RESEARCH ARTICLE

Rice can acclimate to lethal level of salinity by pretreatment with sublethal level of salinity through osmotic adjustment

M. Djanaguiraman · J. Annie Sheeba · Arun K. Shanker · D. Durga Devi · U. Bangarusamy

Abstract The physiological ability to adapt for various environmental changes is known as acclimation. When exposed to sublethal level of stress, plants develop the ability to withstand severe stress, as acquired tolerance. The present study was conducted to explicate the physiological basis of acquired tolerance in rice. Rice seedlings (variety IR 20) were grown in half strength Hoagland solution, and after 22nd day, they were kept in half strength Hoagland solution containing 50 mM NaCl (sublethal dose) for 7 days followed by half strength Hoagland solution containing 100 mM NaCl (lethal dose) for another 7 days. The non-pretreated 29 days old rice seedlings maintained in half strength Hoagland solution were directly transferred to half strength Hoagland solution containing 100 mM NaCl (lethal dose) solution for 7 days. The control plants were maintained in half strength Hoagland solution without NaCl. Various morphological and physiological parameters were recorded on 29th and 36th days old seedlings from control, pretreated and non-pretreated plants. The results revealed significant reduction in growth parameters (shoot length, root length, leaf area and total dry matter production) of non-pretreated plants below that of pretreated plants. The pretreated plants showed increased values to the extreme of 19.8 per cent in leaf water potential (ψ_w), 9 per cent in relative water content (RWC), 26 per cent in photosynthetic rate (P_N), 28 per cent in leaf stomatal conductance, and 47 per cent in chlorophyll a over non-pretreated plants. The same trend was also observed in chlorophyll a/b ratio (6.6%) and F_v/F_m ratio (19.3%). However, a reverse trend was seen in F_o value. The pretreated plants showed improved ionic regulation as evident from low Na^+, Cl^- and high K^+ contents, which is attributed to enhanced plant water status and photosynthesis. Both pretreated and non-pretreated plants had higher contents of osmolytes viz., sucrose, leaf soluble sugars and proline contents than control plants. However, starch content revealed an inverse trend. Therefore, the present study reveals that rice can acclimate to lethal dose of salinity stress by pretreatment with sublethal dose of NaCl.

Keywords Salinity · Pretreatment · Acclimation · Sublethal level · Lethal level · Ions · Osmotic adjustment

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Abbreviations

RWC	relative water content
F_o	baselevel fluorescence
F_v/F_m	variable to maximum fluorescence ratio
P_N	photosynthetic rate
Ψ_w	leaf water potential

Introduction

Rice is a main staple crop around the world, feeding and providing the necessary daily calories for millions of people (Kush 1997). Major environmental limitations on rice production are salinity and drought (Toenissen 1995). Soil salinity is a complex effect causing disturbance of membrane integrity, nutrient imbalance and disturbances on general metabolic activities. The accumulation of salts in the soils of arid and semi arid regions is a continuing threat to crop production. Attempts by soil scientists to adopt management practices to reduce soil salt concentration, which are too expensive, cannot be contemplated in most of the underdeveloped and developing countries like India. A possible alternative is the introduction of crop species/cultivars capable of tolerating the higher soil salinities with moderate economic yield (Yeo and Flowers 1986). Two types of plant responses to salinity have been distinguished: preexisting resistance mechanisms and adaptation or acquired tolerance (Amzallag et al. 1990). Response of plants to any stress agent is particularly of adaptive nature when the stress is sublethal. On the other hand, response shown may be biased towards cell death if the stress is lethal (Grover et al. 2001).

Amzallag et al. (1990) showed that sorghum supplemented with 150 mM NaCl at the seedling stage for 20 days, could survive and produce seeds in even 300 mM NaCl; a concentration that was lethal for normal plants. Similar type of result was also observed by Umezawa et al. (2000) in soybean. Several studies (Uma et al. 1995; Jayaprakash et al. 1998; Kumar et al. 1999; Burke 2001; Srikanthbabu et al. 2002; Senthil-Kumar et al. 2003) have clearly shown that genetic variability for the stress response could only be seen upon exposure to induction stress. The rice variety white ponni completed its life cycle in a lethal NaCl concentration of 300 mM, when it was preceded by treatment to sublethal NaCl concentration (150 mM) for 7 days. However, without this sublethal NaCl treatment, the plant was unable to complete its life cycle (Djanaguiraman et al. 2003). However, the physiological basis of this acquired tolerance was not known. Hence, it is hypothesised that exposure to sublethal stress would bring the required changes in the plant metabolism necessary for withstanding the subsequent severe stress (lethal).

