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Research Article

PROTEOMICS OF BARLEY GRAINS UNDER VARYING SALINITY LEVELS

Yogesh Kumar[1], Arvinder Singh[2] and N. K. Matta[1]*

[1]*Department of Botany, Kurukshetra University, Kurukshetra-136119, India*
[2]*Department of Botany, Akal University, Talwandi Sabo, Bathinda- 151302, India*

Abstract: Alterations in electrophoretic and other protein characteristics appearing at relatively lower salinity level in barley line 'BHS 352' and at higher salinity level in line 'Karan16' reflected their sensitivity and tolerance respectively which these two lines are known for. While major polypeptides in globulin fraction exhibited enhanced accumulation up to 8 dSm^{-1} in salt sensitive line 'BHS 352', those in line 'Karan16' increased in intensity up to the higher salinity level of 12 dS m^{-1}; further increase in salinity led to a negative effect on globulin polypeptides in both the lines. The intensity of major glutelin polypeptides reduced continuously with increase in salinity level, the effect being more pronounced in the salt sensitive line. On the other hand, prolamin polypeptides of the two lines were affected differently at increasing salinity levels, salt sensitive line showing a continuous increase in B-hordein polypeptides and a decrease in C and D-hordein polypeptides. Many more new albumins polypeptides appearing in the salt sensitive line and only three new albumin polypeptides in the salt tolerant line point towards their differential metabolic requirements. Uniform and simultaneous changes in the major polypeptides of a given storage protein fraction indicate their coordinated regulation while varying effects of salinity stress on three storage protein fractions point towards the occurrence of independent regulatory pathways for each fraction.

Keywords: Albumins; globulins; glutelins; prolamins; salinity stress; barley.

Introduction

Barley (*Hordeum vulgare* L.) is one of the important cereals cultivated worldwide under varied growing conditions. With a small proportion of barley production being used for human consumption, it is mainly grown for feed and malting. Nutritionally, barley is rich in fibres, β-glucan, phenolics, antioxidants, minerals and vitamins, and its seed protein content has been reported to vary from 10 to 15% (MacGregor and Fincher, 1993). Seed proteins, in general, have been classified into four groups on the basis of their solubility differences (Osborne, 1924). The proteins soluble in water are called albumins, those soluble in salt solutions are termed as globulins, proteins extracted in aqueous alcohol are called prolamins and those soluble in dilute alkali or acids are named as glutelins. While albumins are known for their metabolic function, the remaining three are considered to perform the storage function. The seed storage proteins are named so because these are synthesized at one stage i.e. seed development and stored for utilization as a source of nitrogen and sulphur at the time of seed germination. In barley, four protein fractions are known to vary as 3-4% albumins, 10-20% globulins, 35-45% prolamins and 35-45% glutelins (Shewry *et al.*, 1978b; Linko *et al.*, 1989).

The alcohol soluble prolamins called as hordeins in barley, have been extensively characterized by different workers. These have been variously classified as B-hordeins (35-46 kDa), C-hordeins (55-70 kDa) and D-hordeins (105 kDa) (Shewry and Miflin, 1983) on the basis of their molecular weight, and as sulphur-rich (S-rich), sulphur-poor (S-poor)

Corresponding Author: **N. K. Matta**
E-mail: nk_matta@yahoo.com

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and high molecular weight (HMW) prolamins on the basis of their amino acid composition and amino acid sequence. The S-rich prolamins include B-hordeins and account for about 80% of the total barley prolamins. These have very high glutamine-proline content and relatively higher cysteine content. The S-poor prolamins include monomeric C-hordeins which lack cysteine and have very low methionine and lysine. The HMW prolamins, represented by polypeptide of *Mr* 105 kDa include D-hordeins and are rich in lysine, glutamine and proline (Shewry and Tatham, 1990). The distribution and quantity of B-hordeins have been reported to be an important factor for malting i.e. cultivars with high B-hordein content in the subaleurone region are not considered suitable for malting (Molina-Cano *et al.,* 2002). Hordeins are reported to exhibit considerable variation in their polypeptide patterns and have been recommended for varietal identification by different workers (Shewry *et al.,* 1978a; Heisel *et al.,* 1986; Radovic and Vapa, 1996). These have also been used for understanding the phylogenetic relationships of different *Hordeum* species (Moralejo *et al.,* 1994). The glutelin fraction, constituting a major proportion of barley seed proteins i.e. 35-45% of total proteins, are poor in cysteine but like prolamins have higher proportion of glutamine and proline (Brandt, 1976; Linko *et al.,* 1989).

With 831 million ha of land at the global level (Geressu and Gezaghegne, 2008) and about 6 million ha land under salt-stress in India, problem of salinization is becoming widespread due to improper agricultural practices (Bhattacharyya *et al.*, 2015). Aimed at enhancing the performance of crops through genetic and environmental/soil manipulations, constant efforts have been made by the plant scientists towards studying the effect and mechanism of action of salt stress. Various studies on the effect of salinity stress on different crops have focused on changes in physiological and biochemical characteristics and these have been mainly explained in terms of the ionic toxicity, osmotic stress, production of reactive oxygen species etc. Soils with high salt concentration are known to cause reduction in growth and yield of different crops (Murumkar and Chavan, 1986; Soliman *et al.,* 1994; Ghassemi-Golezani *et al.,* 2010; Kumar *et al.,* 2010; Baxter *et al.,* 2011). This reduction in growth and productivity is explained as resulting from decrease in the photosynthetic activity which in turn is attributed to the reduced chlorophyll content (Reddy and Vora, 1986; Jamil *et al.,* 2007). Mechanism of tolerance to the salinity stress is not clearly understood. While salt sensitive lines work through preventing the uptake of Na^+ ions and occurrence of osmoprotectants like mannitol, glycinebetaine and proline, the salt tolerant lines possess the mechanism to compartmentalize Na^+ ions in organelles like vacuoles. In addition to ion homeostasis and osmotic homeostasis, a number of enzymes such as catalse, glutathione reductase, superoxide dismutase etc. are also reported towards enabling the plant against different oxidative stresses.

As compared to other cereals, barley is more tolerant to varying conditions of abiotic stresses including salinity (Bothmer *et al.,* 1995). Salinity stress in barley is known to produce detrimental effects on the growth of seedling, leaf area, shoot length and fresh weight of root and shoot (Taghipour and Salehi, 2008). Investigation by Mckenzie *et al.,* (1983) showed that the increase in salinity beyond a threshold level lead to reduction in yield of barley grains. Studies on proteins of root and shoot tissues by Ramagopal (1987) have revealed that certain new proteins are formed under salt stress while synthesis of certain proteins was enhanced and that of other depressed. Barley embryos cultured on MS medium under increasing concentration of Nacl exhibited a reduction in protein content (Demirkiran *et al.,* 2013). Synthesis of protective proteins has been observed in roots of barley grown under the influence of salinity stress (Mostek *et al.,* 2015). Effect of salinity on seed storage proteins has been studied by different workers in major food crops such as chickpea (Murumkar and Chavan, 1986), wheat (Soliman *et al.,* 1994), soybean (Ghassemi-Golezani *et al.,* 2010), oats (Kumar *et al.,* 2010), rice (Baxter *et al.,* 2011) etc. However, no such studies on the effect of salinity on accumulation of seed storage proteins have been reported in barley. Present paper describes the results of such studies on alterations in seed proteins of barley grown under varying levels of salinity.

Materials and Methods

Growing the plants

Plants of the salt sensitive line 'BHS 352' and the salt tolerant line 'Karan 16' were grown in the net house at the botanical gardens of the university. For this purpose, seeds were sown in polythene bags filled with thoroughly washed sand in the end of

October. Plants were watered as per requirement and supplied with nutrient solution as per formulations of Machlis and Torrey (1956). Keeping in view the range of salinity tolerance of barley, four different salinity levels (mean EC) i.e. 4 dS m^{-1}, 8 dS m^{-1}, 12 dS m^{-1} and 15 dS m^{-1}, desired salinity levels were created by adding sodium chloride following the method described in U.S. Salinity Laboratory Hand Book No. 60 (Richards, 1954) to the nutrient solution. The salt solution was supplied just before the onset of flowering and desired salinity levels were maintained till maturity. The mature seeds were harvested in the first week of April.

