Indian Journal of Agricultural Sciences **85** (12): 1626–32, December 2015/Article
https://doi.org/10.56093/ijas.v85i12.54331

Combined effect of boron and salt on polypeptide resolutions in wheat (*Triticum aestivum*) varieties differing in their tolerance

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Received: 8 May 2014; Accepted: 22 June 2015

ABSTRACT

Salinity aggravates toxicity symptoms of boron in wheat. Four wheat *(Triticum aestivum* L.) varieties differing in tolerance to these stresses were subjected to five stress treatments [control (2.5 ppm B), 50 ppm B + 6 dS/m, 100 ppm B + 6 dS/m, 50 ppm B + 10 dS/m and 100 ppm B + 10 dS/m]. Higher reductions for root length, fresh and dry weight were observed in Schomburgk and HD 2009 varieties at 100 ppm B + 10 dS/m NaCl in comparison to KRL 35 and BT-Schomburgk. Results indicated that combined boron and salt stresses significantly increased soluble B and proline concentrations in the roots. At the highest level of stress (100 ppm B + 10 dS/m), maximum proline accumulation was evident in HD 2009 (18.6 mg/g) and minimum in KRL 35 (13.5 mg/g). Protein profile expressions of boron tolerant and intolerant varieties of wheat showed de novo synthesis of two specific polypeptides (35.73 and 31.10 KDa) in boron tolerant variety and one (16.98 kDa) in boron intolerant variety. Likewise, KRL 35 (salt tolerant) showed 4 specific polypeptides of 89.13, 58.4, 46.21 and 31.10 kDa, whereas three specific polypeptides (24.05, 19.13 and 17.52 kDa) appeared in the salt intolerant variety (HD 2009). Appearance of 5 common polypeptides bands of MW 89.13, 53.4, 46.21. 31.10 and 25.12 kDa in both the tolerant varieties, i.e. BT-Schomburgk (boron tolerant) and KRL 35 (salt tolerant) is of special interest and could have possible use as markers for tolerance. The synthesis of common polypeptide of MW 25.12 kDa was observed in all the four varieties with increase in stress treatments.

Key words: Boron, Intolerant variety, Polypeptides, Protein profile, Salinity, Tolerant variety

Boron is toxic to many plant species at levels slightly above those required for normal growth and development. It is found to be in toxic concentrations in soils and ground waters in arid and semi-arid environment throughout the world. Saline irrigation water often contains high boron concentrations, therefore, contributes in accumulation of excessive levels of B in the soil (Nable *et al.* 1997).

B is an important constituent of ground waters of many areas in India, Australia, USA and other countries. Ground waters of Agra (Uttar Pradesh), Gurgaon and Hisar (Haryana), Bhatinda, Sangrur and Amritsar (Punjab) have high B content (up to 7.3 ppm) (ACIAR Project CS1/1996/025). Ground waters of the coastal regions may also have high B content. Kanzaria and Patel (1985) reported that 11% of waters in north Gujarat, Kutch, Kaira, Vadodara and Bharauch had B concentration beyond the tolerance limit of most crop plants. B concentration in waters has also been reported to have significant positive correlation with salinity and sodicity (Minhas and Samra 2003). Yaduvanshi *et al.* (2012, Personnel communication) also reported that 85% of irrigation water samples over 100 locations in Haryana had B concentrations above toxic threshold for wheat (*Triticun aestivum* L.) production. Interactions between salinity and B toxicity are very complex and mechanisms of boron uptake under saline conditions and the way various metabolic and growth processes are affected are not well understood.

Considering the importance of the mechanism of B toxicity in wheat particularly under saline conditions, the present study on the qualitative resolution in protein profiles and boron uptake by roots of four wheat varieties differing in their tolerance was undertaken to delimit the combined effect of boron and salinity.

