

Morpho-physiological variations in response to NaCl stress during vegetative and reproductive development of rice

Shantanu Devidas Wankhade · Maria-Jesus Cornejo · Isabel Mateu-Andrés · Amparo Sanz

Received: 23 November 2011 / Revised: 20 July 2012 / Accepted: 1 August 2012 / Published online: 18 August 2012
© Franciszek Górski Institute of Plant Physiology, Polish Academy of Sciences, Kraków 2012

Abstract The complex nature of plant resistance to adverse environmental conditions, such as salinity and drought requires a better understanding of the stress-induced changes that may be involved in tolerance mechanisms. Here we investigate stress-related morpho-physiological effects during vegetative and reproductive growth in two *Japonica* rice cultivars (Bomba and Bahia) exposed to a range of NaCl concentrations from the seedling stage. The stress-related detrimental effects were observed either earlier or to a higher extent in cv. Bomba than in Bahia. Damages to the photosynthetic apparatus were related to loss of chlorophyll (Chl) and to a decrease of the maximum potential efficiency of PSII (F_v/F_m), affecting negatively net CO₂ assimilation rate (P_N). Stress-related leaf anatomical alterations were analysed during the vegetative and reproductive stages. The size of bulliform cells as well as dimensions related to the vascular system increased under mild stress but decreased in the longer term or under higher stress level. The pattern of the anatomical alterations observed at the reproductive stage under 20 mM NaCl was reflected in poor panicle development and yield loss, with effects more pronounced in cv. Bomba than in Bahia. In summary, our results show that some physiological and,

particularly, leaf anatomical responses induced by NaCl stress are distinctive indicators of sensitivity to salt stress in rice cultivars.

Keywords Chlorophyll content and fluorescence · CO₂ uptake · Leaf anatomy · *Oryza sativa* L. · Salinity · Stress responses

Introduction

Salinity is among the major abiotic stresses that hinder plant growth and development, causing important reductions in productivity of field crops. More than 800 M ha of global salt-affected land stands as an ever existing threat to food production (Munns and Tester 2008; Rengasamy 2006). Resistance to salt stress does not rely on a single trait but, on the contrary, it has a very complex nature. A better understanding of the morpho-physiological variations induced by salinity in crop plants should facilitate the identification of those involved in tolerance mechanisms. Rice (*Oryza sativa* L.) has been shown to be more salt-sensitive at the seedling and reproductive stages than during the vegetative growth and active tillering (Abdullah et al. 2001; Lutts et al. 1995; Moradi and Ismail 2007; Rao et al. 2008; Zeng and Shannon 2000b). Screening the pattern of responses to NaCl, therefore, should include not only seedlings but also the vegetative and reproductive stages.

The initial osmotic effect of NaCl stress inhibits growth and in further developmental stages, ion accumulation at toxic level results in premature leaf senescence (Munns and Tester 2008) which is reflected by leaf rolling, chlorosis/necrosis at the tip and in marginal parts of older leaves, as well as in stunted shoot growth (Alam et al. 2004; Munns

Communicated by S. Renault.

S. D. Wankhade · M.-J. Cornejo · A. Sanz (✉)
Departamento de Biología Vegetal, Facultad de Biología,
Universitat de València, Avda. Dr Moliner 50, Burjassot 46100,
València, Spain
e-mail: Amparo.Sanz@uv.es

S. D. Wankhade · I. Mateu-Andrés
Instituto Cavanilles de Biodiversidad y Biología Evolutiva,
y Departamento de Botánica, Facultad de Biología, Universitat
de València, Avda. Dr Moliner 50, Burjassot, 46100 València,
Spain

2002). The remaining functional (green) leaf area and younger leaves are thus maintained by the stressed plants, probably to cope with the stress.

Leaf tissues such as epidermal and/or bulliform cells possess large vacuoles (Alvarez et al. 2008; Moulia 2000) which serve as a dump for high amount of Na^+/Cl^- ions that are transported to the leaves under stress conditions, to protect physiologically active mesophyll cells (Tester and Davenport 2003). Ion accumulation, when reaching toxic levels in stressed plants, inhibit photosynthetic activity, whether grown in vitro or in combination with other culture conditions in the greenhouse (Cha-um et al. 2006; Chaves et al. 2009; Dionisio-Sese and Tobita 2000; Djanaguiraman et al. 2006; Moradi and Ismail 2007; Netondo et al. 2004).

Cell organelles such as chloroplasts and mitochondria in rice leaves are damaged by salinity stress (Rahman et al. 2000). Thus, reduction in chlorophyll content and in the maximum potential quantum efficiency of PSII, measured as changes in chlorophyll fluorescence (F_v/F_m) is related to the salinity-induced swelling and distortion of thylakoids, grana stacking, stroma and chloroplast envelope (Barhoumi et al. 2007; Rahman et al. 2000; Wang et al. 2009; Yamane et al. 2003, 2008). Therefore, these parameters are referred as efficient indicators for the evaluation of stress related effects on photosystem II (PSII) (Baker 2008; Netondo et al. 2004).

Salinity-induced disturbances in metabolic processes cause delay in flowering and impair fertility, with partial or complete grain loss. This is associated with poor panicle development, which collectively reduces crop yield (Abdullah et al. 2001; Kato et al. 2008; Rao et al. 2008; Zeng and Shannon 2000b).

Among rice subspecies, *Indica* cultivars, which are adapted to tropical environment, are generally more tolerant than *Japonica* rice, mainly adapted to temperate areas (Lee et al. 2003). Soil salinity is the major constraint to increasing rice production in both tropics and temperate regions. Therefore, a number of investigations on physiological responses to salinity have been performed using jointly *Japonica* and *Indica* rice. Thus, Lutts et al. (1995) studied salinity responses in cultivars from both subspecies during germination, young seedling, tillering and reproductive stages. However, most investigations were confined either to seedlings (Dionisio-Sese and Tobita 1998, 2000; Lee et al. 2003; Lutts et al. 1996a, 1999) or to a given developmental stage (Asch et al. 2000; Lutts et al. 1996b; Moradi et al. 2003; Ul Haq et al. 2009; Zeng et al. 2002, 2003). Within *Japonica* cultivars, most investigations have been performed at seedling stage (Bahaji et al. 2002; Sohn et al. 2005; Wankhade et al. 2010) or at flowering (Sultana et al. 1999; Zeng and Shannon 2000a; Zeng et al. 2001) but very seldom at both the vegetative and reproductive stages (Zeng and Shannon 2000b).

We previously reported on the effects of high salinity levels on leaf anatomy in rice seedlings grown in vitro (Bahaji et al. 2002) or in the greenhouse (Wankhade et al. 2010). As the salinity levels used did not allow development to subsequent stages, we have studied long-term anatomical and physiological responses induced by lower salinity levels applied since germination in rice plants at latter developmental stages, including flowering and compared the responses obtained with those caused by higher concentrations but shorter time of exposure. To our knowledge, this is the first report on the long-term impact of salinity in leaf anatomy along the rice life cycle.

Materials and methods

Two *Japonica* rice cultivars (Bomba and Bahia) were used to evaluate morphological and physiological responses in plants subjected to long-term salinity stress. Measurements were performed during the vegetative growth (48–52 days after germination) and at panicle initiation (130–140 days after germination).