Materials and method

Plant material

Rice seeds (*Oryza sativa* L.) variety IR 20 were obtained from the Department of Rice, Tamil Nadu Agricultural University, Coimbatore, India and used as experimental material.

Growth condition and salt treatment

The rice seeds were sterilized with 70% ethanol for 5 min and 10% H_2O_2 for 10 min. They were then washed thoroughly, soaked in distilled water for 48 h and then incubated for 24 h at 30°C under moist, dark conditions. The germinated seeds were grown hydrophonically; four plants per container of 8 L aerated half strength Hoagland nutrient solution in three sets upto 40 days. In each set, a minimum of 20 containers were maintained, with one set as control. The control plants received half strength Hoagland's solution and no NaCl was added.

In the second set, salinity treatment was started on 22 DAS. Uniform seedlings with four leaves were used for various assays. Plants were exposed to 50 mM NaCl added to the half strength Hoagland solution for one week. This constitutes pretreatment (Djanaguiraman et al. 2003). After the expiry of one week, the plants were transferred to half strength Hoagland solution containing 100 mM NaCl and maintained for one week. In the third set, plants of 29 days old were directly transferred to half strength Hoagland solution containing 100 mM NaCl, and maintained for one week. The nutrient solution was renewed every 2 days. The experiment was conducted in a growth chamber. The photoperiod was 14 h and the day/night temperatures were 28/22°C. The light source was fluorescent incandescent lamp with a PAR of 450 μM m^{-2} s^{-1} and the relative humidity was 70%. The plants of all the three sets were analysed for growth, water relations and osmolytes production at 29th DAS and 36th DAS in 2nd and 3rd leaves. A minimum of twelve plants per treatment were analysed.

Growth parameters

After measuring shoot and root lengths (cm), and leaf area (cm^2 $plant^{-1}$) the samples were oven dried

at 80°C for 24 h and weighed for arriving total dry matter production (g plant^{-1}) from all the three sets.

Leaf water potential and relative water content (RWC)

Leaf water potential (Ψ_w) was estimated using a Pressure Bomb Apparatus (Soil Moisture Equipment Corporation, USA). The readings were taken between 11.00 and 11.30 A.M, and expressed as MPa (Turner 1988). Leaf relative water content (RWC) was estimated according to the methodology of Weatherly (1950).

Chlorophyll content, fluorescence and gas exchange parameters

Chlorophyll content was estimated according to Yoshida et al. (1971), by extracting the chlorophyll in 80% acetone. Chlorophyll fluorescence measurements were made with Plant Efficiency Analyzer (PEA) (Hansatech, UK) following the method advocated by Lu and Zhang (1998). Measurement was made on third leaf, which was dark adapted for 30 min prior to measurement. Using both light and dark fluorescence parameters the maximal efficiency of PS II photochemistry (F_v/F_m) in the dark adapted state was calculated ($F_v/F_m = (F_m−F_o)/F_m$). The rate of photosynthesis and stomatal conductance of the seedlings were measured at photosynthetic photon flux (PPF) >600 μE m^{-2} s^{-1} (saturating light), using a Portable Photosynthetic System (LICOR, Li-coln). Gas measurements were made on all the three sets between 10 and 11 A.M. Ten observations on the rate of photosynthesis (μmol m^{-2} s^{-1}) and stomatal conductance (mol m^{-2} s^{-1}) were recorded for each replication.

Ion analysis

Dried and powdered root and shoot samples were used for ion analysis. Na$^+$ and K$^+$ were quantified by atomic absorption spectrometry (Perkin-Elmer, 5500), and Cl$^-$ was quantified by titration method according to Ross (1984), and expressed as mmol g^{-1} DW.

Osmolytes quantification

Sugars and its metabolizing enzymes

Sucrose content of leaf was estimated from the soluble sugar fraction as described by Cardini et al. (1955), and expressed as mmol g^{-1} DW. Starch and soluble sugars contents of the leaf were estimated according to Hedge and Hofreiter (1962) and expressed as mg g^{-1} DW. Sucrose synthase and sucrose phosphate synthase were analysed according to the methodology of Miron and Schaffer (1991). The absorbance was measured at 520 nm against the blank made in the absence of enzyme. The enzyme activity was expressed in μmol mg^{-1} protein h^{-1} FW.

Proline content

Proline was extracted in 3% sulfosalicylic acid and estimated by using acid ninhydrin reagent and measuring the absorbance of the toluene chromophore at 520 nm (Bates et al. 1973) and expressed as μmol g^{-1} DW.