MATERIALS AND METHODS

Four varieties of wheat, i.e. BT-Schomburgk (boron tolerant), Schomburgk (boron intolerant), KRL 35 (salt tolerant), and HD 2009 (salt intolerant) were evaluated for interactive responses to boron (B) and sodium chloride (NaCl) stresses in relation to germination. Seeds of each variety were surface sterilized using 0.1% bavistin and were pre-germinated in petri-dishes at 4°C for 48 hr and then transferred to the bench at room temperature (15-20 °C) for 24 hours. These pre-germinated seeds were then placed in pots (capacity 2.0 L) covered with thick black sheet (to

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prevent exposure of sunlight). After pre-germination, the following treatments were established: control (2.5 ppm B, no salt), low boron and low salt (50 ppm B and 6 dS/m), high boron and low salt (100 ppm B and 6 dS/m), low boron and high salt (50 ppm B and 10 dS/m), high boron and high salt (100 ppm B and 10 dS/m). In order to avoid osmotic shock, NaCl was applied gradually in four equal doses over a period of four days. Control treatment (no salt and 2.5 ppm boron) was kept for each variety. The seedlings were grown for a period of 20 days. The nutrient solution was changed every five days. The pH of nutrient solution was checked and maintained around 7.0 (1N NaOH/1N HCl) daily. Using aquarium pumps and air stones, aeration of pots (to prevent stagnation) was done. Experiment was laid in a complete randomized design with five replicates using OPSTAT software (CCS HAU, Hisar). Plants were harvested after 20 days of treatments.

Freshly harvested roots were oven-dried at 70ºC for 48 hr in order to determine the dry weight (DW) and analyzed for proline content (Bates *et al*. 1973) and boron uptake by roots. For boron, 1.0 g samples were boiled with double distilled water contained 10% glacial acetic acid and kept overnight at 70ºC and the final volume was made to 10 ml. Boron concentration was measured by Atomic Absorption Spectrophotometer (Zeenit 700P, Analytik Jena, Germany).

The 25 µl of crude protein extract, containing 50 µg of protein extract was transferred to an equal volume of Laemmli's 2X sample buffer (0.5 M Tris-HCl, pH 6.8) containing 20% glycerol, 4% SDS, 0.5% bromophenol blue (w/v) and 10% β-mercaptoethanol and heated at 100°C for 3 minutes and cooled. Electrophoresis was carried out by the method of Laemmli (1970). The cooled samples were then loaded on SDS-discontinuous gel system with 0.1 mm thick stacking gel of 4% polyacrylamide in Tris-HCl buffer (pH 6.8) and a resolving gel of 10% polyacrylamide in Tris-HCl buffer (pH 8.8). The gels were run at 15mA in the stacking gel and 25mA in the resolving gel. After electrophoresis, gels were fixed and stained with 0.25% (w/v) Coomassie Brilliant Blue R-250 in 40% (v/v) methanol with 7% glacial acetic acid (v/v) and then destained in 10% methanol (v/v) with 7.5% glacial acetic acid (v/v). After destaining, the gels were stored in 7% glacial acetic acid (v/v).

RESULTS AND DISCUSSION

Growth parameters

Boron toxicity symptoms appeared at 50 ppm B and increased in severity at 100 ppm. Toxicity symptoms first appeared on the tips and margins of leaves. Significant interactive effects of B and salinity were observed on plant growth parameters. Our earlier studies have shown that higher reductions were observed for root length at 100 ppm B + 10 dS/m in Schomburgk and HD 2009 varieties (B intolerant and salt intolerant, respectively) in comparison to KRL 35 (Salt tolerant) and BT-Schomburgk (Boron tolerant). Root biomass showed a linear decrease in response to the increase in B and NaCl concentrations. KRL 35 showed minimum reduction in fresh and dry weights of root at high B and NaCl levels, whereas maximum per cent reductions were observed in Schomburgk and HD 2009 (Table 1).

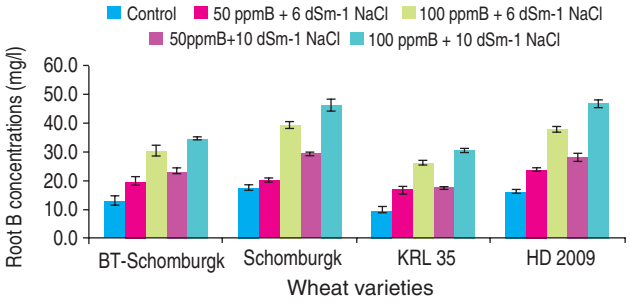

Fig 1 Combined effect of B and Salinity on root boron concentration (mg/l) in wheat varieties.