Culture conditions and plant development

Rice caryopses were sown in pots (20 cm height \times 22 cm diameter) filled with a mixture of peat substrate (Kekkilä Iberia S.L.) and perlite (3:1) in the greenhouse with day/night temperature of 27/18 °C. Pots were irrigated by water or by NaCl solutions as described by Wankhade et al. (2010). Plants were subjected to stress when the first leaves were fully extended (approximately 2 days after germination) using salinity treatments of 10, 20 and 150 mM NaCl. Plant height, number of leaves and leaf development were measured in five plants per pot of each cultivar and salinity level. Upon maturity, panicles were counted, harvested and allowed to dry at room temperature before determining their dry weight.

Natural illumination was supplemented with high pressure sodium vapour lamps from 6.00 a.m. to 10.00 p.m. programmed for radiation intensity of minimum $500 \mu\text{mol m}^{-2} \text{s}^{-1}$ and maximum $1500 \mu\text{mol m}^{-2} \text{s}^{-1}$. The relative humidity was maintained between 60 and 80 %. The pots were individually placed in plastic trays and their position was randomised every 2 weeks to minimise any variations inside the greenhouse chamber during the experiments.

Two independent experiments were performed, one for low NaCl concentrations, 10 and 20 mM (November–April), and another for 150 mM NaCl (March–August). In both experiments two simultaneous independent replicates, each consisting of 15 plants per cultivar and salinity level, including controls, were carried out. For a better

comparison and to avoid duplicity in control values, data for 150 mM NaCl have been scaled proportionally to the difference, if any, between controls of both experiments.

Chlorophyll (Chl) content and Chl fluorescence measurements

Chl content (as CCI: chlorophyll content index) and Chl fluorescence (F_v/F_m) determinations were performed on the basal, mid and tip of each analysed leaf blade with a chlorophyll meter (CCM-220, Opti-Sciences, Inc., USA) and a chlorophyll fluorometer (OS-30P, Opti-Sciences, Inc., USA), respectively. Data were obtained from five plants per pot of each cultivar and salinity level using the youngest fully expanded leaf in the vegetative growth and last two leaves (flag and penultimate leaves) at the reproductive stage. To determine F_v/F_m values, leaves were pre-darkened with aluminium foil for 30 min prior to the measurement.

Leaf gas exchange

A portable infrared gas analyzer (IRGA) photosynthesis system (LI-COR, Model LI-6200) was used to measure net CO_2 assimilation rate (P_N) in five plants per pot of each cultivar and salinity level. The youngest fully expanded leaf was used for experiments at the vegetative stage, and the flag or the penultimate leaves were measured at the reproductive stage. All measurements were carried out around midday (10.00 a.m. to 2.00 p.m.). The initial CO_2 concentration within the leaf chamber was adjusted to ca. $350 \mu\text{L L}^{-1}$ before each measurement and lighting was kept constant during measurements, at approximately $500 \mu\text{mol m}^{-2} \text{s}^{-1}$ PAR, using an additional light source (KL-2500 LCD, WALZ).

Leaf anatomy and image analysis

The anatomical analyses were performed in fully extended leaf blades collected at the vegetative and the reproductive growth stages, as indicated above. Anatomical parameters in each leaf section were estimated in the central ridge, in an area containing the central vascular bundle (CVB), as well as in one of the adjacent lateral ridges, in an area containing a lateral vascular bundle (LVB, Fig. 6a). Leaf thickness, as cross-sectional length at the central ridge, the number of epidermal cells (EC) per mm as well as the size of the largest epidermal and bulliform cells (BC) were also measured.

A total of 70 transverse sections were prepared using leaves from five plants per cultivar, developmental stage and salinity level. Fixation of leaf samples, sectioning, staining with safranin and image analyses of transverse

sections were performed as described previously (Wankhade et al. 2010).

Statistical analysis

Statistical analyses of data were carried out with the PASW Statistics programme (version. 17.0.3). Significant differences were established by post hoc comparisons (HSD test of Tukey) at $P < 0.05$ after ANOVA tests.

Results

Vegetative development under NaCl stress

Control plants of cv. Bomba were taller than those of cv. Bahia at the late vegetative growth and the reproductive stage (40 and 96 %, respectively). Under low NaCl concentrations (10 and 20 mM), shoot height of both cultivars was unaffected during the vegetative growth. At the reproductive stage, though there were no statistically significant differences among mean values, a significant trend to decrease in plant growth with increasing NaCl concentration was noticeable, particularly in cv. Bomba but also in Bahia ($r = -0.883$, $P < 0.02$ and $r = -0.819$, $P < 0.05$, respectively; Fig. 1). The effect on plant height was more pronounced in plants of both cultivars subjected to 150 mM NaCl in which a significant decrease, to <75 % of their controls, was measured at the vegetative growth (Fig. 1). These plants did not reach the reproductive stage.

During the vegetative growth, the leaves of control plants of cv. Bomba were wider and more abundant than those of cv. Bahia (Fig. 2). Growth under 20 mM NaCl stress caused a significant reduction of the number of leaves at this developmental stage in cv. Bomba but not in cv. Bahia. Mean values obtained under 10 mM NaCl were intermediate between controls and 20 mM. In contrast, leaf width decreased significantly under 20 mM NaCl in cv. Bahia. Severe inhibition in both leaf number and leaf width was observed in Bomba and of leaf width in Bahia grown under 150 mM NaCl (Fig. 2). In addition, this treatment increased leaf thickness, particularly in cv. Bomba, in which a 45 % increase over controls was measured (323 ± 8 vs. $222 \pm 6 \mu\text{m}$, at the central ridge) while in cv. Bahia it increased 24 % (350 ± 7 vs. $283 \pm 20 \mu\text{m}$).

Neither of the cultivars withstood 150 mM NaCl stress after the vegetative growth; however, under 10 and 20 mM NaCl stress they progressed till the reproductive stage. At this stage, flag leaves of control plants were longer and wider in cv. Bomba than in cv. Bahia (Table 1). In both cultivars, the length of flag leaves was unaffected under 10 mM NaCl and decreased significantly under 20 mM NaCl. The width decreased under 10 mM NaCl only in cv.

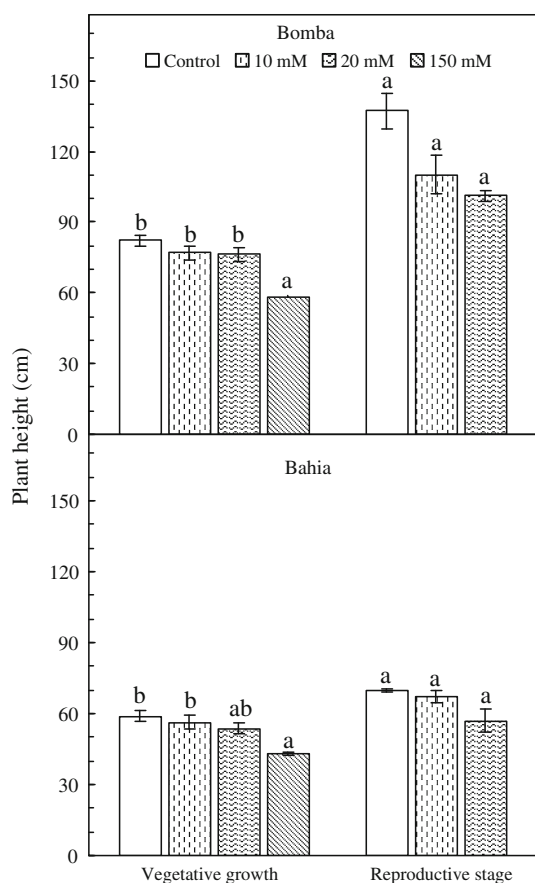


Fig. 1 Effects of NaCl on plant height during vegetative growth and reproductive stage in two rice cultivars. Within each cultivar and developmental stage, *different letters* indicate significant differences ($P < 0.05$). Values are mean \pm SE

