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Evaluation of salinity tolerance in sorghum (*Sorghum bicolor* L.) using ion accumulation, proline and peroxidase criteria

Vahid Bavei · Behrouz Shiran · Ahmad Arzani

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Abstract Salt tolerance of sorghum varieties in terms of fresh weight, ion accumulations, proline content and peroxidase activity was analyzed in this study. Three sorghum varieties, Payam, Kimia, and Jambo, differing in salt tolerance, were grown in a greenhouse-hydroponic culture with a complete nutrition solution to which 0, 50, 100, 150 and 200 mM NaCl was added. Plant roots and leaves were harvested at 15 and 30 days after treatment and subjected to analysis. Clear decline in K^+ and Ca^{2+} concentrations and increase in Na^+ and proline contents were observed in the root and leaf tissues at each NaCl concentration in all varieties during the NaCl treatment. The Ca^{2+} concentration in leaves was higher than in roots, and had the following order in the tested cultivars: Jambo, Kimia, and Payam. Total peroxidase activity increased under salinity stress and it was proportional with the salt concentration. Payam had the largest decrease (46.95%) in fresh weight caused by NaCl, while Jambo had the lowest decrease, 28.63%. Linear regression analysis revealed significant relationships between the estimated factors and fresh weight. The profiles of isoperoxidases were modified under stress conditions. Two isoforms, A1 and A2, were detected in all three varieties with different intensities. Under NaCl stress, isoperoxidases were strongly expressed and a third isoform, A3, was specifically found in variety Jambo suggesting that A3 is implicated in salt adaptation of this variety.

Keywords Salt tolerance · Sorghum · Fresh weight · Proline · Proxidase activity

V. Bavei (✉) · B. Shiran
Department of Agronomy and Plant Breeding,
College of Agriculture, University of Shahrekord,
Shahrekord, Iran
e-mail: vahidbavei@gmail.com

A. Arzani
Department of Agronomy and Plant Breeding,
College of Agriculture, Isfahan University of Technology,
84156 83111 Isfahan, Iran

Introduction

Sorghum (*Sorghum bicolor* [L.] Moench) is often grown in areas of relatively low rainfall, high temperatures and saline soils (Boursier and Läuchli 1990). Because salinity stress is becoming one of the major constrains in agricultural productivity particularly in arid and semiarid areas of the world, breeding for plant tolerance to salinity stress should be given a high research priority in future research programs (Arzani 2008). Therefore, the development of salt tolerant plants using physiological, biochemical and molecular markers are recommended and may offer mechanistic understanding of tolerance (El-Baz et al. 2003).

Many metabolic changes are known to occur in plants subjected to salt stress, among which physiological parameters, such as ionic contents, are salinity tolerance indicators (Ashraf and Waheed 1993; El-Baz et al. 2003; Netondo et al. 2004). Nevertheless, generalizations of plant responses to salinity are difficult and could not be adequate. The deleterious effects of salinity on plant growth are associated with the decreased osmotic potential of the growing medium, specific ion toxicity, and nutrient ion deficiency (Hasegawa et al. 2000; Luo et al. 2005; Netondo et al. 2004). The control of ion accumulations under salt stress in higher plants are usually caused by either ion exclusions at the root cortex (Jeschke 1984) or

redistribution of excess ions to senescing leaves (Yeo and Flowers 1984).

One of the symptoms of salt stress is a substantial K^+ efflux from cells reducing the intracellular K^+ pool, affecting the cytosolic K^+ homeostasis (Cuin et al. 2003; Shabala et al. 2006), and therefore the growth and survival of the plant. Reduction of this efflux correlates with increased salt tolerance (Flowers and Hajibagheri 2001; Chen et al. 2005). Metabolic toxicity of Na^+ is largely a result of its ability to compete with K^+ for binding sites essential for cellular function. More than 50 enzymes are activated by K^+, and Na^+ cannot substitute K^+ in this role (Bhandal and Malik 1988). Thus, high levels of Na^+, or high Na^+/K^+ ratios can disrupt various enzymatic processes in the cytoplasm. Moreover, protein synthesis requires high concentrations of K^+, due to the K^+ requirement for the binding of tRNA to ribosomes (Blaha et al. 2000) and probably due to other aspects of ribosome function (Wyn Jones et al. 1979). The disruption of protein synthesis by elevated concentrations of Na^+ is an important cause of damage by Na^+ as suggested by Tester and Davenport (2003) and Bavei et al. (in press).

An early response to the sudden addition of extracellular Na^+ is a temporary rise in cytosolic Ca^{2+} (Knight et al. 1997). Salinity-induced changes in activity of Ca^{2+} transporters and components of Ca^{2+}-related signal transduction pathways suggest that Ca^{2+} plays a physiologically relevant role in plant responses to salinity (Tester and Davenport 2003). Especially, it was observed in many plants that Ca^{2+} inhibition of initial Na^+ influx is directly correlated with the Ca^{2+}-induced decrease in shoot Na^+ accumulation, and these are correlated with a reduction in growth inhibition by Na^+ upon addition of Ca^{2+} (Cramer 2002). Calcium has been known for some time to alleviate the effects of salt (LaHaye and Epstein 1969), possibly by maintaining the ion selectivity of membranes (Greenway and Munns 1980). It has been shown, however, that the high ionic strength of saline solutions displaces Ca^{2+} from the membranes of root cells (Cramer et al. 1985; Lynch and Läuchli 1985).

