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SALT INDUCED CHANGES IN LEAF PHENOLOGY OF WHEAT PLANTS ARE REGULATED BY ACCUMULATION AND DISTRIBUTION PATTERN OF Na^+ ION

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Salinity is one of the major factors reducing agricultural productivity and natural resources worldwide. In Pakistan about 40 per cent of the irrigated cropped land, which produces around 90% of the total agricultural output of the country, has come under salinity. Field losses in yield of wheat cultivated in moderately salt affected areas of Pakistan are estimated about 64%. This study assesses changes in bread wheat (*Triticum aestivum* L.) genotypes that are likely to affect leaf growth and phenology under salt stress, specifically focusing on accumulation and distribution of sodium (Na^+), potassium (K^+) and Chloride (Cl^-) ions. Four bread wheat genotypes were grown in nutrient solution at two treatment levels (control and 150 mM NaCl). The bread wheat genotypes responded differently at higher salt stress especially at the 2nd phase of plant growth. Salt stress not only reduced leaf elongation and leaf length but also caused reduction in plastochron index, rate of leaf formation and its development. Accumulation of Na^+ and Cl^- ions increased during periods of intense leaf growth, while proline accumulation was quite low in the salt sensitive genotype. In contrast, tolerant genotypes had lower Na^+ and Cl^- concentration in leaves along with higher proline contents throughout salt stress. An increase in proline content was also observed in the older and more injured leaves which occurred at later stages of leaf growth. No direct relationship was found between leaf injury and inorganic ions (Na^+ and Cl^-) concentrations. However, intensity of injury was related to the duration of salt stress as the size of injured zones on leaves increased while inhibition of leaf elongation and toxic ions content decreased with leaf age. This suggests that salt tolerance could also be associated with plant's capacity to regulate the rate of ion transport or accumulation in leaf tissues and it is concluded that whole-plant response system should not be overlooked when breeding wheat for salt tolerance to improve salt tolerance in plants.

Keywords: Leaf development, leaf ontogeny, salt, solute, stress time, wheat

INTRODUCTION

The use of single physiological traits in breeding has not yet proved fruitful as expected (Jackson *et al*., 1996), because of the complexity of traits and that no single phenomenon can account for the improvement in salt tolerance of plants. Thus, identification of key salt tolerance determinants (Zhu, 2001) along with investigation of cause-effect relationships between physiological responses and their potential benefits in stress adaptation are important (Munns, 2002);Qarshi *et al1996*.In monocots, salt tolerance depends on the ability of plant to exclude sodium from the photosynthetically active part of the shoot (Tester and Davenport, 2003). There is also indication that salinity induces reduction of photosynthesis in leaves (Munns, 2002) or their premature senescence (Yeo *et al*., 1990) and these salt specific effects are mostly related to accumulation of Na^+ and Cl^-at toxic concentrations, or depletion of K^+ or Ca^{2+} (Yeo *et al*., 1990; Leidi and Saiz, 1997). Salt stress increases the plastochron and reduces final number of leaves initiated on the main stem (Maas and Grieve, 1990). Therefore, maintaining low concentration of Na^+ and other potentially toxic ions, and their distribution pattern in the growing zone of the leaf, has been correlated to salt tolerance (Lauchli *et al*., 1994; Lacerda *et al*., 2003). Although there is change in physiological processes with growth stages and age of the plant under stress conditions, little or no genotypic variation has been observed in wheat during osmotic effect (James *et al*., 2002; Munns and James, 2003). However, ion specific effect due to higher accumulation of Na^+ on plant growth and leaf senescence showed substantial genotypic variation (Munns and Taster, 2008). Salt tolerance is also related to oxidative stress tolerance (Farhoudi *et al.* 2012). Some investigations made on rice (Lutts *et al*., 1996) and sorghum (Lacerda *et al*., 2003) showed that accumulation of organic solutes increased with salt treatment but this accumulation occurred especially in more injured leaf blade zones of the salt sensitive genotype. They also observed a negative correlation between leaf proline accumulation and salt tolerance. However, such relationships are not still well established in wheat. Thus this study was aimed to quantify the differences in salt tolerance and its relationships with leaf development and senescence as a function of duration of salt stress in contrasting bread wheat genotypes. It was also evaluated that how differential

ion accumulation and distribution patterns within plant tissues and quantify the progressive accumulation and distribution of Na^+ and Cl^- ions in leaves during salt stress.