Statistical analysis

Duplicate samples from six replications of all the three treatments viz., control, pretreated and non-pretreated plants were taken for all the enzyme assays and non enzymatic analysis ($n=12$). The mean values ±S.E. are given in all figures. The data were analysed statistically using Systat for Windows version 6 (Wilkinson et al. 1996). Significance between control and treatment was compared at 0.05 probability levels using student t test.

Results

Growth

The NaCl treatment significantly decreased the leaf area (Fig. 1c) and total dry matter production (Fig. 1d) at 29th DAS in pretreated plants to an extent of 16.0 and 13.4% in that order over control. However at 36th DAS, both pretreated and non pretreated plants showed significant reductions in shoot length (Fig. 1a), leaf area (Fig. 1c) and total dry matter

Fig. 1 Effect of NaCl pretreatment on (**a**) shoot length (cm) and (**b**) root length (cm) (**c**) leaf area (cm^2 plant^{-1}) and (**d**) TDMP (g plant^{-1}), in rice variety IR20
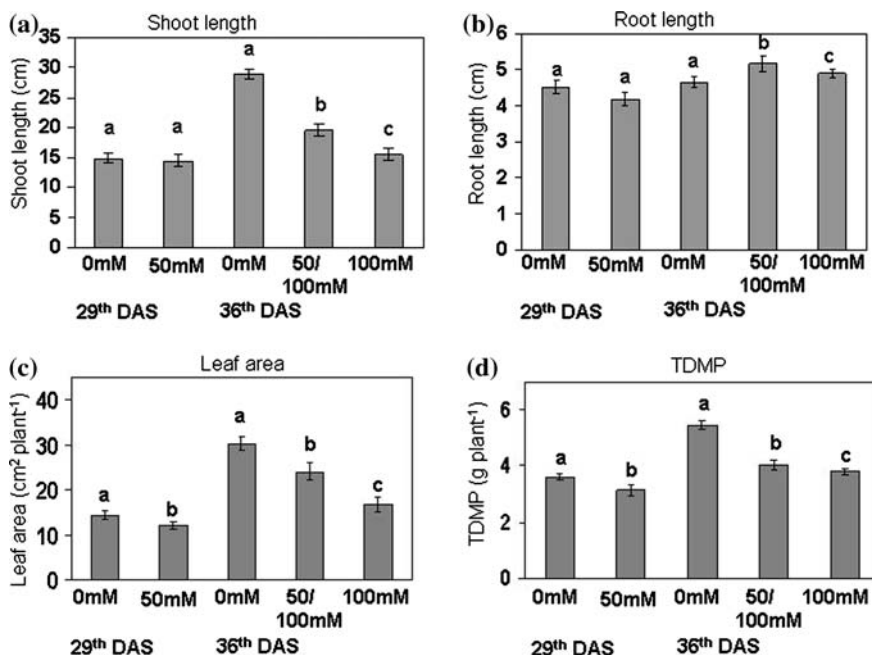
production (Fig. 1d) over control. The root length (Fig. 1b) increased significantly in pretreated and non-pretreated plants over control at 36th DAS only. That the pretreated plants have adapted well to lethal level of NaCl stress compared to non-pretreated plants was revealed by higher values in growth parameters of pretreated plants over non-pretreated ones at the same NaCl concentration (100 mM).

Leaf water potential and RWC

Salinity significantly affected leaf water potential (ψ_w) in pretreated and non-pretreated rice plants at 29th and 36th DAS (Fig. 2a). The pretreated plants had the lowest leaf water potential of −1.33 MPa when the ψ_w of non-pretreated plants was −1.11 MPa in 100 mM NaCl concentration at 36th DAS. Salinity did decrease relative water content (Fig. 2b), but to a lesser extent in pretreated plants than in non-pretreated plants. At 36th DAS the pretreated plants showed a decrease of 20.2% in RWC whereas non-pretreated plants had 29.1% decrease at same NaCl concentration.

Gas exchange

The photosynthetic rate (Fig. 2c) and leaf stomatal conductance (Fig. 2d) decreased significantly with

salinity treatment. Leaf photosynthetic rate decreased to about 14 and 32% in pretreated and non-pretreated plants respectively at 100 mM NaCl concentration. At the same concentration, the reductions in stomatal conductance were 35 and 63% over control for pretreated and non-pretreated plants respectively. The large decrease in stomatal conductance revealed its vulnerability to salinity.

