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Tissue architecture changes of expanding barley (*Hordeum vulgare* L.) leaf under salt stress

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Abstract

Salinity is an important external factor reducing shoot growth and consequently productivity of crops. The leaf cross-sectional area of barley during its development may reduce under salt stress due to architectural changes of leaves. To investigate the effects of salt stress on tissue architecture of barley leaf, a laboratory experiment was conducted hydroponically. Eight seedlings of barley plants were compared at two concentrations of NaCl (0 and 150 mM NaCl) in a completely randomized design in four replications. Under salt stress, mean leaf length of third leaf decreased 32% compared to control 14 days after germination (DAG). The highest elongation rate obtained at 14 and 15 DAG in control and salt stress, respectively. At 25 mm above the leaf base, 33% reduction in cross sectional area was observed under salt stress. Under salinity stress, mean leaf width decreased 45% along the leaf base compared to control. The leaf cross-section of control consisted of one midrib, four large and 13-21 small veins, while it included one midrib, five large and 6-12 small veins under salt stress. In both treatments, the number of small veins increased up to 25 mm above the leaf base and then decreased with distance from leaf base. Also, a considerable reduction was observed in total vein number under salt stress due to a reduction in the number of small veins. In both treatments, the size of midribs decreased sharply from 5 to 100 mm above the leaf base in spite of large and small veins. Likewise, salt stress reduced the area of midrib between 31 to 53% along the leaf axis. Overall, the reduced area of protoxylem and metaxylem in midrib and reduction in the number of small veins may be resulted to lower growth in the growth zone (25-50 mm above the leaf base) under saline conditions**.**

Keywords: Elongation rate, leaf width, metaxylem vessels, midrib area, small veins.
Abbreviation: ANOVA_analysis of variance; DAG_days after germination; Lv_large vein; MX_metaxylem; Mr_midrib; PBS_ phosphate buffered saline; PX_protoxylem; Sv_small vein.

Introduction

Salinity is an environmental stress that limits plant growth and productivity all over the world. This stress affects 7% of the world's land area which is equivalent to 930 million ha and the area in increasing (Szabolcs, 1994; Munns, 2002). Ghassemi et al. (1995) found that 6% of the global land area under cultivation had become saline over 45 years. This problem has become more severe in arid and semi–arid regions. Salinity induces specific physiological, morphological and anatomical changes in cell (Isla et al., 1998), or tissue and organ levels (Munns, 2002). Many studies have revealed that high salinity levels reduce the size of the vein segments and number of small veins (Hu et al., 2005), stomata number (Hwang and Chen 1995; Cavusoglu et al., 2007), and leaf thickness (Cavusoglu et al., 2008). Other structural changes such as inhibition of differentiation, diameter and number of xylem vessels also occur in salt stressed plants (Ola et al., 2012). Barley (*Hordeum vulgare* L.) is a major crop in countries where saline soils exist or may develop (Fricke and Peters, 2002). However, the effects of salt on tissue architecture of barley leaves are still poorly understood. Similar to other cereals, leaf growth at early stages of barley development largely determines the rate of plant growth (Emam, 2007). Under saline conditions, smaller final leaf size is not only due to a shorter leaf length, but also to a narrower leaf (Hu et al., 2005). Likewise, Hu et al., (2000a) reported that the length and width of wheat leaves on the main stem reduced approximately by 20–30%, and width reduction mainly occurred at the leaf base when plants were exposed to 120 mM NaCl. Hu and Schmidhalter (2000) estimated the effect of salinity on leaf width and leaf cellular cross sectional area using leaf water content. However, these changes were not explicable. Leaf growth in barley is limited to a small region near the leaf base or the older leaf sheath (Emam, 2007). The leaf length in grasses increases only because the width remains unchanged after leaf emergence (Dale, 1988). Rademacher and Nelson (2001) declared that the increase in the cross-sectional area along the leaf axis of growing leaves in tall fescue is most rapid near the base, and is independent of genotype and nitrogen amount. According to Hu and Schmidhalter (2000 and 2001), the inhibition of growth in the cross-sectional area or width in a growing wheat leaf by salinity occurs mainly between the leaf base and 5 mm above the base. The vascular tissue of cereals' leaves such as barley consist of a series of roughly parallel longitudinal vein segments that are grouped into dermal, ground and vascular tissues associated with a vein in the cross-section. Each vein segment consists of different types of cells, such as epidermal cells, mesophyll cells, xylem and phloem cells, and intercellular space (Cavusoglu et al., 2007). Hu et al. (2005) showed that a total of 34 veins are present in a mature wheat leaf (cv. Thasos) in the region between the ligule and 15 cm above it. Of these, 11 were of large or medium size. Kuo et al. (1974) reported that 11 large and medium-size veins in wheat would account for 96% of the total water flow in the cross-section. Trivett and Evert (1998) mentioned that first, large and medium veins are initiated in barley and differentiate acropetally; small veins are initiated later in development and differentiate basipetally. Most of axial water transport through the elongation zone occurs through protoxylem vessels. In maize, large and fastconducting metaxylem vessels are found only in mature tissues and beyond the distal end of the elongation zone (Tang and Boyer, 2002). Martre et al., 2000 declared that the switch from a low-conducting protoxylem path (elongation zone) to a high-conducting metaxylem path (maturation zone) may explain the 10-fold lower axial hydraulic conductance in the elongation zone compared to the mature zone of blade. Knowledge of the effect of salinity on the number of large or small veins will contribute to a better understanding of the processes of leaf development and characteristics of water transport. However, little information is available about how the veins in barley leaves respond to salinity or other stresses. Studying the relation between the cross-section of grass leaves and the leaf architectural changes under saline conditions will provide an opportunity to obtain new insights into the mechanisms of salt limitation to plant growth because the physiological functions of the leaf are linked to its architectural properties. The main objective of this study was to determine the effect of salinity on spatial distribution of components in a cross-section of the growing leaf axis including number of veins, the area of midrib, large and small veins, and protoxylem and metaxylem vessels of barley leaf (cv. Valfajr).