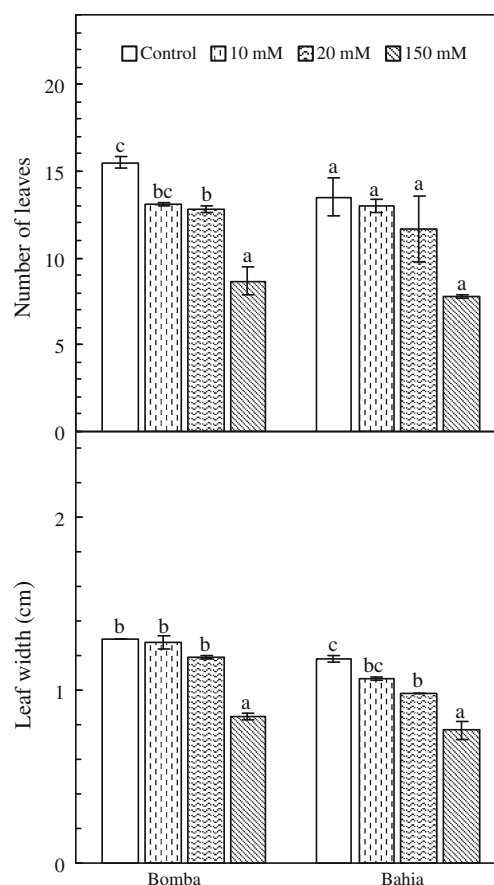


Fig. 2 Effects of NaCl on number of leaves and leaf width in two rice cultivars during vegetative growth. Within each cultivar, *different letters* indicate significant differences ($P < 0.05$). Values are mean \pm SE

Bomba while the decrease was significant in both cultivars under 20 mM NaCl (Table 1).

Leaves from stressed plants exhibited rolled margins with partial or complete chlorosis/necrosis of apical parts in emerging and/or older leaves. These symptoms were evident under 150 mM NaCl during the vegetative growth and under 20 mM NaCl during the reproductive stage. Moreover, leaves from the stressed plants had epidermal layers with thick cuticles and, in general, were more rigid than their control counterparts (personal observation).

Reproductive development under saline stress

The effects of 10 and 20 mM NaCl on the morphology and development of panicles from cultivars Bomba and Bahia are presented in Fig. 3. Quantitative data on the effect of salinity on yield components of these cultivars have been reported earlier (Wankhade et al. 2010). Rice plants delayed panicle emergence (flowering) and showed several deleterious effects on panicle development that became more severe under 20 mM NaCl. In this case, emerging

panicles exhibited more tightly rolled flag leaf-sheaths in cv. Bomba than in cv. Bahia and once developed, panicles showed sterile/aborted spikelets with partial or complete grain loss (Fig. 3). Concomitantly, average panicle weight decreased significantly with respect to controls. In cv. Bomba the decrease was approximately four times more pronounced than in cv. Bahia. Likewise, the reduction caused by NaCl on the number of panicles was four to five times stronger in cv. Bomba (data not shown). Therefore, differences in NaCl sensitivity between these two cultivars became evident at the reproductive stage.

Chlorophyll content and fluorescence

During the vegetative growth, chlorophyll (Chl) content in the leaves of both cultivars declined under all three stress levels, particularly under 150 mM NaCl, with a significant decline in pigment concentration down to values $<70\%$ of their controls in both cultivars (Fig. 4). At the reproductive stage, 10 and 20 mM NaCl-stressed plants showed a reduction in Chl content compared with

Table 1 Effects of NaCl on length and width of the flag leaves during reproductive stage in two rice cultivars

Cultivar	Length			Width		
	Control	10 mM	20 mM	Control	10 mM	20 mM
Dimensions of flag leaves (cm)						
Bomba	32.7 ± 1.9 ^b	29.3 ± 0.4 ^{ab}	27.5 ± 2.9 ^a	1.47 ± 0.07 ^b	1.23 ± 0.01 ^a	1.15 ± 0.01 ^a
Bahia	20.7 ± 0.9 ^b	18.6 ± 0.5 ^{ab}	17.6 ± 0.2 ^a	1.31 ± 0.05 ^b	1.22 ± 0.06 ^{ab}	1.16 ± 0.04 ^a

Within each cultivar, dimension, and culture condition, values followed by different superscript letters indicate significant differences ($P < 0.05$); values are mean ± SE

Fig. 3 Effects of NaCl on panicle development in two rice cultivars; arrows indicate sterile/aborted spikelets with partial or complete grain loss. Values are average panicle weight (g). Different letters indicate significant differences ($P < 0.05$). Values are mean ± SE



control plants, which were statistically significant in cv. Bomba at both concentrations (Fig. 4). The comparison of CCI values of control plants of both cultivars showed that cv. Bahia possessed higher Chl content (around 10 %) than cv. Bomba during both phenological stages, though this difference did not reach levels of statistical significance.

The Chl fluorescence (F_v/F_m) of dark-adapted leaves, which indicates the efficiency of excitation energy transfer of photosystem II (PSII), declined significantly in cv. Bomba under all the three stress levels in the vegetative growth, while in cv. Bahia a significant reduction was noticed only under 150 mM NaCl (Fig. 4). At the reproductive stage, F_v/F_m of cv. Bomba decreased significantly only under 20 mM NaCl stress, while in cv. Bahia it did not differ from controls (Fig. 4). Thus, plants of cv. Bomba showed a higher sensitivity than cv. Bahia with respect to changes in F_v/F_m under stress.

Gas exchange

Net CO₂ assimilation rate (P_N) was strongly reduced in both cultivars by high levels of salinity (150 mM NaCl). In