Chlorophyll contents and fluorescence

The content of chlorophyll *a* decreased significantly under salinity at both 29th and 36th DAS (Fig. 3a). In the pretreated plants, the decrease in chlorophyll *a* was 13.5% when the decrease in non-pretreated plants was 60.8% at 100 mM NaCl concentration. Differences in chlorophyll *a/b* ratio between control, pretreated and non pretreated plants was evident only at 36th DAS (Fig. 3b). Salinity increased the chlorophyll *a/b* ratio of pretreated and non-pretreated plants. The pretreated and non-pretreated plants had increases of 6.7 and 11.6% respectively over control at lethal NaCl concentration (100 mM).

Fig. 3c shows that F_o was considerably enhanced by the NaCl treatment at 36th DAS in both pretreated and non-pretreated plants. At lethal NaCl concentration, the pretreated plants showed an increase of

Fig. 2 Effect of NaCl pretreatment on (**a**) leaf water potential (MPa) and (**b**) relative water content (%), (**c**) photosynthetic rate (μmol m^{-2} s^{-1}) and (**d**) stomatal conductance (mol m^{-2} s^{-1}), in rice variety IR20
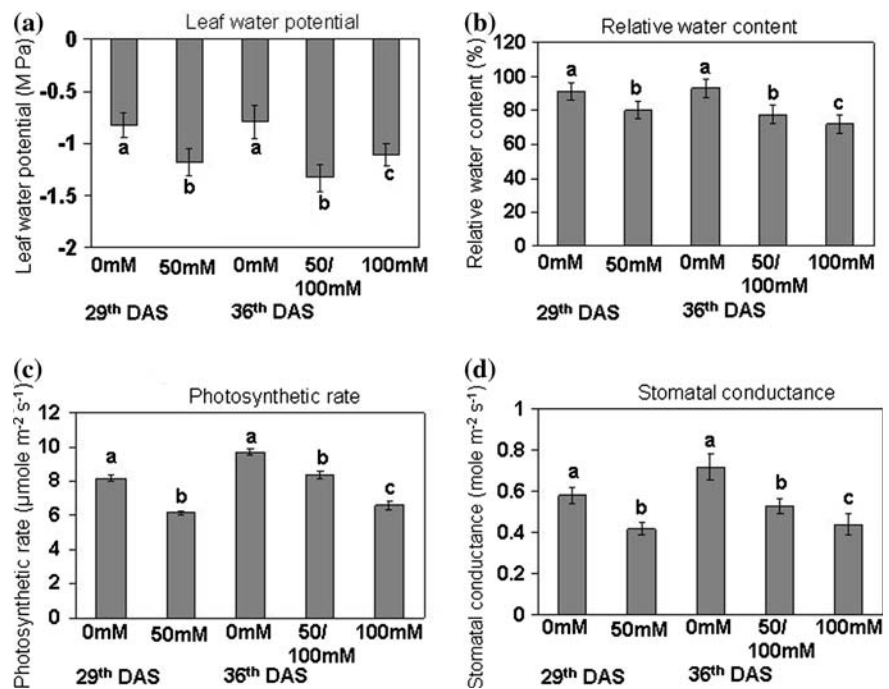
5.8% in F_o over control, whereas, the non-pretreated plants recorded an increase of 26%. Likewise, the pretreated plants also recorded a subtle decrease (9.2%) in F_v/F_m (Fig. 3d) as compared to non-pretreated plants (28.5%) at lethal NaCl concentration (100 mM).
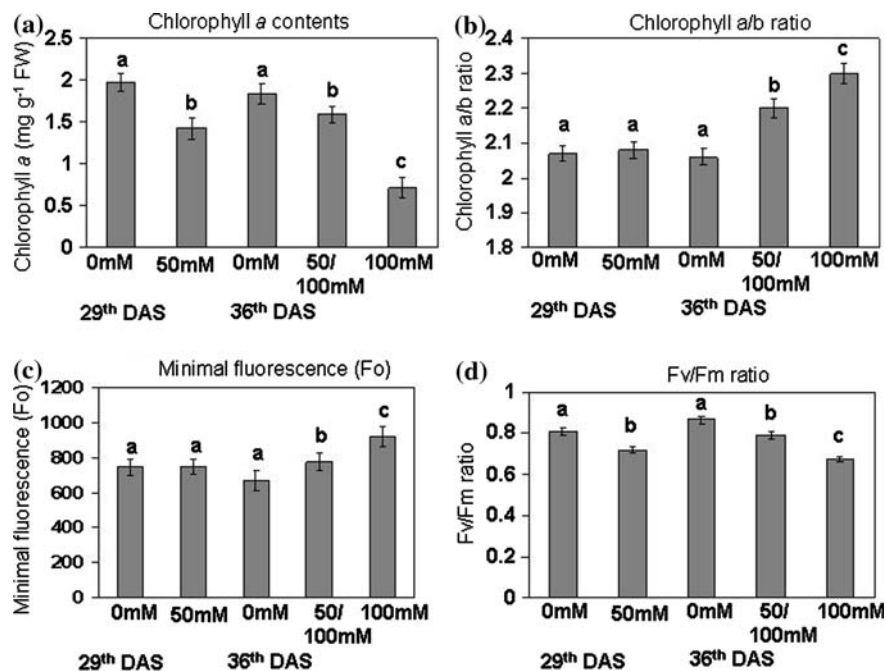