MATERIALS AND METHODS

Plant material: Four bread wheat genotypes from different sources and origin, having variation in salt tolerance were used in this study. Three genotypes were from Pakistan with different sources; SARC-3 salt-tolerant was from Saline Agriculture Research Centre and already used in many trials (Hollington, 2000; Munns, 2005); S-9476 (salt tolerant genotype and showed good yield potential in our previous yield trials in saline conditions (Saqib et al., 2010); S-8189 (salt-sensitive) from Department of Plant Breeding and Genetics, University of Agriculture, Faisalabad, Pakistan and Kharchia-65 was used as reference salt tolerant genotype (Hollington, 2000; El-Hendawy, 2005) for comparing and understanding growth and physiological differences for salt tolerance and collected from CAZS-Natural Resources, Bangor-UK.

***Growth conditions*:** Seeds of selected genotypes were surface sterilized with 1.0% sodium hypochlorite solution and germinated in polyethylene lined iron trays of 4 cm height, and moistened with nutrient solution. Seedlings at two leaf stage were transplanted in foam plugged holes in polystyrene sheet floating on 25 L plastic tubs containing ½ strength Hoagland's nutrient solution (Hoagland and Arnon, 1950). Solution pH was maintained at 6.0-6.5 by adding H_2SO_4 and NaOH as required and the solution was continuously aerated with air pumps fitted with a filter to prevent oil contamination. The salt treatment commenced on the 3rd day after seedlings were transplanted in the tubs. Two treatments; control (only nutrient solution) and 150 mM NaCl were used with five replicates. Salts were added gradually in three installments to achieve the desired NaCl concentration and nutritive solution was renewed weekly.

***Leaf development and anatomical changes*:**

Shoot development: Shoot development was expressed by the plastochron index (Erickson and Michelini, 1957) determined on the basis of leaf length measurements at the time in which successive leaves reached a reference length of 7 mm (Bernstein et al., 1993a,b)

$$PI = n + (\log L_n - \log\lambda) / (\log L_n - \log L_n{+}1)$$

λ = reference value leaf length (e.g. 7 mm); L_n = length of leaf n; n = the number of the leaf equal to or just longer than the reference leaf; i = number of the i^{th} leaf

***Leaf elongation and senescence*:** Leaf elongation was measured daily using a ruler. The length of leaf-1 and leaf-2 was measured from a fixed reference point (almost 5 mm above the base of the stalk) to the leaf tip (Bernstein et al., 1993a). Leaf-3 was not used for measurement of leaf elongation and final leaf length (Lacerda et al., 2003). This leaf may not be affected by salt stress because it had practically reached its final size when salt stress was imposed.

The number of green leaves was recorded weekly and leaf senescence was evaluated by counting the number of leaves dropped from the plant. Leaf formation was determined weekly by counting all newly emerged green leaves (a leaf was considered green if >75% of the leaf was green).

At the end of the experimental period, the total length of leaf blade and of the non-viable area (chlorotic and necrotic region at the leaf tip) of the three fully expanded and mature leaves were measured: leaf 1, leaf 2 and leaf 3 (2nd, 3rd and 4th leaf from the top respectively). The youngest not fully expanded leaf, and leaves older than leaf 3 were not measured.

***Leaf chlorophyll contents*:** Salt stress reduces photosynthesis in plants and plant photosynthetic capacity is directly linked with chlorophyll contents in leaves (Munns et al., 2006), thus measuring chlorophyll using SPAD meter is a more cost-effective, rapid and non-destructive technique. Leaf Chlorophyll contents were measured by using a chlorophyll meter (Minolta SPAD-502 Meter). Three leaves from the shoot apex were measured at four different positions for chlorophyll contents and then averaged.

