not determined as it was not possible to recover intact root systems after the treatments. However, the water contents of both shoot and root were measured although an incomplete root system was used.

Determination of proline

Proline was measured as described by Bates *et al*. (1973). 100 mg of frozen plant material was homogenized in 1.5 ml of 3% sulphosalicylic acid and the residue was removed by centrifugation. 100 μl of the extract was reacted with 2 ml glacial acetic acid and 2 ml acid ninhydrin (1.25 g ninhydrin warmed in 30 ml glacial acetic acid and 20 ml 6 M phosphoric acid until dissolved) for 1 h at 100 °C and the reaction was then terminated in an ice bath. The reaction mixture was extracted with 1 ml toluene. The chromophore-containing toluene was warmed to room temperature and its optical density was measured at 520 nm. The amount of proline was determined from a standard curve in the range of 20–100 μg.

Western analysis of stress proteins

Proteins were resolved by 13% SDS-PAGE and then transferred to PVDF membranes (Millipore) using the BioRad mini protean transblotter. The transfer buffer consisted of 5.8 g l^{-1} TRIS base and 2.9 g l^{-1} glycine. Proteins were transferred for 1 h at 4 °C and 100 V. The membranes were then blocked for 1 h with 5% dried non-fat milk in TBS (TRIS-buffered saline: 150 mM NaCl in 20 mM TRIS-HCl pH 7.4). The blots were used for immunochemical detection of ubiquitin and dehydrins.

Ubiquitin was detected using rabbit anti-ubiquitin (Sigma) according to the instructions of the manufacturer. Blots were incubated for 4 h in 1:100 dilution of the primary antibody in 5% milk-TTBS (TBS containing 0.1% Tween 20). After 5×3 min washes in TTBS, the membranes were incubated for 1 h in 1:1000 dilution of the secondary antibody (goat anti-rabbit IgG horseradish peroxidase conjugate) in 5% milk-TTBS. After 5×3 min washes in TTBS, the signal was detected using the ECL reagent kit as per the manufacturer's instructions (Amersham). The blots were then washed with TTBS for 1 h, immersed in 100% methanol, allowed to dry and kept for subsequent hybridizations.

Dehydrins were detected according to the method of Close *et al*. (1993). The blots were wetted with 100% methanol, then washed briefly with TBS and incubated in 1:1000 dilution of rabbit anti-dehydrin in 5% milk-TBS for 3 h. The antibody was developed in rabbit against the conserved consensus sequence TGEKKG-IMDKIKEKLPGQH (Stressgen Ltd, USA). The blots were washed with five changes of TBS for 25 min and incubated with 1:1000 dilution of goat anti-rabbit IgG horseradish peroxidase conjugate in 5% milk-TBS for 1 h. After 25 min washing, the signals were detected using the ECL reagent kit (Amersham).

Analysis of antioxidant enzymes

Two antioxidant enzymes, namely catalase and peroxidase, were analysed using Native-polyacrylamide gel electrophoresis (Native-PAGE) and spectrophotometry.

For Native-PAGE of antioxidant enzymes, total soluble proteins extracted for SDS-PAGE were separated on 11% acrylamide Native gels without SDS and mercaptoethanol. Electrophoretic separation was performed at 4 °C using a mini protean 3 electrophoresis unit (BioRad Laboratories, Hercules, CA, USA). 10 μg protein samples in native sample buffer (without SDS and mercaptoethanol) were loaded onto the gel.

Staining for catalase was performed using the method described by Woodbury *et al*. (1971). Gels were briefly washed in distilled water followed by incubation in 0.003% (v/v) H_2O_2 for 10 min. After a brief rinse, catalase was detected by incubating the gels in 1% ferric chloride and 1% potassium ferricyanide until the bands appeared (a few seconds).

Staining for peroxidase was achieved as described by Cochrane *et al*. (2000). Gels were washed in distilled water and soaked in 1 mM 3-amino-9-ethyl carbazole in 100 mM acetate buffer pH 5.0 for 45 min. Then 0.3% (v/v) H_2O_2 was added to give a final concentration of 1 mM and allowed to stand for a few minutes at room temperature until the bands appeared. Each experiment was repeated three times.

Catalase (EC 1.11.1.6) activity was measured by following the consumption of H_2O_2 at 240 nm using a UV spectrophotometer (Aebi, 1984). The 1 ml reaction mixture contained 20 μg total protein, 50 mM sodium phosphate buffer (pH 7.0) and 10 mM H_2O_2. For each measurement, the blank corresponds to the absorbance of the mixture at zero time and the actual reading corresponds to the absorbance after 30 s. Each measurement was repeated five times.

Peroxidase (EC 1.11.1.7) activity was determined as described by Chance and Maehly (1955). The 1 ml reaction mixture contained 50 mM sodium phosphate buffer (pH 6.9), 3.2 mM guaiacol, 0.4 mM H_2O_2 and 20 μg total protein. The mixture was allowed to stand for 4 min and the absorbance was measured at 470 nm against a blank without the enzyme. The use of a blank with boiled enzyme did not show significant differences from that with no enzyme.