Protein characterization

For analysis of different seed protein characteristics viz. proportion of four protein fractions, protein content, SDS-gel electrophoresis etc., mature seeds were pulverized and the seed meal was defatted by stirring it in hexane (10 ml/g seed meal) for 2h at 4°C. The contents were centrifuged in a bench centrifuge for 10 minutes and the supernatant decanted. The process was repeated and seed meal in the pellet dried under vacuum.

(a) Protein fractionation

Separation of four seed protein fractions was carried out following the methods given by Shewry *et al.*, (1978b) and Blethen *et al.*, (1990) with slight modifications. A given fraction was extracted by continuous stirring 100 mg of the seed meal in 1 ml of the solvent for 2h. The supernatant was separated and extraction repeated (for complete extraction of a fraction) to have a final volume 2 ml for each protein fraction. Albumins were extracted in distilled water at 4°C and the contents centrifuged at 23000g in a Remi centrifuge C-24 high speed centrifuge at 4°C. This was followed for extraction of globulins from the residual pellet in 1 M NaCl using the procedure as followed for albumins. The alcohol soluble prolamins were extracted using 55% propanol having 2% 2-mercaptoethanol at 60°C followed by centrifugation at 9000g. Finally, glutelins were separated in 50 mM borate buffer (pH 10) containing 1% SDS and 0.6% 2-mercaptoethanol by centrifugation at 14000g.

(b) Protein estimation

Semi-micro Kjeldhal method (Vogel, 1960) was followed for estimation of protein content in the seed meal. Seed meal was digested with concentrated sulphuric acid in the presence of a catalytic mixture of copper sulphate, selenium dioxide and potassium dichromate. The digest was heated with 40% NaOH in Markham's distillation assembly and the ammonia so evolved was volumetrically titrated with N/40 HCl to determine the nitrogen present in the sample. The so determined nitrogen was multiplied by 6.25 to get the seed protein content value.

The protein concentration in four seed protein fractions was estimated using method given by Bradford (1976). A volume of 100 µl of the given fraction representing an extract from 100 mg of seed meal was used and protein content calculated as 'mg protein/g seed meal'.

SDS-polyacrylamide gel electrophoresis

Electrophoretic analysis of four protein fractions was carried out on 14% polyacrylamide gels under reducing conditions (Laemmli, 1970). For the gels were prepared using 1.5 mm thick perspex spacers and glass plates of 24x21 cm size. A current of 28 mA was used for electrophoresis of proteins in stacking gel of pH 6.8 and was increased to 32 mA in the separation gel of pH 8.8.

Molecular weight determination and densitometry of polypeptides

For determination of molecular weights of polypeptides separated on gels, standard protein markers (b-galactosidase- 116 kDa; bovine serum albumin- 66.2 kDa, Ovalbumins- 45 kDa, Lactate dehydrogenase- 35 kDa, REase Bsp981- 25 kDa, b-lactoglobulin- 18.4 kDa, Lysozyme- 14.4 kDa.) were run on the gels and calibration curve so prepared by plotting the relative mobilities of these standard proteins against the log of their molecular weights was used for the purpose (Weber and Osborn, 1969). Concentration of different polypeptides in the electrophoresed protein fractions was determined by densitometric scanning of gels. For this purpose, the gel picture was generated using 'ImageQuant 100' image capture system and analyzed using the 'TL-100' TotalLab software which was downloaded from 'totallab-tl100.software.informer.com' (Nonlinear Dynamics Ltd.). The relative area under the scanned peaks as calculated using the software represented the relative concentration of the corresponding polypeptide.

Results and Discussion

Seed protein content and four protein fractions

The salt sensitive line 'BHS 352' and the salt tolerant line 'Karan 16' responded to the changing levels of salinity for seed protein content and four protein fractions with very little difference. In line 'BHS 352', seed protein content (8.0%) as observed in plants grown under controlled conditions exhibited an increase with initial increase in salt concentration. After reaching 8.2% at 8 dS m^{-1} from 8.0% at 4 dS m^{-1}, it declined to 7.9% at 12 dS m^{-1} and to 7.0% at 15 dS m^{-1}. Following slight increase at initial levels of salinity treatment, protein content in line 'Karan16' was also seen to decrease at further higher salinity levels. In this way, both the lines were found to exhibit a reduction of approx. 10% in protein content at the highest level of salt concentration. As can be seen in Figure 1, accumulation of four protein fractions on mg protein/g seed meal basis was also affected differently at four levels of salinity in the two lines. While albumins registered an increase at salinity levels increasing up to 8 dS m^{-1} in line 'BHS 352' and up to 12 dS m^{-1} in line 'Karan 16', these were seen to decrease on further rise in the salinity level. Similarly, concentration of globulins was also highest at 8 dS m^{-1} in salt sensitive 'BHS 352' and at 12 dS m^{-1} in line 'Karan 16'. Line 'BHS 352' exhibited an increase in the amount of albumins and globulins up to 8 dS m^{-1} salinity level. In this way, tolerance of the line 'Karan 16' was visible in the increasing concentration of these fractions at a higher salinity level of 12 dS m^{-1}. On the other hand, a constant decrease in glutelin concentration was observed to occur with increasing levels of salinity (4 dS m^{-1} to 15 dS m^{-1}) in the salt sensitive line as well as in the salt tolerant line. In case of the alcohol soluble prolamins, increasing the salt concentration level resulted in their higher accumulation in the salt sensitive line while a decrease was observed in the salt tolerant line. The decreasing effect on glutelin concentration noticed to be more pronounced in line 'BHS 352' as compared to the much lesser decline in line 'Karan 16' may also be taken as indicator of sensitivity to salt stress. However, the salt sensitive line exhibited an increase in the prolamins while salt tolerant line showed a decreasing trend with increase in salt concentration.

As compared to other major cereals, barley is known to be relatively more tolerant to salinity; grain yield in barley is reported to decline after 8 dSm^{-1} salinity level (Mckenzie *et al.*, 1983) while in other cereals such as rice and wheat the similar effect was visible above 3 dS m^{-1} and 6 dS m^{-1} respectively (Munns *et al.*, 2006; Kumar *et al.*, 2010; Baxter *et al.*, 2011). In the present study, sensitivity and tolerance of barley lines, 'BHS 352' and 'Karan 16' respectively, is reflected from the differences observed in their response under increasing salinity levels. With an initial increase in seed protein

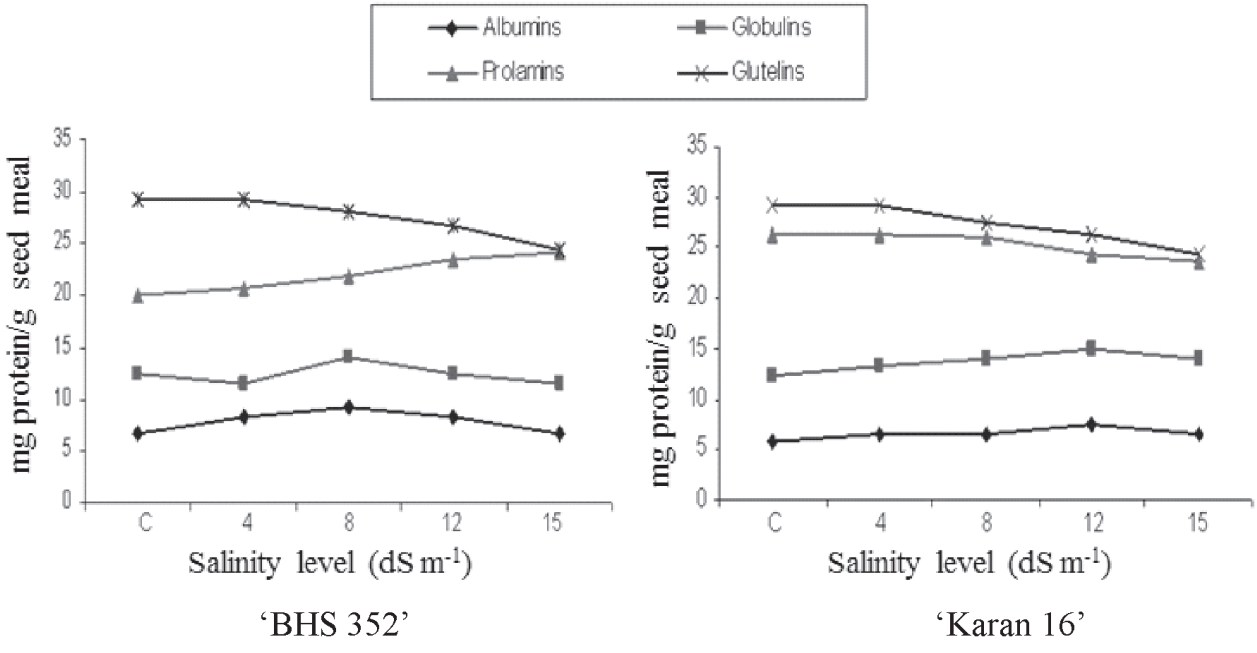

Figure 1: **Protein concentration in four seed protein fractions at different salinity levels**

