



Relative importance of Na⁺, Cl⁻, and abscisic acid in NaCl induced inhibition of root growth of rice seedlings

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Abstract

The relative importance of endogenous abscisic acid (ABA), as well as Na⁺ and Cl⁻ in NaCl-induced responses related to growth in roots of rice seedlings were investigated. The increase in ammonium, proline and H₂O₂ levels, and cell wall peroxidase (POD) activity has been shown to be related to NaCl-inhibited root growth of rice seedlings. Increasing concentrations of NaCl from 50 to 150 mM progressively decreased root growth and increased both Na⁺ and Cl⁻. Treatment with NaCl in the presence of 4,4'-diisothiocyano-2,2'-disulfonic acid (DIDS, a nonpermeating amino-reactive disulfonic acid known to inhibit the uptake of Cl⁻) had less Cl⁻ level in roots than that in the absence of DIDS, but did not affect the levels of Na⁺, and responses related to growth in roots. Treatment with 50 mM Na-gluconate (the anion of which is not permeable to membrane) had similar Na⁺ level in roots as that with 100 mM NaCl. It was found that treatment with 50 mM Na-gluconate effected growth reduction and growth-related responses in roots in the same way as 100 mM NaCl. All these results suggest that Cl⁻ is not required for NaCl-induced responses in root of rice seedlings. Endogenous ABA level showed no increase in roots of rice seedlings exposed to 150 mM NaCl. It is unlikely that ABA is associated with NaCl-inhibited root growth of rice seedlings.

Abbreviations: ABA – abscisic acid; DIDS – 4,4'-diisothiocyano-2,2'-disulfonic acid; DW – dry weight; ELISA – enzyme-linked immunosorbent assay; FW – fresh weight; POD – peroxidase

Introduction

Soil salinity, particularly due to NaCl stress, can be considered as the single most widespread soil toxicity problem that global rice production faces at present. In rice, it was shown that during vegetative growth the young seedling stage is the most sensitive to NaCl, in comparison to germination and tillering stages (Lutts et al., 1995). Roots play a number of important roles during plant growth and development and typically are the first and critical part of the plant to encounter soil salinity. The inhibition of root growth by salinity is a widespread problem in agricultural practice. However, the mechanisms underlying this inhibition are

not yet clear (Greenway and Munns, 1980; Munns and Termaat, 1986; Rengel, 1992).

When growing in saline soils, roots have to cope with two types of stresses. The first of these is an osmotic stress resulting from salt concentration in the soil that results in lowered water potential and a consequent loss of cell turgor in roots. The second is ionic stress induced by changes in the concentrations of Na⁺, Cl⁻ or both in the root-growing medium and within root tissues. These stresses in turn caused a reduction in the water uptake and inhibition of root growth (Kafkafi and Bernstein, 1995).

Recent studies in our laboratory show that NaCl-inhibited root growth of rice seedlings is associated with increase in H₂O₂ level and cell wall peroxidase (POD) activity (Lin and Kao, 1999, 2001a). This suggests that NaCl-induced cell wall stiffening is a

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Figure 1. Photograph of rice seedlings treated with NaCl. Photograph was taken after 5 days of treatment. NaCl concentrations from left were 0, 50, 100 and 150 mM, respectively.

possible mechanism of NaCl-inhibited root growth of rice seedlings. We also reported that increase in cell wall POD activity in NaCl-treated roots is associated with ionic rather than osmotic component of NaCl stress (Lin and Kao, 1999). However, we do not know whether this ionic effect is caused by Na^+ , Cl^- or both.

High tissue Cl^- content found in salt-treated beans has been considered as the principal causes for the salt-induced growth reduction (Marschner, 1995). Cramer et al. (1994) reported early variety differences in leaf growth rates of maize plants. They showed that maize variety which was more sensitive to early growth inhibition by salinity accumulated less Na^+ than the salt-resistant variety. Thus, greater inhibition of leaf growth was not related to proportionately higher rates of salt accumulation. Recent investigations by Montero et al. (1998) and Sibole et al. (1998) strongly suggest that bean is also extremely sensitive to Na^+ . Munns et al. (1988) reported that leaf growth of barley was reduced by the higher NaCl treatments, but is not directly controlled by local concentration of Na^+ or Cl^- , suggesting that some influence originating elsewhere in the plants. It has been observed that ABA increases in plants under salt stress and inhibits growth when applied to plants under control conditions (Munns and Sharp, 1993). Thus, growth reduction under stress is possibly related to higher

rates of ABA accumulation. In fact, data reported by Montero et al. (1997) suggest that salt-inhibited leaf expansion growth in bush beans is mediated by ABA rather than Na^+ or Cl^- toxicity. In this study, we investigated the relative importance of Na^+ , Cl^- and ABA in the inhibition of root growth of rice seedlings caused by NaCl.