Proline is a very influential regulator of K^+ permeable ion channels and, hence, has direct impact on intracellular K^+/Na^+ homeostasis (Cuin and Shabala 2005, 2007a) and reduces the levels of reactive oxygene species (ROS) generated during osmotic stress (Hong et al. 2000). Furthermore, in addition to the conventional osmoprotective role of proline in plants, a beneficial effect of over-expressed levels of proline in salt-stressed plants may be the result of protecting the integrity and transporter proteins of plasma membranes (Cuin and Shabala 2007b). Moreover, proline also protects thylakoid membranes against free radical-induced photodamage by stabilizing the photosystem II complex and preserving the structure of enzymes and proteins (Sivakumar et al. 2000). Activities of the enzymes catalase, peroxidase, and polyphenoloxidase were promoted by proline in vivo. Accumulations of free proline under salt, drought, and freezing stresses have been reported for several plants (Potluri and Devi Prasad 1996). While some researchers have reported positive correlations between the capacity for proline accumulation and salinity tolerance (Aspinall and Paleg 1981; Almansouri et al. 1999), others have challenged the value of this solute as positive indicator for resistance to salt stress (Delauney and Verma 1993; Heuer 2003). Thus, it is controversial that hyper accumulation of proline is essential for improving salinity tolerance, or it is just a symptom of salt stress. In addition, it cannot be excluded that both mechanisms may coexist, providing some effective ROS scavenging in sensitive cultivars or species, while indicating a symptom of salt stress in tolerant ones (Chen et al. 2007).

Active oxygen radicals ($O_2^{\bullet -}$, $OH^{\bullet}$, and H_2O_2) are commonly generated under salt stress, and considered as a major peroxidative damaging factor and they need to be scavenged for maintenance of normal growth (El-Baz et al. 2003). Plant cells possess different antioxidant enzymes such as catalase, peroxidase (POD) and superoxide dismutase, which eliminate these reactive free radicals or suppress their formation. Apparently, the high value of antioxidant enzymes activity in NaCl tolerant plant could be related to salt adaptation process (Piqueras et al. 1996; Sreenivasulu et al. 1999).

There are various reports on the different ionic contents, accumulation of amino acids and their contributions to salinity tolerance among genotypes of particular species, and, hence, high degree of diversity of plants' responses to salinity. For example, results from some studies suggest that there is a positive correlation between the capacity for proline accumulation and salinity tolerance. Nevertheless, some researchers reported a negative correlation between proline accumulation and salt tolerance, or showed that a highly salt-tolerant wild relative of tomato, *Lycopersicon peruvianum*, accumulates higher concentrations of Na^+ than the salt-sensitive domesticated tomato, *L. esculentum* (Tal 1971; Santa-Cruz et al. 1999). There are some more inconsistent results too. In *Arabidopsis thaliana* there is no relationship between Na^+ accumulation and salt sensitivity (Tester and Davenport 2003), while in some brassicas, Na^+ accumulation is correlated with toxicity (Ashraf and Naqvi 1992; Cramer 2002; Rengel 1992; Schmidt et al. 1993). Thus it seems likely that plants respond to salinity in diverse form that necessitates the discovery of individual metabolic acclimation strategies at species or even at genotype level. Taken all these into consideration we have studied salinity tolerance in sorghum with relevance to ion accumulations, proline content and peroxidase activity.

Materials and methods

Three varieties of sorghum differing in salt tolerance, Payam (sensitive), Kimia (moderately tolerant), and Jambo (tolerant), previously investigated by Bavei et al. (in press), were used in this study. These varieties were evaluated under five concentrations of salinity (0, 50, 100, 150, and 200 mM) and at two time points (sampling at 15 and 30 days after salt application). Seeds were grown in plastic pots containing a peat-sand-perlite mix in three replications in 2007 in the greenhouse of the University of Shahrekord, Iran. Treatments were arranged according to a split-split plot design with randomized complete blocks layout.

After 7 days of seed planting, the seedlings were thinned to one plant per pot. During the first 8 days, pots were watered twice daily with deionized water. Thereafter, seedlings were irrigated with Hoagland nutrient solution (Hoagland and Arnon 1959). Application of nutrients started at low concentrations (10%) and increased gradually to the full concentration by 10 days after planting, at which time the seedlings were treated with NaCl. Day and night temperatures, natural illumination and the relative humidity in the greenhouse were set to similar to field conditions. To avoid osmotic shock, saline treatments were initiated by adding 50 mM of NaCl per liter of culture solution per every second day. Plants were harvested at 15 and 30 days after the final salinity level was reached. The control was irrigated with NaCl-free nutrient solution, whose electrical conductivity was 0.01 dS m^{-1}.

Analysis of minerals

Inorganic solute concentrations were measured in three replicates of the roots and leaves, which were harvested at 15 and 30 days after salt application. From each plant, three leaves of different ages were used for elemental analysis. Samples were weighed, carefully rinsed with distilled water, and then dried at 75°C for 72 h. After drying, 0.2 g of dried sample was put into porcelain crucible inside a furnace at 550°C for 2 h. After removing samples from the furnace and before completely cooling, 10 ml of 2 N HCl was added and heated for several minutes for completely dissolve the ash. After filtering the solution with ash-less filter paper, the filtered solution volume was diluted to 100 ml with distilled water and the resulting solution was analyzed for Na^+, K^+ and Ca^{2+} with flame photometer (Genway PFP-7 model). Results were expressed as mg g^{-1} dry weight of leaf or root tissue.