Results and Discussion

Leaf length and leaf elongation rate of barley

The results of non-destructive daily leaf length measurements under control and salt stress (150 mM NaCl) are given in Fig 1a. Mean leaf length of the third leaf decreased 33% under salt stress 14 DAG compared to control. Under control treatment, the leaf length started to increase from 14 DAG and reached to maximum at 16 DAG while under salt stress, maximum leaf length was observed at 17 DAG. In a similar study, Fricke et al., (2006) observed leaf elongation rate decreased 0.11 mm/hr or 6% less at 11 DAG than control and the elongation rate suddenly recovered to 1.13 mm/hr at 13 DAG, when the barley (cv. Golf) exposed to 100 mM NaCl. Vysotskaya et al., (2010) showed that the mean third leaf length declined 20% compared to control in wild barley (*Hordeum spantaneum*) under 75 mM NaCl, and the leaf length started to increase at 12 DAG and maximized at 15 DAG. They concluded that the type of cultivar, salinity level and seedling growth stage had the main role in salt tolerance of wild barley. The highest leaf elongation rate obtained at 14 DAG (1.2 mm/hr) and 15 DAG (0.72 mm/hr) in control and salt stress, respectively (Fig 1b).When the genotype was exposed to 150 mM NaCl, elongation rate of third leaf decreased 52% at 14 DAG compared to control. Generally, development of the third leaf is delayed one day under salt stress. The decreasing effect of salt stress on barley leaf growth is similar to its effect on maize (Neves-Piestun and Bernstein, 2001), sorghum (Bernstein et al., 1993).