content at lower salinity levels, the salt tolerant and salt sensitive lines exhibited a decrease in protein content at higher salinity (12 dS m^{-1} and 15 dS m^{-1}). As reported in earlier studies on changes in seed protein content under salinity treatment in different crops like wheat (Soliman *et al*., 1994; Abdul Qados, 2009), oats (Kumar *et al*., 2010), soybean (Ghassemi-Golezani *et al*., 2010), chickpea (Murumkar and Chavan, 1986) *Pennisetum* (Reddy and Vora, 1985) etc., the seed protein content in our studies also decreased with increase in the level of salinity. Seed protein content, in wheat, has been reported to decrease under varying (0.5 dS m^{-1}, 4.0 dS m^{-1}, 8.2 dS m^{-1} and 12.5 dS m^{-1}) salinity levels (Soliman *et al.,* 1994). In rice, whereas the concentration of albumins and glutelins have been studied to increase under saline conditions, the globulins and prolamins revealed no definite pattern i.e. these two fractions showed increase in their concentrations in some cultivars and a decrease in others (Baxter *et al.,* 2011). Protein accumulation during seed development is known to result from the interaction of various genetic and environmental factors. Changes in accumulation patterns and composition of seed proteins under the influence of varying mineral supply, salinity, water stress, phytohormones etc. have been reported in different crops such as barley, wheat, rice, chickpea etc. (Kirkman *et al.,* 1982; Rahman *et al.,* 1983; Murumkar and Chavan, 1986; Jacobsen and Shaw, 1989; Soliman *et al.,* 1994; Shewry *et al.,* 2001; Baxter *et al.,* 2011). A decrease in protein content beyond the respective tolerance levels may be due to alteration in nitrate absorption as explained earlier by Ghassemi-Golezani *et al*. (2010). Salinity stress also creates oxidative stresses which result in production of reactive oxygen species (ROS) such as O_2, H_2O_2 and OH^- (Moran *et al*., 1994; Mittler, 2002) that bind non-specifically with the nucleic acids, proteins, lipids and trigger their destruction by peroxidative reactions.

Polypeptides constituting seed protein fractions

Four protein fractions separated from seeds of plants grown under different salinity levels were analyzed on SDS-gels for comparative analysis of the changes occurring in their polypeptide composition (Figure 2; Figure 3; Figure 4; Figure 5) and in concentration of their polypeptides; concentration of polypeptides in terms of area under their respective peaks as determined by densitometric scanning (Table 1; Figure 6; Figure 7). The two lines i.e. 'BHS 352' and 'Karan 16' differed in their response for polypeptide changes to the varying salinity levels. Alterations in respect of polypeptide patterns under saline conditions were noticed only in the albumin fraction; other three fractions i.e. globulins, prolamins and glutelins did not show any qualitative change in their polypeptides. In line 'BHS 352', albumin fraction was seen to be formed by polypeptides of *Mr* 70 kDa, 62 kDa, 57 kDa, 54 kDa, 52 kDa, 48 kDa, 45 kDa, 42 kDa, 40 kDa, 37 kDa, 36 kDa, 34 kDa, 33 kDa, 30.5 kDa, 29 kDa, 27 kDa, 23 kDa, 21 kDa, 17 kDa, 15 kDa and 13 kDa under controlled conditions. New albumin polypeptides of *Mr* 59 kDa, 56 kDa, 53 kDa, 39 kDa, 31 kDa, 25.5 kDa and 24.5 kDa were seen as distinct dark bands at 4 dS m^{-1} and 8 dS m^{-1} salinity levels; on further increase in salinity to 12 dS m^{-1} and 15 dS m^{-1} levels, some of these polypeptides decreased in their concentration while others were seen to have almost disappeared. On densitometric scanning of gels, it was seen that alterations in a number of albumin polypeptides under salt stress also amounted to increase or decrease in their concentration (Figure 2; Table 1). Whereas polypeptides of *Mr* 62 kDa, 45 kDa, 30.5 kDa and 23 kDa stayed darkly staining at all levels of salinity, others such as those of *Mr* 70 kDa 59 kDa 57 kDa, 54 kDa, 53 kDa, 52 kDa, 48 kDa, 42 kDa, 40 kDa, 37 kDa, 36 kDa, 33 kDa, 27 kDa, 21 kDa and 13 kDa decreased in their concentration with increasing salinity, many of these going invisible at 15 dS m^{-1}. With appearance of new polypeptides of *Mr* 70 kDa, 45 kDa and 26 kDa, albumins of the salt tolerant line 'Karan 16' showed fewer qualitative changes as compared to those seen in the salt sensitive line 'BHS 352'. Also, very little changes were observed in their concentration under saline conditions. The polypeptides of *Mr* 62 kDa, 57 kDa, 37 kDa, 30.5 kDa and 23 kDa exhibited enhanced intensity at all salinity levels. On the other hand, polypeptides of *Mr* 48 kDa, 42 kDa, 40 kDa, 34 kDa, 21 kDa and 13 kDa followed a decrease in their intensity at the highest salinity level i.e. 15 dS m^{-1}. However, the intensity of *Mr* 13 kDa polypeptide was severely affected and found to be considerably low at the 15 dS m^{-1}. A common and distinct response of both the lines was for the albumin polypeptides of *Mr* 17 kDa and 15 kDa which showed enhanced concentration at the highest salt concentration of 15 dS m^{-1}.

Under controlled conditions, globulins of line 'BHS 352' included polypeptides of *Mr* 67 kDa, 64

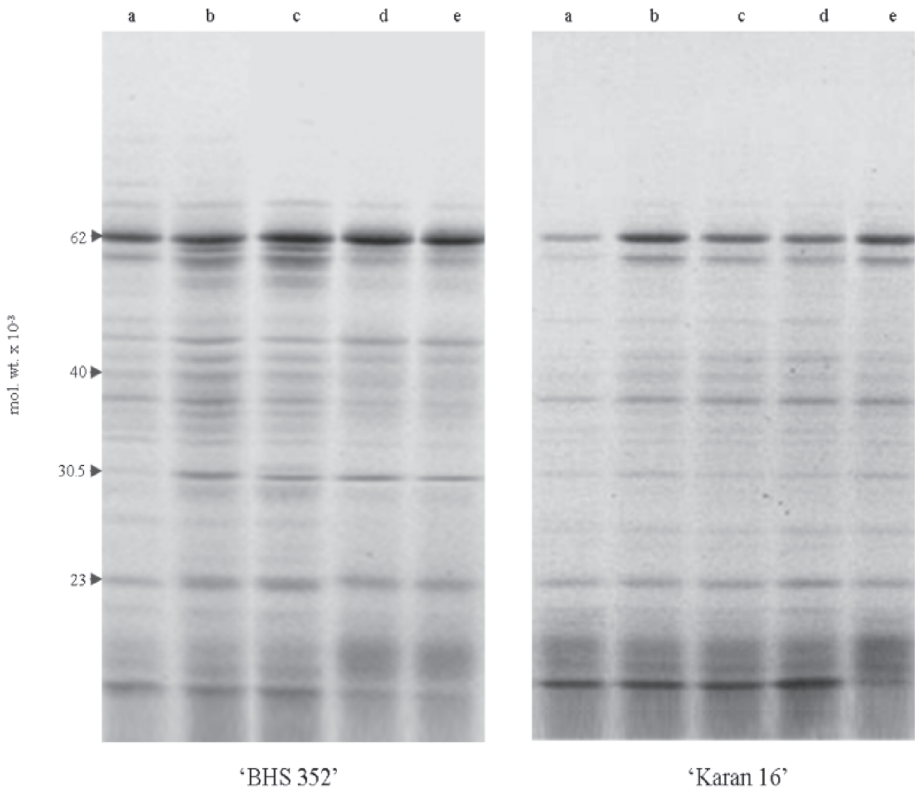

Figure 2: SDS-polyacrylamide gel electrophoresis of albumins at different salinity levels (Lane: a**-control;** b**- 4 dS m^{-1};** c**- 8 dS m^{-1};** d**- 12 dS m^{-1} and** e**- 15 dS m^{-1})**