Proline content

Proline accumulation was determined as described by Bates et al. (1973). 0.5 g of fresh leaf tissues from each treatment were homogenized in 10 ml of 3% w/v sulphosalicylic acid and the homogenate was filtrated. The resulting solution was treated with 2.5% ninhidrine solution and glacial acetic acid. In test tubes, the reaction mixtures were kept in a water bath at 100°C for 60 min to develop the colors. Soon after removal from the water bath, the test tubes were cooled in ice bath and toluene was added to separate chromophores. Optical density was read at 520 nm using UV–VIS spectrophotometer. Proline content was expressed as mg g^{-1} fresh weight of leaf tissue.

Enzyme extraction

Leaf and root tissues of each variety were homogenized separately in 50 mM Tris–HCl buffer pH 7.4 at 4°C. The homogenate was centrifuged at $9{,}000 \times g$ for 20 min in a refrigerated high-speed centrifuge. The pellet was washed with the same extraction buffer and centrifuged in the same way. The resultant supernatants were assayed for peroxidase activity.

Assay of peroxidase activity

Peroxidase (POD) activity (EC 1.11.1.7) was determined by the absorbance at 430 nm of pyrogallol according to Amako et al. (1994). Reaction mixtures contained 1.5 ml of 100 mM K-phosphate buffer (pH 6.8), 1 ml of 60 mM pyrogallol, 0.48 ml of 0.6 mM H_2O_2 and 20 μ1 of the enzyme extract. The increase in ΔA430 was measured for 3 min and POD activity expressed as ΔA430 min^{-1} mg^{-1} protein. Proteins in the extracts were quantified by the method of Bradford (1976) using BSA as standard.

Peroxidase electrophoresis

Non-denaturing discontinuous polyacrylamide gel electrophoresis (PAGE) was done in 10% separating and 5% stacking gels according to Davis (1964). Enzyme extracts (30 µg protein) from leaf tissues were loaded into the slots. After electrophoresis, the gels were stained with benzidine for peroxidase isozymes as described by Schrauwen (1966).

Statistical analysis

The experiment was carried out in a split-split plot design with completely randomized blocks layout of five salt levels (0, 50, 100, 150, and 200 mM NaCl), three varieties (Payam, Kimia and Jambo), two sampling times (15 and 30 days after salt application), and three replicates. Analysis of variance (ANOVA) was performed by Minitab statistical program (Minitab Inc., State College, PA).

Means were separated using the least significant difference (LSD) test at 5% level.

Results

Plant fresh weight

Fresh weight of the plants grown under control conditions ranged from 46.26 (Kimia) to 48.00 g (Jambo). Under these conditions, there were no significant differences in fresh weights of the three varieties. In all plants, fresh weight decreased under salt stress that ranged from 46.94 (50 mM) to 22.48 g (200 mM) in Jambo, from 41.09 (50 mM) to 14.89 g (200 mM) in Kimia, and from 42.27 (50 mM) to 9.51 g (200 mM) in Payam. The greatest decrease, 46.95%, was observed with Payam, while Jambo had the lowest decrease, 28.63%. These differences were significant at $P \leq 0.5$ level (Table 1).

Effect of salinity stress on the concentration of mineral ions

An immediate and primary effect of the imposition of salt stress is a perturbation in tissue cation levels. To determine how Na^+ accumulated under our experimental conditions, and how the concentrations of other cations (such as Ca^{2+} or K^+) might be affected by Na^+ accumulation, cation contents of sorghum roots and leaves were measured at 15 and 30 days after NaCl treatment. The Na^+ concentration was higher in roots than in leaves (Fig. 1). The root Na^+ concentration of var. Kimia and Jambo became saturated at about 150 mM external NaCl concentration and did not substantially increase at higher salt concentration. Jambo accumulated significantly ($P \leq 0.01$) less Na^+ in roots and leaves than did other varieties at each NaCl concentration. Increasing levels of NaCl led to a significant ($P \leq 0.01$) rise in Na^+ concentration of leaves with no indication of reaching a saturation level as it was found in roots.

Table 1 Fresh weight (g) of the plants and relative reduction of fresh weights of three sorghum varieties under salinity stress

	Salinity levels (mM)					Percent reduction†
	0 (Control)	50	100	150	200	
Payam	47.71 a	42.27 b	29.70 c	19.76 c	9.51 c	46.95 a
Kimia	46.26 a	41.09 b	33.68 b	24.46 b	14.89 b	38.33 b
Jambo	48.00 a	46.94 a	39.05 a	28.56 a	22.48 a	28.63 c

Means followed by the same letter are not significantly different at the 5% probability level

† Reductions are related to average fresh weights of plants subjected to NaCl (50–200 mM) compared to fresh weights of control plants