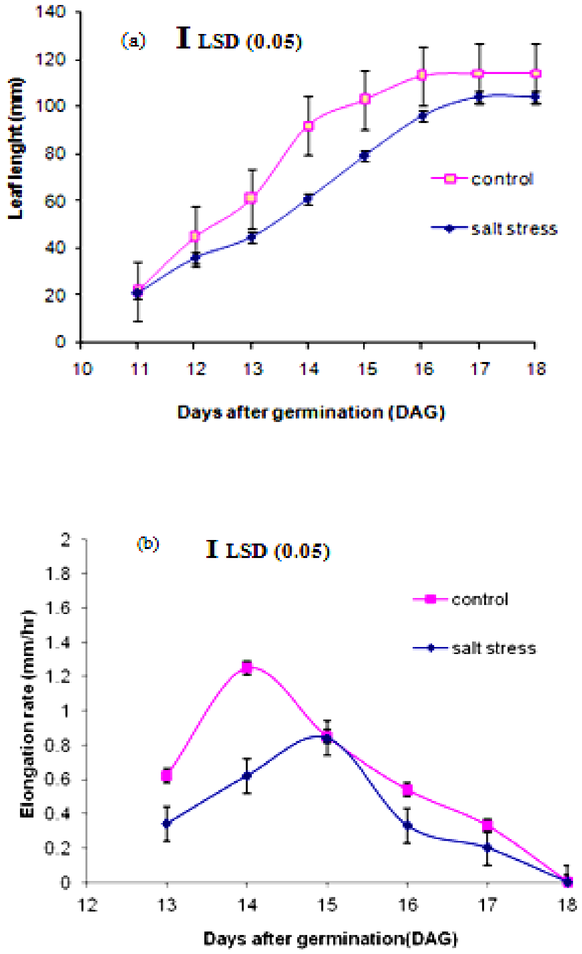


Fig 1. Effect of 150 mM NaCl on leaf length (a) and elongation rate (b) of barley third leaf. Data represent results of non-destructive daily leaf length measurements. Vertical bars represent standard error and data was compared using LSD at 5% probability level.

Leaf tissue architecture

The tissue and vascular architecture of barley third leaf showed using light micrographs of cross-sections at 5, 25, 50, 75, and 100 mm above the leaf base for control and 150 mM NaCl treatments in Fig 2 and 3. The following results were obtained from these measurements.

Cross sectional area and width leaf

In both treatments, cross sectional area increased with a distance up to 25 mm above the leaf base and then decreased up to 100 mm above the leaf base (Figs 2 and 4a). Also, at 25 mm above the leaf base, 33% reduction in cross sectional area was observed under salt stress compared to control (Fig 4a). In both treatments, the pattern of spatial distribution of width along the leaf axis was similar to that of cross sectional area. Maximum leaf width was observed at growth zone (25 mm above the leaf base; Fig 4b). Under salt stress, leaf width decreased 48, 40, 36, 50 and 54% in 5, 25, 50, 75, and 100 mm above the leaf base of third leaf compared to control, respectively.

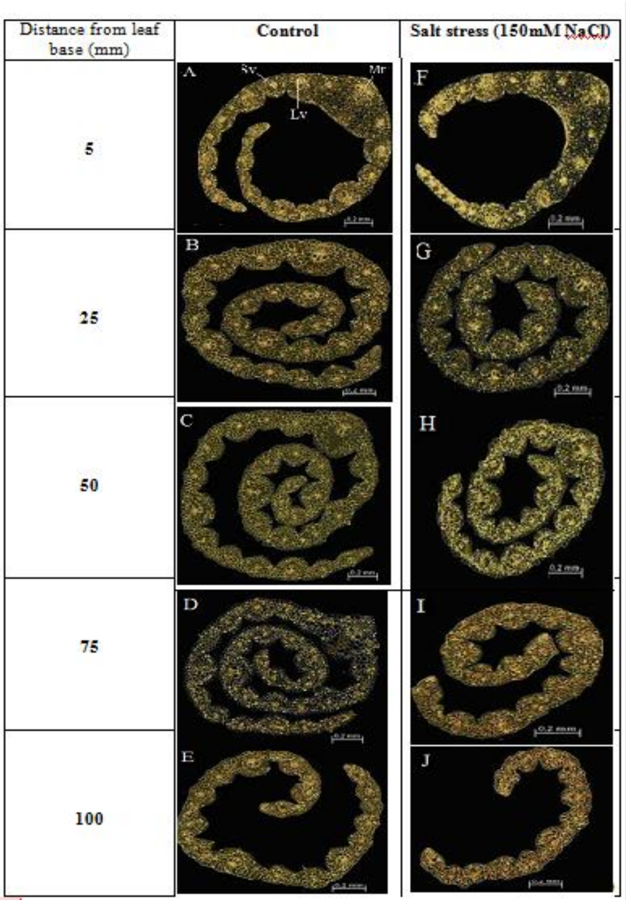


Fig 2. Cross-sections micrographs of barley third leaf at 5 mm (start of elongation zone; A, F), 25 mm (middle of the growth zone; B, G), 50 mm (end of the growth zone; C, H), 75 mm (zone of secondary cell wall deposition; D, I), and 100 mm (photosynthetic tissues; E, J) above the leaf base for 0 (left) and 150 mM NaCl (right) treatments. The structures of midrib, large vein and small vein have been indicated with arrows in cross section. Mr: midrib, Lv: large vein, Sv: small vein. Scale bar is 0.5 mm.