Fig. 3 Effect of NaCl pretreatment on (**a**) chlorophyll a (mg g^{-1} FW) contents (**b**) chlorophyll a/b ratio (**c**) minimal fluorescence (F_o) and (**d**) F_v/F_m ratio, in rice variety IR20

Ion analysis

Pretreatment with NaCl at 29th DAS increased the Na$^+$ content in leaves (Fig. 4a). Leaf accumulated more of Na$^+$ and K$^+$ than root (Fig. 4c). In leaf, Na$^+$ accounted for a raise of 84% to that of control, while, root showed a boost of 24.2% only (Fig. 4b). At 36th DAS, the pretreated plants had lesser increase in leaf Na$^+$ content (84%), whereas, non-pretreated plants had a higher increase (141%) over control. In root, the sodium content increased to 80.2 and 170 per cent over control by pretreatment and non-pretreatment respectively. The reverse trend was followed in potassium. The chloride contents in leaf and root followed sodium trend (Fig. 5a and b).

Sucrose synthase and sucrose phosphate synthase

Sucrose synthase activity showed a tendency to decrease with salinity regimes, but this response was more pronounced in non-pretreated plants than in pretreated plants at 36th DAS (Fig. 5c). At 100 mM NaCl concentration, sucrose synthase activity decreased to 38.4% in non-pretreated plants, while, in pretreated plants it decreased to 15.3%, as compared

to control. Sucrose phosphate synthase activity increased with increasing salinity level (Fig. 5d). The pretreated and non-pretreated plants recorded an increase of 33 and 60%, respectively over control at 100 mM NaCl concentration.

Osmolytes

The contents of sucrose (Fig. 6a), starch (Fig. 6b), soluble sugars (Fig. 6c) and proline (Fig. 6d) were distinctly affected by NaCl stress. The starch content was generally lower in the salinity regime than control, and the pretreated plants exhibited a higher value than non-pretreated plants at lethal NaCl concentration (100 mM). However, a reverse trend was observed in soluble sugars content. Sucrose content increased progressively from sublethal to lethal NaCl concentration. The pretreated plants had lower sucrose content than non-pretreated plants at the same NaCl level (100 mM). The pretreated plants had a higher proline content than non-pretreated and control plants. At lethal NaCl concentration, the pretreated plants showed 77.8 and 120% increase in proline and soluble sugar contents over control, respectively.
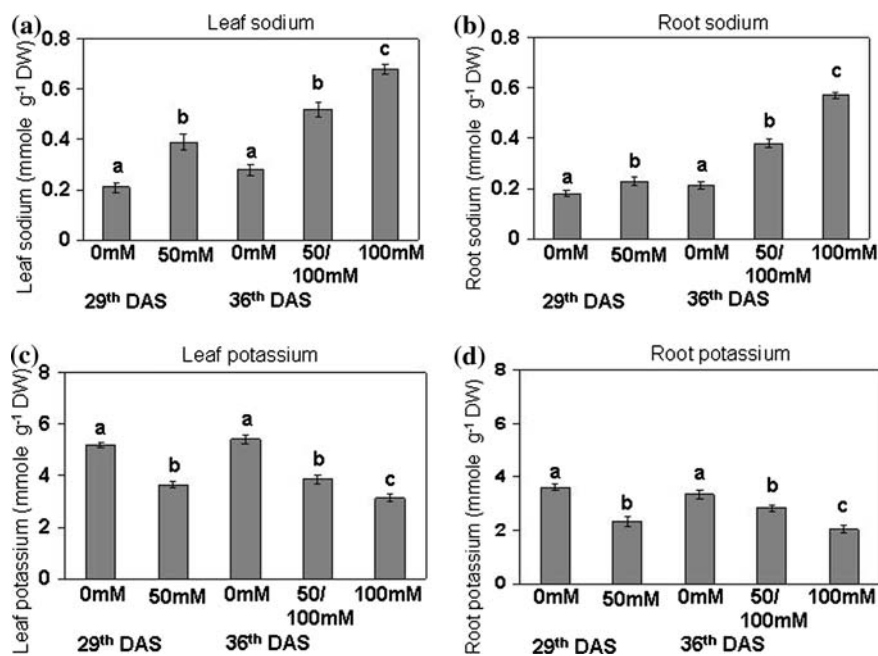

