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IN VITRO AND GREENHOUSE RESPONSE OF DIFFERENT WHEAT LINES AGAINST SALINITY STRESS

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ABSTRACT

The current study provides information on the response of different wheat lines to salinity stress under both in vitro and green house conditions. The root and shoot response of three different wheat varieties, Siran, Atta-Habib and Pir-Sabaq was evaluated against salt stress under *in vitro* condition. In the next phase, the same lines were grown under greenhouse conditions and the response was studied on morphological and biochemical traits. Under *in vitro* conditions, the Siran variety performed better than Atta-Habib in terms of shoot and root morphological data. Under green house condition, the Siran variety showed significantly high ($P \leq 0.05$) root length and number, fresh and dry mass, and moisture content than that of Atta-Habib and Pir-Sabaq under salt stress (100 mM NaCl). The Siran variety maintained significantly ($P \leq 0.05$) high chlorophyll and proline content than that of Atta-Habib and Pir-Sabaq under salt stress. The tolerance mechanism in the Siran variety is proposed to be the high chlorophyll and proline content under salt stress. These results also suggest that *in vitro* screening provides useful preliminary information for evaluation of salt tolerance in different varieties of wheat.

Key words: Salt stress, *in vitro* conditions, wheat varieties, physiological effects.

INTRODUCTION

Wheat is the leading grain crop and ranks first among the cereal crops in Pakistan (Hussain *et al*., 2013). Several high yielding and tolerant varieties have been developed through the breeders efforts and are being extensively grown throughout the country including irrigated and non-irrigated areas of the Khyber Pakhtunkhwa province (Muhammad *et al*., 2010).

In Pakistan the total area occupied by wheat during 2011-2012 was 8.70 million hectares with estimated production of 23.50 million tonnes and average yield of 2833kg/hectares (Govt of Pakistan, 2012). In the KPK, wheat was grown on 724.5 thousand hectare with a total production of 1155.8 thousand tonnes giving an average of only 1595 kg/ha (Agriculture Statistics of Pakistan, 2010-2011). Due to increasing population and the growing dependency on land and water resources, the demand for wheat is increasing day by day. Despite of immense importance of wheat, its per hectare yield is below than its yield potential, which might be due to several reasons like, shortage of good quality water, poor nutrients management, poor weeds and pest management, water logging, drought, and salinity (Jamal *et al*., 2011).

Salt stress is one of the most prevalent abiotic stresses in the world that negatively impacts plant growth (Munns and Tester, 2008; Khan, 2011). The saline area is three times larger than the land used for agriculture (Munns and Tester, 2008). Salinity has been a serious problem in several parts of the world including Pakistan (Mehmood *et al*., 2009). In Pakistan alone, the total land lost due to salinity is around 40,000 hectares (Ashraf *et al*., 2008). Salt stress imposes a major environmental threat to agriculture by limiting plant growth and reducing crop yield. In the next 25 years, the increasing salinization of arable land will result into 30% land loss (Wang *et al*., 2003). Therefore the efforts to find salt tolerant varieties of different crops are important for sustainable agriculture. Like other crops, wheat is adversely affected by salt stress resulting huge losses in productivity around the world (Saboora and Kiarostami, 2006; Mehmet *et al*., 2006). Therefore, the conventional biotechnology techniques coupled with more advanced methods are required to screen the natural salt tolerance in the existing wheat germplasm.

Prolonged exposure to salt stress may affect plant growth and productivity by hampering the key physiological and biochemical pathways such as photosynthesis, respiration, nitrogen fixation and carbohydrate metabolism (Azizpour *et al*., 2010). High levels of soil salinity can significantly inhibit seed germination and seedling growth, due to the combined effects of high osmotic potential and specific ion toxicity. Seed germination and early seedling growth are the most sensitive stages to salinity stress (Muhammad and Hussain, 2010). *In vitro* tissue culture conditions provide the easy, reliable and speedy means for preliminary germplasm screening for salt stress responses at the seedling and germination stages (Aghaei *et al*., 2008).