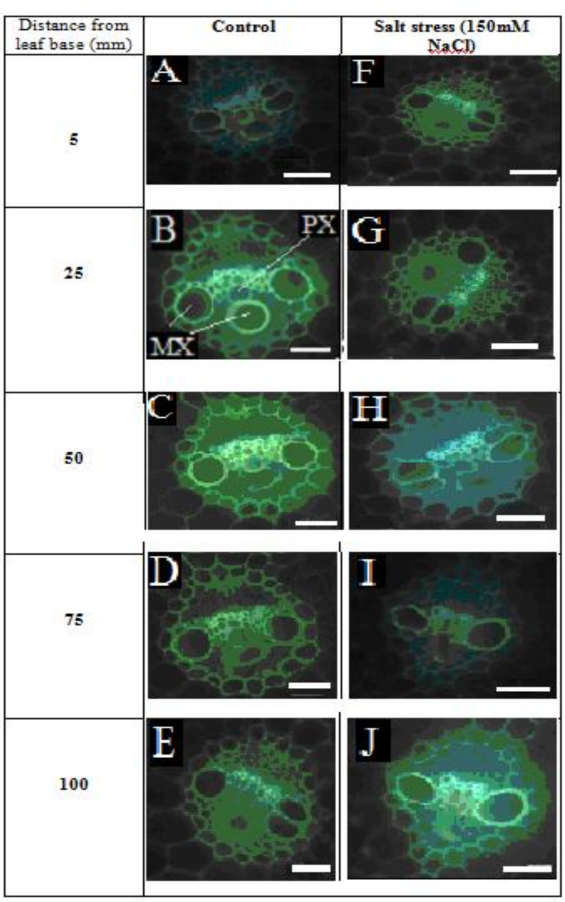


Fig 3. Cross-sections micrographs of xylem vessels including metaxylem and protoxylem vessels for third leaf of barley at 5 mm (start of elongation zone; A, F), 25 mm (middle of the growth zone; B, G), 50 mm (end of the growth zone; C, H), 75 mm (zone of secondary cell wall deposition; D, I), and 100 mm (photosynthetic tissues; E, J) above the leaf base for control (left) and 150 mM NaCl (right). MX: metaxylem, PX: protoxylem. Scale bar is 0.5 mm.

In a similar study, Hu et al., (2005) attributed the reduction in cross-sectional area to a decrease in the size of the vein segments and a reduced number of medium and small veins of wheat leaf. They approximately observed 35% reduction in the number of veins under saline conditions (mainly in the number of small veins) which likely suggest reduction in the capacity of re-translocation of mineral nutrients and assimilates due to salinity. Hu and Schmidshalter (2000) observed cross sectional area or width along the leaf axis mainly at the leaf base and elongation zone. This indicates that salinity affects the initiation of leaf cross section, possibly by altering the processes of recruiting founder cells from the shoot apical meristem. Taleisink et al., (2009) stated that tissue expansion and cell division in monocotyledons during leaf development tend to become unidirectional, resulting in strap-like organs. Most studies have focused on leaf length expansion under salinity in grasses, although narrower leaves are common under saline conditions and reduction in cross section also occurs at the leaf base (Hu et al., 2005; Taleisink et al., 2009). Smith and Hake (1992) reported that the orientation of cell division is primarily responsible for the increase in leaf width at early leaf development. In a study on sorghum (*Sorghum bicolor*), Baum et al., (2000) reported that salinity stress caused narrower midribs and smaller leaf width and cross sectional area. Under these conditions, only half of the veins were functional in water transport.

Number and area of midrib and veins

The cross-sectional area of a leaf is dependent on both the number of veins and the size of vein section (Fig 2). The total number of veins includes midrib and all large, medium and small veins. Figs 5 and 6 show only the trend of spatial distribution of cross-sectional areas of the midrib, and large and small vein sections along the leaf axis. This is because leaf cross-sections of control and salt stress treatments did not contain medium veins and the size of the medium vein segment was similar to that of the small vein segments. Except the medium veins, the leaf cross-section of the control consisted of one midrib, four large and 13-21 small veins, while it included one midrib, 5 large and 6-12 small veins