Fig. 4 Effect of NaCl pretreatment on (a) leaf sodium (mmol g^{-1} DW) (b) root sodium (mmol g^{-1} DW) (c) leaf potassium (mmol g^{-1} DW) (d) root potassium (mmol g^{-1} DW), in rice variety IR20
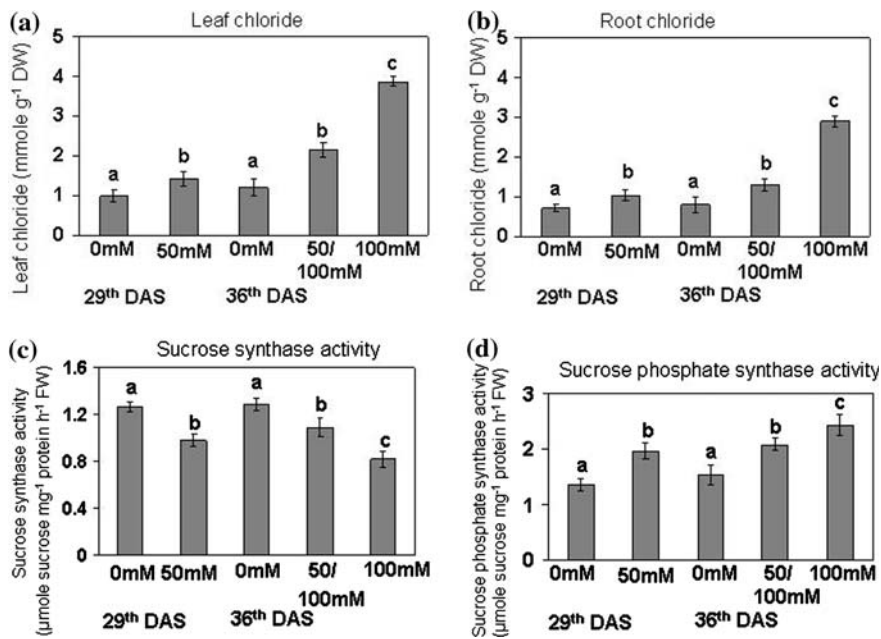
Fig. 5 Effect of NaCl pretreatment on (**a**) leaf chloride (mmol g^{-1} DW) (**b**) root chloride (mmol g^{-1} DW) (**c**) sucrose synthase activity (μmol sucrose mg^{-1} protein h^{-1} FW) (**d**) sucrose phosphate synthase activity (mol sucrose mg^{-1} protein h^{-1} FW), in rice variety IR20

Discussion

Sub lethal induction stress which the plants experience under natural conditions before being subjected to severe stress activates an array of physiological and biochemical process that modifies the crop yield. The reduction in growth of many crop plants by salinity may result from its effects on dry matter
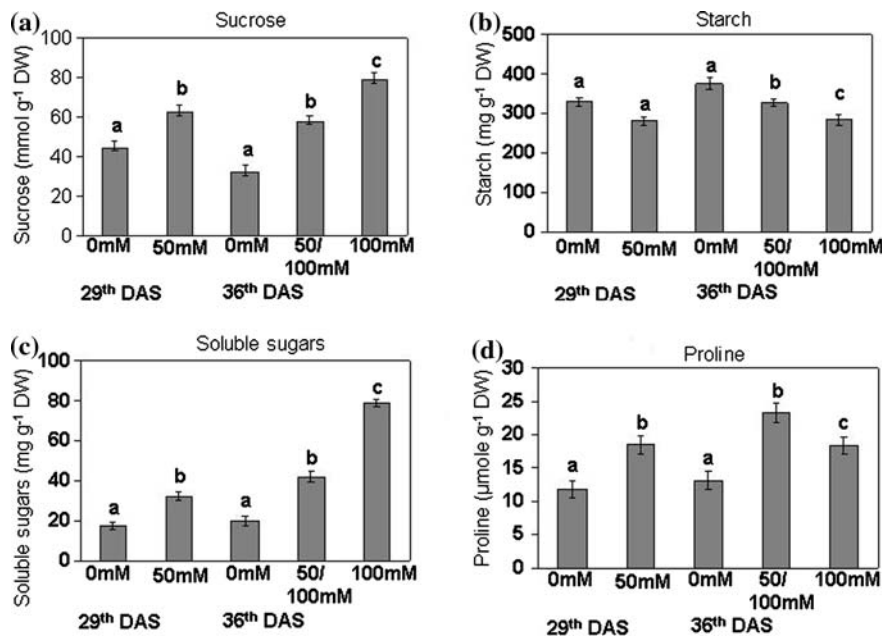

