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ALLEVIATION OF BORON-SALT TOXICITY BY CALCIUM IN WHEAT THROUGH ASSOCIATED CHANGES IN ANTI-OXIDANT DEFENSE SYSTEM

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SUMMARY

Two cultivars of wheat (salt tolerant KRL 1-4 and salt sensitive HD 2329) were exposed to salinity levels of 0, 40, 120 mM of NaCl and 0, 0.3, 0.9 mM of Boron (B) and two levels of Ca (0 and 20 mM as $CaCl_2$). Various growth parameters such as plant height, tiller number, leaf number, leaf area, root volume and total dry weight of plant decreased with increase in salt and B concentrations. It was found that ascorbic acid content and the activity of ascorbate peroxidase, catalase, glutathione reductase, peroxidase and superoxide dismutase either remained constant or increased slightly up to 40 mM NaCl (S_{40}) and 0.3 mM boron ($B_{0.3}$), but further increase in salt and B caused a significant decrease in the enzymatic activity. The decline in enzyme activity was, in general, more in salt sensitive than in salt tolerant cultivar. Application of Ca caused a partial reversal of the decline in the activity of above mentioned enzymes. The efficacy of alleviation by Ca was also more in salt sensitive cultivar (HD 2329) than in salt tolerant cultivar (KRL 1-4).

***Key words*: Anti-oxidant enzymes, boron, Ca, morpho-physiology, salinity, toxicity.**

INTRODUCTION

Soil salinity affects various plant physiological processes depending on salt type and concentrations, plant genotype, growth stage and environmental conditions (Ahmed and Jhon 2005). The alteration in plant growth in saline environment may be due to either unspecific osmotic effects and/or specific ion toxicity. These primary effects lead to secondary stresses such as oxidative damage (Zhu 2001, Benlloch-Gonzales *et al*. 2005). Although B is an essential micronutrient, it can be phytotoxic to plants if present in high concentration. B toxicity problem has been recognized in low rainfall areas of Southern Australia, West Asia and North Africa (Cartwright *et al*. 1984, 1986) and semiarid tracts of Northern India (Manchanda 1976). As a matter of fact under arid conditions salinity and boron often co-exist (Tanji 1990).

A common feature of different stress factors is their potential to increase the reactive oxygen species production in plant tissues (Hernandez *et al.* 2000, Apel and Hirt 2004). When the reactive oxygen species production increased damage may occur. To prevent this damage, plants possess an antioxidant system composed of low molecular weight antioxidants (glutathione, ascorbate) and protective enzymes, *i.e.* superoxide dismutase, ascorbate peroxidase, monodehydro ascorbate reductase, glutathione reductase etc. (Morita *et al.* 1999, Sgherri *et al.* 2000). Accumulation of H_2O_2 during stress sometimes induces the synthesis of antioxidants protective enzymes like cytosolic ascorbate peroxidase in rice, thus participating in oxidative stress signalling (Morita *et al.* 1999). Plants subjected to salt stress display complex molecular responses including the production of stress proteins and compatible osmolytes. Many stress proteins with unknown function probably detoxify the

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plants by scavenging reactive oxygen species (Zhu 2001).

Furthermore, Ca is known to alleviate the toxic effects of salinity and boron. The alleviation of salinity-boron toxicity effects by supplemental Ca has been reported in many crops like tomato (Lopez and Satti 1996), kiwifruit (Sotiropoulos *et al*. 1999), rice (Alam *et al*. 2002) and guava seedlings (Elbert *et al*. 2002). The present investigations were undertaken with the contention that when B was superimposed along with salinity, it accentuated the deleterious effects manifolds. Therefore, it was considered worthwhile to study the morpho-physiological and associated antioxidant responses to delimit such changes under B-salt toxicity effects and its alleviation by Ca application.

MATERIALS AND METHODS

Seeds of two cultivars of wheat, a salt tolerant (KRL 1-4) and salt sensitive (HD 2329) were sown in polythene sealed earthen pots filled with 5 kg of dune sand having least amount of negligible components of nutrients, salinity or boron (B). The sandy soil was artificially salinized by different levels of salt (0, 40, 120 mM NaCl) designated as S_0, S_{40}, and $S_{120,}$ respectively. Further each of these salinities and their respective levels were superimposed with three levels of B (0, 0.3 and 0.9 mM) designated as B_0, $B_{0.3}$, $B_{0.9}$ and two levels of Ca (0 and 20 mM as $CaCl_2$) denoted as Ca_0 and $Ca_{20,}$ respectively. Ten seeds of each cultivar, i.e. KRL 1-4 and HD 2329 were sown in each pot. Four plants of uniform size were retained in each pot after 10 days of emergence. The sampling of plants for morphological observations and biochemical analyses were done at 60 days after sowing (DAS) at the vegetative stage and 90 DAS at flowering stage. However, as the trends of results were similar at both the stages of growth, the data at 60 DAS are presented here.