***Plant biomass*:** Five plants from each replicate were harvested after every one week of salt addition at vegetative stage. Fresh and dry weight of plant shoots and roots were measured and tissue dry weight was recorded after drying samples in a forced-draft oven at 65±5 °C until constant weight was obtained.

***Organic and inorganic ions determination*:** Inorganic solute (Na^+, K^+ and Cl^-) concentrations were measured in the leaf-1, 2 and 3 after each week starting from the 1st day of salt application. Solute accumulation and distribution in leaves of salt stressed seedlings, throughout the experimental period, were also studied. For leaf ionic concentration, leaf sap was extracted by using the freeze-thaw method (Gorham, 1994). Thawed leaf samples were put into micro centrifuge tubes having a basal opening which allow leaf sap, but no tissue fragments, to pass through into a collection tube. Each leaf sample was then centrifuged for 3 min at 11,000 rpm. Measurement of K^+ and Na^+ concentration in extracted leaf sap was undertaken using a flame photometer (Sherwood Flame photometer, Model-410; Sherwood Scientifics, Ltd, Cambridge UK). For Cl^-, the extract was diluted with distilled water and Cl^- was determined using a chloride analyzer (Sherwood, Model, MK-323, Sherwood Scientifics, Ltd, Cambridge UK).

Free proline contents in fresh leaf (100 mg) samples were determined on a fresh weight basis following the methods of Bates et al. (1973).

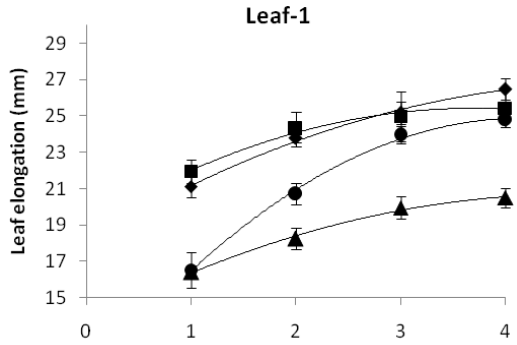
***Experimental design and statistical analysis*:** The experimental layout was a completely randomized design with factorial arrangement using five replicates. The data were subjected to statistical analysis by analysis of variance

technique (Steel and Torrie, 1980). Treatment means were compared at 5% probability and the data were subjected to regression analysis for establishing relationships of the observed data by using Genstat® Discovery edition (Pyne *et al.*, 2005).

RESULTS

***Leaf phenological changes as a function of salt accumulation and stress time*:** The immediate response of plants to elevated salinity was a decrease in the rate of leaf expansion, a key factor in reduction of total leaf area of the plant in stress conditions. Results showed that addition of NaCl to the growing medium reduced leaf elongation (Fig.1) in both the leaves-1 and 2). In the tolerant wheat genotype, decrease in leaf elongation was 20-25% in leaf-1 and 16-21% in leaf-2 with minimum reduction in SARC-3 (20%) in leaf-1 and Kharchia-65 (16%) in leaf-2, while for the salt sensitive genotype, reduction in leaf elongation was 29 and 26%, respectively, for leaves-1 and 2.

(a)



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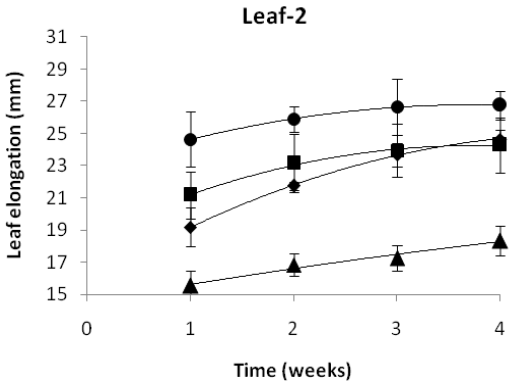

Figure 1. Length of leaf-1 (a) and leaf-2 (b) of four contrasting bread wheat genotypes grown at 150 mM NaCl for 4 weeks. (Means ± SE; n=5)

The development of shoot and leaf was inhibited during salt stress, and leaf senescence occurred in plants in the saline treatment (Fig. 2b).