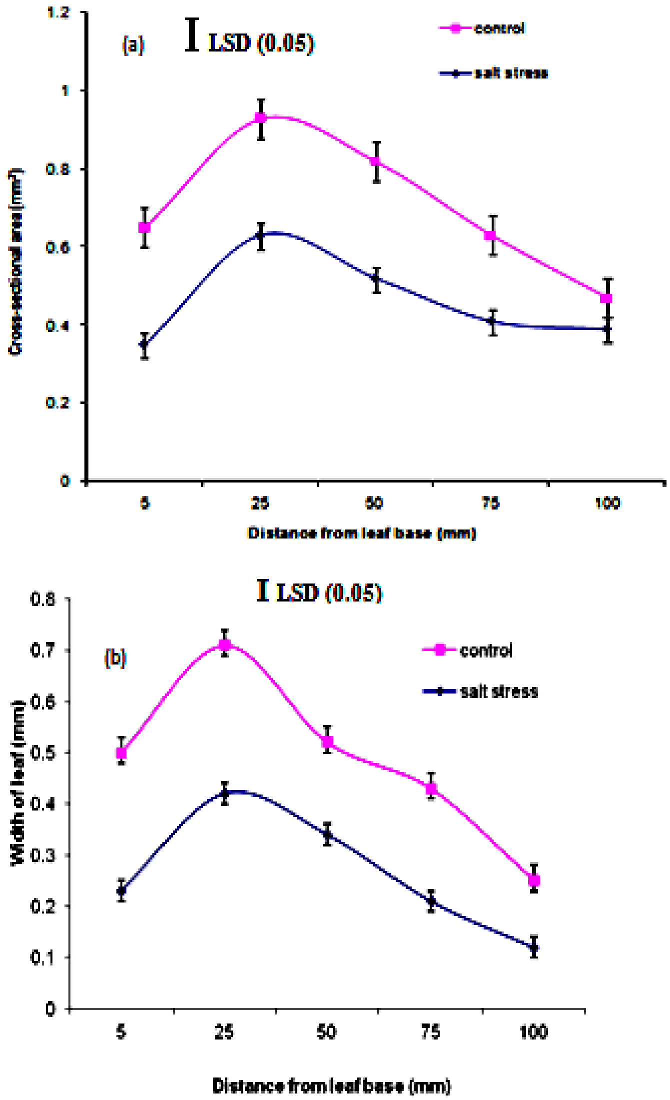


Fig 4. Distribution of the cross-sectional area (a) and width (b) of barley third leaf grown under 0 and 150 mM NaCl treatments. The measurements were based on light micrographs.Vertical bars represent standard error and data was compared using LSD at 5% probability level.

under salt stress (Fig 5). A significant reduction was observed in total vein number due to a reduction in the number of small veins (Fig 5). The number of large veins did not change with distance for neither of treatments. In both treatments, the number of small veins increased up to 25 mm above the leaf base and then decreased with distance from leaf base. Also, a considerable reduction was observed in total vein number due to a reduction in the number of small veins. Hu et al., (2005) reported that the leaf cross-section of control in wheat consisted of one midrib, four large and 14–23 small veins in addition to medium veins whereas it contained one midrib, five large and 11–15 small veins (totalling 16–20 veins) for 120 mM NaCl treatment. This indicated a 30% reduction in the number of small veins under salt stress. In our study, 47% reduction was observed in small veins under salt stress. Trivett and Evert (1998) reported that, large veins are initiated and differentiated acropetally in monocotolydons, after which medium and small veins are initiated basipetally. Thus the greater reduction in the number of small veins suggests that salinity affects the development of cross section well after the formation of large veins. In a similar study, Kuo et al., (1974) reported that the total number of veins in the transverse leaf section was about 32 in control plants and remained constant up to 25 mm above the leaf base. Under both treatments, the size of the midrib decreased sharply from 5 to 100mm above the leaf base in spite of large and small veins (Fig 6). Also, salt stress reduced the area of the midrib between 31 to 53% along the leaf axis. In both treatments, the maximum size of large vein was observed 25 mm above the leaf base and decreased up to the end of the growth zone. The size of small vein increased up to 50 and 75 mm for the control and salt stress treatments, respectively whereas there was no difference for small vein segments between these treatments.