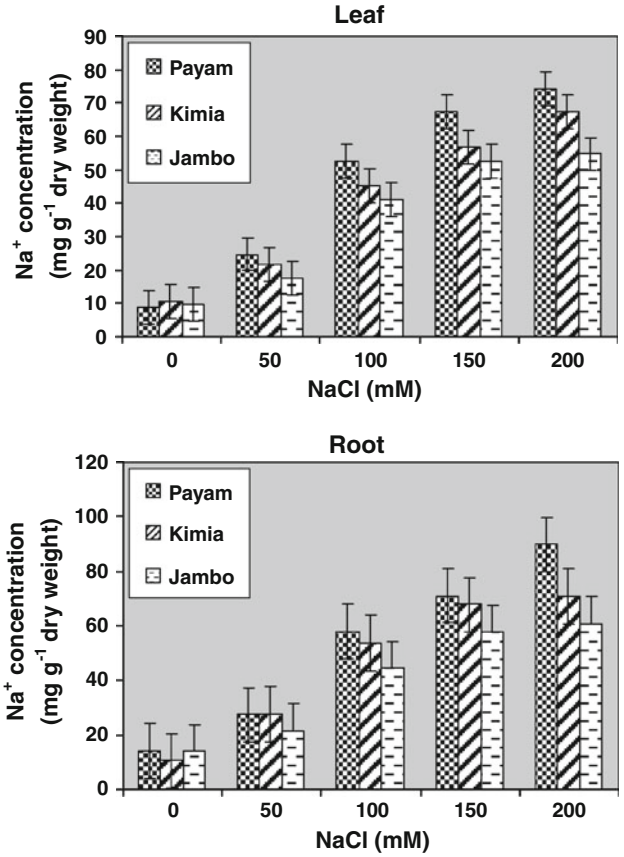

Fig. 1 Effects of increasing NaCl concentration in the irrigation medium on sodium ions of leaf and root of sorghum varieties Payam, Kimia, and Jambo. Means of three replicates (leaf, $LSD_{0.05} = 0.67$; root, $LSD_{0.05} = 3.48$)

There was a significant ($P \leq 0.01$) decrease in K^+ concentration in roots and leaves of all varieties with increasing salinity (Fig. 2). Jambo accumulated more K^+ at all levels of NaCl concentration both in roots and leaves than the other two varieties. Concentrations of K^+ in leaves of all varieties were higher than in roots under stress conditions.

There was a clear proportionate decline of Ca^{2+} in roots and leaves with increasing NaCl concentration in the growth medium (Fig. 3). The Ca^{2+} concentration in leaves was higher than in roots in the following sequence: Jambo, Kimia, and Payam.

Clear declines in K^+ and Ca^{2+} concentrations with advancing time of NaCl treatment were observed in the root and leaf tissues at each NaCl concentration in each variety (Table 2).

Effect of NaCl on proline accumulation

The accumulation of proline in response to salt stress in leaves of sorghum plants is presented in Fig. 4. The var. Jambo had significantly higher proline concentration

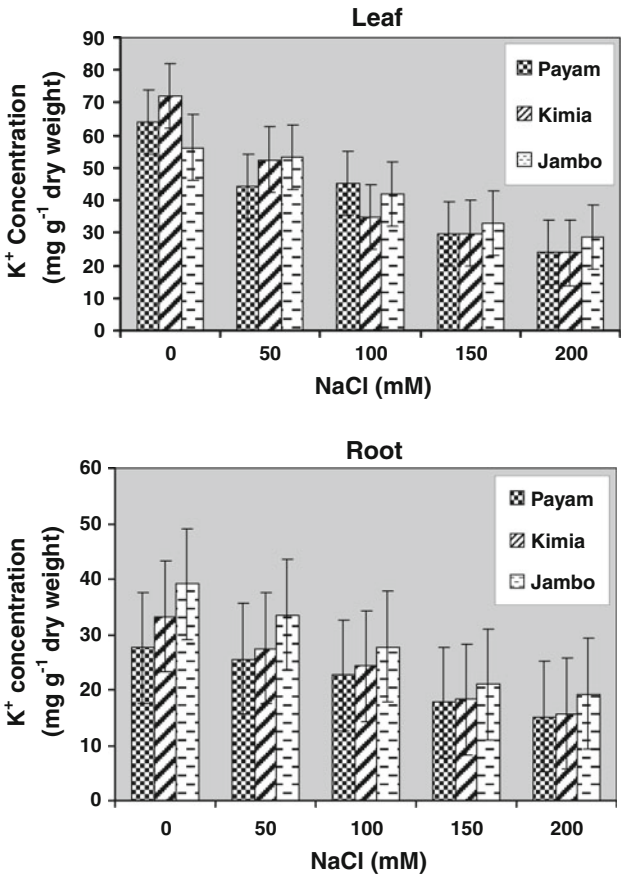

Fig. 2 Effects of increasing NaCl concentration in the irrigation medium on potassium ions of leaf and root of sorghum varieties Payam, Kimia, and Jambo. Means of three replicates (leaf, $LSD_{0.05} = 7.65$; root, $LSD_{0.05} = 0.52$)

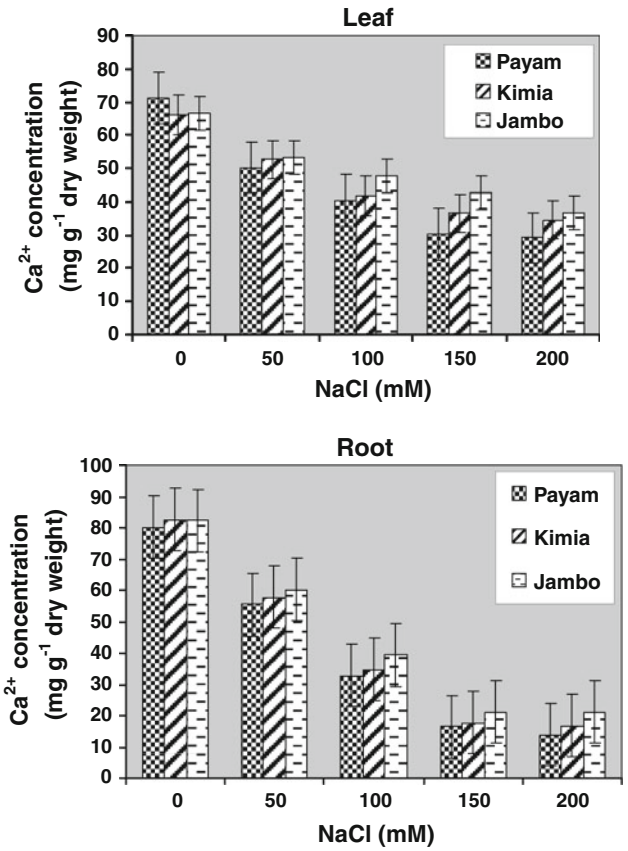

Fig. 3 Effects of increasing NaCl concentration in the irrigation medium on calcium ions of leaf and root of sorghum varieties Payam, Kimia, and Jambo. Means of three replicates (leaf, $LSD_{0.05} = 0.69$; root, $LSD_{0.05} = 0.86$)

compared to var. Kimia and var. Payam in all salt treatments. Increasing concentrations of NaCl from 50 to 200 mM progressively increased the proline concentration in the leaf tissue of var. Payam and var. Kimia by about fourfold, while in var. Jambo, the increase was about sixfold compared to the non-treated control.