Materials and methods

Rice (*Oryza sativa* L., cv. Taichung Native 1) seeds were sterilized with 2.5% sodium hypochlorite for 15 min and washed extensively with distilled water. In order to get more uniformly germinated seeds, rice seeds in Petri dish (20 cm) containing distilled water were pretreated at 37 °C for 1-day under dark condition. Uniformly germinated seeds were then selected and transferred to a Petri dish (9.0 cm) containing two sheets of Whatman No. 1 filter paper moistened with 10 ml of distilled water or NaCl at the desired concentration as specified in the individual experiments. Root growth of seedlings grown in distilled water is similar to that grown in medium containing inorganic salts, thus seedlings grown in distilled were used as the controls. For the experiments to examine the relative importance of Cl^- in regulating NaCl-inhibited

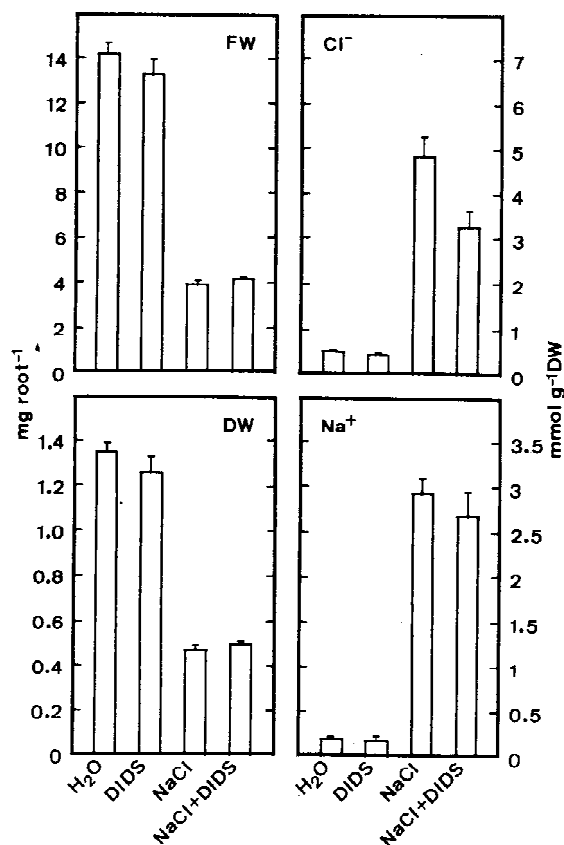


Figure 2. Effects of NaCl (150 mM) on root growth and Na⁺ and Cl⁻ levels in roots of seedlings in the presence or absence of DIDS (0.05 mM). Root growth and Na⁺ and Cl⁻ levels were measured after 5 days of treatment. Bars represent standard errors ($n=4$).

root growth, Na-gluconate and DIDS were used. Each Petri dish contained 20 germinated seeds. Each treatment was replicated four times. The germinated seeds were allowed to grow at 27 °C in darkness and 3 ml of distilled water or test solutions was added to each Petri dish on day 3 of the growth.

Cell walls were prepared by homogenizing roots in ice-cold phosphate buffer (50 mM, pH 5.8) using a pestle and mortar. The homogenate was centrifuged at 1000 *g*, and washed at least four times with 50 mM phosphate buffer (Lee and Lin, 1995). The pellet was collected and used as a cell wall fraction.

POD ionically bound to the walls was extracted with 1 M NaCl. Cell walls were prepared as described above and incubated in 1 M NaCl for 2 h with shaking at 30 °C and centrifuged at 1000 *g*. The supernatant was considered as the cell wall fraction.