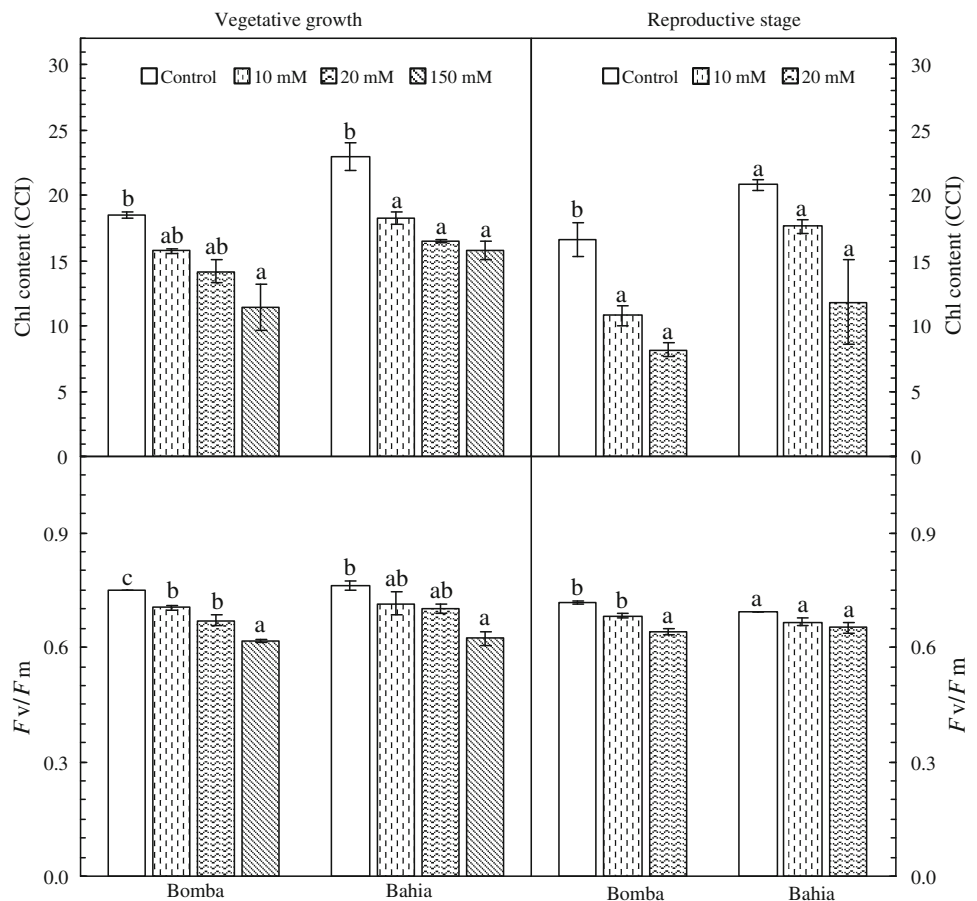
both, a comparable reduction of P_N during the vegetative growth was measured, yielding values around 25 % of controls (Fig. 5). In the longer term, however, differences in sensitivity to NaCl were found between cultivars. Thus, at panicle initiation (reproductive stage), low NaCl concentrations did not affect (10 mM) or induced a small decrease (20 mM) in photosynthetic activity of cv. Bahia, while in cv. Bomba P_N was reduced significantly at both concentrations, down to values corresponding to 35 and 20 % of controls, respectively (Fig. 5).

Leaf anatomy

Figure 6 shows transverse leaf sections of an area which includes a lateral vascular bundle from control and NaCl-stressed leaves of cv. Bomba at the reproductive stage. Quantitative data are included in Table 2.

During the vegetative growth, xylem element vessels as well as dimensions of the central and lateral vascular bundles increased significantly under NaCl stress in both cultivars. The magnitude of the increase was higher under 20 mM than under 150 mM stress (Table 2). All the parameters measured were altered by salinity in cv.

Fig. 4 Effects of NaCl on chlorophyll content (CCI) and fluorescence (F_v/F_m) during vegetative growth and reproductive stage in two rice cultivars. Within each cultivar and developmental stage, different letters indicate significant differences ($P < 0.05$). Values are mean \pm SE



Bomba. A lower number of leaf features increased significantly in cv. Bahia, but they were also related to the length of vascular bundles and the area of xylem element vessels and bulliform cells in central and lateral vascular bundles.

At the reproductive stage, stress-induced alterations in leaf anatomy were more prominent under 10 mM than under 20 mM NaCl. The latter stress level appeared to be excessive at this developmental stage, as also occurred with 150 mM NaCl at the vegetative growth (Table 2). In cv. Bomba, values of most anatomical parameters either overlapped (7 out of 12) with control values or increased (5 out of 12) significantly under 10 mM NaCl. The increased parameters were area of xylem element vessels and length of lateral vascular bundles as well as size of bulliform cells in central and lateral vascular bundles. Under a higher stress level (20 mM NaCl), values of most leaf features remained unaffected (9 out of 12) or decreased (3 out of 12) in comparison to control values (Table 2).

With respect to cv. Bahia, values of several leaf features either overlapped (8 out of 12) with control counterparts or increased (4 out of 12) significantly in 10 mM NaCl-stressed leaves during the reproductive stage. As occurred with cv. Bomba, stress-induced increases in size were mostly related to xylem element vessels and bulliform

cells. Under 20 mM NaCl, most parameters (8 out of 12) decreased significantly in comparison to control values (Table 2).

Discussion

In these investigations, different NaCl stress levels were applied after germination to evaluate variations in physiological and morphological responses at two different developmental stages of the life cycle of rice (cvs. Bomba and Bahia), i.e. the late vegetative growth and the reproductive stage, which has been shown to present different sensitivity to salt stress (Lutts et al. 1995; Moradi and Ismail 2007; Rao et al. 2008).

According to our results, values of growth parameters declined significantly during the vegetative growth under high stress level (150 mM NaCl) and the effects of lower NaCl concentrations (10 and 20 mM) varied between cultivars, the decline being greater in cv. Bomba than in cv. Bahia (Figs. 1, 2). At the reproductive stage, all growth parameters except shoot height decreased significantly under 20 mM NaCl in both cultivars (Fig. 1; Table 1). This suggests that 150 and 20 mM NaCl are excessive

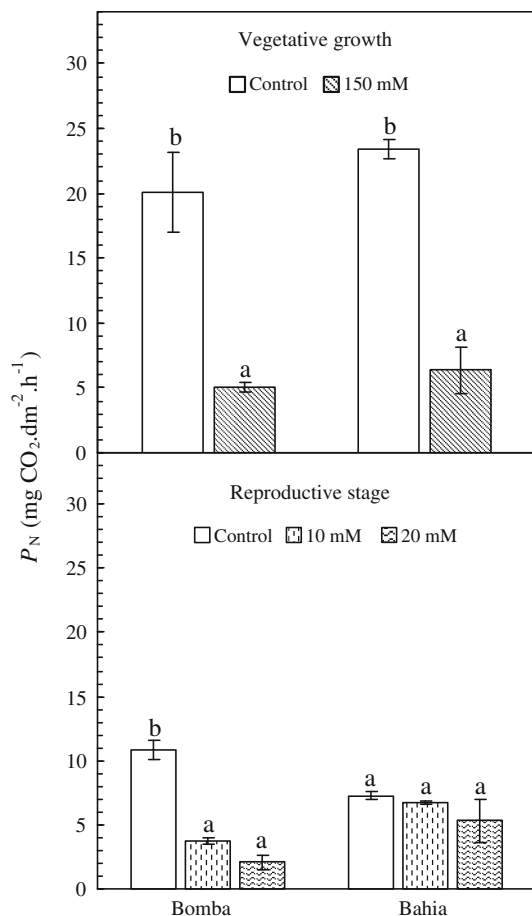


Fig. 5 Effects of NaCl on net photosynthesis (P_N) during vegetative growth and reproductive stage in two rice cultivars. Within each cultivar and developmental stage, different letters indicate significant differences ($P < 0.05$). Values are mean \pm SE

concentrations, with deleterious effects, during the vegetative growth and the reproductive stage, respectively. These results are in accordance with data obtained with other rice cultivars using various stress levels (Alam et al. 2004; Alamgir and Ali 2006; Khan et al. 1997; Welfare et al. 1996).