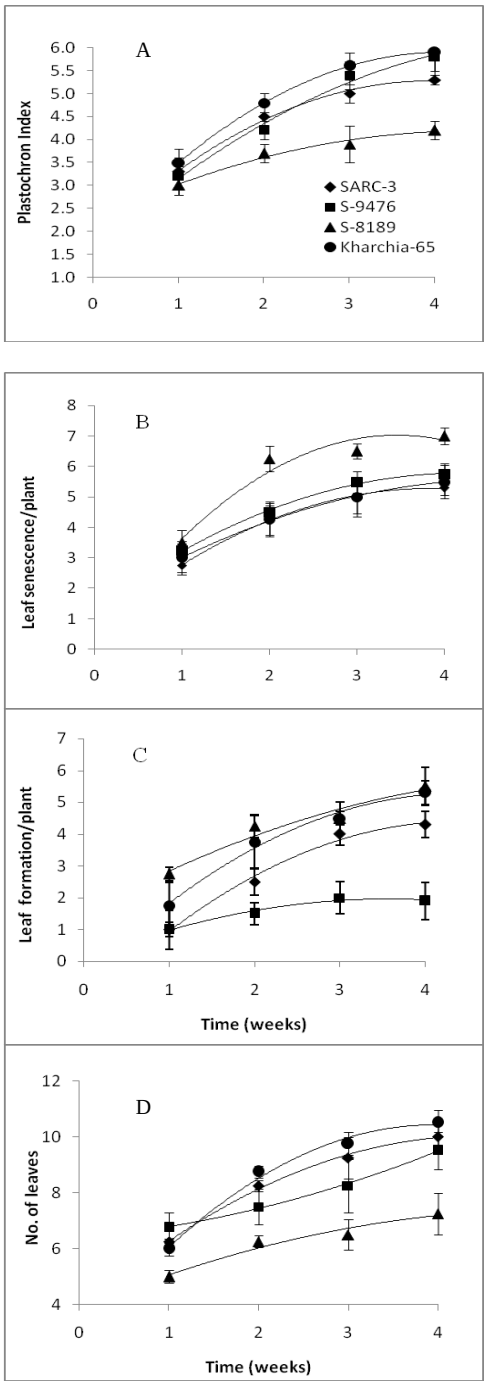

Figure 2. Plastochron index (A), leaf senescence (B), leaf formation (C) and Number of leaves per plant (D) in four contrasting bread wheat genotypes grown at 150 mM NaCl salinity for 4 weeks. (Means ± SE; n=5)

The salt sensitive genotype S-8189 had higher rates of leaf senescence than the other genotypes, and there were

insignificant differences among salt tolerant genotypes. The effect on leaf senescence was prominent after the first week, and continued to the third week of salt stress. Injury of leaves of stressed plants was more obvious in the salt-sensitive genotype where the injured area was 30-65% of the leaf length. Maximum injury on the 2nd leaf (1st fully expanded) leaf of the salt-tolerant genotype was 22%.

The reduction in leaf formation (Fig. 2) was more pronounced in the salt sensitive genotype (S-8189) with increase in salt stress duration when compared to salt tolerant genotypes, however, the trend was opposite for leaf senescence. This interesting observation accentuated that leaf senescence is more important determinant as compared to leaf formation/no of leaves, when plant are under stress environment.

Salinity stress affected the rate of new leaf formation and it was slowed down with time (Fig. 2d). The salt tolerant genotypes retained more number of leaves as compared to salt sensitive genotype at 150 mM NaCl. At first there was negligible differences in number of leaves of all four genotypes, however, after 2 weeks, a significant reduction was apparent in number of leaves and differences between salt tolerant and sensitive genotypes. At final harvest (4 weeks), the salt sensitive genotype had a significant reduction (60%) in the total number of leaves when compared to the salt tolerant genotypes (on average of 51%), when compared with control.

Leaf chlorophyll index was increased at the start of salt stress but declined after two weeks (Fig. 3). The decline in chlorophyll was greater in the salt sensitive genotype (S-8189) than other wheat genotypes and had shown 67 and 51% reduction in leaf-1 and leaf-2, respectively. However, differences in chlorophyll index among all genotypes were not significant.