Fig. 6 Effect of NaCl pretreatment contents on (**a**) sucrose (mmol g^{-1} DW) (**b**) starch (mg g^{-1} DW) contents (**c**) soluble sugars (mg g^{-1} DW) (**d**) proline (μmol g^{-1} DW), in rice variety IR20

production, ion relations, water status and other physiological and biochemical reactions. It has been observed that genetic variability in rice for yield is only seen upon an induction treatment prior to severe stress and the observed variability was marginal when the seedlings were exposed directly to the severe stress (Djanaguiraman et al. 2003). The results of the present investigation indicate the physiological and biochemical basis of this acquired tolerance.

Growth

Salt stress has been generally considered to exert both osmotic and ionic effects. The accumulation of toxic ions, Na^+ and Cl^- in the plants is often claimed to be toxic and the main cause of growth inhibition induced by salinity (Muscolo et al. 2003). Improved growth rate under salinity is essential for salt acclimation (Amzallag et al. 1990, 1993). This could be accomplished by maintenance of optimum level of Na^+ and K^+ concentrations under salinity (Boursier and Lauchli 1990). Plants pretreated with sublethal level of NaCl showed a higher accumulation of K^+ in roots and shoots over non-pretreated plants. Hence, the stimulation of growth under pretreatment than non-pretreated plants could have been due to enhanced K^+ accumulation. The growth reduction in non-pretreatment might be due to the toxic effect created by the presence of excess amount of Na^+ and Cl^- coupled with low K^+ concentration.

The enhanced growth in pretreated plants over non-pretreated plants at lethal dose indicates that the low concentration of NaCl pretreatment had a stimulative effect in acclimation process as reported by Umezawa et al. (2000) in soybean. Salinity causes an increase in root length of pretreated plants at 36th DAS, whereas, at 29th DAS the pretreated plants did not showed any variation over control, indicating that shoots were more sensitive than the roots to lethal dose of salinity.

Photosynthesis, pigment and chlorophyll fluorescence

Both salinity treatments reduced stomatal conductance (g_s) and photosynthetic carbon assimilation (P_N). However, the pretreated plants had higher g_s than non-pretreated plants. The reduced photosynthetic carbon assimilation was due to reduced stomatal conductance as reported by Brugnoli and Lauteri (1991) and Bayuelo-Jimenez et al. (2003). Higher stomatal conductance in the leaves of pretreated plants could be due to lower leaf water potential (Liang et al. 2002). The lower P_N may also be due to the cumulative effects of other non-stomatal and biochemical components. Apart from this, higher concentrations of both Na^+ and Cl^- ions are capable of reducing CO_2 assimilation because of ion toxicity (Cachorro et al. 1993; Bayuelo-Jimenez et al. 2003). The reduced Na^+ and Cl^- ions content in pretreated plants than non-pretreated plants may be due to better ion compartmentation or an efficient mechanism to exclude toxic ions from the photosynthetic apparatus, which deserves further attention.

Photosynthetic parameters such as chlorophyll content and chlorophyll fluorescence were also reduced by salinity treatments. Chlorophyll b was being degraded at a higher rate than chlorophyll a in leaves exposed to lethal NaCl level because chlorophyll a/b ratio increased due to salinity treatment. This can be explained by the fact that the first step in chlorophyll b degradation involves its conversion to chlorophyll a (Fang et al. 1998). The reduced chlorophyll pigments under salinity treatments could be attributed to the increased activity of chlorophyllase enzyme (Reddy and Vora 1985), or disruption of fine structure of the chloroplast and instability of pigment and protein complex by ions (Djanaguiraman and Ramadass 2004).

The stability of F_o, found for sublethal NaCl concentration at 29th DAS, indicates that this low concentration of salt has no significant changes in the photosynthetic reaction centres. However, the variations found for lethal NaCl concentration indicates loss of energy transfer from pigments to the reaction centre. The reduction of F_v/F_m ratio in NaCl stressed plants may also be due to reduction in F_m value (data not shown), which indicates increased energy dissipation, dissociation of the light harvesting antennae from the PSII core, denaturation of the PS II reaction centre (Maxwell and Johnson 2000; Lutts et al. 1996; Santos et al. 2001), accumulation of excess ions viz., Na^+ and Cl^- or dehydration (Muranaka et al. 2002). In our study, the leaf RWC and Na^+ and Cl^- ion contents were reduced in higher percentage in non-pretreated plants than in pretreated plants. Thus the

more reduction in RWC and excess ion accumulation in non-pretreated plants could be the main factor for the photochemical inactivation.