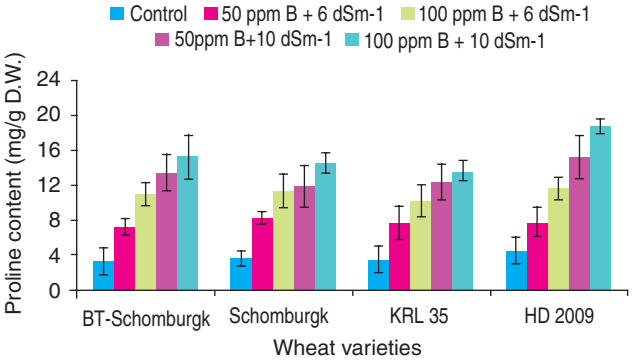

Fig 2 Combined effect of B and salinity on proline content (mg/g WD) in wheat varieties.

Boron uptake and proline accumulation

The intolerant varieties accumulated more B in the roots, with the increase in concentration of boron and salt in the nutrient medium. Maximum accumulation was found in roots of wheat variety HD 2009 while minimum in KRL 35 (Fig 1) at 100 ppm B + 10 DS/m.

The accumulation of proline reveals how much degree of stress, a genotype is able to tolerate. The proline content increased with increasing levels of boron and salt in all varieties (Fig 2). Even the lowest levels of boron and salt (50 ppm B + 6 DS/m) induced proline accumulation, the increase being least in BT-Schomburgk (7.2 mg/g) and maximum in Schomburgk (8.2 mg/g) over control. At highest level of boron and salt stress (100 ppm B + 10 DS/m), maximum proline accumulation was evident in HD 2009 (18.6 mg/g) and minimum in KRL 35 (13.5 mg/g).

Protein profiling/Polypeptide resolutions

Plants have a multitude of mechanisms which help them to survive and propagate under different stresses. Although protein biosynthesis generally declines under stress conditions, cells preferentially synthesized specific stress proteins. Results of protein profile pattern using SDS-

PAGE (10%) revealed definite quantitative and qualitative alterations in polypeptide banding pattern in all the varieties. In case of BT-Schomburgk (boron tolerant), a total number of 9 polypeptides bands ranging from 83.21 to 13.63 kDa were observed in control (Fig 3A). At 50 ppm B + 6 dS/m salinity, the number of bands increased to 12 of which three new polypeptides were synthesized *de novo,* i.e. 58.4, 46.21 and 25.12 kDa. With increase in the concentration of B in the nutrient medium, two new polypeptides, i.e. 89.13 and 31.10 kDa appeared. There was appearance of one new polypeptide bands of MW 35.73 kDa, whereas 2 polypeptides, of 42.19 and 16.16 kDa disappeared at 50 ppm B + 10 dS/m. Out of total polypeptide bands, 13 remain the same at 100 ppm B + 10 dS/m level in wheat variety BT-Schomburgk.

Differential polypeptides expression was observed in Schomburgk (B intolerant variety) as compared to BT-Schomburgk (Fig 3B). A total number of 12 polypeptides appeared in control. *De novo* synthesis of 5 new polypeptides (89.13, 58.4, 46.21, 25.12 and 16.98 kDa) was observed with the addition of 50 ppm B + 6 dS/m. Further increase in the concentration of B and salt caused disappearance of polypeptides bands, i.e. one band of 66.89 kDa molecular weight at 100 ppm B + 6 dS/m, one 42.19 kDa at 50 ppm B + 10 dS/m and four bands of 37.12, 35.73, 32.35 and 25.12 kDa at 100 ppm B + 10 dS/m respectively. Disappearance of 6 polypeptides with increasing stress conditions, suggested that polypeptide expression varied depending upon the different developmental stages and the differential gene expression of concerned structural or regulatory gene (s).