POD activities were measured using a modification of the procedure described by Curtis (1971). The assay

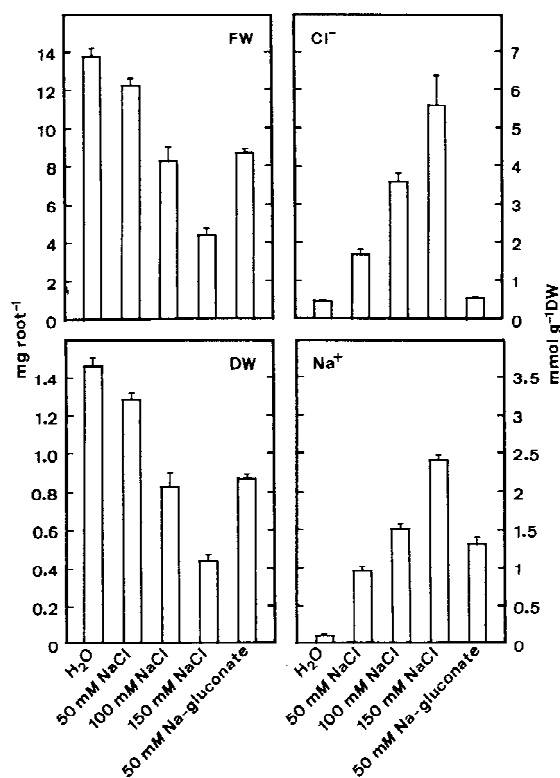


Figure 3. Effects of NaCl and Na-gluconate on root growth and Na⁺ and Cl⁻ levels in roots of rice seedlings. Root growth and Na⁺ and Cl⁻ levels were measured after 5 days of treatment. Bars represent standard errors ($n=4$).

medium contained 0.05 M phosphate buffer (pH 5.8), 7.2 mM guaiacol, 11.8 mM H₂O₂ and 0.1 ml enzyme extract in a final assay volume of 3.0 ml. The reaction was initiated by the addition on H₂O₂ and the change in absorbance at 470 nm was measured. Activity was calculated using the extinction coefficient (26.6 mM⁻¹ cm⁻¹ at 470 nm) for tetraguaiacol. One unit of POD was defined as the amount of enzyme that causes the formation of 1 μmol tetraguaiacol per min. The activity of cell wall POD was expressed on the basis of DW.

The H₂O₂ level was colorimetrically measured as described by Jana and Choudhuri (1981). H₂O₂ was extracted by homogenizing 10 roots with 3 ml of phosphate buffer (50 mM, pH 6.8). The homogenate was centrifuged at 6000 *g* for 25 min. To determine H₂O₂ levels, 3 ml of extracted solution was mixed with 1 ml of 0.1% titanium chloride (Aldrich) in 20% (v/v) H₂SO₄ and the mixture was then centrifuged at 6000 *g* for 15 min. The intensity of yellow color of supernatant was measured at 410 nm. H₂O₂ level

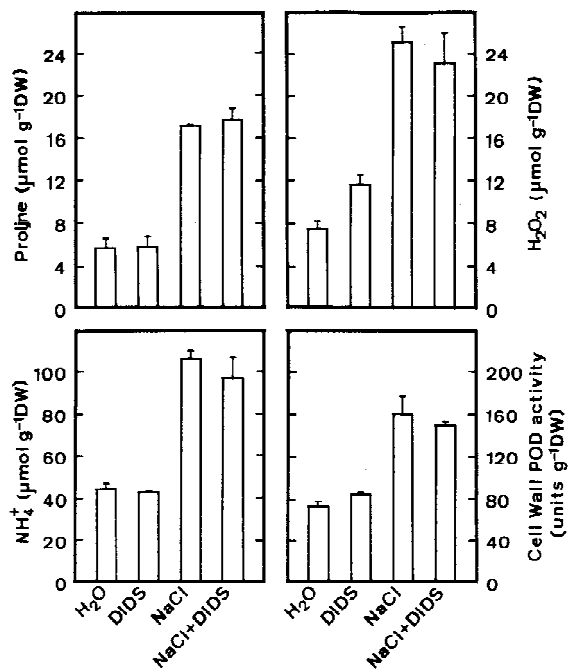


Figure 4. Effects of NaCl (150 mM) on ammonium, proline and H_2O_2 levels, and cell wall POD activity in roots of rice seedlings in the presence or absence of DIDS (0.05 mM). All measurements were made after 5 days of treatment. Bars represent standard errors ($n=4$).