Effect of NaCl on peroxidase activity

The activity of the antioxidative peroxidase enzyme in leaves and roots of different sorghum varieties was estimated. Total peroxidase activity of the crude enzyme extracts by using pyrogallol, which reacts with all peroxidases, was measured in root and leaf tissues at 15 and 30 days after application of different NaCl concentrations. The time course of appearance of peroxidase was similar in all varieties. As shown in Fig. 5, the activity of peroxidase was significantly enhanced by NaCl treatments. POD activity in leaves of var. Jambo and Kimia was higher than in leaves of var. Payam at each salinity level. The activity of peroxidase in root tissue was more pronounced than in leaves. The pattern of POD activity in roots was similar to that in leaves (Fig. 5).

Differential expression of peroxidase isoforms

Peroxidase isoforms were detected in the extracts of both control and NaCl-treated seedlings of sorghum varieties by using 10% PAGE. Since the same amounts of soluble proteins (30 μg protein) from each preparation was loaded on gel, the intensity of isoform bands reflects the amounts of individual peroxidase isoforms in each variety. Two isoperoxidases, designated A1 and A2, were detected in non-treated control extract of all varieties (Fig. 6). The amounts of these isoperoxidases were progressively enhanced with increasing NaCl concentrations and were intensified especially at the second time point. The amounts of isoperoxidases A1 and A2 were relatively higher in the var. Jambo than in the two other varieties. One isoperoxidase, designated A3, was expressed only in the var. Jambo. It appeared at the first time point at 200 mM NaCl concentration, but expressed with higher intensity at the second time point both at 150 and 200 mM NaCl concentrations.

Table 2 Analysis of variance of mineral ions, plant fresh weight, POD activity of leaf and root and leaf proline content recorded in three varieties of sorghum of exposure to five salinity levels at two time points

Source of variation		Mean squares									
	df	Leaf					Plant fresh weight	Root			
		Na^+	K^+	Ca^{2+}	POD act.	Proline		Na^+	K^+	Ca^{2+}	POD act.
Salinity	4	1,219.8**	1,465.0**	69.18**	9.6**	10.8**	1,605.64**	1,864.2**	730.8**	15.89**	13.8**
Genotype	2	1,116.5**	647.0**	23.63**	3.56**	2.5**	153.15**	1,441.2**	423.2**	11.16**	9.5**
Sampling	1	1,376.4**	1,393.7**	103.36**	7.5**	4.6**	471.49**	576.3**	1,411.7**	64.81**	10.6**

ns non-significant; * significant at $P \leq 0.05$; ** significant at $P \leq 0.01$

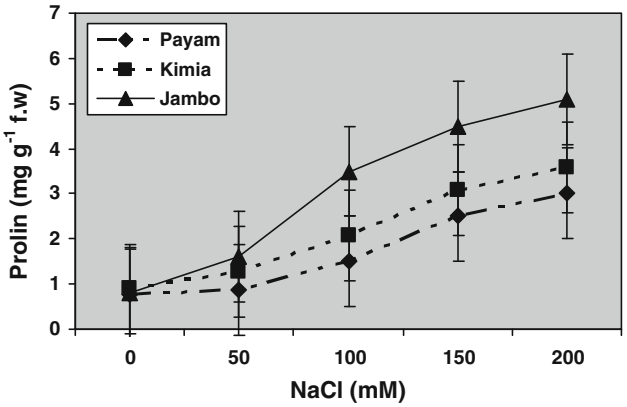

Fig. 4 Effects of increasing NaCl concentration in the irrigation medium on proline accumulation in leaves of sorghum varieties Payam, Kimia, and Jambo. Means of three replicates ($LSD_{0.05} = 0.48$)

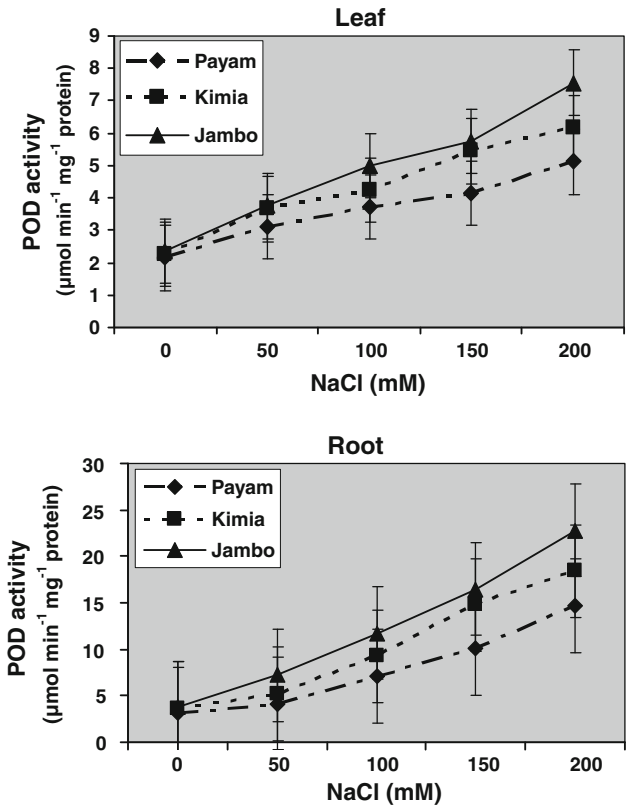

Fig. 5 Effects of increasing NaCl concentration in the irrigation medium on peroxidase (POD) activity of leaf and root of sorghum varieties Payam, Kimia, and Jambo. Means of three replicates (leaf, $LSD_{0.05} = 0.75$; root, $LSD_{0.05} = 0.55$)