Results

Effect of stress treatments on the growth of Pancratium

At the beginning of the experiments, seedlings were homogeneous in terms of length. Treatment with NaCl brought about a decrease in the fresh weight of the shoots, where it was reduced to about one-third by 300 mM NaCl (Fig. 1A). Feeding of proline to the NaCl-stressed plants did not exhibit an effect on the growth except at 300 mM NaCl where the fresh weight of shoots was significantly higher (P=0.05) in the presence of proline than in its absence. Proline did not significantly alter the fresh weights of shoots of non-stressed seedlings.

The dry weight of shoots was reduced down to about 50% of the control by the highest levels of salt-stress (Fig. 1B). Feeding of proline did not significantly affect the dry weight of NaCl-stressed plants or the dry weights of shoots of non-stressed seedlings.

The water content of the shoot (Fig. 2A) decreased after treatment with NaCl although it was higher at 75 mM NaCl than that of the control. Stressed plants fed with proline had slightly lower water contents compared to those stressed with NaCl alone, except at 300 mM NaCl where the water content was significantly higher in presence of proline (P=0.001) than in its absence.

The water content of the root (Fig. 2B) increased slightly after salt stress up to 225 mM, then decreased at 300 mM. Feeding proline also significantly increased the water content of the roots at 300 mM NaCl (P=0.001).

The protein content of shoots (Fig. 3A) was increased slightly by salt treatment up to 150 mM NaCl and thereafter declined until it was lower than the zero salt control at 300 mM NaCl. In the roots (Fig. 3B) the presence of salt increased the protein content at all concentrations tested, although the beginning of a decline was apparent at 300 mM. Nevertheless, the protein contents of severely stressed roots were lower than those of moderately stressed ones, but still higher than the control (Fig. 3B). Protein contents of salt-stressed shoots and roots were significantly higher in the presence of proline (P=0.001) than in its absence.

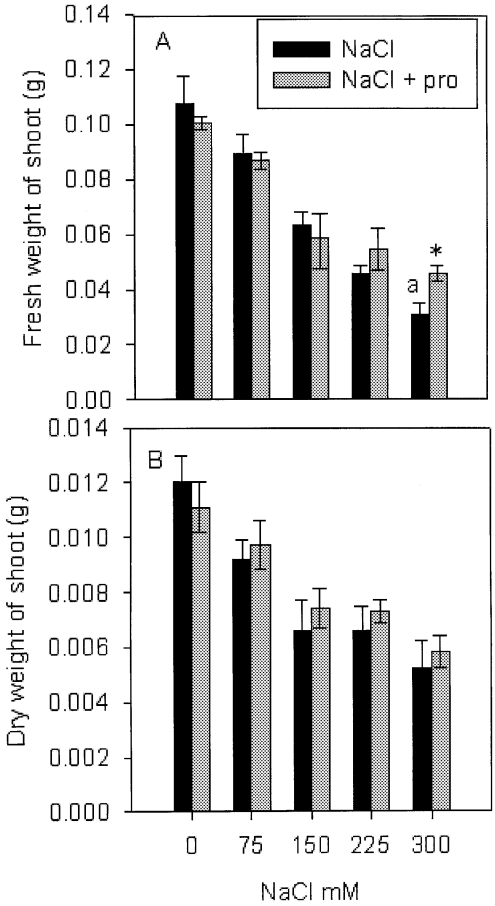

Fig. 1. Changes in fresh weight (A) and dry weight (B) of shoots after treatment with NaCl and proline. Seedlings of *Pancratium* were salt-stressed for 10 d with NaCl (0–300 mM) and individual seedlings were analysed for fresh and dry weights. Seven seedlings from each treatment were analysed. Values are weights of shoots of individual seedlings in grams ±SE. An asterisk indicates significant from 'a' at P=0.05.

Internal proline contents increase in response to exogenous proline in non-stressed and stressed plants

To verify whether exogenous proline modifies the internal amino acid content, the proline content of shoot and root was measured (Fig. 4). Up to a 3-fold increase in proline content of the shoot was measured in salt-stressed shoots at moderate and severe salt-stress conditions. Stressed roots accumulated less proline than the shoot, where proline increased slightly at 150 mM NaCl and up to 1.5-fold at 300 mM NaCl. The internal proline content increased in response to exogenous proline in non-stressed and stressed plants. When proline was fed to non-stressed plants, a 2-fold increase in the internal proline content was detected, particularly in the roots. Proline accumulation was