During the vegetative growth both cultivars showed a significant decline in chlorophyll content (CCI) and fluorescence (F_v/F_m) with increasing NaCl concentrations. At the reproductive stage, the effects were different depending on the cultivar, as both concentrations tested at this stage (10 and 20 mM) induced significant variations in one or both parameters in cv. Bomba while any of these parameters was not affected significantly in cv. Bahia (Fig. 4). Loss of chlorophyll pigment (Bahaji et al. 2002; Siringam et al. 2009) and reductions in the F_v/F_m ratio under salinity stress were also reported for other rice cultivars (Abdullah et al. 2001; Cha-um et al. 2007; Djanaguiraman et al. 2006; Siringam et al. 2009; Yamane et al. 2008) as well as in sorghum (Netondo et al. 2004), naked oat (Zhao et al.

2007), wheat (Zheng et al. 2009) and chickpea (Eyidogan and Öz 2007).

Stress-induced damages to the photosynthetic pigments are coupled with destruction of various enzymatic pathways responsible for CO_2 harvesting during photosynthesis (Chaves et al. 2009). This is also related to ion accumulation at toxic levels in photosynthetically active leaf cells (Tester and Davenport 2003). A decline in P_N as a consequence of NaCl toxicity has been reported by several authors in different rice cultivars (Abdullah et al. 2001; Dionisio-Sese and Tobita 2000; Djanaguiraman et al. 2006; Moradi and Ismail 2007; Pieters and Nuñez 2008; Walia et al. 2005; Welfare et al. 1996) as well as in other cereal crops such as sorghum (Netondo et al. 2004), wheat (Sharma et al. 2005; Zheng et al. 2009) and naked oat (Zhao et al. 2007). In accordance with this, we observed that P_N of both cultivars declined significantly under 150 mM NaCl during the vegetative growth (Fig. 5).

Data on the effect of NaCl at low concentrations are scarce even though conditions of low level, but extended NaCl stress are common in the natural environment. James et al. (2006), working with barley and wheat plants, observed a decrease of P_N under elevated NaCl levels (more than 150 mM) but an increase at low NaCl concentration (25 mM). They also noticed that the accumulation of Na^+ in the leaves of 25 mM NaCl-stressed plant is proportional to the duration of the stress. In this regard, prolonged exposure of cv. Bomba to mild stress levels (10 and 20 mM NaCl, from seedling to reproductive stage) and of both cultivars to higher (150 mM NaCl) stress level for shorter period (from seedling to late vegetative growth), demonstrate clear symptoms of salt toxicity by reducing P_N significantly (Fig. 5). Apart from the possible cumulative effects of NaCl with time, it cannot be ruled out that the response observed at the flowering stage could be consequence of damages occurring at previous developmental stages.

Stress-induced alterations caused by 150 mM NaCl in the anatomy of leaves during the vegetative growth appear related to ion toxicity, since previous results showed that during the seedling stage isosmolar concentrations of sorbitol (300 mM) had less pronounced effects than 150 mM NaCl (Wankhade et al. 2010). In the present work, salinity-related symptoms such as leaf rolling, chlorosis/necrosis of leaves and reduced shoot growth were progressively noticed along the duration of NaCl stress and plants did not survive after, approximately, 70 days of stress.

In both cultivars, the size of bulliform and epidermal cells increased under NaCl stress during the vegetative growth (Table 2). Similar observations have been reported previously for a different *Japonica* rice cultivar (Taipei 309) subjected to stresses causing dehydration, such as freezing (Moukadiri et al. 1999) or saline and osmotic stresses (Bahaji et al. 2002). It has also been shown to

Table 2 Effects of NaCl on several leaf anatomical parameters during vegetative growth and reproductive stage in two rice cultivars

Leaf blade features	Vegetative growth			Reproductive stage		
	Control	20 mM	150 mM	Control	10 mM	20 mM
Bomba						
Vertical length of CVB (μm)	99 \pm 3 ^a	122 \pm 5 ^b	120 \pm 1.6 ^b	127 \pm 3 ^b	127 \pm 3 ^b	114 \pm 3 ^a
Horizontal length of CVB (μm)	106 \pm 2 ^a	123 \pm 5 ^b	121 \pm 1.9 ^b	134 \pm 5 ^b	129 \pm 3 ^{ab}	116 \pm 2 ^a
Large xylem vessel in CVB (μm^2)	872 \pm 22 ^a	1,270 \pm 103 ^b	1,087 \pm 22 ^{ab}	1,546 \pm 72 ^b	1,602 \pm 53 ^b	1,285 \pm 31 ^a
Small xylem vessel in CVB (μm^2)	828 \pm 26 ^a	1,112 \pm 67 ^b	966 \pm 6 ^{ab}	1,340 \pm 64 ^{ab}	1,375 \pm 39 ^b	1,192 \pm 28 ^a
Largest BC near CVB (μm^2)	1,069 \pm 58 ^a	1,516 \pm 83 ^b	1,540 \pm 21 ^b	1,789 \pm 35 ^a	2,050 \pm 81 ^b	1,610 \pm 63 ^a
Vertical length of LVB (μm)	80 \pm 1.0 ^a	98 \pm 3 ^b	101 \pm 1.7 ^b	106 \pm 3 ^{ab}	111 \pm 1.7 ^b	102 \pm 1.3 ^a
Horizontal length of LVB (μm)	85 \pm 1.4 ^a	109 \pm 1.9 ^c	102 \pm 0.2 ^b	113 \pm 3 ^a	122 \pm 2 ^b	112 \pm 1.3 ^a
Large xylem vessel in LVB (μm^2)	743 \pm 13 ^a	1,160 \pm 72 ^b	1,085 \pm 25 ^b	1,240 \pm 32 ^a	1,433 \pm 32 ^b	1,244 \pm 24 ^a
Small xylem vessel in LVB (μm^2)	684 \pm 10 ^a	1,041 \pm 49 ^b	978 \pm 16 ^b	1,199 \pm 30 ^a	1,350 \pm 29 ^b	1,122 \pm 34 ^a
Largest BC near LVB (μm^2)	882 \pm 36 ^a	1,192 \pm 70 ^b	1,176 \pm 21 ^b	1,467 \pm 26 ^a	1,675 \pm 53 ^b	1,473 \pm 56 ^a
Largest epidermal cell (μm^2)	121 \pm 2 ^a	164 \pm 11 ^b	160 \pm 3 ^b	151 \pm 7 ^{ab}	176 \pm 10 ^b	126 \pm 4 ^a
ECs/mm	75 \pm 2 ^a	71 \pm 3 ^a	75 \pm 3 ^a	65 \pm 1.5 ^{ab}	63 \pm 2 ^a	71 \pm 2 ^b
Bahia						
Vertical length of CVB (μm)	104 \pm 2 ^a	116 \pm 4 ^b	109 \pm 1.4 ^{ab}	126 \pm 3 ^b	122 \pm 1.5 ^{ab}	114 \pm 4 ^a
Horizontal length of CVB (μm)	103 \pm 3 ^a	121 \pm 4 ^b	104 \pm 0.7 ^a	127 \pm 2 ^a	125 \pm 3 ^a	120 \pm 4 ^a
Large xylem vessel in CVB (μm^2)	896 \pm 28 ^a	1,205 \pm 52 ^c	1,055 \pm 16 ^b	1,497 \pm 74 ^b	1,691 \pm 45 ^c	1,120 \pm 53 ^a
Small xylem vessel in CVB (μm^2)	795 \pm 27 ^a	1,081 \pm 63 ^b	938 \pm 22 ^{ab}	1,296 \pm 46 ^b	1,570 \pm 59 ^c	963 \pm 35 ^a
Largest BC near CVB (μm^2)	1,253 \pm 36 ^a	1,640 \pm 79 ^b	1,666 \pm 19 ^b	2,253 \pm 59 ^b	2,565 \pm 99 ^c	1,928 \pm 28 ^a
Vertical length of LVB (μm)	91 \pm 0.8 ^a	96 \pm 1.5 ^b	96 \pm 0.6 ^b	107 \pm 5 ^b	105 \pm 2 ^b	92.3 \pm 1.7 ^a
Horizontal length of LVB (μm)	97 \pm 1.2 ^a	102 \pm 3 ^a	95 \pm 2 ^a	119 \pm 3 ^a	116 \pm 3 ^a	109 \pm 2 ^a
Large xylem vessel in LVB (μm^2)	851 \pm 3 ^a	1,109 \pm 27 ^c	959 \pm 23 ^b	1,506 \pm 54 ^b	1,606 \pm 52 ^b	1,067 \pm 30 ^a
Small xylem vessel in LVB (μm^2)	793 \pm 4 ^a	1,050 \pm 38 ^c	916 \pm 14 ^b	1,329 \pm 55 ^b	1,471 \pm 72 ^b	938 \pm 30 ^a
Largest BC near LVB (μm^2)	1,073 \pm 33 ^a	1,413 \pm 98 ^b	1,304 \pm 19 ^b	2,005 \pm 54 ^b	2,035 \pm 47 ^b	1,820 \pm 38 ^a
Largest epidermal cell (μm^2)	132 \pm 1.8 ^a	156 \pm 8 ^b	152 \pm 4 ^{ab}	181 \pm 4 ^a	234 \pm 6 ^b	173 \pm 4 ^a
ECs/mm	70 \pm 3 ^a	66 \pm 2 ^a	69 \pm 1.5 ^a	57 \pm 2 ^a	59 \pm 3 ^a	62 \pm 2 ^a