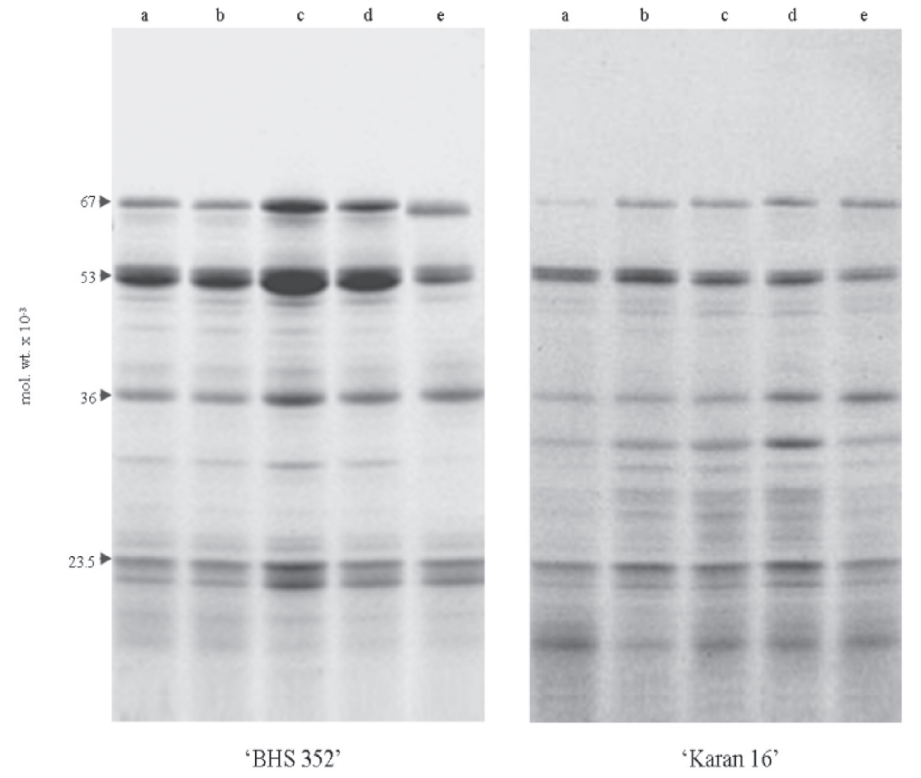

Figure 3: **SDS-polyacrylamide gel electrophoresis of globulins at different salinity levels (Lane:** a**-control;** b**- 4 dS m^{-1};** c**- 8 dS m^{-1};** d**- 12 dS m^{-1} and** e**- 15 dS m^{-1})**

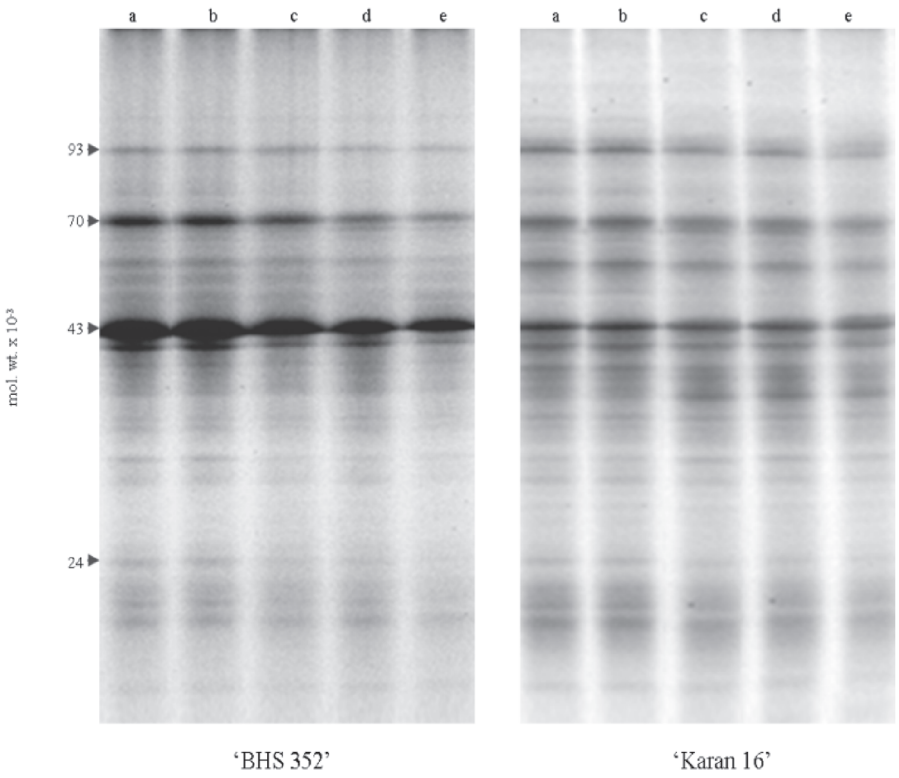

Figure 4: **SDS-polyacrylamide gel electrophoresis of glutelins at different salinity levels (Lane:** a**-control;** b**- 4 dS m^{-1};** c**- 8 dS m^{-1};** d**- 12 dS m^{-1} and** e**- 15 dS m^{-1})**

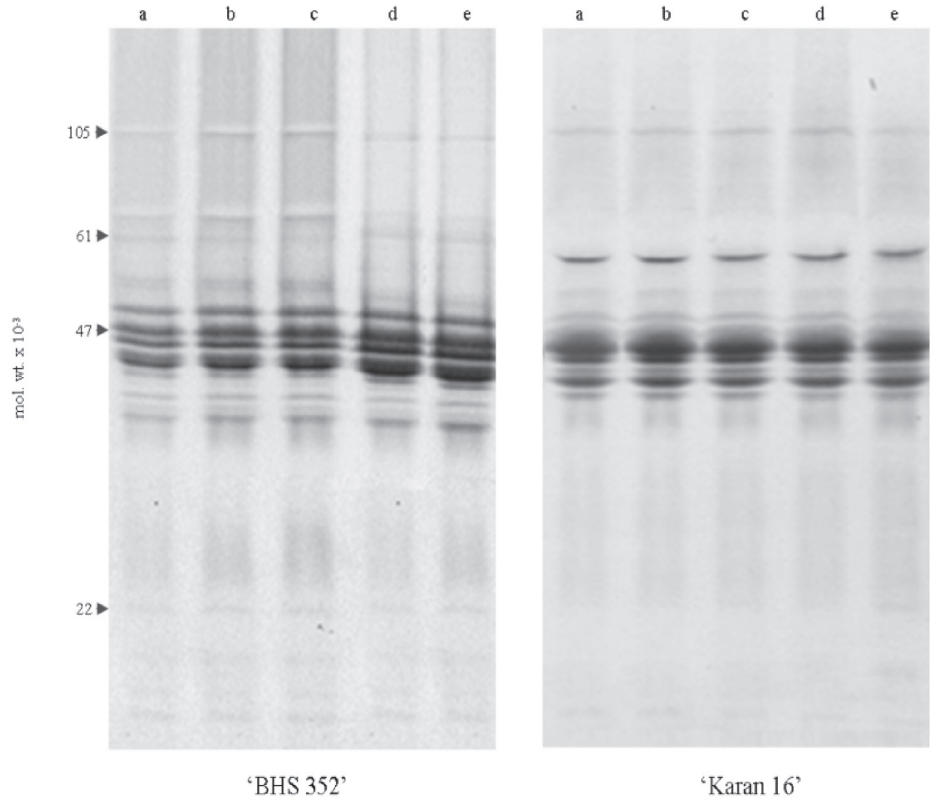

Figure 5: **SDS-polyacrylamide gel electrophoresis of prolamins at different salinity levels (Lane:** a**-control;** b**- 4 dS m^{-1};** c**- 8 dS m^{-1};** d**- 12 dS m^{-1} and** e**- 15 dS m^{-1})**