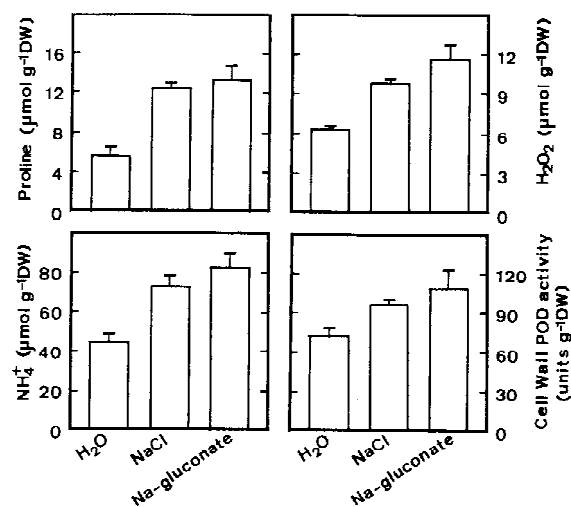


Figure 5. Effects of NaCl (100 mM) and Na-gluconate (50 mM) on ammonium, proline and H_2O_2 levels, and cell wall POD activity in roots of rice seedlings. All measurements were made after 5 days of treatment. Bars represent standard errors ($n=4$).

was calculated using the extinction coefficient $0.28 \mu\text{mol}^{-1} \text{cm}^{-1}$.

Ammonium and proline were extracted and determined according to the methods described previ-

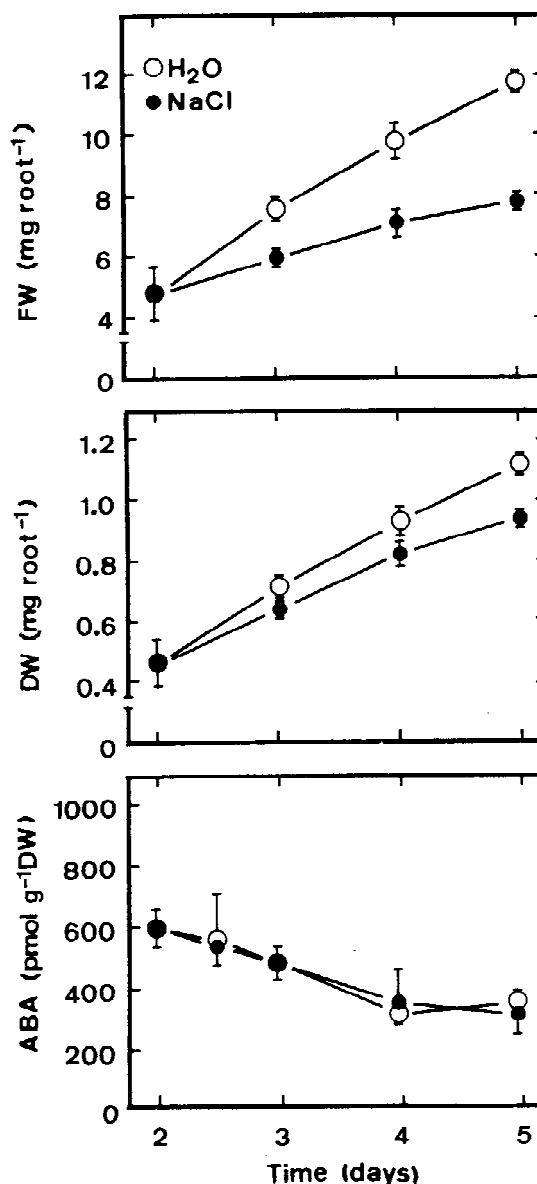


Figure 6. Changes in root growth and ABA levels in roots of rice seedlings treated with distilled water or NaCl. Two-day-old rice seedlings were treated with distilled or 150 mM NaCl. Bars represent standard errors ($n=4$).

ously (Lin and Kao, 1996a, b). For Na^+ determination, harvested roots were washed three times (with each 1 min) with distilled water, dried at 65°C for 2 days, extracted in 1 N HCl at room temperature (Hunt, 1982) and analyzed with a flame photometer (Evans Electro Selenium Ltd., UK). Chloride was estimated in a separate extract made according to the method described by Hodson et al. (1985) and estimated using

an ion meter (Mittler Delta 350, UK) equipped with chloride ion electrode. The levels of ammonium, proline, H_2O_2 , Na^+ and Cl^- were expressed on the basis of DW.