Discussion

By increasing salinity in root medium, sodium was the major cation that accumulated in plant tissues. In this study, the preferential accumulation of Na^+ in roots over leaves may be interpreted as a mechanism of tolerance in at least two ways: (1) plants need to maintain internal water potential below that of the soil to maintain turgor and water uptake into the roots for growth and (2), restricting Na^+ transport to shoot (Renault et al. 2001). Moreover, the less Na^+ accumulation in leaves could be due either to minimized Na^+ loading to the xylem because of reduced transpiration (Wang 2001) or to maximized retrieval before reaching the leaves (Tester and Davenport 2003). This implies that roots and leaves experience different primary stresses at the initial stage of high salinity stress. We propose that within short time of salt stress at moderate severity (100 mM NaCl) during the day, leaves mainly experience osmotic stress, whereas roots experience both osmotic and ionic stresses. The conclusion is supported by the analysis of K^+ content, which had significantly decreased level in root even at the first time point of salt stress, while it was not significantly different in leaves compared to the non-treated control at this time point (data not shown).

According to our results, high Na^+ concentration strongly inhibited the uptake and accumulation of K^+ and Ca^{2+} in roots. Because K^+ is a macronutrient involved in turgor control, inhibition of K^+ uptake should inhibit growth (Renault et al. 2001) and changes in the cytosolic K^+/Na^+ ratio that induce early senescence by triggering programmed cell death (Shabala 2009). Salinity decreased root Ca^{2+} concentration as reported by Colmer et al. (1996). High Na^+ levels in the external medium greatly reduce the

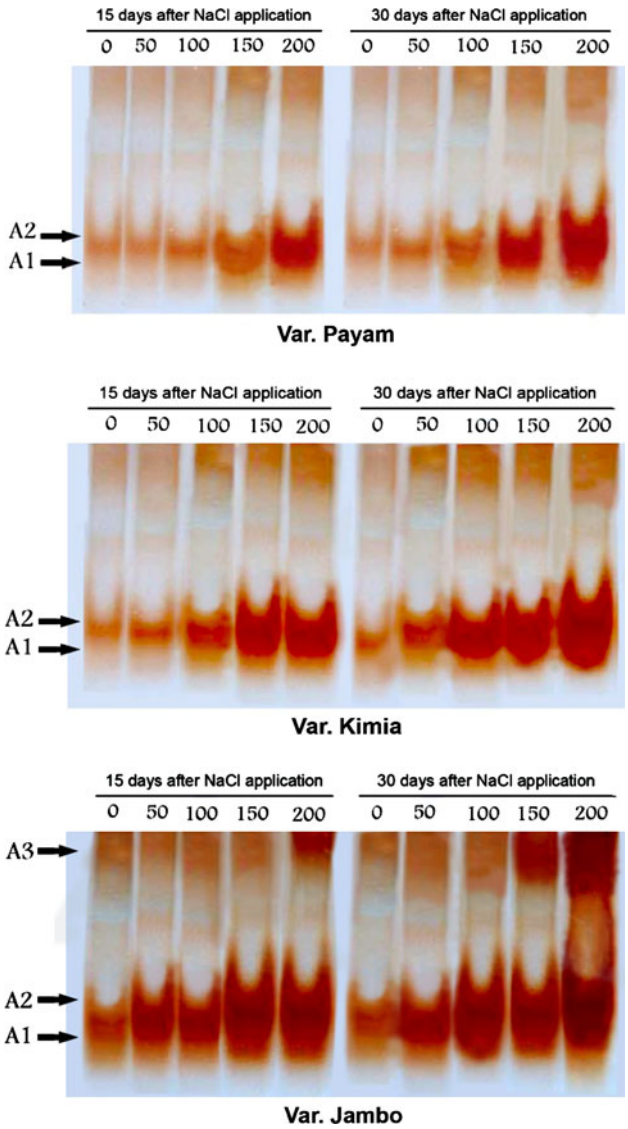

Fig. 6 Peroxidase isoforms in the leaf tissues of sorghum varieties Payam, Kimia, and Jambo at different NaCl concentrations (control, 50, 100, 150 and 200 mM) in samples collected at 15 and 30 days after salt application