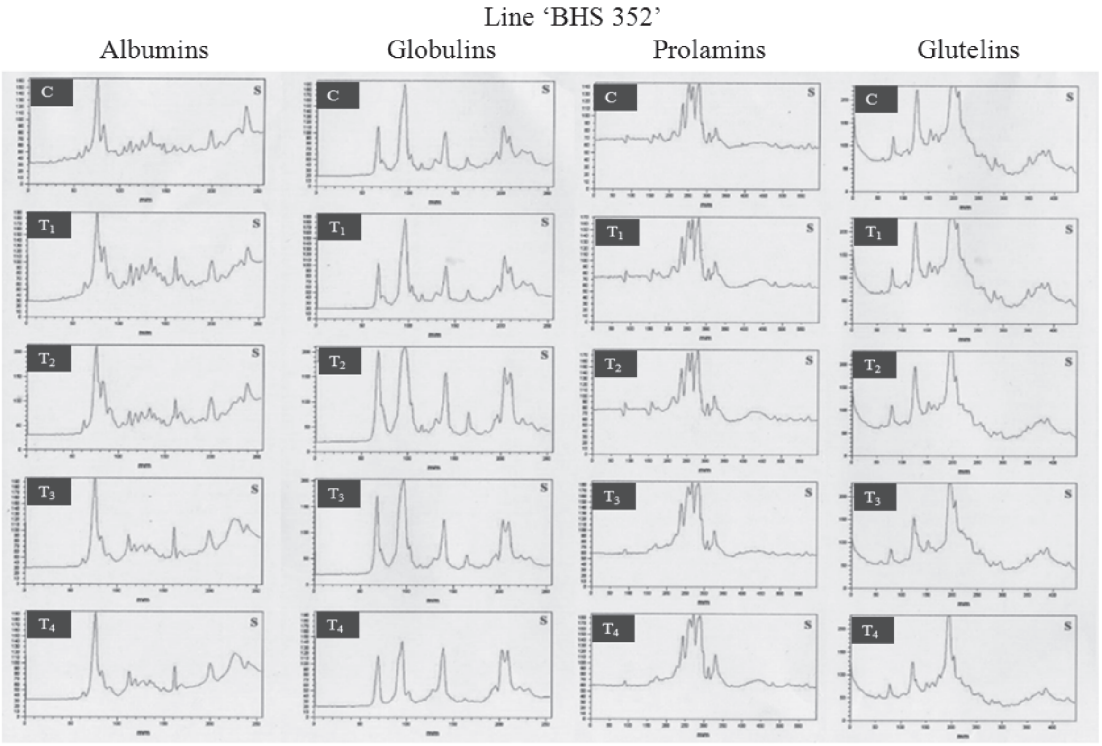

Figure 6: **Densitometric scanning profiles of four seed protein fractions at different salinity levels in salt sensitive line (s) 'BHS 352'**

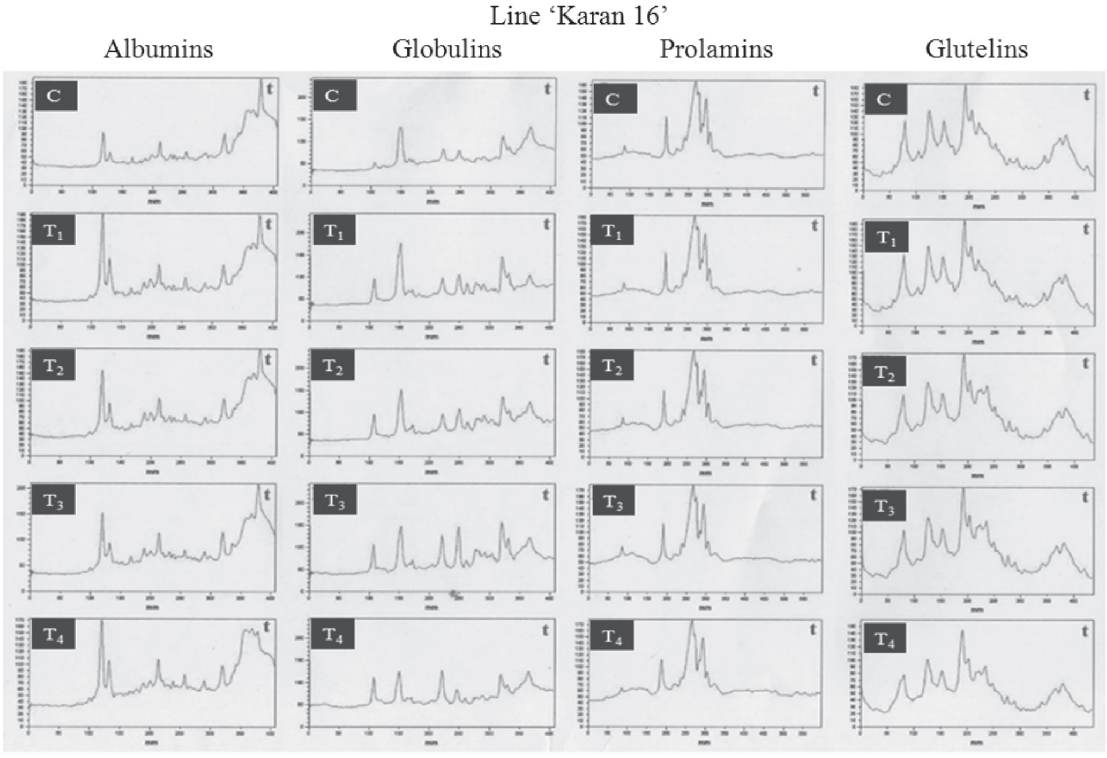

Figure 7: **Densitometric scanning profiles of four seed protein fractions at different salinity levels in salt tolerant line (t) 'Karan 16'**

Table 1
Concentration of major polypeptides (corresponding to area under respective densitometrically scanned polypeptides) of four seed protein fractions in barley lines 'BHS 352' and 'Karan 16' at different levels of salinity

		Densitometrically scanned area / intesity (%)									
Protein Fraction	*Polypeptide (kDa)*	*Barley line 'BHS 352' Salinity levels*					*Barley line 'Karan 16' Salinity levels*				
		Control	*4*	*8*	*12*	*15*	*Control*	*4*	*8*	*12*	*15*
Albumins	70	320	318	380	345	318	———	576	624	576	528
	62	656	688	734	746	641	560	956	804	804	936
	59	———	152	190	112	102	———	———	———	———	———
	57	304	390	412	230	142	216	468	420	420	454
	56	———	190	190	———	———	———	———	———	———	———
	54	114	152	152	103	103	———	———	———	———	———
	53	———	216	232	182	182	———	———	———	———	———
	52	136	128	———	———	———	———	———	———	———	———
	48	262	180	149	———	———	217	230	225	285	110
	45	338	618	592	580	572	———	176	108	191	191
	42	250	542	484	304	266	136	368	383	324	284
	40	336	380	356	316	316	182	394	376	272	220
	39	———	———	———	124	121	———	———	———	———	———
	37	462	480	380	342	304	424	620	520	520	494
	36	210	248	228	194	118	122	174	156	162	187
	34	236	304	284	———	———	216	228	228	224	129
	33	252	278	228	228	194	214	234	234	234	278
	31	———	———	228	———	———	———	———	———	———	———
	30.5	320	518	490	480	480	480	579	550	562	536
	29	246	532	570	464	564	118	140	104	104	104
	27	324	354	342	187	———	435	462	424	486	472
	26	———	———	———	———	———	———	210	190	219	110
	25.5	———	192	203	———	———	———	———	———	———	———
	24.5	———	215	231	———	———	———	———	———	———	———
	23	488	686	694	660	674	864	812	812	864	812
	21	304	380	356	190	———	224	214	212	432	216
	20	———	———	———	———	———	228	136	146	101	280
	17	316	438	444	532	370	364	356	360	342	598
	15	342	394	408	568	376	342	356	363	376	508
	13	546	594	546	356	251	868	916	968	1112	532
Globulins	67	470	470	946	608	———	324	568	568	596	520
	65	———	———	———	———	378	———	———	———	———	———
	64	280	242	336	256	———	———	———	———	———	———
	63	232	223	246	———	———	———	———	———	———	———
	55	418	418	669	518	380	416	572	376	376	264
	53	970	823	1234	1086	470	476	720	668	624	320
	49	228	228	396	319	304	232	232	232	232	232
	48	266	266	315	186	102	228	332	332	332	232
	45	224	212	304	294	118	186	220	232	273	212
	43	219	212	266	228	228	———	———	———	———	———
	41	———	———	190	152	———	———	———	———	———	———
	40	421	456	542	380	394	———	———	———	———	———
	36	532	646	836	798	820	406	436	436	572	588
	32	———	———	———	———	———	323	482	482	‴	334
	30	356	380	470	380	128	184	336	371	337	232
	28.5	———	———	———	———	———	122	154	154	182	121
	28	———	———	———	———	———	118	154	154	174	110
	27	284	266	304	228	———	299	365	352	292	242
	25	304	304	342	304	304	118	128	324	236	120
	24.5	266	228	266	266	228	112	112	101	112	112
	23.5	418	456	580	442	442	361	416	368	524	364
	22	418	380	646	470	510	235	384	384	720	384
	21	———	———	———	———	———	284	180	218	192	192
	17.5	384	308	418	304	304	352	184	223	223	210
	16	494	532	394	370	332	532	256	328	386	422