Like photosynthesis, water status is also highly sensitive to salinity and is, therefore, dominant in determining the plant responses to stress (Yeo et al. 1985). There is substantial evidence that glycophytic as well as halophytic species adjust to high salt concentrations by lowering tissue osmotic potentials with an increase of inorganic ions from the external solution and/or compatible solutes (Cachorro et al. 1995). This generalization appears to hold for rice because of accumulation of high levels of inorganic ions and sugars in their leaves in response to salt stress.

The pretreated plants under lethal NaCl concentration accumulated lower contents of Na^+ and Cl^- and higher compatible solutes viz., sugars and proline. Leaves of non-pretreated plants accumulated more inorganic ions as opposed to compatible solutes. Although osmotic adjustment can be achieved by the uptake of inorganic ions, this could lead to impairment in ion compartmentation and subsequently a decline in leaf function in non-pretreated plants. Inorganic ions in the non-pretreated plants may be in the short term, involved in osmotic adjustment. However, in the long term, plant growth depression may be caused by Na^+ and/or chloride toxicity (Munns 1993). Moreover, high Na^+ and Cl^- concentrations may also reduce plant growth by altering mineral nutrient uptake. The non-pretreated plants had lower K content than pretreated plants, suggesting probable retranslocation of K^+ from roots to leaves or a sustained acquisition despite considerable overall Na^+ uptake (Bayuelo-Jimenez et al. 2003). The greater CO_2 assimilation rates in NaCl pretreated plants at lethal NaCl stress than non-pretreated plants could be in part due to high K^+ in leaves, which could contribute to regulation of stomatal opening under salt stress. This adaptation is worthy of further exploration because it may provide a means for control of transpiration (the amount of salt delivered to the shoot). This mechanism may be very important, since it may be a potential contributor to the development of salt tolerance in pretreated plants.

As salinity adversely influenced the photosynthetic process, photosynthates production was inhibited. The non-pretreated plants had higher sucrose and soluble sugars content than pretreated plants. Increased accumulation of sugars has been reported in many plant species exposed to salinity (Flowers et al. 1977). The decrease in starch content in non-pretreated plants was associated with an increase in soluble sugars concentration, suggesting that it was related to an attempt of osmotic adjustment.

Pretreated plants had lower sucrose content than non-pretreated plants. Present results agree with those of Sacher and Staples (1985) and Liu and Van Staden (2001), that the utilization of carbohydrates could be the limiting factor of growth under salinity. Higher sucrose content in non-pretreated plants, linked with their decreasing growth rate in lethal NaCl concentration, and also it could exert a feed-back inhibition on carbon metabolism. Morris and Arthur (1984) reported higher sucrose synthase and low sucrose phosphate synthase levels with rapidly growing cells having a high demand for sucrose. The present results showed pretreated plants had more demand for sucrose than non-pretreated plants as it has low sucrose phosphate synthase (SPS) and higher sucrose synthase (SS) activity. The possible explanation for higher sucrose content in the leaves of non-pretreated plants may be due to inhibition of translocation of sugars from leaves to various plant parts, as it reveals a lower SPS and higher SS enzyme activity. The greater accumulation of carbohydrates in the cytoplasm was to serve as an osmoticum to balance the enhanced ion accumulation in the non-pretreated plants. This has been justified by enhanced ion contents in non-pretreated plants.

An increase in leaf proline in the presence of NaCl observed in the present study is in agreement with the findings of other researchers (Colmer et al. 1996; Kafi et al. 2003; Singh et al. 1996). The enhanced proline content under salinity stress is a consequence of a reduction in the osmotic potential (Chu et al. 1976). Thus a higher amount of proline is necessary to make a balance between cytosol and vacuolar osmotic potential. The pretreated plants accumulated this organic solute at higher proportion than did the non-pretreated plants. Apparently, proline has a specific role in the adaptation of plant cells to water stress (Handa et al. 1986) and it has been reported that elevated levels of proline confers increased tolerance to hyperosmotic stress (Kishor et al. 1995). This might be the role of proline in pretreated plants.

Our data, therefore, support the hypothesis that the adaptation to lethal dose of NaCl in pretreated plants is a consequence of better control of ion absorption and its transport to the leaves and enhanced accumulation of osmolytes for osmotic adjustment.

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