Area of metaxylem and protoxylem of large veins

Fig 7 shows the areas of metaxylem and protoxylem from a large vascular bundle in the third leaf of barley grown under control and 150 mM NaCl**.** In control, the area of metaxylem in large veins maximized approximately 25 mm above the leaf base and decreased sharply up to 100 mm under non-salinity conditions whereas maximum area of metaxylem was observed at 50 mm above the leaf base and decreased slightly up to 100 mm (Fig 7a). Under salt stress treatment, both leaf axis and metaxylem area reduced between 41 to 70% compared to control. Protoxylem area in large veins decreased along the leaf base, with a distance from the leaf base (Fig 7b). Overall, comparison of control and salt stress treatments showed that the reduction in protoxylem area was greater at 5mm than 100 mm above the leaf base. In contrast, Baum et al., (2000) reported that salt treatment had no effect on the protoxylem area of sixth leaf. Hu et al., (2005) reported that the reduced area of protoxylem and metaxylem in midrib and large vein segments in growing tissues may be responsible for lower shoot growth under saline conditions. Our results were in disagreement with those reported by Ola et al., (2012) in Kallar grass (*Leptochloa fusca* L. Kunth) who reported that metaxyleme area and number of veins were not affected by salinity up to 100 mM NaCl. Generally, some researchers suggested that tissue architecture changes depend on salinity level and type of crop and cultivar (Hu and Schmidhalter, 2000; Martre and Durand, 2000; Munns, 2002).

Materials and Methods

Plant material

The effect of salt stress on the leaf tissue architecture of barley (*Hordeum vulgare* L.) was studied in Valfajr cultivar, hydroponically. After soaking seeds in distilled water for one day, they were floated in 1.0 mM $CaSO_4$ solution for three days until germinated.

Hydroponic culture

The germinated seeds were transferred into a hydroponic system, containing a modified half-strength Hoagland nutrient solution [KH_2PO_4 (1.5 mM), KNO_3 (2.0 mM), $CaCl_2$ (1.0 mM), $MgSO_4$ (1.0 mM), $FeNa_2$ (18.0 µM), H_3BO_3 (8.1 µM), $MnCl_2$ (1.5 µM)] (Fricke et al., 1997). Four seedlings

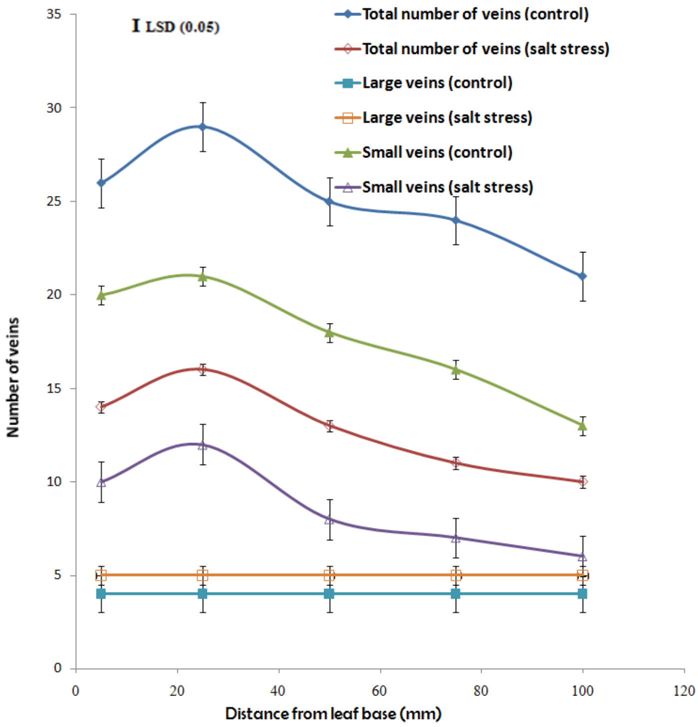


Fig 5. The numbers of veins in third leaf of barley grown under 0 and 150 mM NaCl. The measurements were based on the light micrographs. Vertical bars represent standard error and data was compared using LSD at 5% probability level.