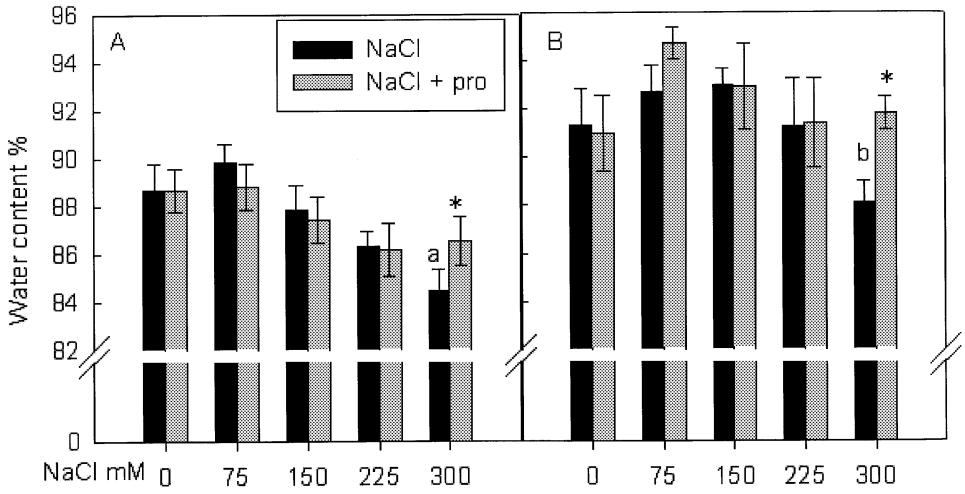

Fig. 2. Changes in water content of shoot (A) and root (B) of *Pancratium* seedlings after treatment with NaCl and proline (pro). Seedlings were dried at 70 °C for 2 d and the loss of weight was recorded as water content. Seven seedlings from each treatment were analysed. Values are water contents ±SE. An asterisk indicates significantly different from 'a' or 'b' at P=0.05. See legend to Fig. 1 for treatments.

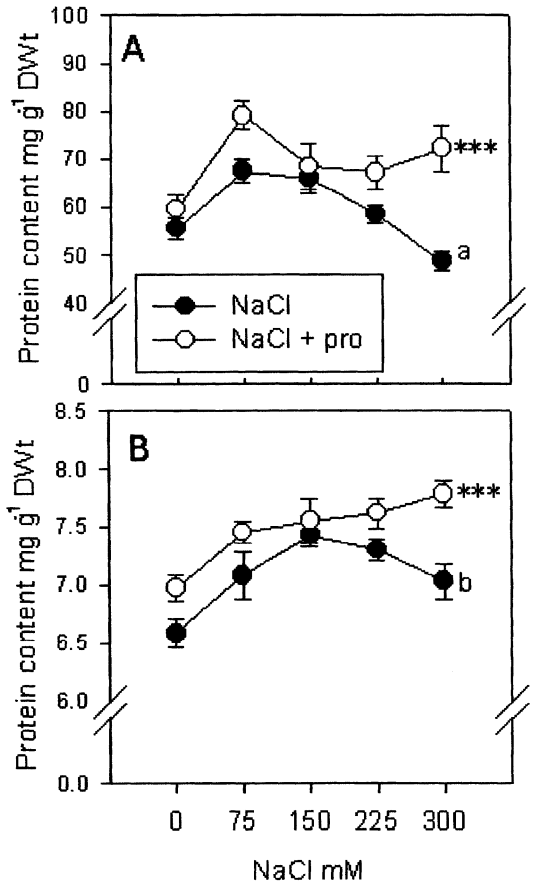

Fig. 3. Effect of salt stress on protein content of *Pancratium* seedlings. (A) Shoot, three asterisks indicates significant from 'a' at P=0.001. (B) Root, three asterisks indicates significant from 'b' at P=0.001. Each measurement was repeated three times. Values are protein contents ±SE. See legend to Fig. 1 for stress treatments.

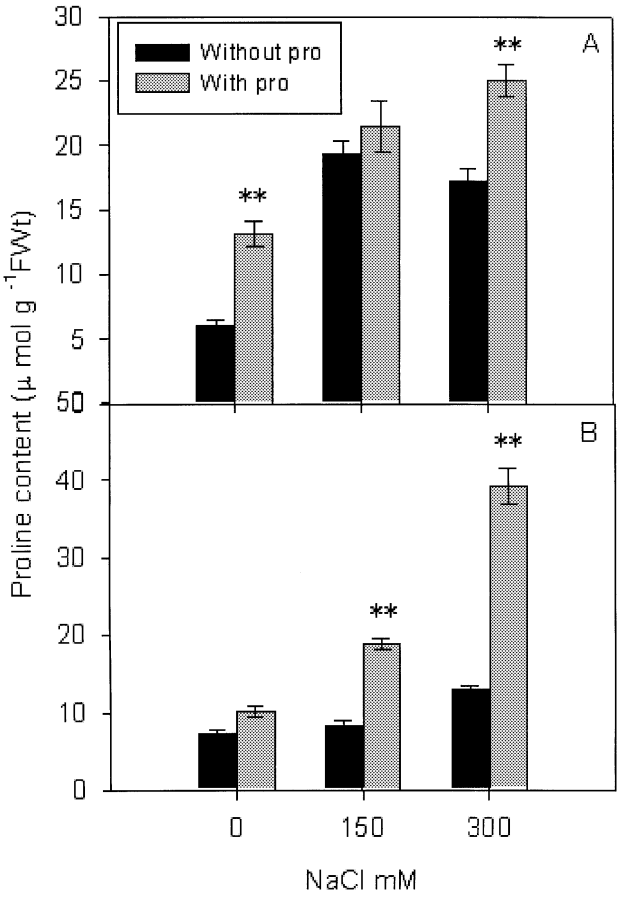

Fig. 4. Proline contents of shoot (A) and root (B) of *Pancratium* seedlings after treatment with NaCl and proline. Values are internal proline content ±SE. Two asterisks indicates significant from the corresponding black bar at P=0.01. Each measurement was repeated three times. See legend to Fig. 1 for stress treatments.

increased by 30% in severely stressed (300 mM) shoots and by 300% in severely stressed (300 mM) roots.