Leaf area was measured by leaf area meter (Li-Cor Lambda Instrument Corporation, USA). Root volume was measured by water displacement method using a graduated cylinder and expressed as ml of water displaced by roots per plant. Dry weight of the plants was measured by drying the plants to a constant weight in a forced air circulation oven at 70+1°C for 96 h.

Ascorbic acid content was estimated by the method of Schopfer (1966). For the extraction of various enzymes, 500 mg leaves were crushed in 5 ml of 0.1 M potassium phosphate buffer (pH 7.0) and centrifuged at 7000 x g at 4°C for 20 minutes. The supernatant was then used for the estimation of different enzymes like catalase activity by the UV method of Aebi (1983). Similarly, peroxidase activity was estimated by the method of Shannon *et al*. (1996), glutathione reductase by the method of Goldberg and Spooner (1983) and ascorbate peroxidase by the method of Nakano and Asada (1981). The superoxide dismutase activity was determined by the method of Giannopolitis and Ries (1977). The enzyme activity was expressed as units per mg protein per minute. The enzymatic protein was measured by Folin-cio-calteau reagent (Lowry *et al*. 1951).

RESULTS AND DISCUSSION

The results on plant height, tiller number, leaf number, leaf area, and root volume showed that at vegetative stage two cultivars (KRL 1-4 and HD 2329) of wheat were adversely affected by salt, B as well as with salt + B interaction (Table 1). Interestingly, the toxicity of salinity increased in the presence of B. These findings were in consonance with the various workers who also observed the reduced plant height, stem diameter, number of branches and number of leaves particularly under salinity (Gorham and Bridge 1995). Mor (1991) found 21% decrease in root growth of pea at 6 dSm^{-1} ECe as compared to controls. Further, addition of B in the soil up to 2 and 4 mg kg^{-1} levels decreased it by 38 and 52 %. It has been reported in pea (Bagheri *et al*. 1993) and broad bean (Doila *et al*. 1998) that plant height and number of nodes were reduced and the severity of symptom expression increased at higher B treatments correlated to shoot boron level.

Another significant observation was that application of Ca partly reversed the deleterious effects of salinity and B by causing enhancement in the accumulation of total plant dry weight and increase in plant height, leaf number, leaf area, root volume and total dry weight in both the wheat cultivars particularly at higher salt and B levels. The cultivar KRL 1-4 responded better than HD 2329 particularly at higher salt and B levels.

Table 1. NaCl (S) and Boron (B) toxicity effects and its alleviation by calcium (Ca) on plant height (cm), tiller number, leaf number, leaf area (cm^2), root volume and total plant dry weight (gm/plant) at vegetative stage, i.e. 60 days after sowing (DAS) in two cultivars KRL 1-4 (Salt tolerant) and HD 2329 (Salt sensitive) of wheat.