The aim of the present research work was to investigate the response of different wheat lines/varieties in terms of morphological and biochemical changes against various concentrations of salt (NaCl) stress under *in vitro* and greenhouse conditions.

MATERIALS AND METHODS

The research was conducted at Recombinant DNA Technology Laboratory, Institute of Biotechnology and Genetic Engineering (IBGE), The University of Agriculture Peshawar, during 2013-2014.

Sterilization method

The sterilization method was followed as described by Sauer and Burroughs (Sauer and Burroughs, 1986). All seeds were washed with tap water, and then with 70% ethanol. Seeds were then washed with 30% bleach and then with distilled water.

Plant material

For *in* vitro study, seeds of wheat varieties i.e. Siran, Atta-Habib and Pir-Sabaq were grown on MS media Murashige and Skoog, 1962), and the root and shoot morphological data was recorded.

In vitro experiment

In the laminar flow chamber, the sterilized seeds were put in the test tubes with MS media provided with (0 mM and 100 mM NaCl). Test tubes with seeds were closed with cotton buds and were placed in the growth chamber under controlled light and temperature conditions. For this experiment, 10 test tube plants per variety were kept for each salt concentration. After two weeks of seed culturing on the media, the individual plants were taken out of the test tubes. Analysis of variance (ANOVA) was applied to determine the effect of salt stress and varietal differences on the tested parameters. The experiment was repeated three times.

Greenhouse experiment

Seeds of all the three wheat varieties were grown in soil pots in a Complete Randomized Design (CRD) in the green house during the month of February. Two salt concentrations i.e. control (0 mM) and high salt stress (100 mM) was set for the experiment under greenhouse condition. Ten pots were used for each variety in which five pots were used for control (0mM NaCl) and five for 100 mM NaCl concentrations. Pots were prepared and equal number of seeds per variety was sown. The soil pots were watered with normal tape water for some days until the seeds germinated and reached to 5-7 inches in height. After early seed germination and attaining a plant height up to 5-7 inches, the salt stress evaluation was started. The salt test was continued for three months (March to May). The control (0 mM) plant pots were watered with normal tape water. The other set of plants were watered with saline water (100 mM NaCl) with intervals at the rate of I irrigation/week. Soil electric conductivity was measured. Initial ECe was 3.5 ds/m. By applying 100 mM NaCl, the ECe value of the soil was recorded in the range of 4.5-5.0 ds/m. ANOVA was applied to analyze the effect of salt stress and varietal differences on the tested parameters. The experiment was repeated three times.

Root and shoot data

In the greenhouse experiment, the three months old plants in soil pots were socked with water and the soil was carefully removed. Roots were washed with tape water and then the root morphology was studied. Total number of roots per plant was counted and the total average length was calculated. The data was analyzed statistically. For fresh and dry mass determination of the whole plant, the individual plants per treatment per replication were weighed, air dried and then placed in an oven at 70^{o}C for 24 h as described by Windham *et al*. (1987). Average fresh and dry mass were then calculated by weighing the samples in an electronic balance. The average values were then compared statistically.

Moisture content

The moisture content was determined by an oven dry method (Windham *et al*., 1987). Total plant samples were weighed before oven drying. After complete drying at 70^{o}C for 24 h, the moisture content in individual plant samples was determined with the following equation. Average values were compared statistically and expressed as percentage by weight.

Moisture Content = Wet Weight – Dry Weight (Yang and Miyazaki, 2003).

Proline content

For proline extraction, the protocol developed by Bates *et al*. (1973) was used. A sample of 500 mg of frozen plant material was homogenized with 3% aqueous sulfo- salicyclic acid and then centrifuged at 9000 g. After that supernatant was collected and 1 ml of acid ninhydrin and glacial acetic acid was added to 250 µl of extract. The sample was then boiled at 100ºC for 1hr in water bath; the reaction was terminated on ice. For extraction 4 ml of toluene was added and optical density of proline was measured at 520 nm. From standard curve in the range of 0-20 ug/ml of L-proline the amount of proline was determined.