For extraction of ABA, root samples were homogenized with a mortar and pestle in extraction solution (80% methanol containing 2% glacial acetic acid). To remove plant pigments and other non-polar compounds which could interfere in the immunoassay, extracts were first passed through polyvinylpyrrolidone column and C18 cartridges. The eluates were concentrated to dryness by vacuum-evaporation and resuspended in Tris-buffered saline before enzyme-linked immunosorbent assay (ELISA). ABA was quantitated by ELISA (Walker–Simmons, 1987). ABA immunoassay detection kit (PGR-1) was purchased from Sigma Chemical Co. (St Louis, MO, USA), is specific for (+)-ABA.

For biochemical analysis, four independent extractions were performed for each treatment, and for each extraction 20 seedlings were used. All experiments described here were repeated at least three times. The data reported here are from a single experiment.

Results and discussion

Figure 1 shows the effect of NaCl on the growth of rice seedlings. Increasing concentrations of NaCl from 50 to 150 mM progressively decreased root length. Since the pattern of root growth of rice seedlings in response to NaCl is similar to that of FW or DW of roots, FW and DW of roots were used as an indicator of root growth for the subsequent experiments. Figure 2 shows the effect of NaCl on root growth and Na^+ and Cl^- levels in roots of rice seedlings in the presence or absence of DIDS, a nonpermeating amino-reactive disulfonic acid known to inhibit the uptake of Cl^- (Lin, 1981). NaCl at 150 mM decreased root growth and increased both Na^+ and Cl^- levels in roots. Treatment with NaCl in the presence of DIDS had less Cl^- level in roots than that in the absence of DIDS, but did not affect Na^+ level and root growth when compared with NaCl treatment. This suggests that Cl^- is not involved in NaCl-inhibited root growth of rice seedlings. DIDS treatment resulted in about 40% reduction of Cl^- (Figure 2). Thus, the lack effect of DIDS on root growth of rice seedlings treated with NaCl can be explained by the possibility that the level of Cl^- is still high enough to exert its effect. In order to test further the involvement of Cl^- , experiments were performed to compare

the effect of NaCl with that of Na-gluconate, the anion of which is not able to permeate the membrane (Blumwald and Poole, 1985). The results are shown in Figure 3. Increasing concentrations of NaCl from 50 to 150 mM progressively decreased FW and DW of roots and increased both Na^+ and Cl^- levels in roots. The reduction of root growth is closely correlated with the increase of Na^+ and Cl^- levels in roots. Treatment with 50 mM Na-gluconate had similar Na^+ level but had much less Cl^- level in roots as that with 100 mM NaCl. The reduction of root growth by 50 mM Na-gluconate is similar to that by 100 mM NaCl. These results strongly suggest that Cl^- is not required for NaCl-inhibited root growth of rice seedlings.

In previous studies, we have shown that proline accumulation is correlated with root growth inhibition of rice seedlings induced by NaCl (Lin and Kao, 1996b). In the present study, we demonstrated that proline content in roots of rice seedlings treated with NaCl in the presence of DIDS was similar to that in the absence of DIDS (Figure 4). We also demonstrated that roots of rice seedlings treated with 50 mM Na-gluconate accumulated similar level of proline as those with 100 mM NaCl (Figure 5).

It is known that ammonium inhibits the growth of many plants (Haynes and Goh, 1978). It has been shown that NaCl was effective in stimulating the accumulation of ammonium in roots of rice seedlings, and that accumulation of ammonium in roots preceded inhibition of root growth caused by NaCl (Lin and Kao, 1996a). If Na^+ rather than Cl^- is important in regulating growth reduction of roots caused by NaCl, then ammonium accumulation in roots treated with NaCl is expected to be induced by Na^+ instead of Cl^- . The results reported in Figures 4 and 5 demonstrate that this is, in fact, correct.