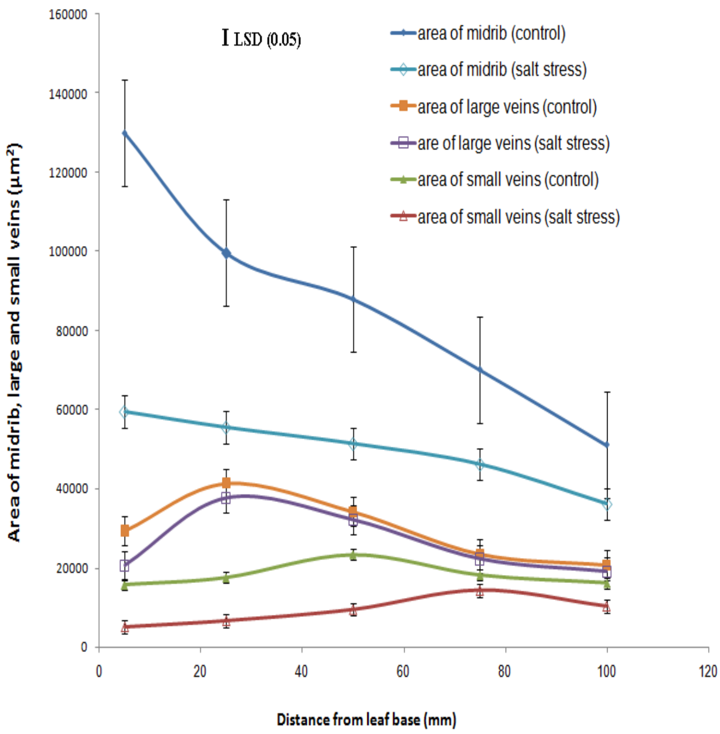


Fig 6. The area of a midrib, large vein and small vein segment in third leaf of barley grown under 0 and 150 mM NaCl. The measurements were based on light micrographs. Vertical bars represent standard error and data was compared using LSD at 5% probability level.

were grown in 1-liter glass beakers of nutrient solution ventilated by a gas exchange pump at a flow rate of 400 mL min^{-1} in growth chamber at 16/8 hours day/night photoperiod , temperature cycle of 21/15°C, and relative humidity of 70%. Plants were sampled 18 days after germination (DAG) corresponding to the three leaf stage (ZGS13, Zadoks et al., 1974).

Determination of leaf length and elongation rate

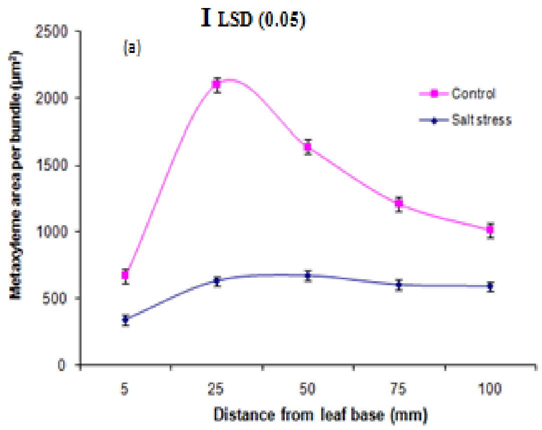
Leaf length and elongation rate were measured daily and expressed to the nearest 0.5 mm from the base of the third leaf to the tip of the leaf according to Bernstein et al., 1993.

Tissue sampling

The third leaves of about 11-15 cm in length were selected for tissue sampling (Fig 8). Leaf elongation is approximately steady during this stage (Hu et al., 2000a). The elongating leaf was carefully freed from the surrounding leaf sheaths, and then cut from the base with a razor blade. The leaf was sectioned into five 5-mm-long segments above the base: from the leaf base (5 mm above the leaf base), at the middle of the growth zone (25 mm), at the end of the growth zone (50 mm), in the zone of secondary cell wall deposition (75 mm), and photosynthetic tissues (100 mm); definition of these zones is based on the study by Hu et al., (2000b) (See Fig 8). Sampling was done quickly (within 2-3 min) under low light intensity to prevent disturbances in water status of tissue. After sampling, segments were immediately transferred to phosphate buffered saline (PBS) supplemented with 3% formaldehyde and incubated overnight. Then samples were washed in PBS and dehydrated in a graded series of ethanol (Hu et al., 2005). After embedding in white acrylic resin, semi-thin sections at 5, 25, 50, 75 and 100 mm above the leaf base were cut with a razor blade and stained with Toluidine blue for one minute (Brundrett et al., 1988; Hachez et al., 2006) and leaf components were detected under bright light microscope (Canon 2022, Japan). All measurements were performed in three replicates. The longitudinal veins in barley leaves were classified as large, intermediate and small. However in this study, to include both their appearance and functions, these were referred to as ‘‘large vein segments’’, ‘‘medium vein segments’’ and ‘‘small vein segments’’, respectively. These veins are characterized by differential presence of metaxylems and protoxylems. Large veins have a large metaxylem vessel on either side of the protoxylem while medium veins have smaller metaxylem, and small veins lack protoxylem and the metaxylem vessels. Light micrographs showing cross-sections of vascular vessels and xylem vessels (Fig 2 and 3). According to light micrographs, the number and the area of midrib, large, medium and small veins were determined. Also, the areas of vascular bundles were determined for large and small veins and areas of metaxylem and protoxylem in a large vein.