Comparison of protein profiles of boron tolerant and boron intolerant wheat varieties indicated that there was synthesis of two specific polypeptides in boron tolerant variety (35.73 and 31.10 KDa) and one in boron intolerant (16.98 kDa). Synthesis of common polypeptide of MW 89.13, 58.4, 46.21 and 25.12 kDa observed with increase in B and salt, needs special mention.

Salt tolerant variety KRL 35 showed total number of 6 polypeptides under control treatment which increased to 9 with addition of low B and low salt (50 ppm B + 6 d S/m), i.e. appearance of 3 new polypeptide bands of MW 58.4, 46.21 and 25.12 kDa (Fig 4A). Increase in boron causes appearance of two new polypeptide bands (89.13 and 31.10 kDa). There was appearance of one new polypeptide band of MW 32.35 kDa, whereas 2 polypeptides of 46.21 and 16.16 kDa disappeared with increase of salt concentration with low boron at 50 ppm B + 10 dS/m. At higher salinity level of 10 dS/m with high B, pattern of resolution remained unaffected. While in case of salt intolerant variety HD 2009, total numbers of 9 bands were present in the control treatment and were not affected at 50 ppm B + 6 dS/m. Disappearance of 2 polypeptides of 89.13 and 27.43 kDa MW and appearance of 3 new polypeptide bands of MW 25.12, 24.05 and 17.52 kDa was observed at 100 ppm B + 6 dS/m. One new polypeptide of 19.13 kDa appeared and one polypeptide of 35.73 kDa disappeared with increase in

Table 1 Combined effect of B and Salinity on fresh and dry weight of root (mg) in wheat varieties

Varieties/ Treatment	Fresh weight of root (mg)				Dry weight of root (mg)			
	Control	50 ppm B + 6 dS/m NaCl	100 ppm B + 6 dS/m NaCl	50 ppm B + 10 dS/m NaCl	100 ppm B + 10 dS/m NaCl	Control	50 ppm B + 6 dS/m NaCl	100 ppm B + 6 dS/m NaCl	50 ppm B + 10 dS/m NaCl	100 ppm B+10 dSm NaCl
BT-Schomburgk	741	260 (64.9)	85 (88.5)	118 (84.1)	71 (90.4)	58.47	19.4 (66.7)	17.0 (71.0)	15.2 (74.0)	11.1 (81.0)
Schomburgk	856	201 (76.5)	48 (94.4)	99 (88.4)	19 (97.8)	75.10	17.8 (76.3)	8.5 (88.6)	11.9 (84.2)	6.1 (92.0)
KRL 35	685	625 (8.8)	100 (85.4)	221 (67.7)	82 (88.0)	53.57	55.8 (-4.2)	14.9 (72.1)	29.4 (45.1)	11.4 (78.7)
HD 2009	695	175 (74.8)	33 (95.35)	83 (88.2)	14 (98.0)	57.47	17.3 (69.9)	8.7 (84.9)	12.8 (77.8)	3.2 (94.4)
Mean	744.25	315.42	66.62	130.17	46.75	61.15	27.59	12.28	17.32	7.97
	CD(P=0.05)	Varieties – 68.49 ± 23.92				CD (P=0.05)	Varieties – 7.75 ± 2.71			
		Treatment – 76.57 ± 26.75					Treatment – 8.67 ± 3.03			
		Varieties × Treatment – 152.14 ± 53.48					Varieties × Treatment – 17.35 ± 6.05			