Expression of ubiquitin protein

Ubiquitin was detected in the form of free monomers at low molecular weight (*c*. 8.6 kDa) and protein-conjugates at a range of molecular weights (80–209 kDa) and seen as a darker grey stain on the immunoblots. In shoots, the free ubiquitin content increased proportionately to increasing NaCl concentrations (Fig. 5a) (up to 5-fold compared to the control at 300 mM NaCl; Fig. 5e). However, proline was also able to induce a similar increase in free ubiquitin in the absence of NaCl and this was maintained when NaCl concentration was increased (Fig. 5b, e). The amounts of high molecular weight ubiquitin-conjugates (cellular proteins conjugated to ubiquitin) increased in response to NaCl. In the presence of proline, ubiquitin-conjugates decreased in NaCl-stressed plants. In plants fed with proline in the absence of NaCl (Fig. 5b), the ubiquitin-conjugate content was slightly increased compared with the control.

In roots, only small amounts of free ubiquitin monomers were detected (Fig. 5c, d). The monomer content declined in response to NaCl-stress both in the absence and presence of proline. However, proline fed to non-stressed plants induced higher levels of free ubiquitin compared with the control. Roots contained high amounts of ubiquitin-conjugates. Ubiquitin was detected predominantly in the root in the form of high molecular weight ubiquitin-conjugates and larger amounts of ubiquitin-conjugates were detected in roots (Fig. 5c, d) compared with shoots (Fig. 5a, b). Increasing NaCl concentration resulted in a decline of conjugate content of roots (Fig. 5c, f). The amounts of conjugates were higher in salt-stressed roots fed with proline than in those stressed with salt alone at all concentrations of salt. Moreover, exogenous proline increased the amounts of conjugates in non-stressed roots compared to the control (Fig. 5d, f).

Antioxidative activity

Salt stress resulted in a decrease in the activity of catalase of the shoot especially at 300 mM NaCl where it declined to less than 50% compared to the control (Fig. 6a). Proline fed to NaCl-stressed plants was able to maintain the catalase activity significantly higher than that of plants stressed with NaCl alone particularly, at 300 mM NaCl (P=0.01).

In the case of roots, catalase activity declined to about one-third of the control in response to NaCl (Fig. 6b). In presence of proline, the catalase activity was slightly higher in the case of NaCl-stress than in its absence although statistical analysis did not show that this difference was significant.

Activity gels showed that there is one catalase band both in shoots and in roots. The band intensity, as a measure of activity, showed the same trends as the spectrophotometric measurements (Fig. 6c–f) indicating that this band is probably responsible for most of the change in catalase activity.