Treatments		Plant height				Tiller number				Leaf number				Leaf area				Root volume				Total plant dry weight			
Salt (S) levels (mM)	B levels (mM)	Ca_0		Ca_{20}		Ca_0		Ca_{20}		Ca_0		Ca_{20}		Ca_0		Ca_{20}		Ca_0		Ca_{20}		Ca_0		Ca_{20}	
		KRL 1-4	HD 2329	KRL 1-4	HD 2329	KRL 1-4	HD 2329	KRL 1-4	HD 2329	KRL 1-4	HD 2329	KRL 1-4	HD 2329	KRL 1-4	HD 2329	KRL 1-4	HD 2329	KRL 1-4	HD 2329	KRL 1-4	HD 2329	KRL 1-4	HD 2329	KRL 1-4	HD 2329
S_0	B_0	56.80	61.05	54.05	62.15	3.65	3.90	2.65	2.90	7.00	7.80	5.90	7.15	210.00	224.20	200.00	229.54	7.20	7.73	7.50	8.13	3.50	3.70	3.12	3.50
	$B_{0.3}$	56.80	55.55	53.30	57.15	3.00	3.15	2.65	2.75	6.65	7.15	6.25	7.00	180.00	219.40	170.00	211.47	7.40	7.98	7.40	7.48	3.24	3.28	3.03	3.16
	$B_{0.9}$	48.70	39.75	47.80	37.10	2.80	1.90	2.55	1.25	6.00	6.65	5.90	6.40	160.00	182.95	140.00	197.71	6.30	7.23	6.10	6.98	2.41	1.84	2.27	1.71
S_{40}	B_0	53.25	58.80	49.20	49.30	3.20	3.75	2.40	3.15	6.25	7.40	5.65	6.50	148.16	172.63	142.18	164.82	6.20	6.73	6.10	6.98	3.55	3.36	3.25	2.90
	$B_{0.3}$	49.00	55.15	48.80	46.70	2.90	3.25	2.50	1.55	6.15	6.90	5.90	6.15	130.79	148.75	133.57	142.16	5.90	4.99	5.70	5.24	2.82	2.48	2.32	2.00
	$B_{0.9}$	47.40	34.05	39.70	33.85	2.00	1.15	1.85	1.00	6.15	6.40	5.40	5.65	109.86	116.95	105.88	114.91	4.99	4.24	4.74	4.49	1.51	1.10	1.47	1.11
S_{120}	B_0	29.50	41.65	22.00	28.05	1.00	1.00	1.00	1.00	5.00	6.25	5.00	5.65	68.00	38.97	65.00	40.90	2.34	2.49	2.24	2.00	1.03	0.42	0.92	0.39
	$B_{0.3}$	31.95	32.60	35.35	30.90	1.00	1.00	1.00	1.00	5.25	6.25	5.65	6.15	50.00	32.07	55.00	33.93	2.00	1.25	1.88	1.50	0.86	0.26	0.80	0.29
	$B_{0.9}$	29.20	25.35	30.75	26.15	1.00	1.00	1.00	1.15	5.15	4.90	5.10	5.15	30.00	17.10	35.00	19.80	1.50	0.75	2.12	1.50	0.40	0.17	0.47	0.27
CD at 5% level of significance: Cultivar (A), Salinity level (B), Boron level (C), Calcium level (D)		A = NS B = 0.94 C = 0.94 D = 0.77				A = NS B = 0.16 C = 0.16 D = 0.13				A = 0.15 B = 0.19 C = 0.19 D = 0.15				A = 0.22 B = 0.27 C = 0.27 D = 0.22				A = 0.003 B = 0.010 C = 0.010 D = NS				A = 0.10 B = 0.12 C = 0.12 D = 0.02			

Therefore, the foregoing results clearly showed that Ca partially offsets the deleterious effects on growth when depressed to a large extent. These results are in accord with Abd-Ella and Shalaby (1993) who have clearly depicted decrease in fruit growth, fruit number and seed yield per plant in blueberry with increasing salinity and increased with $CaCl_2$, primarily due to increased leaf area and number. Likewise alleviatory effects of Ca under salinity stress have been reported by different workers in tomato (Lopez and Satti 1996), kiwifruit (Sotiropoulos *et al*. 1999), rice (Alam *et al*. 2002) and guava seedlings (Elbert *et al.* 2002).

Perturbations in the reactive oxygen species scavenging system in response to salt-B stress showed that ascorbic acid content in leaves at vegetative stage (60 DAS) was rather enhanced or remain unchanged up to S_{40} salt and $B_{0.3}$ concentration, but the activity decreased drastically at S_{120} concentration and $B_{0.9}$ concentration (Table 2). The maximum reduction in activity occurred when both S_{120} and $B_{0.9}$ were used in conjunction with each other. Another important observation was a marginal increase in ascorbic acid content in tolerant cultivars KRL 1-4 with Ca application, but it remained statistically insignificant. Reduction in total ascorbate was also observed in salt tolerant and salt sensitive cultivars of cotton (Gossette *et al*. 1996), *Pisum sativum* L. (Hernandez *et al.* 2000) and Foxtail millet (Sreenivasulu *et al*. 2000) in response to long term salt stress but the decline was more pronounced in sensitive variety. The increase in reactive oxygen species production was observed in roots and shoots of lentil (Bandeoglu *et al*. 2004), which may result from stomatal closure causing a decrease in CO_2 concentration in the chloroplast. This in turn might cause a decrease in concentration of $NADP^+$ available to accept electrons from PSI/II and thus initiate oxygen reduction in concomitant with generation of active oxygen species (Foyer and Noctor 2005).

The activity of all enzymes of antioxidant defense system, i.e. catalase, ascorbate peroxidase, peroxidase, superoxide dismutase, and glutathione reductase decreased with an increase in salt and B concentrations and maximum inhibition occurred at $S_{120} + B_{0.9}$ in leaves at vegetative stage in both KRL 1-4 and HD 2329 cultivars, respectively. Ca application partially alleviated the deleterious effects of salt + B treatments as was apparent from a partial reversal of decrease in enzyme activity occurring in salt + B treatment on Ca application. Many research workers like Swamy and Reddy (1991) conform to these findings who observed more lipid peroxidation on increasing levels of NaCl, while SOD and catalase activities decreased. A reverse trend as observed with Ca by modulating lipid peroxidation through maintaining higher levels of enzymes such as SOD and catalase as was observed in the present investigation with Ca application especially at higher salt + B levels (Table 2).