Chlorophyll content

Leaf material (0.1-0.3g) was taken from sample. Liquid nitrogen was used for crushing and then extraction for 15 minutes on ice in 700 uL of 80% acetone, cell debris was layered by centrifugation at 18000 g at 4ºC for 10 minutes then transfer upper layer to new tube. The pellet was re-suspended in same volume of 80% acetone as before, mix thorough vortexing and after sedimentation of cell debris using centrifuge machine, the supernatant was combined with first extraction. Using photometer, the OD of extract (against 80% acetone) was determined at 645 nm and 663 nm. Total chlorophyll in mg/g FW was calculated according to the (Litehtenhaler and Wellburn, 1983) formula.

$$C = 20.2*OD645+8.02*OD663$$

RESULTS

In vitro experiment

Seed germination was observed only in Siran and Atta-Habib varieties in both control and salt stressed plants. Seeds of Pir-Sabaq variety failed to germinate under *in vitro* conditions due to unknown reasons.

Siran and Atta-Habib varieties performed better in terms of root and shoot growth under salt stress. Siran variety showed higher ($P \leq 0.05$) shoot growth than that of Atta-Habib (Table-1). On MS media with 100 mM NaCl concentration, Siran and Atta-Habib attained about 22 and 8 cm average shoot length, respectively. Under control condition, root length was significantly ($P \leq 0.05$) higher (16.3cm ± 2.0) in Siran than that of Atta-Habib (Table-1). Salt stress (50 and 100 mM) reduced root length in Siran than that of Atta-Habib. Under 100 mM salt concentration, Siran exhibited significantly ($P \leq 0.05$) high (7.3cm ± 0.8) root number.

In this *in vitro* study, Siran variety showed salt tolerance in terms of better root and shoot growth as compared to that of Pir-Sabaq. Under 100 mM salt concentration, the shoot length in Siran (22.5cm ± 1.5) increased significantly ($P \leq 0.05$) than at control and 50 mM salt stress. On the contrary, the shoot length in Atta-Habib significantly ($P \leq 0.05$) decreased under 100 mM than control and 50 mM concentrations. The Siran variety might have attained comparatively high salt tolerance and shoot growth by efficiently overcoming the osmotic stress imposed by the increasing salt concentration in MS media.

Table 1. Analysis of shoot and root morphology of wheat varieties subjected to salt stress under *in vitro* conditions.

Varieties	Salt concentrations used	*In vitro* growth parameters		
		Shoot length	Root length	Root number
Siran	0 mM	16.3 ± 2.0^{b}	5.7 ± 1.0^{a}	4.8 ± 0.8^{b}
	50 mM	15.9 ± 2.3^{b}	3.4 ± 0.9^{b}	3.8 ± 0.6^{c}
	100 mM	22.5 ± 1.5^{a}	2.1 ± 0.6^{c}	7.3 ± 0.8^{a}
Atta-Habib	0 mM	16.0 ± 1.0^{b}	$2.3.\pm 0.7^{c}$	5.3 ± 1.0^{b}
	50 mM	17.5 ± 2.5^{b}	3.0 ± 0.8^{b}	5.3 ± 0.8^{b}
	100 mM	9.5 ± 2.0^{c}	2.1 ± 0.6^{c}	4.6 ± 0.5^{b}

Mean values within the same column with different letters are significantly ($P \leq 0.05$) different from one another using LSD; The ± sign shows standard deviation values.