Values in parenthesis are per cent reduction over control

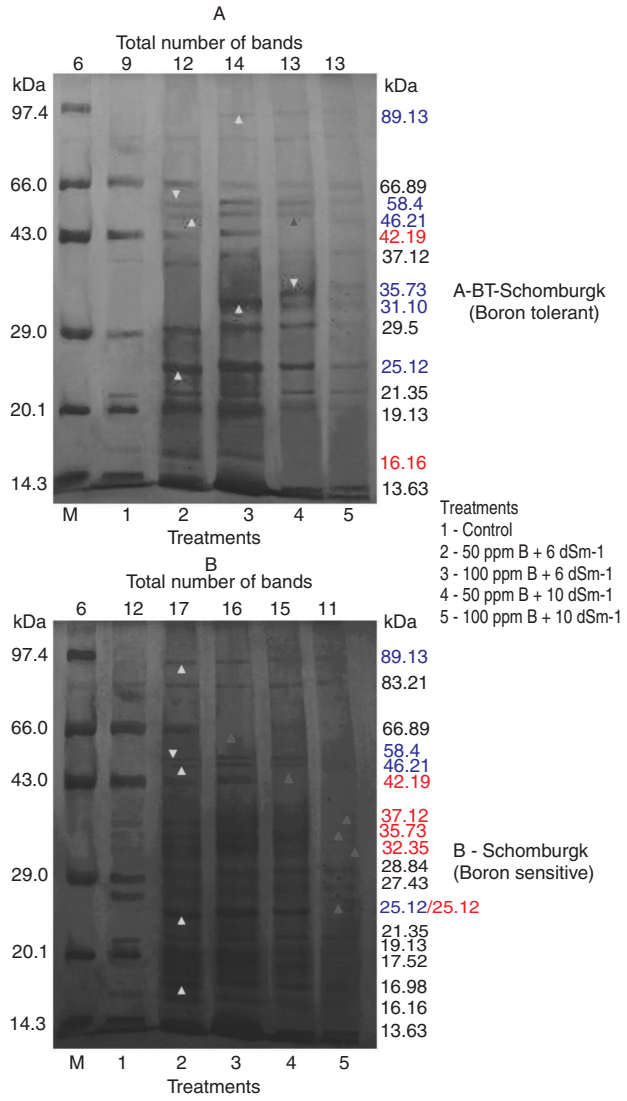

Fig 3 Combined effect of boron and salinity on protein profile expression in roots of BT-Schomburgk (A) and Schomburgk (B) through SDS-PAGE (10%M – Protein molecular weight marker).