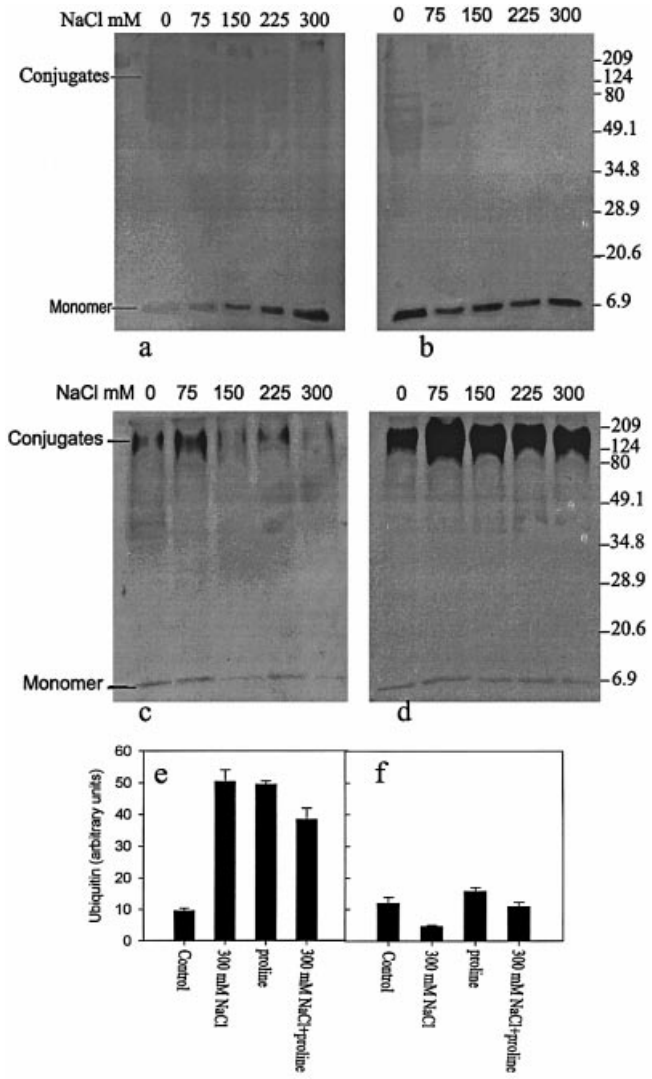

Fig. 5. Effect of salt stress on the expression of ubiquitin and the accumulation of ubiquitin-conjugates in *Pancratium* leaves (a, b) and roots (c, d) in the absence (a, c) and presence (b, d) of 5 mM proline as revealed by western blot analysis of total leaf and root proteins, respectively. Seedlings were stressed with NaCl for 10 d. Concentrations of NaCl are shown at the top of each treatment. (e, f) Quantification of ubiquitin in leaves and roots, respectively. Values are ubiquitin content in arbitrary units ±SE. the detection and quantification were repeated three times.

The total peroxidase activity of the shoot remained almost unchanged at low concentrations of NaCl, but increased at higher concentrations (Fig. 7a). By contrast, it decreased continually in the roots in response to increasing concentrations of both NaCl (Fig. 7b).

The addition of proline to non-stressed plants significantly decreased the total peroxidase activity of the roots compared with the control. By contrast, the total

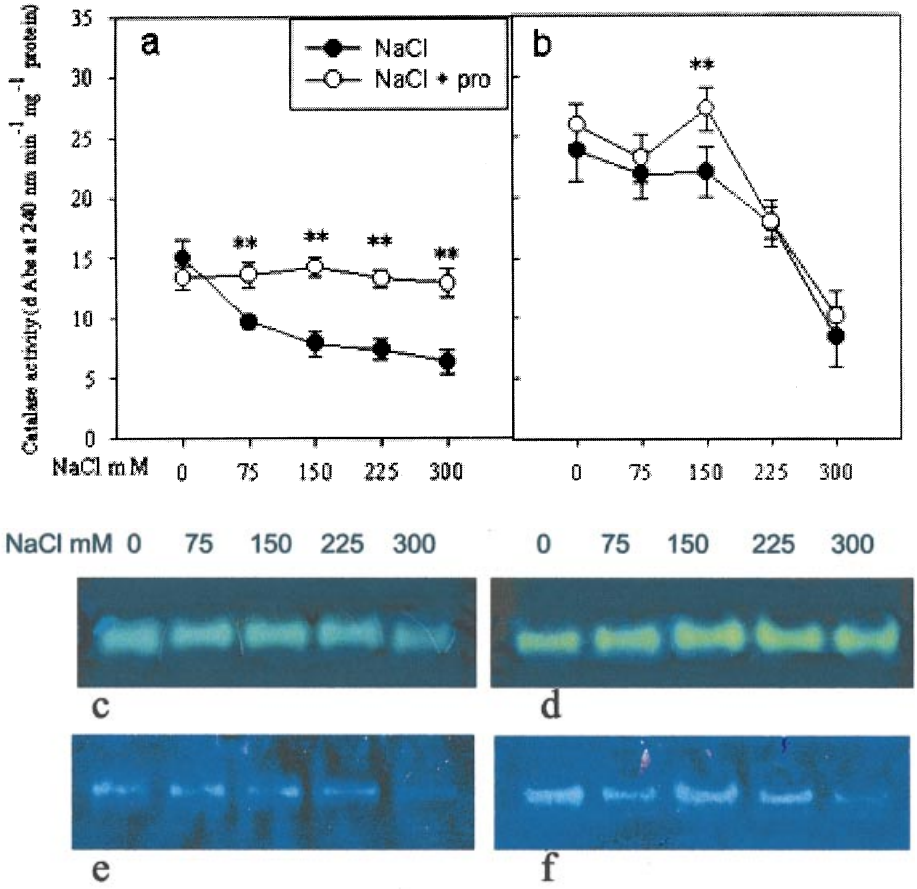

Fig. 6. Changes in catalase activity of the shoot (a) and root (b) of *Pancratium* seedlings after treatment with NaCl (0–300 mM) for 10 d in the absence and presence of proline. Values are catalase activity ±SE. Two asterisks indicates significant from the corresponding closed circle at P=0.01. (c–f) Activity gels of shoot (c, d) and root (e, f) catalase after treatment with NaCl in the absence (c, e) and presence of proline (d, f). The concentrations of NaCl are shown on top of each gel. Each measurement was repeated three times.

peroxidase activity of stressed shoots and roots was significantly higher in the presence of proline, particularly at higher NaCl concentrations (Fig. 7a, b). Activity gels of peroxidases showed that various bands exist in both shoots and root. Three bands were detected in both shoots and roots (bands 1, 2 and 3; Fig. 7c–f). The three bands were differentially regulated by stress treatments. Table 1 summarizes the response of the three shoot and root peroxidase bands to various treatments.