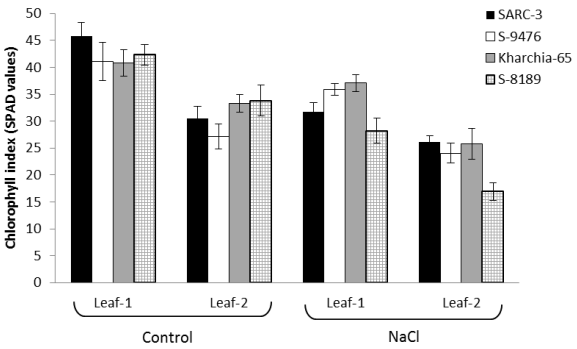

Figure 3. Chlorophyll index (SPAD values) of leaf 1& 2 of four contrasting bread wheat genotypes grown under 150 mM NaCl salt stress for 4 weeks (Means ± SE; n=5)

***Shoot fresh and dry biomass*:** Shoot fresh and dry biomass was similar in the control, but variation between genotypes was apparent at 150 mM NaCl (Fig. 4). Differences were also observed between salt tolerant and sensitive genotypes at 150 mM NaCl. At the beginning of salt stress, shoot biomass (fresh and dry weight) increased with time in all genotypes, but as the duration of salt stress increased, a continuous decrease in absolute biomass and progressive differences between contrasting (salt tolerant and sensitive) genotypes became apparent. Moreover, the salt sensitive genotype (S-8189) exhibited a greater decline in biomass accumulation than the salt tolerant genotypes. At final harvest, the salt sensitive genotype (S-8189) showed a reduction of 76 and 67% in fresh and dry weight respectively, whereas the salt tolerant genotypes had average reductions of 67 and 53% at higher NaCl level when compared to the control.

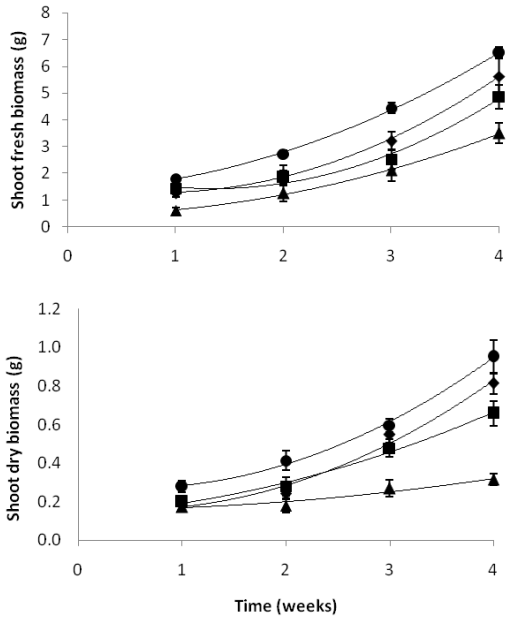

Figure 4. The shoot fresh and dry biomass of four contrasting bread wheat genotypes grown at 150 mM NaCl salinity for 4 weeks. (Means ± SE; n=5)

Proline and inorganic solute accumulation as a function of salt stress and time: Leaf sap Na^+ concentration increased with increase in NaCl stress in all genotypes, however, the salt tolerant genotypes maintained a significantly lower Na^+ in the leaf with higher salinity (Fig. 5). The leaf K^+ content after first week of salt treatment were the same in the salt tolerant and sensitive genotypes and there was variation among genotypes for K concentration in leaf. The salt sensitive genotypes showed a sharp decline in its K concentration of leaf when compared with others. A different trend in proline concentration was observed (Fig. 5) while in 2nd leaf it increased rapidly for about two weeks and then decreased slightly until final harvest.