Within each cultivar and culture condition, values followed by different superscript letters indicate significant differences ($P < 0.05$); values are mean \pm SE

BC bulliform cell, EC epidermal cell, VB vascular bundle (C central or L lateral)

occur in cogon grass under salinity (Hameed et al. 2009). This suggests a common response to different dehydration stresses in epidermal and bulliform cells, which are largely vacuolated and with low metabolic activity (Alvarez et al. 2008; Moulia 2000). The presence of large vacuoles may serve as a dumping system for toxic ions that helps to protect mesophyll cells under stress (Epimashko et al. 2004; Tester and Davenport 2003).

During the vegetative growth, anatomical features were increased significantly by mild saline stress (20 mM NaCl). However, this pattern varied drastically at the reproductive stage in both cultivars as most features either were unaffected or decreased in size (Table 2). This reflects the toxicity of 20 mM NaCl, applied after germination, at the reproductive stage. Under lower NaCl concentration (10 mM NaCl) most parameters increased in cv. Bomba and the effects were less pronounced in cv. Bahia, as

occurred during the vegetative growth under 20 mM NaCl (Table 2). Boughalleb et al. (2009) studying two xerohalophyte and one glycophyte species reported that the sizes of xylem element vessels in the leaf's main vein were unaffected under salinity, but the number of vessels increased. This may be related to changes in IAA contents in salt-stressed plants, as IAA concentrations control xylem differentiation. Thus, low auxin concentrations result in fewer and larger vessels (Aloni 2010) and, in rice, salinity has been reported to lower IAA biosynthesis (Kim et al. 2006; Prakash and Prathasenan 1990). An increase in diameter of xylem vessels may be advantageous under conditions of mild saline stress, as this reduces friction with cell walls and improves water conductance, but not under a stronger water shortage. In this case, a reduction of the effective area for transport and, therefore, in water supply to the above-ground parts of the plant would result in a

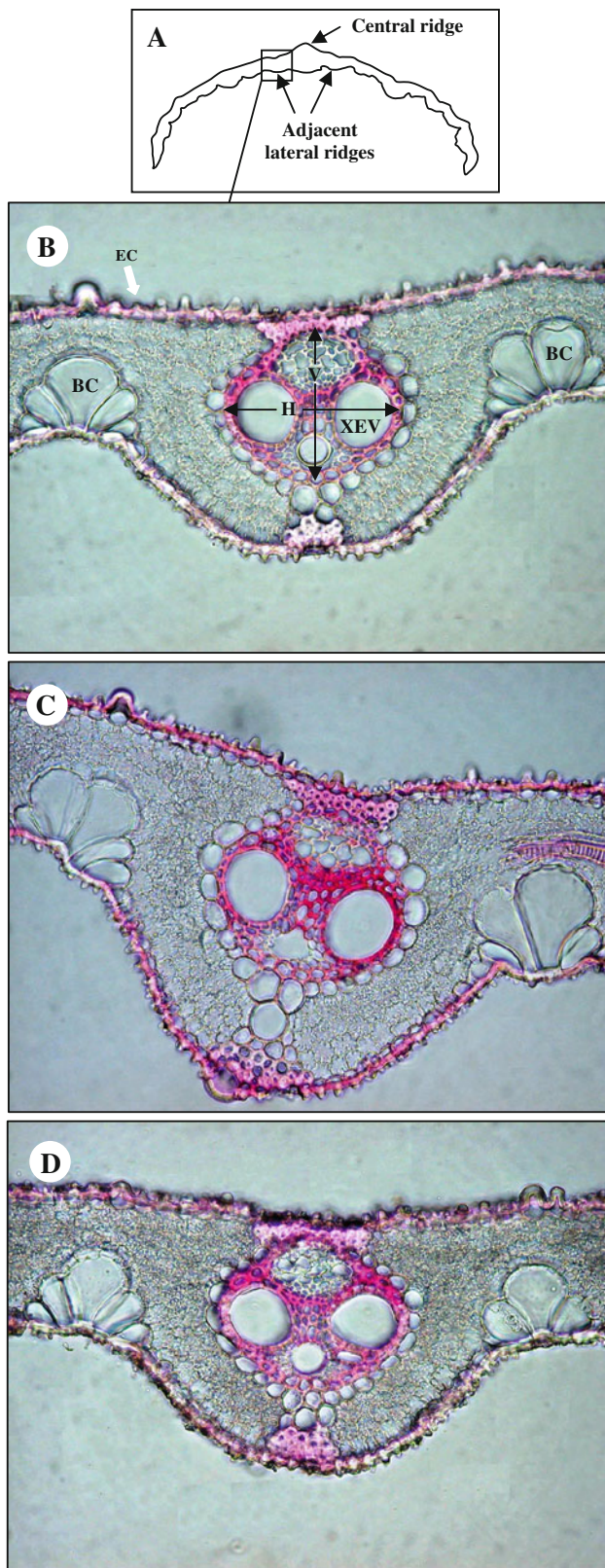


Fig. 6 Diagram of a transverse leaf section showing the analysed areas (a). Transverse sections from fully developed flag leaves (cv. Bomba) showing a leaf area with a lateral vascular bundle (LVB). Sections were obtained from control (b), 10 mM (c) and 20 mM NaCl-stressed plants (d). XEV xylem element vessel, BC largest bulliform cell, EC epidermal cell, H horizontal length of LVB, V vertical length of LVB. Magnification $\times 400$