Recent studies in our laboratory show that NaCl-inhibited root growth of rice seedlings is associated with increase in H_2O_2 level and cell wall POD activity (Lin and Kao, 1999, 2001a), suggesting that NaCl-induced cell wall stiffening is a possible mechanism of NaCl-inhibited root growth of rice seedlings. If the proposed mechanism of NaCl-inhibited roots growth of rice seedlings is correct, then the increase in H_2O_2 level and cell wall POD activity is expected to be caused by Na^+ rather than Cl^- . It was found that treatment with NaCl in the presence of DIDS or with Na-gluconate increased H_2O_2 level and cell wall POD activity in roots, in the same way as that NaCl did (Figures 4 and 5). The data reported in the present study clearly indicate that growth-related responses in

roots treated with NaCl are caused by Na⁺ rather than Cl⁻.

It is usually accepted that ABA is a potent growth inhibitor (Creelman, 1989). Inhibition of cell expansion growth by ABA has been related to ABA-induced inhibition of the synthesis of some cell wall components, to decreased cell wall extensibility and to effects on enzymes involved in cell wall-loosening and cell elongation (Kutschera and Schopfer, 1989; Van Volkenburg and Davies, 1983; Wakabayashi et al., 1989). Recently, we demonstrated that cell wall stiffening catalyzed by H₂O₂-dependent POD is involved in the regulation of root growth reduction of rice seedlings caused by ABA, a mechanism similar to that of NaCl-inhibited root growth (Lin and Kao, 1999, 2001a, b). Thus, it is of great interest to know whether NaCl-inhibited root growth of rice seedlings is mediated through the increase of endogenous ABA level. However, there is no increase in ABA level in the roots of rice seedlings treated with NaCl when compared with the control (Figure 6). Moons et al. (1995) observed that ABA level increased slightly in the roots of salt-stressed Taichung Native 1 rice seedlings (the investigated variety of this study). It appears that NaCl-inhibited root growth of rice seedlings is unlikely to be mediated through ABA. However, Montero et al. (1997) reported that endogenous ABA levels are linked to decreased leaf growth of bean plants treated with NaCl.

The fact that ABA did not increase in NaCl-treated roots does not mean that it is not overproduced in roots in response to NaCl if the translocation of ABA from roots to shoot is also stimulated by NaCl. It is not known whether NaCl stimulates the translocation of ABA from roots to shoots. Further work is needed to clarify this issue.

On the basis of the results reported in the present investigation, it can be concluded that Cl⁻ is not involved in NaCl-induced growth reduction and growth-related responses of roots of rice seedlings.