Regulation of dehydrin expression

Six major dehydrin bands were detected in the control shoots (bands 1–6; Fig. 8a, b). However, it is not clear whether each band contains one or more proteins. Dehydrins were specifically regulated by salt-stress, where some bands were up- and some others were down-regulated. The major bands (bands 4, 5 and 6) were up-regulated by salt-stress. In addition, dehydrin bands number 5 and 6 were induced by proline in non-stressed and in salt-stressed shoots. The up-regulation of bands 5 and 6 by proline was not reversed by NaCl, therefore proline increased them in salt-stressed shoots. The response of dehydrins to stress treatments is summarized in Table 2.

In the roots, three dehydrin bands were detected in the control (bands 1–3; Fig. 8c, d). The effect of salt stress was somewhat variable, but the presence of proline clearly increased activity especially at higher salt concentrations.

Discussion

The growth of *Pancratium* seedlings in terms of fresh weight of the shoot was reduced by salt-stress, but this reduction was alleviated in part by the addition of exogenous proline (Fig. 1). Measurements of dry weight (Fig. 1) and water content (Fig. 2) of seedlings indicated that proline did not improve the dry weight yield of salt-stressed seedlings. Therefore exogenous proline could only maintain a higher water content under salt stress and this is further emphasized by measurements that showed that the

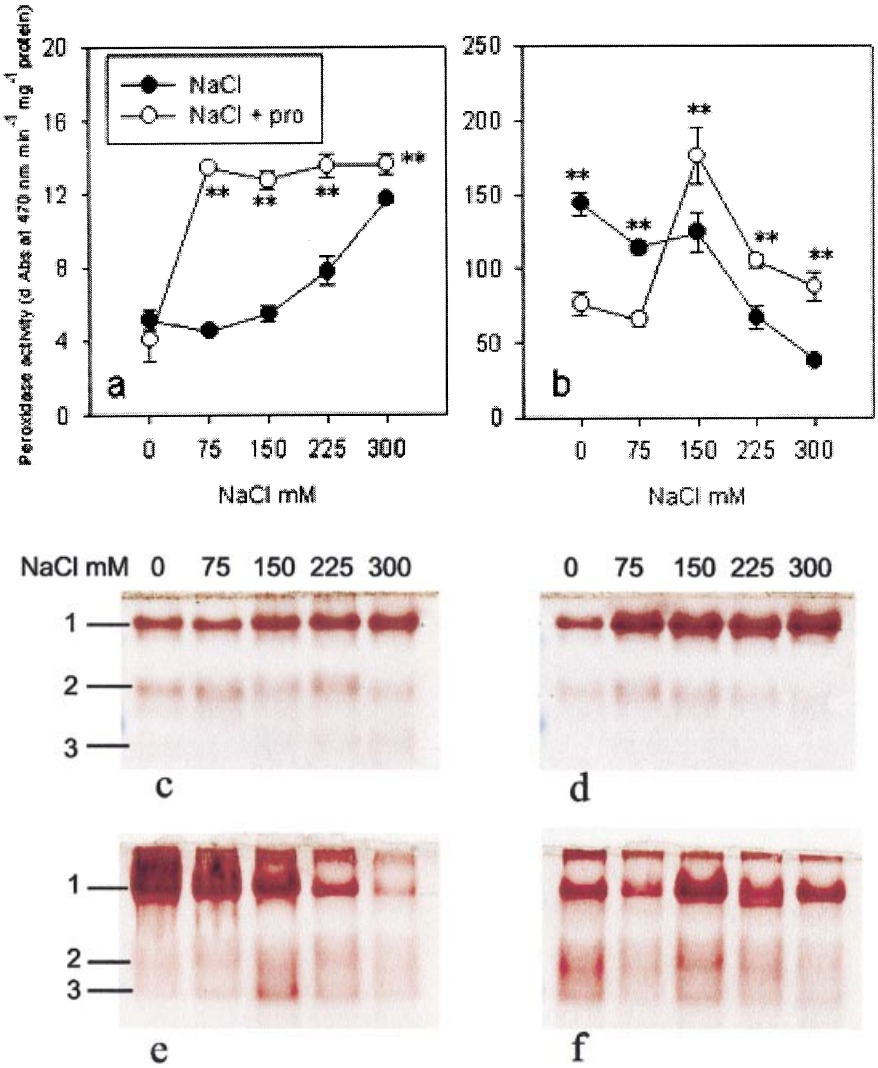

Fig. 7. Changes in peroxidase activity of shoot (a) and root (b) of *Pancratium* seedlings after treatment with NaCl (0–300 mM) for 10 d in the absence and presence of proline. Values are peroxidase activity ±SE. Two asterisks indicates significant from the corresponding closed or opened circle at P=0.01. (c–f) Activity gels of shoot (c, d) and root (e, f) peroxidase after treatment with NaCl in the absence (c, e) and presence of proline (d, f). The concentrations of NaCl are shown on the top of each gel. Each measurement was repeated three times.

water content of salt-stressed seedlings was higher in the presence of proline. In the roots, the water content decreased in response to salt stress, however, exogenous proline kept it comparable to that of the control in salt-stressed roots. The ability of exogenous proline to maintain higher water content in severely stressed seedlings might be attributed to its contribution to osmotic adjustment both directly by increasing the internal proline content (Fig. 4) and indirectly by increasing the internal contents of other amino acids (data not shown).