Greenhouse experiment

In the greenhouse experiment, the physiochemical properties of the soil used is summarized (Table-2). Soil was composed of sand, silt and clay in a ratio of 35, 32 and 33 grams respectively, of the total 100 g soil per pot. In this experiment, wheat varieties showed different responses to control and 100 mM salt concentrations. Shoot length increased in all varieties under 100 mM salt concentration as compared to control (Table-3). Siran showed comparatively high shoot length (45.3± 4.4) under control and 100 mM than that of Pir-Sabaq. For some crop plants, it has been observed that medium salt concentration up to 50 mM may increase root and shoot length as an adaptation response (Rewald *et al*., 2013). But when salt concentration increases to high levels (100 mM and above), may drastically affect the shoot and root length by physiological and biochemical changes (Rewald *et al*., 2013). It was observed that Pir-sabaq variety showed lower shoot length as compared to that of Siran and Atta-Habib under both control and 100 mM concentrations. Pir-Sabaq variety was less responsive under *in vitro* condition and had low salt tolerance that resulted reduction in vegetative growth under greenhouse condition.

Table 2**.** Physiochemical properties of soil used in greenhouse experiment.

Soil pH	Soil color	Soil texture	% Quantity
5.8	brown	sand	35
		silt	32
		clay	33

Under control condition, no significant difference was observed for the three varieties for root length (Table 3). However, root length increased (9.3cm ± 2.0) for Siran and Atta-Habib while non-significantly for Pir-Sabaq under 100 mM. Similar trend was observed in case of root number. Siran showed significant increase in root number as compared to that of Pir-Sabaq (Table 3). In this context, the two varieties Siran and Atta-Habib have good response to salinity as compared to Pir-Sabaq.

Higher fresh mass was observed in Siran as compared to Atta-Habib and Pir-Sabaq under both control and 100 mM salt concentrations (Table 3). Maximum dry weight was observed in Siran as compared to that of Atta-Habib and Pir-Sabaq (Table-3). Siran showed higher moisture content (10%) than that of Atta-Habib (7.5%) and Pir-Sabaq (5.1%) under control (0 mM NaCl) (Table 3). High salt stress (100 mM NaCl) significantly reduced moisture content in Atta-Habib (6.2%) and Pir-Sabaq (4.7%). However, the Siran variety showed comparatively high moisture content (9%) even at high salt stress. Overall, the Siran variety maintained maximum moisture content under both control and salt stress conditions compared to the other two varieties (Table 3).

Chlorophyll content

Under control (0 mM NaCl) conditions, the total chlorophyll content was observed in the range of 1.4-1.6 mg. g^{-1} fresh weight for all the three wheat varieties. In comparison to Atta-Habib and Pir-Sabaq, the Siran variety showed high proline content (1.6 mg g^{-1} FW) even under control conditions (Table 3). When plants were subjected to salt stress (100 mM NaCl), the total chlorophyll content decreased in all the three varieties. However, the Siran variety maintained significantly ($P \leq 0.05$) higher chlorophyll content (1.4 mg g^{-1} FW) than that of Atta-Habib (0.83 mg g^{-1} FW) and Pir-Sabaq (0.53 mg g^{-1} FW).

Proline content

All the three varieties showed proline content in the range of 3.5-4.3 µM g^{-1} FW (Table 3). Under control conditions, Siran showed 4.2 µM g^{-1} FW proline content, while Atta-Habib and Pir-Sabaq accumulated 4.3 and 3.5 µM g^{-1} FW proline, respectively. No significant variation was observed for proline content among the three varieties under control conditions. However, when the plants were exposed to salt stress (100 mM NaCl), drastic variation in proline content was observed. The Siran variety showed significantly ($P \leq 0.05$) high proline content (5.8 µM g^{-1} FW) than that of Atta-Habib (4.6 µM g^{-1} FW), and Pir-Sabaq (3.8 µM g^{-1} FW).

Table 3. The effect of different salt concentrations on various morphological and physiological parameters of wheat varieties grown under greenhouse conditions.