		Densitometrically scanned area / intesity (%)									
Protein Fraction	*Polypeptide (kDa)*	*Barley line 'BHS 352' Salinity levels*					*Barley line 'Karan 16' Salinity levels*				
		Control	*4*	*8*	*12*	*15*	*Control*	*4*	*8*	*12*	*15*
Glutelins	93	416	416	374	358	343	434	434	360	422	332
	70	341	318	282	124	124	284	284	224	232	176
	61	764	696	589	364	364	804	804	632	656	514
	53	412	412	401	362	299	450	485	416	460	384
	50	408	408	341	278	260	228	228	198	210	144
	46	402	396	396	244	368	———	———	———	———	———
	43	1232	1136	968	896	304	754	754	754	754	460
	41	516	486	414	486	366	384	384	366	384	304
	39	370	346	284	370	228	452	452	394	304	272
	38	204	216	228	266	194	292	292	314	372	316
	36	224	224	217	313	217	272	226	465	465	419
	34	278	278	252	296	210	315	315	344	344	321
	30	440	452	396	328	172	288	304	412	427	296
	28	388	384	374	384	240	342	342	304	304	292
	24	392	392	317	342	360	394	394	339	370	304
	22	284	284	215	220	312	360	360	217	217	166
	21	240	240	328	360	218	396	394	356	356	330
	20	360	364	352	364	352	456	456	415	434	392
	13	332	388	300	312	284	392	392	306	330	268
Prolamins	105	272	304	341	314	218	356	460	494	570	248
	65	312	360	416	282	114	———	———	———	———	———
	61	384	384	264	245	232	———	———	———	———	———
	58	———	———	———	———	———	598	812	812	931	712
	55	216	240	296	252	216	———	———	———	———	———
	54	216	216	216	182	184	456	456	468	418	312
	49	563	616	686	686	713	356	357	418	418	380
	48	———	———	———	———	———	252	252	190	190	190
	47	652	692	746	796	883	———	———	———	———	———
	46	———	———	———	———	———	570	593	570	543	432
	45	614	664	664	789	865	———	———	———	———	———
	43	340	428	428	540	540	328	374	374	190	228
	42.5	484	496	496	546	596	———	———	———	———	———
	41.5	240	240	240	496	496	152	152	190	172	152
	40	267	281	292	322	346	342	342	342	342	380
	39	———	———	———	———	———	120	134	132	132	144
	38	244	264	297	252	286	———	———	———	———	———
	35	362	378	378	362	550	560	699	522	398	322
	22	272	260	290	284	228	208	274	208	298	342
	19	216	228	272	260	228	228	342	532	532	684
	14.5	230	248	292	292	248	260	532	456	608	570
	11.5	216	216	260	244	232	280	342	342	456	342

kDa, 63 kDa, 55 kDa, 53 kDa, 49 kDa, 48 kDa, 45 kDa, 43 kDa, 39 kDa, 36 kDa, 30 kDa, 27 kDa, 25 kDa, 24.5 kDa, 23.5 kDa, 22 kDa, 17.5 kDa and 16 kDa (Figure 3). Maximum enhancement in terms of their intensity was visible at 8 dS m^{-1} salinity level when the major polypeptides viz. *Mr* 67 kDa, 55 kDa, 53 kDa, 49 kDa, 36 kDa, 30 kDa, 23.5 kDa and 22 kDa were present as very dark bands. On further increase in salinity, intensity of various polypeptides was reduced, maximum effect being visible on those of *Mr* 64 kDa, 63 kDa and 27 kDa through their disappearance at the highest salinity of 15 dS m^{-1}. In the salt tolerant line, intensity of almost all globulin polypeptides was found to be maximum at 12 dS m^{-1}; that of *Mr* 55 kDa, 53 kDa and 30 kDa polypeptides followed a decrease at salinity levels above 4 dS m^{-1}. On the other hand, *Mr* 16 kDa polypeptide exhibited an increasing trend after undergoing a drop in its concentration at 4 dS m^{-1} level of salinity.

Unlike albumins and globulins which were enhanced in both the lines, most of the glutelin polypeptides showed a decreasing effect under salinity treatment, the effect looking more pronounced in the salt sensitive line 'BHS 352' (Figure 4; Table 1). As is vivid, concentration of polypeptides of *Mr* 93 kDa, 70 kDa, 61 kDa, 53 kDa, 50 kDa, 43 kDa, 41 kDa and 24 kDa decreased continuously in both the lines as the salinity level was increased. In line 'BHS 352', some of the polypeptides viz. *Mr* 39 kDa, 38 kDa and 36 kDa were seen to follow an increase in their concentration at 12 dS m^{-1}. Over the entire range of salinity i.e. 4 to 15 dS m^{-1}, the polypeptide of *Mr* 43 kDa dominated all glutelin polypeptides with respect to its relative concentration at any given level of salinity treatment. In the salt tolerant line 'Karan 16' also, certain polypeptides such as *Mr* 38 kDa, 36 kDa and 30 kDa showed enhanced concentration at elevated levels of salinity i.e. 8 dS m^{-1} to 12 dS m^{-1}; concentration of these polypeptides decreased with further rise in salt concentration.

Prolamins of the salt sensitive and the salt tolerant line also did not undergo any qualitative change at different salt treatments (Figure 5). Of the various polypeptides in line 'BHS 352', low molecular weight (*Mr* 22 kDa, 19 kDa, 14.5 kDa and 11.5 kDa) and high molecular weight (*Mr* 105 kDa, 65 kDa, 55 kDa and 54 kDa) polypeptides were lightly staining and exhibited an increase in their intensity up to 8 dS m^{-1} salinity level; further increase in salinity level to 12 dS m^{-1} and 15 dS m^{-1} resulted in reduction in their concentration (Table 1). On the other hand, concentration of major polypeptides such as those of *Mr* 49 kDa, 47 kDa, 45 kDa, 43 kDa, 42.5 kDa, 41.5 kDa, 40 kDa, 38 kDa and 35 kDa followed a constant increase as the salinity level was raised from 4 dS m^{-1} to 15 dS m^{-1}. In contrast, prolamins of the salt tolerant line underwent lesser changes with respect to concentration of their polypeptides. A decreasing trend was observed for concentration of polypeptides of *Mr* 105 kDa, 54 kDa, 48 kDa, 46 kDa, 43 kDa and 35 kDa at highest salinity levels. On the other hand, polypeptide of *Mr* 40 kDa was unaffected up to 12 dS m^{-1} and showed enhanced concentration at 15 dS m^{-1}. The bands of *Mr* 58 kDa and 41.5 kDa showed their maximum concentration at 12 dS m^{-1} and 8 dS m^{-1} respectively; their intensity was seen to decrease at further increase in salinity level.

A sharper decrease in the intensity of glutelin polypeptides of line 'BHS 352' with increasing salt concentration levels vis-à-vis that of 'Karan 16' reflects its relative sensitivity to the salinity stress. On the other hand, the prolamins showed a regular increasing accumulation in the line 'BHS 352' and this response may be taken as the sensitivity indicator for salt stress in barley. Prolamin has been named so because of the high proline and glutamine content in this protein fraction. It is likely that the enhanced intensity of prolamin polypeptides is driven by increased availability of proline in the organic nitrogen reserve under high salinity. Further, this divergence in favor of prolamin accumulation may be occurring at the cost of glutelin polypeptides which showed reduced accumulation under salt stress. Alterations in the intensity of polypeptides should be a function of various factors regulating the rate of protein synthesis and protein degradation during seed development. These may vary from mRNA stability, various factors of significance in translation, availability of amino acids, inhibitors of specific proteinases etc (Callis, 1995). Decrease in the intensity of polypeptides under high salt concentration may also result from the enhanced activity of certain proteinases which are known to occur in the developing seed (Zhang and Jones, 1995).