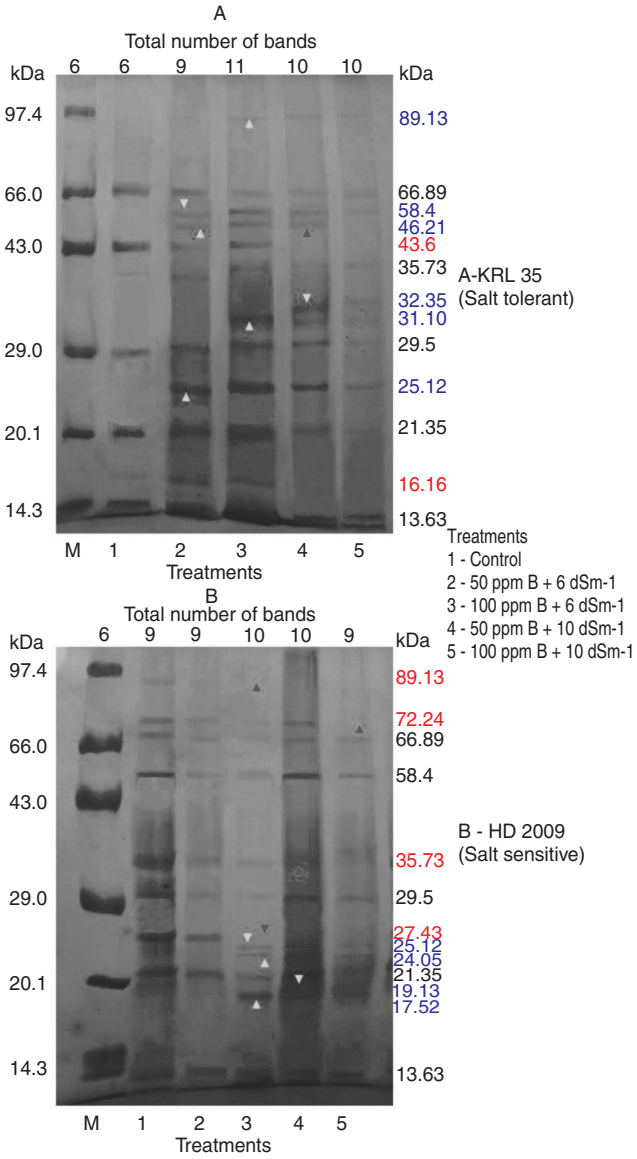

Fig 4 Combined effect of boron and salinity on protein profile expression in roots of KRL 35 (A) and HD 2009 (B) through SDS-PAGE (10 % M – Protein molecular weight marker)

salinity level (Fig 4B). With the disappearance of one polypeptide band of 72.24 kDa, total 9 polypeptide bands were found at 100 ppm B + 10 dS/m level in HD 2009 variety.

Effect of combined stresses on growth parameters

Plants are unable to express their full genetic potential when subjected to stressful environments. Various environmental stresses cause important modifications in gene expressions of plants. Such modifications might led to the accumulation or depletion of certain metabolites, alterations in the behaviors of many enzymes, overall changes in protein synthesis, and of particular interest, synthesis of new sets of proteins which are specific to the particular type of stress (Bhagwat and Apte 1989, Blinda *et al*. 1997). The toxic effects of B and salt appeared to be more detrimental in the intolerant varieties as compared to the tolerant varieties (Sharma *et al*. 2013, Personnel communication) and are in accordance with the reports of Alpaslan and Gunes (2001), Ismail (2003/04) and Hassan (2007).

Effect of combined stresses on boron uptake and proline accumulation

Plant species or genotypes which are tolerant, characterized by a low B concentration in their leaf tissues in comparison with the non-tolerant ones, probably due to a reduced uptake of B into roots and shoots both (Camacho-Cristobal *et al*. 2008). Our results are in conformity with Hu and Brown (1997), Grattan *et al*. (1997) and Wimmer *et al*. (2003) who reported that salinity together with boron toxicity increased soluble B concentrations in inter and intra-cellular compartments of basal leaf sections in wheat when compared to either stress alone. In addition

to this, the ability of tolerant genotypes to resist high boron in the growing medium was not a consequence of the ability to tolerate high B concentrations in the plant tissues and was due to the capacity of the plant to restrict B uptake by roots and transport to shoots (Nable *et al*. 1990, Paull *et al.* 1992).

Accumulation of compatible non-toxic solutes especially proline, glycine-betaine and sugars is a common observation under stress condition (Ashraf *et al*. 1994, Qasim 2003). Such an accumulation of proline allows additional water uptake from the environment to maintain turgor (Sairam *et al*. 2002, Misra and Gupta 2005). Proline has been reported to increase the stress tolerance of the plants via osmoregulation, the protection of enzymes against denaturation, and the stabilization of protein (Bohnert *et al*. 2005). With the increase in NaCl, the proline content in all the varieties increased significantly. Higher level of proline content in stem and leaf might be due to expression of genes encoding key enzymes of proline synthesis pyrroline-5-carboxylate and low activity of the oxidizing enzymes (proline dehydrogenase) which is controlled by osmotic and salinity stress (Maggio *et al*. 2002). These compounds buffer redox potential, preserve quaternary structure of proteins, highly ordered state of membranes and stabilize many functional units such as oxygen evolving PS-II complex (Chinnusamy *et al*. 2005), activity of enzymes, and the ability to detoxicate ROS (Yokoi *et al*. 2002). Storey and Wyn Jones (1975) reported that the proline concentration was 10-fold in shoots and 18-fold higher in roots of plants grown at 100 mM NaCl than in plants grown in the absence of salt. Accumulation of proline is not sufficient enough to play significant role in osmotic adjustment; it might be involved in protection of cell structure (Viegas and Silviera 1999), as high concentration of proline is not required for plasma membrane protection. So, proline accumulation may be consequence of salinity induced disturbance in protein and amino acid metabolism rather than adaptive mechanism.