Statistical analysis

Eight seedlings of barley plants at two concentrations of NaCl (0 and 150 mM NaCl) in four replications were compared in a completely randomized design. Data were



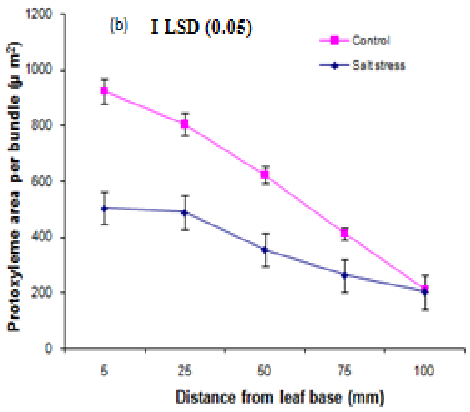


Fig 7. The areas of the metaxylem (a) and protoxylem (b) from a large vascular bundle in third leaf of barley grown under 0 and 150 mM NaCl. The measurements were based on light micrographs. Vertical bars represent standard error and data was compared using LSD at 5% probability level.

subjected to analysis of variance (ANOVA) and the means were compared using LSD test ($p \leq 0.05$) by SAS software.

Conclusion

Restriction of leaf growth is among the earliest visible effects of many stress conditions, including salinity. Because leaves determine radiation interception and are the main photosynthetic organs, salinity effects on leaf expansion and function are directly related to yield constraints under saline conditions. It can be concluded that the reduction in the cross-section of barley is mainly due to a decreased number of small veins. On the other hand, the reduced area of protoxylem and metaxylem in midrib and veins especially at 0-25 mm from the leaf base may be responsible for lower growth under saline conditions. Because the architectural properties of the leaf vein system are related to physiological leaf functions, further work is needed to determine the water flow of nutrients in relation to leaf anatomical structure in growing leaves using different barley cultivars and salinity levels.

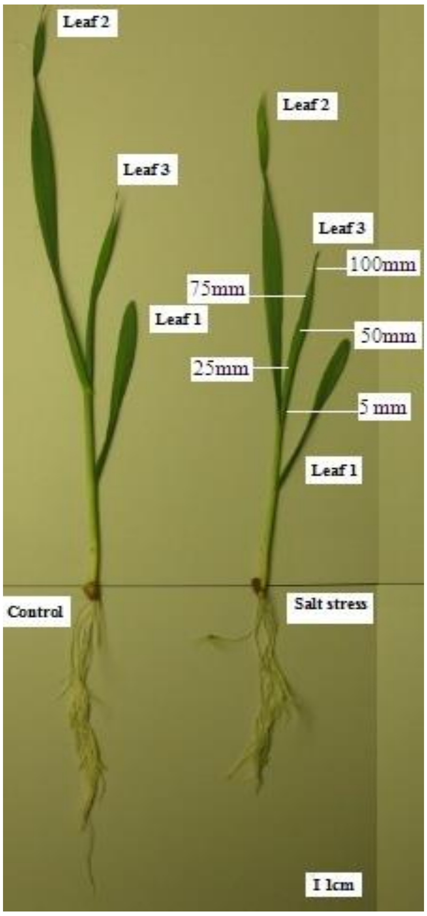


Fig 8. Seedling of the barley (Valfajr cultivar) at the third leaf stage grown under 0 and 150 mM NaCl conditions. Scale bar is 1cm. Above the third leaf base, the leaf was sectioned into five segments: from the leaf base (5 mm above the leaf base), at the middle of the growth zone (25 mm), at the end of the growth zone (50 mm), in the zone of secondary cell wall deposition (75 mm), and photosynthetic tissues (100 mm).

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