physicochemical activity of dissolved calcium and may thus displace Ca^{2+} from the plasma membrane of root cells (Cramer and Läuchli 1986). In turn, displacement of Ca^{2+} from root membranes by Na^+ affects Na^+/K^+ uptake selectivity in favor of sodium (Boursier and Läuchli 1990; Netondo et al. 2004). High extracellular Ca^{2+} inhibits the plasma membrane inwardly directed Na^+-permeable non-selective cation channels (NSCCs) that mediate toxic Na^+ influx, and apoplastic Ca^{2+} also prevents K^+ loss from the cell by regulating K^+ efflux channels (Shabala et al. 2006). Our results indicate that Na^+ preferentially accumulated to higher concentrations in older leaves and roots (the leaves and roots harvested after 30 days salt treatment), especially in treatments of 100 mM NaCl and above. This phenomenon is common in glycophytes (Nakamura et al. 1996) and could be explained by the fact that older leaves have been exposed to salt for a longer period than the younger ones. In addition, their vacuoles are bigger and thus can accumulate more Na^+ than those of younger leaves (Netondo et al. 2004). Increased NaCl concentrations in our experiment reduced K^+ and Ca^{2+} contents in leaves and roots of all varieties. However, overall K^+ and Ca^{2+} concentrations in leaf tissues were higher than in roots. Therefore, severe potassium deficiency is unlikely to occur, at least in sorghum leaves, at low to moderate salt concentrations. The results also show that there was a significant difference in K^+ concentrations both in leaves and roots of all varieties and at both time points var. Jambo had the highest amount of K^+ (Fig. 2). This difference would imply that var. Jambo had limited Na^+ transmission to leaf and it is more selective for K^+ than Na^+ uptake. A strong correlation has been observed between NaCl-induced K^+ efflux and salt tolerance based on variety of physiological and agronomical indices (Chen et al. 2005, 2007). This led to the suggestion of using K^+ retention as an indicator for salt tolerance (Ashraf and Tufail 1995; Munns et al. 2003; Santa-Maria and Epstein 2001).

Reduction in Ca^{2+} content was significantly higher at long-term than at short-term salinity stress. This is characteristic of Ca^{2+} whose transport is known to be affected by NaCl. The Ca^{2+} content of Jambo tissues was less affected compared with the other two varieties. The ionic interaction, particularly with Na^+, leads to low Ca^{2+} concentration in the xylem fluid, and in turn, to a lower supply of Ca^{2+} to the leaf tissues. It seems that the intact roots of var. Jambo was capable to increase the translocation of Ca^{2+} ions to the leaves.

Under salt stress, retention of shoot development is observed as delayed leaf emergence and expansion, and decreased leaf size. The mechanism of this increased sensitivity of the shoot to salt stress is not known. However, it is hypothesized that the plant's reduction in leaf growth is an adaptive response to save water in soils with reduced osmotic potential, i.e. dry and saline soils (Munns and Tester 2008). Considering the data obtained on fresh weight reduction under salt stress, Jambo was able to tolerate salt stress better (Table 1). The genotype Payam showed higher fresh weight reduction than Jambo under salt stress and, hence, is considered salt sensitive (Table 3).

The difference in salt tolerance was accompanied by a large variation in organic and inorganic solutes, which consequently promoted differences in the fresh weights of these three sorghum genotypes. This study showed a negative significant relationship between leaf and root content of Na^+ and salt tolerance in term of plant fresh weight. The significant relationships between the plant fresh weight and K^+ and Ca^{2+} contents (Table 4) indicate significant

Table 3 Means of evaluated factors in three sorghum varieties of exposure to five salinity levels at two time points

Var.	Time points	Leaf				Proline (mg g^{-1})	Root			
		Na^+ (mg g^{-1})	K^+ (mg g^{-1})	Ca^{2+} (mg g^{-1})	POD act.*		Na^+ (mg g^{-1})	K^+ (mg g^{-1})	Ca^{2+} (mg g^{-1})	POD act.
Payam	I†	42.86 d	42.68 b	35.81 c	0.376 e	3.45 d	44.98 d	25.96 c	23.70 c	0.725 e
	II‡	68.16 a	36.68 d	25.94 f	0.515 d	4.15 c	70.17 a	17.58 f	23.06 f	0.898 d
Kimia	I	33.1 e	46.53 a	40.53 b	0.524 d	4.43 c	40.71 e	27.34 b	24.34 b	0.90 d
	II	57.57 b	38.72 cd	30.59 e	0.825 b	5.92 b	61.69 b	20.25 e	23.34 e	1.395 b
Jambo	I	29.05 f	47.29 a	45.71 a	0.676 c	5.75 b	39.08 e	30.89 a	24.74 a	1.06 c
	II	49.66 c	40.13 bc	30.85 d	1.216 a	6.48 a	51.34 c	25.35 d	23.60 d	1.734 a
LSD value (5%)	0.33	5.66	0.05	0.10	0.34	2.39	0.35	0.04	0.06	

Data represent means of three replications

Means followed by the same letter are not significantly different at the 5% probability level

† Time point I sampling at 15 days after NaCl treatment

‡ Time point II sampling at 30 days after NaCl treatment

* Peroxidase activity expressed as $\Delta A430\ min^{-1}\ mg^{-1}$ protein

Table 4 Relationship between plant fresh weight and ionic/chemical parameters under salinity stress

Ionic/chemical parameters	Linear regression equation	Regression coefficient (R^2)
Leaf Na^+ content	$Y = -1.4297x + 89.98$	0.96**
Leaf K^+ content	$Y = 0.5374x + 19.06$	0.83*
Leaf Ca^{2+} content	$Y = 0.8207x + 17.24$	0.97**
Leaf proline content	$Y = 0.191x - 2.89$	0.99**
Leaf POD activity	$Y = 0.1602x + 0.10$	0.94**
Root Na^+ content	$Y = -1.4044x + 104.27$	0.99**
Root K^+ content	$Y = 0.5821x + 5.28$	0.98**
Root Ca^{2+} content	$Y = 0.6348x + 13.65$	0.99**
Root POD activity	$Y = 0.6012x - 5.82$	0.96**

** Significant at $P \leq 0.01$; * significant at $P \leq 0.05$

contributions of these ions to fresh weight of sorghum under salt stress. Although leaf K^+ content had the lowest R^2 value, it was still significantly correlated with fresh weight. This could be related to the fact that Ca^{2+} inhibition of initial Na^+ influx is directly correlated with the Ca^{2+}-induced decrease in shoot Na^+ accumulation (Cramer 2002). The highly significant relationships between these ion contents and the rankings among genotypes for fresh weight also confirmed the importance of K^+ and Ca^{2+} selectivity to the salt tolerance.