Albumin polypeptides have been known to be responsible for their metabolic role while globulins, prolamins and glutelins perform the storage functions. In view of this, variations occurring in

the albumin polypeptides probably represent the changing metabolic requirements under salinity stress. A number of barley albumin polypeptides have been characterized by different workers. These include β-amylase (Shewry *et al.*, 1988), Z-protein (Hejgaard, 1982) and a number of cysteine proteinases such as C4, C5 and C6 type and aspartic proteinsases of E group (Zhang and Jones, 1995) that occur in developing and ungerminated seed. Therefore, it will be of interest to further characterize various albumin polypeptides in barley for their specific role and for their relationship with alterations seen in various characteristics under salt stress. Studies by Shewry *et al.* (1988) showed that the albumin polypeptide of *Mr* 60 kDa represented the β-amylase in developing and ungerminated barley seeds. Considering the *Mr* 62 kDa polypeptide as representing the â-amylase band, increased concentration of this polypeptide probably indicates a higher activity of â-amylase under salt stress. Enhanced degradation of starch sink resulting from increased â-amylase activity is likely to cause higher nitrogen to carbon ratio of food reserves thus in turn leading to an increase in protein content at the increased salt concentration level. The albumin polypeptide of *Mr* 40 kDa is likely to represents a polypeptide of the serpin superfamily which is believed to function as protease inhibitor in the seed (Roberts *et al.*, 2003). In the present study, intensity of this albumin polypeptide increases slightly under salt stress and is likely to regulate the concentration level of polypeptides by inhibiting the proteases functional during seed development.

Based on a large number of studies, salt tolerance has been explained to be governed by complex mechanisms involving different morphological, physiological and biochemical changes. Some of these include production of proline, glycine betaine, ion transporters for ionic homeostasis, calcium mediated transduction, ABA dependent and ABA independent pathways etc. which are operated through activation and regulation stress specific genes. (Bray, 1997; Shinozaki and Yamaguchi-Shinozaki, 1997; Thomashow, 1999; Hasegawa *et al.*, 2000; Wang *et al.*, 2003; Sairam and Tyagi, 2004 Batool *et al.*, 2014; Bahmani *et. al.*, 2015). The proteins coded by these genes are classified into two groups. One group of proteins represent the functional proteins which work for salinity tolerance and the other group represents regulatory proteins like transcription factors which participate in gene expression and signal transduction pathways under various mechanisms proposed by different workers. In the present study, major polypeptides constituting a given seed storage protein fractions followed a similar pattern of alteration in their intensity while albumins underwent qualitative and quantitative changes varying on polypeptide to polypeptide basis. Keeping in view such variation in their response, it may be stated that genes for most of the albumin polypeptides are independently regulated under salt stress while those for the three storage protein fractions i.e. globulins, glutelins and prolamins are coordinately regulated with separate regulatory mechanisms for each of the fractions under salt stress.

In view of various findings, improvement of crops for salinity tolerance has been suggested through different methods of classical breeding, molecular assisted selection and genetic engineering. These all focus on manipulation of the genes for changes underlying various mechanisms for salinity tolerance stated above. In addition to focusing on genes induced under stress conditions, importance of work on proteomics has also been suggested as a powerful approach in this direction. Identification and quantification of such proteins at different stages of gene regulation promises the improvement of stress tolerance in different crops. Based on the reports of various salt responsive proteins reviewed by Zhang *et al.* (2012), as many as 2171 proteins from 34 plant species have been identified as salt responsive proteins. These proteins have been further placed in 14 functional groups which are involved in carbohydrate and nitrogen metabolisms, ROS scavenging, protein metabolism, cytoskeleton stability, plant membrane proteins etc. (Gupta and Huang, 2014).

In our studies, the newly seen polypeptides in albumin fraction and polypeptides that showed up-regulation and down-regulation in other protein fractions may be considered as the salinity responsive proteins. In addition, a number of acid and alkaline proteases have been reported to increase under salt stress (Parida *et al.*, 2004). Therefore, increasing activity of these proteases might account for decrease in intensity of certain polypeptides under saline conditions. It will be of interest to further plan studies towards understanding molecular mechanisms which regulate alterations in various polypeptides belonging to different seed protein fractions. The

present study has described alterations in more than 90 polypeptides constituting four protein fractions of barley through the use of single dimension gels. It may be stated that it represents the first study focusing on seed proteins reporting such a large number of changes under varying salinity levels in a major crop. The changes observed reflect the up-regulation and down-regulation of genes for different polypeptides and also expression of certain newly induced genes under salt stress. The use of advanced technique of MALDI-TOF-MS should provide further information on the exact nature of alterations in polypeptides and their relatedness with the metabolic changes under salt stress.

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Conflict of Interest

The authors do not have any conflict of interest with the contents of this manuscript.

Abbreviation

HMW, high molecular weight; kDa, kilodalton; *Mr*, molecular weight; ha, hectare; mM, millimolar; EC, electrical conductivity; dS m^{-1}, deci Siemens per metre; h, hour; g, relative centrifugal force; µl, microlitre; SDS, sodium dodecyl sulfate; mA, milliampere; ABA, abscisic acid.

References

Abdul Qados, A.M.S. (2009). Effect of arginine on growth, yield and chemical constituents of wheat grown under salinity condition. *Academic J. Plant Sci*. 2, 267-268.

Bahmani, K., Noori, S.A.S., Darbandi, A.I. and Akbari, A. (2015). Molecular mechanism of plant salinity tolerance: a review, *Aust. J. Crop Sci.* 9, 321-336.

Batool, N., Shahzad, A., Ilyas, N. and Noor, T. (2014). Plants and Salt stress. *Int. J. Agri. Crop Sci.* 7, 582-589.

Baxter, G., Zhao, J. and Blanchard, C. (2011). Salinity alters the protein composition of rice endosperm and the physicochemical properties of rice flour. *J. Sci. Food Agric.* 91, 2292-2297.

Bhattacharyya, R., Ghosh, B.N., Mishra, P.K,, Mandal, B., Rao, C.S., Sarkar, D., Das, K., Anil, K.S., Lalitha, M., Hati, K.M. and Franzluebbers, A.J. (2015). Soil degradation in India: Challenges and potential solutions. *Sustainability 7*, 3528-3570.

Blethen, D.B., Wohlt, J.E., Jasaitis, D.K. and Evans, J.L. (1990). Feed protein fractions: relationship to nitrogen solubility and degradability. *J. Dairy Sci.* 73, 1544-1551.

Bothmer, R. von, Jacobsen, N., Baden, C., Jorgensen, R.B. and Linde-Laursen, I. (1995). An Ecogeographic Study of the Genus *Hordeum.* Systematic and Ecogeographic Studies on Crop Gene pools 7, International Board for Plant Genetic Resources, Rome, 2nd ed. pp 129.

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of dye binding. *Anal. Biochem*. 72, 248-254.

Brandt, A. (1976). Endosperm protein formation during kernel development of wild type and a high lysine barley mutant. *Cereal Chem*. 53, 890-901.

Bray, E.A. (1997). Plant responses to water deficit. *Trends in Plant Sci*. 2, 48-54.

Callis, J. (1995) Regulation of protein degradation. *Plant Cell* 7, 845-857.

Demirkiran, A., Marakli, S., Temel, A. and Gozukirmizi, N. (2013). Genetic and epigenetic effects of salinity on in vitro growth of barley. *Genet. Mol. Biol*. 36, 566-570.

Geressu, K. and Gezaghegne, M. (2008). Response of some lowland growing sorghum (*Sorghum bicolor* L. Moench) accessions to salt stress during germination and seedling growth. *Afr. J. Agric. Res.* 3, 44-48.

Ghassemi-Golezani, K., Taifeh-Noori, M., Oustan, S., Moghaddam, M. and Seyyed-Rahmani, S. (2010). Oil and protein accumulation in soybean grains under salinity stress. *Not. Sci. Biol*. 2, 64-67.

Gupta, B. and Huang, B. (2014). Mechanism of salinity tolerance in plants: physiological, biochemical and molecular characterization, *Int. J. Plant Genomics,* 2014, 1-18.

Hasegawa, P.M., Bressan, R.A., Zhu, J.K. and Bohnert, H.J. (2000). Plant cellular and molecular responses to high salinity. *Plant Mol. Biol*. 51, 463-499.

Heisel, S.E., Peterson, D.M. and Jones, B.L. (1986). Identification of US barley cultivars by SDS-PAGE of hordeins. *Cereal Chem.* 63, 500–505.