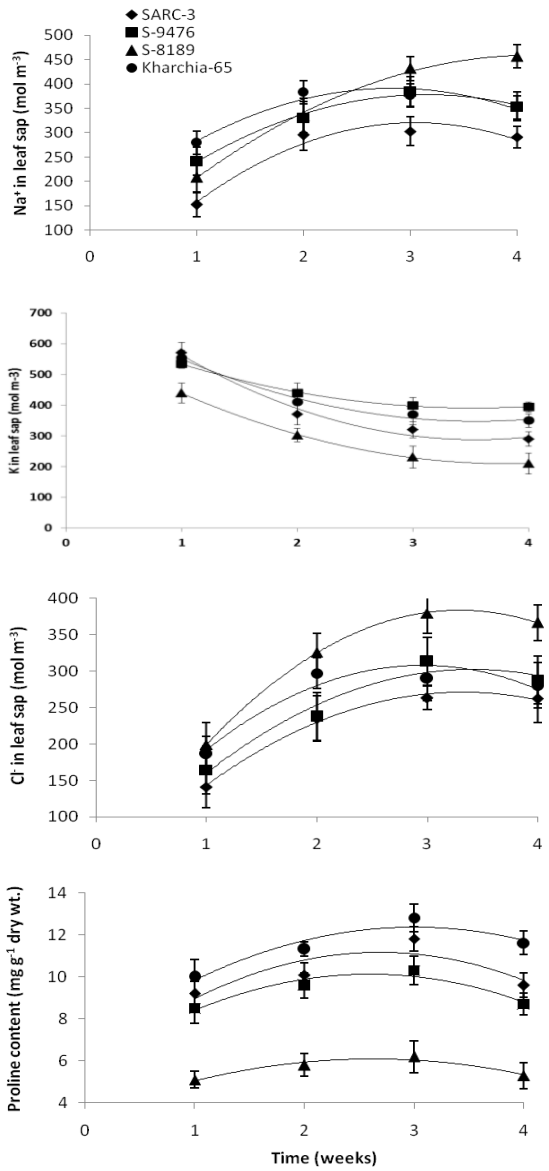

Figure 5. Accumulation of Na, K and Cl and proline in leaf as function of time of salt stress in four contrasting bread wheat genotypes grown at 150 mM NaCl salinity for 4 weeks. (Means ± SE; n=5)

Ion accumulation and distribution in different leaves: The concentration of Na^+ and Cl^- in leaves of all wheat genotypes (Fig. 6) was lowest in the youngest (leaf-1) and highest in the oldest leaf (leaf-3). At final harvest, Na^+ concentrations in leaf-2 and leaf-3 were 3.5 and 3.0 times higher in the salt sensitive genotype when compared to the control, while in case of the salt tolerant genotypes, this magnitude was 3.0 and 2.0 times. An opposite trend was observed for K^+, where higher concentrations were found in younger leaves of control plants (of all genotypes) than older ones (Fig. 7).

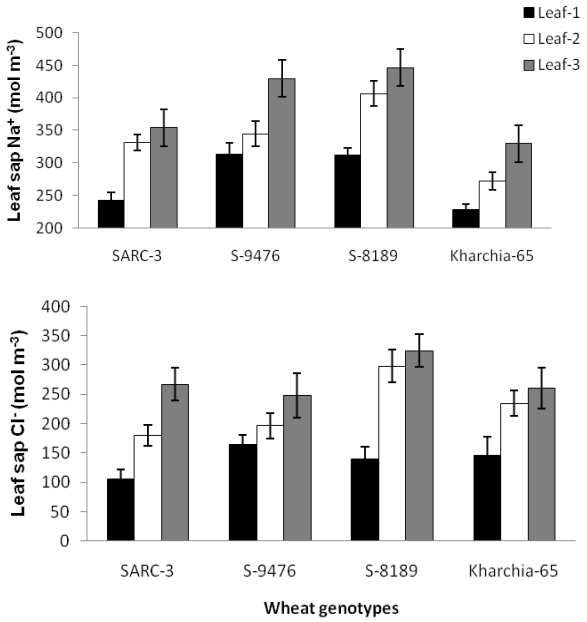

Figure 6. Accumulation of Na^+ and Cl^- ions in different leaves of four contrasting bread wheat genotypes grown at 150 mM NaCl salinity for 4 weeks. (Means ± SE; n=5)