Effect of combined stresses on protein profiling/ Polypeptide resolutions (SDS-PAGE)

Stresses induce quantitative and qualitative changes in protein content of the plant cells. The gene (S) responsible for certain proteins had been completely suppressed as a result of stress might be one possible explanation for disappearance (Elobeidy *et al*. 2001). Depressed protein synthesis and acceleration in its degradation in plants in response to salt stress has been reported by number of workers (Chandershekar *et al*. 1986, Lal and Bhardwaj 1987). The proteins that accumulate under stress conditions may provide a storage form of nitrogen that is re-utilized in post-stress recovery (Singh *et al*. 1987) and also play a role in osmotic adjustment. These proteins may be synthesized either *de novo* in response to stress or may be present constitutively at low concentration and increase when plants are exposed to stress conditions (Pareek *et al*. 1997). This showed that the timing of the synthesis or break down of particular polypeptides, might coincide with the protein content during stress associating their involvement in tolerance process.

Presence or absence of polypeptides could be potentially used as marker to decipher the differential behavior of varieties for boron and salinity. In conformity with our results, Rani *et al.* (2007) observed two protein bands with molecular weight of 83.6 and 28.1 kDa which were present only in salt + B treatments. It showed the expression of these bands require B, i.e. B induced these proteins. These results showed that varietal behavior to either boron (tolerant/intolerant) or salt (tolerant/intolerant) is dependent on nature, concentration and combination of stresses involved. The bands which were present in the treated seedling may be inherently associated with germination and growth processes. Their disappearance may affect the functional capabilities of seeds to perform in the stress environment or it may suggest the negative effect of stress on protein/gene synthetic machinery as advocated by Rani *et al.* (2007). Amzallag and Lerner (1994) reported 76.3 to 14.6 kDa protein bands were apparently synthesized *de novo* that presumably are critical for plant adaptation to stress conditions. Similar results were reported in tobacco, where a 26 kDa polypeptide was found due to salt stress and its possible role in osmoregulation was suggested (Singh *et al*. 1985). Bishnoi *et al*. (2006), who observed disappearance of 54.3 kDa protein in ICPL 88039 plumule, 68.4 kDa in Manak radicle and 28.1 kDa in ICPL 88039 radicle under B and salinity stress, and reported that the genotypes tolerant to salinity are tolerant to B as well. These two stresses seem to be associated with resolution of different polypeptides during the course of given stress.

Results from our other studies showed that the combined effect of B and salt on wheat were more damaging than boron alone, the B-salt interactions are additive and varieties known to be tolerant to one stress show tolerance to other stress also (Sharma *et al.* 2013; unpublished data). There are some other reports as well which indicate that genotypes tolerant to salinity are also tolerant to B (Schuman 1969, Paull *et al.* 1992). These results clearly indicated that polypeptides were either induced *de novo* or their expression level increased under stress in comparably tolerant genotypes than the sensitive one, which might play a protective role in combating higher stress. Further characterization of these proteins and the use of data for understanding the molecular basis of stress tolerance will be worthwhile.

Results of our study revealed that varieties differing in tolerance to B and salt stress showed differential polypeptide resolution behavior in response to the combined B and salt. The toxic effects of B and salt appear to be more detrimental in the intolerant varieties as compared to the tolerant varieties. Disappearance of polypeptide bands may be interpreted as the "turning off" protein synthetic genetic machinery "genes" in response to B and salt. It is more likely that the polypeptides

disappearing in response to stresses are the result of denaturation and the timing of synthesis or break down of particular polypeptides coincides with the protein content during stress associating their involvement in tolerance process. Identification of differentially regulated proteins can lead to the identification of their corresponding genes, which might be involved in the physiology of stress resistance and could be possible to combine tolerance with other desirable traits of crop so as to evolve new varieties better adapted to these abiotic stresses.

ACKNOWLEDGEMENT

The authors are thankful to the Head, Division of Crop Improvement, and Director, Central Soil Salinity Research Institute, Karnal for providing the required research facilities and necessary materials respectively.

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