The specific activity of ascorbate peroxidase (APX) decreased gradually with increase in salt and B levels in both the wheat cultivars. However, KRL 1-4 maintained higher level of this enzyme in comparison to HD 2329 throughout except at higher levels of salt where reverse was true. Ca application enhanced the activity of ascorbate peroxidase either in non-saline or at highest salt + B levels. The decreased enzyme activity may be due to imbalance between the production of active oxygen species and the quenching ability of antioxidants that get upset and oxidative damage may occur. Many scientists while working on the effects of salt stress on various plants support these findings (Morita *et al*. 1999, Kukreja *et al*. 2005). Schwanz and Polle (2001) found that drought avoiding species of pine registered lower activity of APX than tolerant species of oak as has been observed in our studies where invariably high APX activity was observed in salt tolerant KRL 1-4 than salt sensitive HD 2329.

Another significant observation was the decrease in peroxidase (POX) activity with increase in salt and B and their combinations, but Ca application partially decreased POX activity especially at lower concentrations of salt, B and salt + B combinations (Table 2). These results are in consonance with the findings of Santos *et al*. (2001) who have reported a decrease in peroxidase activity in sunflower plants under KCl stress. Even in fig cell lines, salt treatment decreased the activities of SOD, POX, CAT and APX enzymes. SOD activity was negatively correlated with salt tolerance. Thus, enzyme eliminating H_2O_2 produced by SOD might be more important in salt tolerance of fig cells (Wang *et al.* 1999). In cotton salt stress decreased

Table 2. NaCl (S) and Boron (B) toxicity effects and its alleviation by calcium (Ca) on ascorbic acid content, ascorbate peroxidase (APX), peroxidase (POX), catalase (CAT), superoxide dismutase (SOD) and glutathione reductase (GR), activities (units/mg protein/minute) at vegetative stage (60 DAS) in two cultivars KRL 1-4 (salt tolerant) and HD 2329 (Salt sensitive) of wheat.

Treatments		Ascorbic acid				Ascorbate peroxidase				Peroxidase				Catalase				Superoxide dismutase				Glutathione reductase			
Salt (S) levels (mM)	B levels (mM)	Ca_0		Ca_{20}		Ca_0		Ca_{20}		Ca_0		Ca_{20}		Ca_0		Ca_{20}		Ca_0		Ca_{20}		Ca_0		Ca_{20}	
		KRL 1-4	HD 2329	KRL 1-4	HD 2329	KRL 1-4	HD 2329	KRL 1-4	HD 2329	KRL 1-4	HD 2329	KRL 1-4	HD 2329	KRL 1-4	HD 2329	KRL 1-4	HD 2329	KRL 1-4	HD 2329	KRL 1-4	HD 2329	KRL 1-4	HD 2329	KRL 1-4	HD 2329
S_0	B_0	0.60	0.31	0.59	0.32	5.43	2.99	5.40	3.17	82.13	84.70	81.02	81.77	52.68	29.03	50.78	31.33	2.98	3.21	2.83	3.04	6.59	6.71	6.62	6.82
	$B_{0.3}$	0.53	0.28	0.54	0.30	5.34	2.40	5.32	2.92	78.20	80.66	77.31	78.58	47.11	24.50	44.33	25.89	2.45	2.90	2.36	2.80	6.58	6.75	6.32	6.37
	$B_{0.9}$	0.47	0.66	0.48	0.64	5.14	5.71	5.07	5.60	68.13	68.45	66.28	65.89	41.10	57.67	42.81	55.77	2.16	2.80	2.05	2.64	6.02	6.01	5.98	5.93
S_{40}	B_0	0.62	0.64	0.58	0.62	5.72	5.45	5.68	5.50	89.19	82.12	86.68	80.73	51.84	52.10	53.04	49.33	3.08	2.50	3.00	2.35	6.40	6.53	6.28	6.34
	$B_{0.3}$	0.57	0.58	0.58	0.58	5.95	5.10	5.77	5.04	82.03	74.83	80.14	74.21	48.59	45.56	49.07	47.27	2.28	2.30	2.19	2.20	5.96	6.16	5.69	5.71
	$B_{0.9}$	0.55	0.60	0.56	0.59	4.99	5.40	4.88	5.70	75.56	67.08	76.61	69.12	42.08	54.68	42.54	55.87	1.90	1.95	1.81	1.85	4.49	4.32	4.29	4.07
S_{120}	B_0	0.36	0.57	0.38	0.58	3.79	5.34	3.51	5.01	65.34	56.48	64.03	54.11	32.89	51.43	33.76	51.90	1.34	1.25	1.42	1.32	3.12	2.91	3.35	3.33
	$B_{0.3}$	0.31	0.48	0.32	0.47	2.99	4.41	3.17	4.19	55.36	45.20	55.94	44.49	29.03	43.23	31.33	43.69	1.10	1.00	1.14	1.05	2.74	2.43	2.76	2.58
	$B_{0.9}$	0.28	0.34	0.30	0.34	2.40	2.54	2.92	2.69	37.89	28.00	39.27	35.88	24.50	32.77	25.89	33.63	0.70	0.50	0.80	0.61	2.20	1.58	2.29	2.10
CD at 5% level of significance: Cultivar (A), Salinity level (B), Boron level (C), Calcium level (D)		A = NS B = 0.02 C = 0.02 D = NS				A = 0.08 B = 0.10 C = 0.10 D = 0.06				A = 0.73 B = 0.90 C = 0.90 D = 0.73				A = 1.11 B = 1.36 C = 1.36 D = 0.01				A = 0.02 B = 0.02 C = 0.02 D = 0.02				A = 0.10 B = 0.12 C = 0.12 D = 0.02			