As shown in Fig. 4, proline content in sorghum leaves ranged from 0.78 to 5.08 mg g^{-1} of fresh tissue and considering this amino acid, all genotypes were affected by salt stress. In *Brassica juncea* grown under salt stress conditions, proline content increased more than 19-fold compared to control plants (Madan et al. 1994). When the var. Jambo was grown in 200 mM NaCl, its proline content increased to 5.08 mg g^{-1}, while proline content in Kimia and Payam were 3.58 and 3.02 mg g^{-1}, respectively (Fig. 4). This change in proline content in var. Jambo could be correlated with its capacity to tolerate and adapt to salinity condition. Our findings corroborated the results presented in previous reports demonstrating that total free amino acids in the leaves are higher in salt tolerant than in salt sensitive lines of alfalfa (Petrusa and Winicov 1997) sunflower (Ashraf and Tufail 1995), safflower (Ashraf and Fatima 1995), *Eruca sativa* (Ashraf 1994), and *Lens culinaris* (Hurkman et al. 1991). In contrast, some reports showed a negative correlation between proline accumulation and salt tolerance (Aziz et al. 1998; Parida et al. 2004). Alarcon et al. (1994) found low contribution of this amino acid to the increase of cell osmotic potential of tomato plants under salt stress condition. Therefore, some researchers assumed that the increase in proline content is rather a salt stress effect than a cause of tolerance (Widholm 1988; Ashraf 1989). According to Chen et al. (2005) and Cuin and Shabala (2007a), by decreasing the extent of the NaCl-induced K^+ efflux, proline could substantially mitigate the effects of salt-stress on optimal K^+/Na^+ homeostasis in the cell cytosol and, ultimately, enhance plant adaptation to salinity. They reported that increased pools of amino acids may eventually contribute to cell osmotic adjustment via regulating the cellular content of inorganic solutes, specifically that of K^+, which would contribute to osmotolerance. This indicates that the common assumption that increases in free amino acid under abiotic stress is merely a symptom of damage may need

reconsidering. Linear regression revealed positive relationships between proline content and fresh weight of sorghum plants under salinity. Therefore, we suggest that proline accumulation is not merely a symptom of salt-stress injury in sorghum, but rather it can be used as a criterion for salt tolerance. However, proline accumulation cannot be used as an exclusive criterion for salt tolerance, as it also accumulates under other stresses such as high temperature, drought and starvation (Hong et al. 2000). Proline content in sorghum leaves harvested at 30 days after salt treatment was significantly higher than in leaves sampled earlier (Table 3). This is in agreement with the observation of Colmer et al. (1995) that solute accumulation in leaf blades is highly dependent on the plant and leaf age and is higher in older leaves than in younger ones.

In the present study, a significant increase in the activity of peroxidase was recorded in all varieties under NaCl stress conditions, and roots had higher activity than leaves (Fig. 5). Jambo had higher peroxidase activity in both roots and leaves at all NaCl concentration than the other two varieties (Fig. 5). The qualitative and quantitative changes in the activity of peroxidase activity isolated from plants subjected to salinity stress were reported to play a key role in salt tolerance (Heath and Packer 1968; Sreenivasulu et al. 1999). The greater activity of peroxidase in salt-adapted cells may reflect the changed mechanical properties of the cell wall (Heath and Packer 1968). There was also a significant relationship between peroxidase activity and fresh weight. The salt tolerance of Jambo, in term of fresh weight compared to other genotypes, is hypothesized to be the result of better protection from ROS.

The peroxidase system of higher plants exists in multiple isoforms that are developmentally regulated and highly reactive in response to exogenous stimuli (Gaspar et al. 1982). In the present study, NaCl stress caused an induction of isoperoxidases, namely A1, A2, and A3, with different Rf values (Fig. 6). Two bands, A1 and A2, were displayed with different densities and intensities in salt treated plants of all varieties, whereas A3 was specifically found only in the var. Jambo. Similar increases in two isoperoxidases were reported in tobacco associated with resistance to blue mold and were assigned a role in catalyzing cross linking of the cell wall extensions (Ye et al. 1990). As well as POD activity, the amounts of POD isoforms were increased in all varieties by salinity. The highest increase was detected in the salt tolerant var. Jambo (Fig. 6). These peroxidases might be involved in maintenance of cell membrane integrity and regulation of seedling growth under salt stress conditions as demonstrated earlier in some other plant species (Gaspar et al. 1991). This can be further substantiated by the occurrence of a specific isoperoxidase (A3) only in the var. Jambo subjected to 150 and 200 mM NaCl stress (Fig. 6), which could be related to salt adaptation of this variety. Our results are in agreement with the finding of Sreenivasulu et al. (1999) who reported that high peroxidase activity and additional isoperoxidase isoforms were found in a tolerant cultivar compared to a salt susceptible cultivar of *Setaria italica*.

The isoform A3 of var. Jambo belongs to the category of enzymes detected in the leaves of the tolerant cultivar of *Haliminone portulacoides* (Kalir et al. 1984), *Setaria italica* (Sreenivasulu et al. 1999) and *Cucums sativus* (El-Baz et al. 2003) subjected to salinity stress. The present data reveal that the relatively tolerant nature of Jambo variety may be related to the induction of the specific peroxidase isozyme A3.

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