Inhibition of growth and a decrease in water content induced by water stress has been universally observed even in tolerant plants (Bartels and Salamini, 2001; Mittler *et al*., 2001). Although growth is the visible indicator of plant performance under stress, it is considered to result from the sum of the adaptive mechanisms that are adopted by a given species. It would therefore be predicted that exogenous proline can induce adaptive mechanisms in *Pancratium*. In contrast to its effect on *Pancratium*, exogenous proline was found to reduce the growth of roots of rice seedlings and the amount of inhibition increased with increasing the concentration of proline from 1 mM to 4 mM. This inhibition was attributed to the observation that proline induced cell wall bound peroxidases which resulted in increased lignin synthesis, thereby stiffening the cell wall and reducing expansion (Lin and Kao, 2001).

The effect of salt-stress on protein content depended on the concentration of NaCl. At lower levels of NaCl, there was an increase in protein content, but higher

Table 1. *Response of shoot and root peroxidases of* Pancratium *to 300 mM NaCl with and without proline*

Up and down arrows indicate the change in band intensity as revealed by activity gels. ↑: Band intensity induced, ↓: band intensity inhibited, ↔: band intensity remains largely the same.

Band	NaCl		Pro		NaCl+Pro	
	Shoot	Root	Shoot	Root	Shoot	Root
1	↑	↓	↓	↓	↑	↑
2	↓	↔	↓	↑	↓	↔
3	↑	↔	–	↑	–	↔

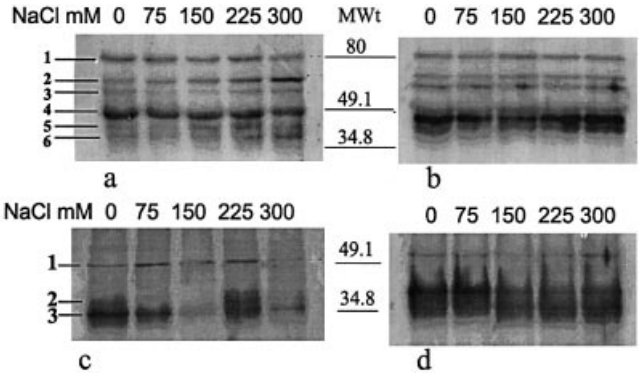

Fig. 8. The regulation of dehydrins of shoot (a, b) and root (c, d) of *Pancratium* seedlings by salt stress in the absence (a, c) and presence (b, d) of proline as revealed by western blot analysis using anti-dehydrins. The concentrations of NaCl are shown on the top of each blot.

Table 2. *Regulation of shoot dehydrins of* Pancratium *by salt stress and proline*

Up and down arrows indicate the change in band intensity as revealed by western blotting of soluble proteins. ↓: Band intensity inhibited, ↑: synthesis induced, –: synthesis unchanged, *: band responds to proline (all compared to the control).

Dehydrin band	Response to		
	NaCl	Pro	NaCl+Pro
1	↓	–	–
2	↑	–	–
3	↑	↑	↑
4	↓	↓	–
5	↑	↑*	↑
6	↑	↑*	↑

concentrations caused it to decline in both shoot and root. This suggests that the initial response to water stress involves increased protein synthesis that is prevented when the stress becomes too severe. Exogenous proline increased the protein content of the shoot and root at all levels of salt stress and, interestingly, also in non-stressed plants (Fig. 3). Changes in growth and protein contents as a result of exogenous proline correlated with increases in the internal content of proline suggesting that proline was taken up into the roots and transported to the shoots (Fig. 4). Proline may perform this adaptive effect by working as an additional nitrogen source and/or decreasing proteolytic activity (data not shown).