Hejgaard, J. (1982). Purification and properties of protein Z - a major albumin of barley endosperm. *Physiol. Plant* 54, 174-182.

Jacobsen, J.V. and Shaw, D.C. (1989). Heat-stable proteins and abscisic acid action in barley aleurone cells. *Plant Physiol.* 91, 1520-1526.

Jamil, M., Rehman, S., Lee, K.J., Kim, J.M., Kim, H.S. and Rha, E.S. (2007). Salinity reduced growth PS II photochemistry and chlorophyll content in radish. *Sci. Agric*. 64, 1-10.

Kirkman, M.A., Shewry, P.R., Miflin, B.J. (1982). The effect of nitrogen nutrition on the lysine content and protein composition of the barley seed. *J. Sci. Food Agric.* 33, 115-127.

Kumar, A., Agarwal, S., Kumar, P. and Singh, A, (2010), Effects of salinity on leaf and grain protein in some genotypes of oat (*Avena sativa* L.). *Rec. Res. Sci. Tech.* 2, 85-87.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature* 227, 680-688.

Linko, R., Lapvetelainen, A., Laakso, P. and Kallio, H. (1989). Protein composition of a high protein barely flour and barley grain. *Cereal Chem.* 66, 478-482.

MacGregor, A.W. and Fincher, G.B. (1993). Carbohydrates of barley grain. In: Barley: Chemistry and Technology, (eds. MacGregor, A.W. and R.S. Bhatty). American Association of Cereal Chemists Inc., St. Paul, Minnesota, USA, pp 73-130.

Machlis, L. and Torrey, J.G. (1956). Mineral nutrition. In: Plants in action. A Laboratory manual of Plant physiology. W.H. Freeman & Com., Inc., San Francisco and Bailey Bros. & Swinfen, Ltd., London.

Mckenzie, R.C., Sprout, C.H. and Clark, N.F. (1983). The relationship of the yield of irrigated barley to soil salinity as measured by several methods. *Can. J. Soil Sci.* 63, 519-528.

Mittler, R. (2002). Oxidative stress, antioxidants and stress tolerance. *Trends Plant Sci.* 7, 405-410.

Molina-Cano, J.L., Sopena. A., Polo, J.P., Bergareche, C., Moralejo, M.A., Swanston, J.S., Glidwell, S.M. (2002). Relationships between barley hordeins and malting quality in a mutant of cv. Triumph. II. Genetic and environmental effects on water uptake. *J. Cereal Sci.* 36, 39-50.

Moralejo, M., Romagosa, I., Salcedo, G., Sanchez-Monge, R. and Molina-Cano, J.L. (1994). On the origin of Spanish two-rowed barleys. *Theor. Appl. Genet.* 87, 829-836.

Moran, J.F., Becana, M., Iturbe-Ormaetxe, I., Frechilla, S., Klucas, R.V., Aparicio-Tejo, P. (1994). Drought induces oxidative stress in pea plants. *Planta* 194, 346–352.

Mostek, A., Borner, A., Badowiec, A. and Weidner, S. (2015). Alterations in root proteome of salt-sensitive and salt-tolerant barley lines under salt stress conditions. *J. Plant Physiol.* 174, 166-176.

Munns, R., James, R.A. and Lauchli, A. (2006). Approaches to increasing the salt tolerance of wheat and other cereals. *J. Exp. Bot.* 57, 1025-1043.

Murumkar, C.V. and Chavan, P.D. (1986). Influence of salt stress on biochemical processes in chickpea, *Cicer arietinum* L. *Plant Soil* 96, 439-443.

Osborne, T.B. (1924). The vegetable proteins. 2nd Edn. Longman Green & Co., London.

Parida, A. K., Das, A. B., Mittra, B. and Mohanty, P. (2004). Salt-stress induced alterations in protein profile and protease activity in the mangrove *Bruguiera parviflora*. *Z. Naturforsch C.* 59, 408-414.

Radovic, D. and Vapa, L. (1996). Hordein composition of Yugoslav barley cultivars. *Cereal Res. Com.* 24, 332-337.

Rahman, S., Shewry, P.R., Forde, B.G., Kreis, M. and Miflin, B.J. (1983). Nutritional control of storage- protein synthesis in developing grain of barley (*Hordeum vulgare* L.). *Planta* 159, 366-372.

Ramagopal, S. (1987). Salinity stress induced tissue-specific proteins in barley seedling. *Plant Physiol.* 84, 324-331.

Reddy, M.P. and Vora, A.B. (1986). Changes in pigment composition, hill reaction activity and saccharides metabolism in bajra (*Pennisetum typhoides* S and H) leaves under NaCl salinity. *Photosynthetica* 20, 50-55.

Richards, L.A. (1954). Diagnosis and improvement of saline and alkali soils. United States Salinity Laboratory Staff, Agriculture Handbook No. 60. USDA, Washington.

Roberts, T.H., Marttila, S., Rasmussen, S.K. and Hejgaard, J. (2003). Differential gene expression for suicide-substrate serine proteinase inhibitors (serpins) in vegetative and grain tissues of barley. *J. Exp. Bot.* 54, 2251-2263.

Sairam, R.K. and Tyagi, A. (2004). Physiology and molecular biology of salinity stress tolerance in plants. *Curr. Sci*. 86, 407-421.

Shewry, P. R., Ellis, J.R.S., Pratt, H.M. and Miflin, B.J. (1978a). A comparison of methods for the extraction and separation of hordein fractions from 29 barley varieties. *J. Sci. Food Agric.* 29, 433-441.

Shewry, P.R. and Tatham, A.S. (1990). The prolamine storage proteins of cereal seeds: structure and evolution. *Biochem. J.* 267, 1-12.

Shewry, P.R., Hill, J.M., Pratt, H.M., Leggatt, M.M. and Miflin, B.J. (1978b). An evaluation of techniques for the extraction of hordein and glutelin from barley seed and a comparison of the protein composition of Bomi and RisØ 1508. *J. Exp. Bot.* 29, 677-692.

Shewry, P.R., Miflin, B.J. (1983). Characterization and synthesis of barley seed proteins. In: Seed Proteins: Biochemistry, Genetics, Nutritive Value, (eds. Gottschalk, W. and H.P. Miller). M. Nijhoff/ W. Junk Publisher, The Hague, London, pp 143-205.

Shewry, P.R., Parmar, S., Buxton, B., Gale, M.D., Liu, C.J., Hejgaard, J. and Kreis, M, (1988). Multiple molecular forms of â-amylase in seeds and vegetative tissues of barley. *Planta* 176, 127-134.

Shewry, P.R., Tatham, A.S. and Halford, N.G. (2001). Nutritional control of storage protein synthesis in developing grain of wheat and barley. *Plant Growth Regul.* 34, 105-111.

Shinozaki, K. and Yamaguchi-Shinozaki, K. (1997). Gene expression and signal transduction in water-stress response. *Plant Physiol.* 115, 327-334.

Soliman, M.S., Shalabi, H.G. and Campbell, W.F. (1994). Interaction of salinity, nitrogen and phosphorous fertilization on wheat. *J. Plant Nutr.* 17, 1163-1173.

Taghipour, F. and Salehi, M. (2008). The study of salt tolerance of Iranian barley (*Hordeum vulgare* L.) genotypes in seedling growth stages. *American-Eurasian J. Agric. Environ. Sci*. 4, 525-529.

Thomashow, M.F. (1999). Plant cold acclimation: freezing tolerance genes and regulatory mechanisms. *Annu. Rev. Plant Physiol. Plant Mol. Biol*. 50, 571-599.

Vogel, A.I. (1960). Quantitative inorganic analysis. Longman, Green and Co. Ltd., London, pp. 248-249.

Wang, W., Vincour, B. and Altman, A. (2003). Plant responses to drought, salinity and extreme temperatures: towards genetic engineering for stress tolerance. *Planta* 218, 1-14.

Weber, K. and Osborn, M. (1969). The reliability of molecular weight determination by dodecyl sulphate polyacrylamide-gel electrophoresis. *J. Biol Chem.* 244, 4406-4412.

Zhang, H., Han, B., Wang, T., Chen, S., Li, H., Zhang, Y. and Dai, S. (2012). Mechanism of plant salt response: insights from proteiomics. *J. Proteome Res.* 1, 49-67.

Zhang, N. and Jones, B.L. (1995). Development of proteolytic activities during barley malting and their localization in the green malt kernel. *J. Cereal Sci.* 22, 147-155.