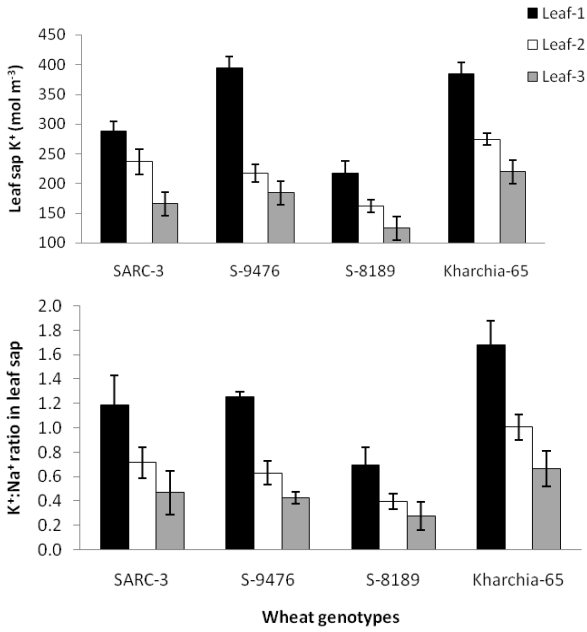

Figure 7. Potassium concentration and K^+/Na^+ ratio in leaf sap of four contrasting bread wheat genotypes grown at 150 mM NaCl salt stress for 4 weeks (Means ± SE; n=5)

Consequently, K^+/Na^+ ratio was higher in salt tolerant genotypes when compared to salt sensitive genotype at 150 mM NaCl (Fig. 7). Leaf K^+/Na^+ ratio demonstrated

significant genotypic differences as a result of salinity stress (Fig. 7). Overall, there was a decreasing trend with increasing NaCl concentration in all three leaves, and the lowest K^+/Na^+ ratio was found in leaf-3. The K^+/Na^+ ratio was 0.43 to 0.67 and 0.28 in leaf-3, 0.63 to 1.01 and 0.40 in leaf-2 and 1.13 to 1.68 and 1.02 in leaf-1 of salt tolerant genotypes and sensitive one, respectively. The genotypic variations were more obvious in leaf-2 when compared to leaf-1 & 3 at 150 mM NaCl level.

Conversely, proline concentration in leaves was higher in older leaves in all wheat genotypes (data not shown). Under salt stress, salt tolerant genotypes had higher proline concentration (about two times) than the sensitive genotype (S-8189), and the highest proline concentration was found in Kharchia-65 (11.6 mg g^{-1} fresh wt).

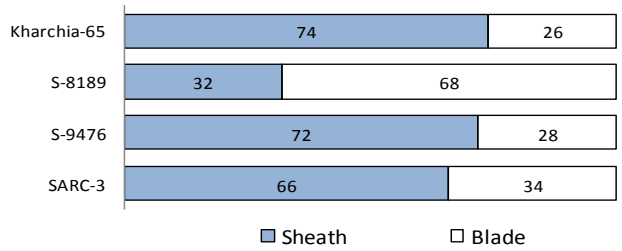

Figure 8. Proportion of Na^+ ion (%) in leaf blade and sheath of 1st fully expanded leaf in four contrasting bread wheat genotypes grown at 150 mM NaCl for 4 weeks. (Means ± SE; n=5)

DISCUSSION

Exposure to high NaCl concentration in growth medium causes a reduction in leaf growth rate, leaf emergence rate and overall shoot development (Bernstein *et al*., 1993a,b). In the present study, salt induced stress resulted in retardation of not only leaf elongation and the final length of leaf 1 and 2 but also caused reduction in the plastochron index (PI). Fewer and smaller leaves were observed in stressed plants than under control conditions, especially in the salt sensitive genotype. Salt stress also resulted in increased senescence of newly emerged leaves because of reduced chlorophyll content and increased leaf injury. The salt sensitive genotype showed severe leaf senescence, might be due to decay of chlorophyll and protein contents and low ribonucleic acid content in leaves of stressed plants, thus causing hormonal imbalance (Dangl *et al*., 2001) and similar relationship of leaf senescence with salt sensitivity was also suggested by Lutts *et al*. (1996) in rice.