It would be predicted that plants under stress would have a powerful protein turnover machinery to degrade stress-damaged and environmentally-regulated proteins (see Introduction). Because ubiquitin is an essential component of this machinery, its availability during stress is therefore vital and hence the up-regulation of ubiquitin by stress has been viewed as an adaptive feature (O'Mahony and Oliver, 1999). The data presented here show that ubiquitin expression is induced by water stress (Fig. 5), but that this is as a result of free ubiquitin accumulation in the shoots and due to ubiquitin-protein conjugates in the roots. One possible explanation for this is that ubiquitin production is increased in response to stress and that the accumulation of conjugates reflects the different allocation of control to enzymes in the protein turnover pathway between the two tissues. Hence the rate of protein ubiquitination may be an important control point in stressed shoots, but in roots the rate of turnover of ubiquitinated proteins is the major control point. Ubiquitin-conjugates decreased in response to salt-stress independently of the free ubiquitin abundance, indicting that free ubiquitin is not rate limiting under these conditions. The accumulation of conjugates depends on at least two factors: first, the efficiency of ubiquitinating enzymes in linking ubiquitin to damaged proteins and, second, the rates of degradation or deconjugation; ubiquitinated proteins may be repaired by chaperones and/or deubiquitnated by cystiene proteases (Yen *et al*., 2000). Exogenous proline increased the amounts of conjugates even in non-stressed plants, suggesting that proline is able to induce similar responses as those induced by water stress. Therefore, exogenous proline seems here to play an adaptive role by up-regulating ubiquitin and presumably by stabilizing ubiquitinating enzymes.

Catalase is involved in scavenging hydrogen peroxide that is produced during photorespiration and β-oxidation of lipids in the peroxisomes under normal conditions and

accumulated under stressful conditions. The induction of catalase activity under water stress is well documented and a positive relationship has been found between its up-regulation and stress tolerance (Hernandez *et al*., 2000; Hamilton and Heckathorn, 2001; Shalata *et al*., 2001; Ushimaru *et al*., 2001).

The down-regulation of catalase by salt-stress (Fig. 6) may indicate that the plant is not able to maintain protection against active oxygen under salt-stress particularly at high salt concentrations. The decrease in catalase activity measured under severe water stress has been reported previously (Baisak *et al*., 1994; Sgherri and Navari-Izzo, 1995; Schwanz *et al*., 1996). Such a decrease may be due to some stress-induced damage to the enzyme. This may be supported by the ability of proline to maintain higher catalase activity under stress, since proline can work as an enzyme protectant under stress (see Introduction).

Like catalase, peroxidase is involved in neutralizing hydrogen peroxide, but at the expense of another substrate being oxidized such as ascorbate. Peroxidase is also reported to be enhanced by water stress and this was positively correlated with water stress tolerance (Hernandez *et al*., 2000; Sairam and Saxena, 2000; Hamilton and Heckathorn, 2001; Lin and Kao, 2001; Shalata *et al*., 2001; Ushimaru *et al*., 2001).

The consistent increase in peroxidase activity in the shoots (Fig. 7) even during severe salt-stress and its differential regulation show that peroxidase may be more stable or more important for stress tolerance than catalase. This is probably dictated by the wide range of metabolic processes in which peroxidases are known to be involved, such as lignin biosynthesis and formation of isodityrosine bridges that are believed to crosslink structural protein molecules, in addition to antioxidative activity. The reduction of peroxidase activity by severe salt-stress may be due to stress damage that can be partially relieved by exogenous proline.

The pattern of expression of dehydrins is now established to be developmentally and environmentally regulated. They are expressed in the late stages of embryogenesis and induced in vegetative tissues in response to different kinds of stress including dehydration, salinity, cold, and heat stress. In barley, one dehydrin was detected in well-hydrated barley seedlings, but, upon water stress, many others were newly synthesized (Close *et al*., 1993). In sunflower, no dehydrin transcripts were detected in well-watered drought-tolerant and drought-sensitive lines (Cellier *et al*., 1998). In the drought-tolerant resurrection plant, *Craterostigma plantagineum*, Schneider *et al*. (1993) detected two proteins that resemble dehydrins in the well-watered leaves. But upon dehydration, more new proteins were detected using the same antibodies. They suggested that some members of these proteins might be involved in normal metabolism. In this study, it was found that many dehydrins are constitutively expressed in both stressed and non-stressed plants and that proline was able to increase expression at all stress levels. This constitutive expression has not been observed previously even in drought-tolerant species.

In *Pancratium*, the expression of the whole set of dehydrin proteins in the shoot and root (Fig. 8) may indicate that these proteins are all required for normal metabolism, but more amounts are required during water stress. However, the pre-existence of dehydrins in the non-stressed tissues may provide more protection upon water stress. The quantitative and qualitative regulation of dehydrins by salt-stress suggests quantitative differences in the requirement of individual dehydrins to cope with the stress and that the protection against stress damage may be attained by the co-ordinated action of the whole set of dehydrins rather than by the action of individual proteins (Bartels and Salamini, 2001).

The induction of dehydrins by proline indicates that it might be a component of the regulatory process(s) that leads to the accumulation of dehydrins as can be deduced from its ability to induce dehydrins of the shoot and root to levels that are comparable to these under stress conditions. Similar results have been found by Garcia *et al*., (1997), who found that the *sal*T gene of rice is induced by proline and it was found previously that this gene is induced by NaCl, drought and ABA (Claes *et al*., 1990). However, Iyer and Caplan (1998) found that pyrroline-5-carboxylate, which is a product of proline catabolism, but not proline itself was able to induce dehydrins in rice.

From these results it is concluded that proline could act as a component of signal transduction pathways that regulate stress responsive genes in addition to its previously described osmoprotective roles, thereby improving the tolerance to salt stress.

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