In fact, our results show that increases in the area of bulliform and epidermal cells as well as changes in the diameter of xylem element vessels are responses to NaCl stress and the latter appears distinctively associated with sensitivity. Thus, a decrease in these parameters seems a response to the toxic effects of excessive salinity, as this effect occurs at high levels of NaCl (150 mM) or after prolonged exposure to milder stress conditions (reproductive stage, 20 mM) and is more prominent in cv. Bahia than in cv. Bomba (Table 2; Fig. 6). Therefore, some stress-induced anatomical alterations are related to the level of sensitivity to stress and appear to predict the effect of salinity on grain yield (Fig. 3).

In summary, the application of saline stress to rice plants, from the seedling stage through the vegetative growth to flowering, allowed us to relate several physiological and, especially anatomical variation to salt sensitivity as a function of both cultivar and developmental stage.

Author contribution This study was carried out thanks to the collaborative work of all authors. MJC defined the work and designed the experiments. SDW performed the lab and greenhouse experiments. Morpho-anatomical and physiological measurements were planned and supervised by IM-A and AS, respectively. AS and SDW analysed the data, searched for literature and wrote the draft. All authors contributed in interpreting the results and approved the manuscript.

Acknowledgments A pre-doctoral fellowship from the Spanish Agency for International Cooperation (AECI) to Shantanu D. Wankhade is gratefully acknowledged. We are thankful to Ms. Cristina Navajo for her help with growing plants in the greenhouse.

References

- Abdullah Z, Khan MA, Flowers TJ (2001) Causes of sterility in seed set of rice under salinity stress. *J Agron Crop Sci* 187:25–32
- Alam MZ, Stuchbury T, Naylor REL, Rashid MA (2004) Effect of salinity on growth of some modern rice cultivars. *J Agron* 3:1–10
- Alamgir ANM, Ali MY (2006) Effects of NaCl salinity on leaf characters and physiological growth attributes of different genotypes of rice (*Oryza sativa* L.). *Bangladesh J Bot* 35:99–107
- Aloni R (2010) The induction of vascular tissues by auxin. In: Davies PJ (ed) *Plant hormone*, 3rd edn. Springer, Berlin, pp 485–506
- Alvarez JM, Rocha JF, Machado SR (2008) Bulliform cells in *Loudetiopsis chrysothrix* (Nees) Conert and *Tristachya leiostachya* Nees (Poaceae): structure in relation to function. *Braz Arch Biol Technol* 51:113–119

decrease in plant growth but probably also in higher survival rates. The threshold, however, may depend on the sensitivity of the plant.