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References

- Blumwald E and Poole R J 1985 Na⁺/H⁺ antiport in isolated tonoplast vesicles from tissue of *Beta vulgaris*. *Plant Physiol.* 78, 163–167.
- Cramer G R, Alberico G J and Schmidt C 1994 Leaf expansion limits dry matter accumulation of salt stressed maize. *Aust. J. Plant Physiol.* 21, 663–674.
- Creelman R A 1989 Abscisic acid physiology and biosynthesis in higher plants. *Physiol. Plant.* 75, 131–136.
- Curtis C R 1971 Disc electrophoretic comparisons of proteins and peroxidases from *Phaseolus vulgaris* leaves infected with *Agrobacterium tumefaciens*. *Can. J. Bot.* 49, 333–337.
- Greenway H and Munns R 1980 Mechanism of salt tolerance in nonhalophytes. *Annu. Rev. Plant Physiol.* 31, 149–190.
- Haynes R I and Goh K M 1978 Ammonium and nitrate nutrition of plants. *Biol. Rev.* 53, 485–510.
- Hodson M J, Opik H and Wainwright S J 1985 Changes in ion and water content of individual shoot organs in a salt-tolerant and a salt-sensitive clone of *Agrostis stolonifera* L. during and subsequent to treatment with sodium chloride. *Plant Cell Environ.* 8, 657–668.
- Hunt J 1982 Dilute hydrochloric acid extraction of plant material for routine cation analysis. *Commun. Soil Sci. Plant Analysis* 13, 49–55.
- Jana S and Choudhuri M A 1981 Glycolate metabolism of three submerged aquatic angiosperms during aging. *Aquat. Bot.* 12, 345–354.
- Kafkafi U and Berstein N 1995 Root growth under salinity stress *In* *Plant Roots: The Hidden Half*. Eds. Y Waisel, A Eiskel and U Kafkafi. pp 435–451. Marcel Dekker, New York.
- Kutschera U and Schopfer P 1986 Effect of auxin and abscisic acid on cell wall extensibility on maize coleoptile. *Planta* 167, 527–535.
- Lee T-M and Lin Y-H 1995 Changes in soluble and cell wall-bound peroxidase activities with growth in anoxia-treated rice (*Oryza sativa* L.) coleoptiles and roots. *Plant Sci.* 106, 1–7.
- Lin C C and Kao C H 1996a Disturbed ammonium assimilation is associated with inhibition of roots in rice seedlings caused by NaCl. *Plant Growth Regul.* 18, 233–238.
- Lin C C and Kao C H 1996b Proline accumulation is associated with inhibition of rice seedling root growth caused by NaCl. *Plant Sci.* 114, 121–128.
- Lin C C and Kao C H 1999 NaCl induced changes in ionically bound peroxidase activity in root of rice seedlings. *Plant Soil* 216, 147–153.
- Lin C C and Kao C H 2001a Cell wall peroxidase activity, hydrogen peroxide level and NaCl-inhibited root growth of rice seedlings. *Plant Soil* 230, 135–143.
- Lin C C and Kao C H 2001b Abscisic acid induced changes in cell wall peroxidase activity and hydrogen peroxide level in roots of rice seedlings. *Plant Sci.* 160, 323–329.
- Lin W 1981 Inhibition of anion transport in corn root protoplast. *Plant Physiol.* 68, 435–438.
- Lutts S, Kinet J M and Bonharmont J 1995 Changes in plant response to NaCl during development of rice (*Oryza sativa* L.) varieties differing in salinity resistance. *J. Exp. Bot.* 46, 1843–1852.
- Marschner H 1995 *Mineral Nutrition of Higher Plants*. 2nd edn. Academic press, London.
- Montero E, Cabot C, Barcelo J and Poschenrieder C 1997 Endogenous abscisic acid levels are linked to decreased growth of bush bean treated NaCl. *Physiol. Plant.* 101, 17–22.

- Montero E, Cabot C, Poschenrieder C and Barcelo J 1998 Relative importance of osmotic-stress and ion-specific effects on ABA-mediated inhibition of leaf expansion growth in *Phaseolus vulgaris*. *Plant Cell Environ.* 21, 54–62.
- Moons A, Bauw G, Priusem E, Van Montagu M and Van Der Straeten D 1995 Molecular and physiological responses to abscisic acid and salts in roots of salt-sensitive and salt-tolerant indica rice. *Plant Physiol.* 107, 177–186.
- Munns R and Sharp R E 1993 Involvement of abscisic acid in controlling growth in soils of low water potential. *Aust. J. Plant Physiol.* 20, 425–437.
- Munns R and Termaat A 1986 Whole plant responses to salinity. *Aust. J. Plant Physiol.* 13, 143–160.
- Munns R, Gardner P A, Tonnet M L and Rawson H M 1988 Growth and development in NaCl-treated plants II. Do Na⁺ or Cl⁻ concentrations in dividing or expanding tissues determine growth in barley? *Aust. J. Plant Physiol.* 15, 529–540.
- Rengel Z 1992 The role of calcium in salt toxicity. *Plant Cell Environ.* 15, 625–632.
- Sibole J V, Montero E, Cabot C, Poschenieder C and Barcelo J 1998 Role of sodium in ABA-mediated long-term growth response of bean to salt stress. *Physiol. Plant.* 104, 299–305.
- Van Volkenburgh E D and Davies W J 1983 Inhibition of light-stimulated leaf expansion by abscisic acid. *J. Exp. Bot.* 34, 835–845.
- Wakabayashi K, Sakurai N and Kuraishi S 1989 Role of the outer tissue in abscisic acid-mediated growth suppression of etiolated squash hypocotyls segments. *Plant Physiol.* 75, 151–156.
- Walker-Simmons M 1987 ABA levels and sensitivity in developing wheat embryos of sprouting resistant and susceptible cultivars. *Plant Physiol.* 84, 61–66.

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