the activity of enzymes SOD, POX and GR (Meloni *et al*. 2003).

Catalase activity was invariably more in salt tolerant KRL 1-4 than HD 2329 especially under non- saline conditions, but converse was true at higher salinities. Boron concentrations decreased the catalase activity in both the cultivars of wheat, but in case of HD 2329 it enhanced the catalase activity especially at higher salinities in both the cultivars (Table 2). These results reportedly corroborate with Corpas *et al*. (1993) who have reported a significant decrease in the catalase activity during salt stress in both salt tolerant and salt sensitive cultivars of pea leaves. Under salinity, conformational changes occur in protein molecule. The reduction in catalase activity may be due to the disruption of the quaternary structure and separation of subunits of the enzyme under salt stress. Likewise, inhibition of catalase activity was also noticed in rice (Lee *et al*. 2001), in sunflower plants (Santos *et al*. 2001), and in common bean (*Phaseolus vulgaris*) nodules (Jebara *et al*. 2005) under salinity.

Antioxidant and free radical scavenging enzyme superoxide dismutase (SOD) activity enhanced in salt tolerant cultivar KRL 1-4 than HD 2329 especially at S_{40} level of stress and Ca application also maintained its higher levels (Table 2). Further increase in salt and B levels caused a decline in SOD activity. It was interesting to note that the overall SOD activity in salt tolerant cv. KRL 1-4 was higher than the sensitive cv. HD 2329 at the higher salt and B levels. Above research findings are in close proximity with Dionisio-sese and Tobita (1998) who also exhibited an elevated level of SOD activity in salt tolerant genotype of rice *Pokkali*, which exhibited higher SOD activity as compared to salt sensitive rice genotypes *Hitomebore* and *IR 28*. In sunflower plants (*Helianthus annuus* cv. *SH 222*) senescence induced by KCl stress decreases the SOD activity which is correlated with increase in lipid peroxidation and membrane permeability (Santos *et al.* 2001). Decreased SOD activity resulted in higher accumulation of H_2O_2, thereby, affecting all subsequent steps of ascorbate-glutathione cycle (Polle 2001).

Glutathione reductase (GR) activity was also decreased with increase in salt, B and salt + B levels. Ca application did not affect the specific activity of this enzyme except in HD 2329 where it was slightly enhanced especially at higher salt and B levels (Table 2). These results are consistent with the observations by Comba *et al*. (1998) who also observed decrease in GR activity in soybean root nodules when exposed to NaCl. On the contrary, higher glutathione reductase activity during salt stress in tolerant pea plants and diminished activity of the enzyme in the sensitive plants was reported by Hernandez *et al*. (2000).

It seemed that the anti-oxidative defense system was more efficient in KRL 1-4 in comparison to HD 2329. Comparison of ascorbic acid levels and activities of enzymes SOD, CAT, POX, APX and GR activity was in general slightly increased at low level of salt and B, whereas, high level of salt and B decreased the enzyme activity drastically. Ca application partially offset the deleterious effects of salt, B and salt + B treatments. It is opined that Ca application acts as a double edged weapon which is capable of reducing the deleterious effects of both salinity as well as B.

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