- Asch F, Dingkuhn M, Dörffling K, Miezian K (2000) Leaf K/Na ratio predicts salinity induced yield loss in irrigated rice. *Euphytica* 113:109–118
- Bahaji A, Mateu I, Sanz A, Cornejo MJ (2002) Common and distinctive responses of rice seedlings to saline- and osmotically-generated stress. *Plant Growth Regul* 38:83–94
- Baker NR (2008) Chlorophyll fluorescence: a probe of photosynthesis in vivo. *Annu Rev Plant Biol* 59:89–113
- Barhouni Z, Djebali W, Chaïbi W, Abdelly C, Smaoui A (2007) Salt impact on photosynthesis and leaf ultrastructure of *Aeluropus litoralis*. *J Plant Res* 120:529–537
- Boughalleb F, Denden M, Tiba BB (2009) Anatomical changes induced by increasing NaCl salinity in three fodder shrubs, *Nitraria retusa*, *Atriplex halimus* and *Medicago arborea*. *Acta Physiol Plant* 31:947–960
- Cha-um S, Supaibulwatana K, Kirdmanee C (2006) Water relation, photosynthetic ability and growth of thai jasmine rice (*Oryza sativa* L. ssp. *indica* cv. KDML 105) to salt stress by application of exogenous glycinebetaine and choline. *J Agron Crop Sci* 192:25–36
- Cha-um S, Supaibulwatana K, Kirdmanee C (2007) Glycinebetaine accumulation, physiological characterizations and growth efficiency in salt-tolerant and salt-sensitive lines of indica rice (*Oryza sativa* L. ssp. *indica*) in response to salt stress. *J Agron Crop Sci* 193:157–166
- Chaves MM, Flexas J, Pinheiro C (2009) Photosynthesis under drought and salt stress: regulation mechanisms from whole plant to cell. *Ann Bot* 103:551–560
- Dionisio-Sese ML, Tobita S (1998) Antioxidant responses of rice seedlings to salinity stress. *Plant Sci* 135:1–9
- Dionisio-Sese ML, Tobita S (2000) Effects of salinity on sodium content and photosynthetic responses of rice seedlings differing in salt tolerance. *J Plant Physiol* 157:54–58
- Djanaguiraman M, Sheeba JA, Shankar AK, Devi DD, Bangarusamy U (2006) Rice can acclimate to lethal level of salinity by pretreatment with sublethal level of salinity through osmotic adjustment. *Plant Soil* 284:363–373
- Epimashko S, Meckel T, Fischer-Schliebs E, Lüttge U, Thiel G (2004) Two functionally different vacuoles for static and dynamic purposes in one plant mesophyll leaf cell. *Plant J* 37:294–300
- Eyidogan F, Öz MT (2007) Effect of salinity on antioxidant responses of chickpea seedlings. *Acta Physiol Plant* 29:485–493
- Hameed M, Ashraf M, Naz N (2009) Anatomical adaptations to salinity in cogon grass [*Imperata cylindrica* (L.) Rauschell] from the salt range, Pakistan. *Plant Soil* 322:229–238
- James RA, Munns R, Caemmerer SV, Trejo C, Miller C, Condon TAG (2006) Photosynthetic capacity is related to the cellular and subcellular partitioning of Na⁺, K⁺ and Cl⁻ in salt-affected barley and durum wheat. *Plant Cell Environ* 29:2185–2197
- Kato Y, Kamoshita A, Yamagishi J (2008) Preflowering abortion reduces spikelet number in upland rice (*Oryza sativa* L.) under water stress. *Crop Sci* 48:2389–2395
- Khan MSA, Hamid A, Salahuddin ABM, Quasem A, Karim MA (1997) Effects of sodium chloride on growth, photosynthesis and mineral ions accumulation of different types of rice (*Oryza sativa* L.). *J Agron Crop Sci* 179:149–161
- Kim SK, Son TK, Park SY, Lee IJ, Lee BH, Kim HY, Lee SC (2006) Influences of gibberellin and auxin on endogenous plant hormone and starch mobilization during rice seed germination under salt stress. *J Environ Biol* 27:181–186
- Lee KS, Choi WY, Ko JC, Kim TS, Gregorio GB (2003) Salinity tolerance of *japonica* and *indica* rice (*Oryza sativa* L.) at seedling stage. *Planta* 216:1043–1046
- Lutts S, Kinet JM, Bouharmont J (1995) Changes in plant responses to NaCl during development of rice (*Oryza sativa* L.) varieties differing in salinity resistance. *J Exp Bot* 46:1843–1852
- Lutts S, Kinet JM, Bouharmont J (1996a) Effects of salt stress on growth, mineral nutrition and proline accumulation in relation to osmotic adjustment in rice (*Oryza sativa* L.) cultivars differing in salinity resistance. *Plant Growth Regul* 19:207–218
- Lutts S, Kinet JM, Bouharmont J (1996b) NaCl-induced senescence in leaves of rice (*Oryza sativa* L.) cultivars differing in salinity resistance. *Ann Bot* 78:389–398
- Lutts S, Majerus V, Kinet JM (1999) NaCl effects on proline metabolism in rice (*Oryza sativa*) seedlings. *Physiol Plant* 105:450–458
- Moradi F, Ismail AM (2007) Responses of photosynthesis, chlorophyll fluorescence and ROS-scavenging systems to salt stress during seedling and reproductive stages in rice. *Ann Bot* 99:1161–1173
- Moradi F, Ismail AM, Gregorio GB, Egdane JA (2003) Salinity tolerance of rice during reproductive development and association with tolerance at the seedling stage. *Indian J Plant Physiol (Special Issue)*:276–287
- Moukadiri O, Deming J, O'Connor JE, Cornejo MJ (1999) Phenotypic characterization of the progenies of rice plants derived from cryopreserved calli. *Plant Cell Rep* 18:625–632
- Mouliya B (2000) Leaves as shell structures: double curvature, auto-stresses, and minimal mechanical energy constraints on leaf rolling in grasses. *J Plant Growth Regul* 19:19–30
- Munns R (2002) Comparative physiology of salt and water stress. *Plant Cell Environ* 25:239–250
- Munns R, Tester M (2008) Mechanisms of salinity tolerance. *Annu Rev Plant Biol* 59:651–681
- Netondo GW, Onyango JC, Beck E (2004) Sorghum and salinity: II. Gas exchange and chlorophyll fluorescence of sorghum under salt stress. *Crop Sci* 44:806–811
- Pieters AJ, Nuñez M (2008) Photosynthesis, water use efficiency, and $\delta^{13}C$ in two rice genotypes with contrasting response to water deficit. *Photosynthetica* 46:574–580
- Prakash L, Prathasan G (1990) NaCl- and gibberellic acid-induced changes in the content of auxin and the activities of cellulase and pectin lyase during leaf growth in rice (*Oryza sativa*). *Ann Bot* 65:251–257
- Rahman MS, Matsumuro T, Miyake H, Takeoka Y (2000) Salinity-induced ultrastructural alterations in leaf cells of rice (*Oryza sativa* L.). *Plant Prod Sci* 3:422–429
- Rao SP, Mishra B, Gupta SR, Rathore A (2008) Reproductive stage tolerance to salinity and alkalinity stresses in rice genotypes. *Plant Breed* 127:256–261
- Rengasamy P (2006) World salinization with emphasis on Australia. *J Exp Bot* 57:1017–1023
- Sharma N, Gupta NK, Gupta S, Hasegawa H (2005) Effects of NaCl salinity on photosynthetic rate, transpiration rate, and oxidative stress tolerance in contrasting wheat genotypes. *Photosynthetica* 43:609–613
- Siringam K, Juntawong N, Cha-um S, Kirdmanee C (2009) Relationships between sodium ion accumulation and physiological characteristics in rice (*Oryza sativa* L. ssp. *indica*) seedlings grown under iso-osmotic salinity stress. *Pak J Bot* 41:1837–1850
- Sohn YG, Lee BH, Kang KY, Lee JJ (2005) Effects of NaCl stress on germination, antioxidant responses, and proline content in two rice cultivars. *J Plant Biol* 48:201–208
- Sultana N, Ikeda T, Itoh R (1999) Effect of NaCl salinity on photosynthesis and dry matter accumulation in developing rice grains. *Environ Exp Bot* 42:211–220
- Tester M, Davenport R (2003) Na⁺ tolerance and Na⁺ transport in higher plants. *Ann Bot* 91:503–527
- Ul Haq T, Akhtar J, Nawaz S, Ahmad R (2009) Morpho-physiological response of rice (*Oryza sativa* L.) varieties to salinity stress. *Pak J Bot* 41:2943–2956

- Walia H, Wilson C, Condamine P, Liu X, Ismail AM, Zeng L, Wanamaker SI, Mandal J, Xu J, Cui X, Close TJ (2005) Comparative transcriptional profiling of two contrasting rice genotypes under salinity stress during the vegetative growth stage. *Plant Physiol* 139:822–835
- Wang RG, Hua C, Zhou F, Zhou QC (2009) Effects of NaCl stress on photochemical activity and thylakoid membrane polypeptide composition of a salt-tolerant and a salt-sensitive rice cultivar. *Photosynthetica* 47:125–127
- Wankhade SD, Bahaji A, Mateu-Andrés I, Cornejo MJ (2010) Phenotypic indicators of NaCl tolerance levels in rice seedlings: variations in development and leaf anatomy. *Acta Physiol Plant* 32:1161–1169
- Welfare K, Flowers TJ, Taylor G, Yeo AR (1996) Additive and antagonistic effects of ozone and salinity on the growth, ion contents and gas exchange of five varieties of rice (*Oryza sativa* L.). *Environ Pollut* 92:257–266
- Yamane K, Kawasaki M, Taniguchi M, Miyake H (2003) Differential effect of NaCl and polyethylene glycol on the ultrastructure of chloroplast in rice seedlings. *J Plant Physiol* 160:573–575
- Yamane K, Kawasaki M, Taniguchi M, Miyake H (2008) Correlation between chloroplast ultrastructure and chlorophyll fluorescence in the leaves of rice (*Oryza sativa* L.) grown under salinity. *Plant Prod Sci* 11:139–145
- Zeng L, Shannon MC (2000a) Effects of salinity on grain yield and yield components of rice at different seeding densities. *Agron J* 92:418–423
- Zeng L, Shannon MC (2000b) Salinity effects on seedling growth and yield components of rice. *Crop Sci* 40:996–1003
- Zeng L, Shannon MC, Lesch SM (2001) Timing of salinity stress affects rice growth and yield components. *Agric Water Manage* 48:191–206
- Zeng L, Shannon MC, Grieve CM (2002) Evaluation of salt tolerance in rice genotypes by multiple agronomic parameters. *Euphytica* 127:235–245
- Zeng L, Poss JA, Wilson C, Draz A-SE, Gregorio GB, Grieve CM (2003) Evaluation of salt tolerance in rice genotypes by physiological characters. *Euphytica* 129:281–292
- Zhao GQ, Ma BL, Ren CZ (2007) Growth, gas exchange, chlorophyll fluorescence, and ion content of naked oat in response to salinity. *Crop Sci* 47:123–131
- Zheng C, Jiang D, Liu F, Dai T, Jing Q, Cao W (2009) Effects of salt and waterlogging stresses and their combination on leaf photosynthesis, chloroplast ATP synthesis, and antioxidant capacity in wheat. *Plant Sci* 176:575–582