Accumulation of Na^+ and Cl^- in leaf 1 and 2 (Figure-6) was increased with salinity stress (high NaCl concentrationin external medium) as K^+ is replaced by either Ca^{2+} or Na^+, and nitrate is replaced by Cl^- especially in the epidermis of the elongation and emerged zone and caused greater reduction in leaf elongation. Accumulation of these ions (Na^+, K^+, and Cl^-) in different leaves was more during period of very intense leaf growth (Fig. 6) and may suggest salt tolerance could be associated with the synchronization between the rate of ion transport to the shoot and the plant capacity to compartmentalize them in different tissues or cells (Boursier and Lauchli, 1989; Lacerda *et al*., 2001). A control mechanism of absorption and transport of these ions to the leaves may be involved, at least in sorghum (Greenway and Munns, 1980; Moya *et al*., 1999). In addition, leaves with most severe inhibition of elongation as a result of salt stress were those which showed the lowest K^+ concentration (Fig. 7), especially in the salt sensitive genotype. The K^+/Na^+ ratio decreased substantially after the plants were exposed to high levels of NaCl, particularly in the sensitive genotype. Therefore, leaf elongation in salt sensitive genotype, was reduced not only by accumulation of Na^+ and Cl^- ions but also by reduced K^+ contents in stressed plant to deficient level (<1.5% in wheat), which is essential to cell enlargement, especially in young leaves (Taleisnik and Grunberg, 1994).

A direct relationship between leaf injury and inorganic (Na^+ and Cl^-) ions concentration was not observed, nor was there a relationship between leaf injury and inhibition of leaf elongation. The injured zone increased with leaf age, while the inhibition of leaf elongation and Na^+ and Cl^- ion concentration decreased with leaf age (data not shown). The intensity (area) of leaf injury was found to be related to the duration of salt treatment. Since the growth of younger leaves is dependent on photosynthates produced by mature leaves (Munns, 2002) and these leaves were more severely injured in the salt sensitive genotype, the degree of injury of mature leaves may be used as a criterion for discrimination of genotypes with differential tolerance to salts (Munns *et al*., 2006).

Chlorophyll index as estimated in term of SPAD values was found effective especially for screening genotypes in many previous studies (Samadure *et al*., 2000; Munns and James, 2003), where a negative relationship was observed between SPAD values and Na^+ accumulation in leaves (Munns and James, 2003). Studies have also shown a linear relationship between SPAD values and maximum net photosynthesis rate in soybean (Ma *et al*., 1995), in rice (Laza *et al*., 1996) and in wheat (Gutierrez-Rodriguez *et al*., 2000).

Salt-induced proline accumulation in leaf was quite low in the salt sensitive genotype, while the tolerant genotypes accumulated proline at higher concentrations throughout the salt treatment as found in other studies by Sairam *et al*. (2002) and Shi and Sheng (2005). The increase in proline content was greater in older and more injured leaves, and occurred at later stages of leaf growth. Contrasting results were also found in sorghum by Lacerda *et al*. (2003). They observed a negative correlation between leaf proline accumulation and salt tolerance. The reason for this negative relationship could be the accumulation of proline may result

from tissue reaction during stress damage rather than a tissue response to salinity (Lacerda *et al*. 2003). A higher accumulation of organic solutes for osmotic adjustment and comparatively lower concentration of Na^+ and Cl^- ions in salt tolerant plant or varieties provide a suitable microenvironment within their cells that facilitate more efficient metabolism and relatively higher growth rates under saline conditions. These results showed that reduction in shoot development and leaf elongation due to salts was due to differential ion accumulation and distribution patterns within plant tissues during shoot and leaf development. Saline conditions intensify the senescence of older leaves but extent was relatively less in salt tolerant genotypes due to the ability to better control the absorption of ions and their transport to/within plant. Higher accumulation of proline also added in improving the salt tolerance in wheat genotypes by protecting protein turnover mechanisms and regulating stress protective proteins along with acting as an well-known osmoprotectant. It was true, especially in salt tolerant genotypes where its production was relatively high.

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