Varieties	NaCl (mM)	Shoot length (cm)	Root length (cm)	Root number	Fresh mass (g)	Dry mass (g)	Moisture content (%)	Chlorophyll Content (mg g^{-1} FW)	Proline Content (μM g^{-1} FW)
Siran	0	38.5±5.0	3.6±0.5	10.0±1.7	1.2±0.2	0.2±0.04	10.0±0.2	1.6±0.15	4.2±0.6
	100	45.3±4.4	9.3±2.0	11.3±1.8	1.3±0.2	0.27±0.03	9.0±0.12	1.36±0.12	5.8±0.62
Atta-Habib	0	36.0±6.0	3.6±1.0	7.0±1.8	0.9±0.2	0.15±0.03	7.5±0.09	1.4±0.1	4.3±0.8
	100	39.5±5.2	6.6±1.5	9.3±1.9	0.8±0.1	0.17±0.04	6.2±0.08	0.83±0.2	4.6±0.85
Pir-Sabaq	0	29.6±4.7	4.5±1.0	4.6±0.7	0.7±0.2	0.18±0.03	5.1±0.12	1.43±0.05	3.5±0.45
	100	37.0±6.1	5.2±0.6	6.0±1.0	0.6±0.2	0.12±0.03	4.7±0.13	0.53±0.05	3.8±0.56

Data are averages with ± standard error (n = 5).

DISCUSSION

Salt stress negatively impacts plant growth and developmental processes with the resultant loss in quality and productivity (Basalah, 2010). Germination and early seedling growth are the most affected stages of crop plants by salt stress. The harmful effects of salt stress on these two stages may arise due to the osmotic stress that prevents water availability to roots (Wakeel *et al*., 2001). Like other crops, growth of wheat seedlings is also negatively affected by salinity stresses (Soltania *et al*., 2006). Hussain *et al*. (2013) investigated the relative salt tolerance of three wheat varieties and reported difference in their responses through germination and seedling growth under laboratory conditions. Increase in root length under salt stress condition has previously been observed in crop plants as an adaptation response to salinity (Rewald *et al*., 2013). Salt stress imposes osmotic and ionic stress that results in lower water uptake by plants from soil (Munns and Tester, 2008). As an adaptation strategy, plants when exposed to salt stress increase their root length to reach maximum water depth. In our results, the Siran variety showed comparatively good response to salinity stress in terms of improved shoot and root growth.

Salt stress evaluation studies have been conducted in wheat varieties to investigate their relative salt tolerance levels (Hussain *et al*., 2013). Salt stress imposes ionic, osmotic and oxidative stresses which collectively restrict water availability to cells, irreversibly damage the cellular organelles through reactive oxygen species (Munns and Tester, 2008). In response to salt stress, plants undergo molecular, biochemical and physiological changes to cope with the damaging effects and achieve adaptation (Wang *et al*., 2003). The adaptation mechanisms include production of osmoprotectants (glycine betaine, proline, trehalose etc.), reactive oxygen species (ROS) scavengers with antioxidant activity, Na^+ sequestration through membrane antiporter proteins, and induction of the transcription factors for expression of stress related gene expression (Vinocur and Altman, 2005). The above studies conducted on the evaluation of relative salt tolerance in wheat cultivars and varieties might have involved the differential induction of the same protective mechanisms. For example, Sairam *et al*. (2002) investigated the relative response of two wheat varieties (Kharchia 65, tolerant, and KRL 19, moderately tolerant) to salt stress. They reported that the high salt tolerance of the Kharchia variety was due to the high accumulation of osmoprotectants, ROS scavengers and lower H_2O_2 levels. Similar protective effects due to the differential activities of ROS scavengers were found in two wheat varieties (Egypt 449, salt tolerant, Syria 371, salt sensitive) (Esfandiari *et al*., 2011).

In a number of studies, it has been shown that salt stress negatively affects chlorophyll and the photosynthesis process (Misra *et al*., 1997; Kumar and Das, 2005; Gomes *et al*., 2011). In a study, Talat *et al*. (2013) investigated the negative effects of salt stress on various physiological and biochemical parameters of two wheat varieties. They reported a significant decrease in chlorophyll content upon 100 mM salt treatment. However, the foliar application of proline ameliorated the negative effects of salt stress on various growth, morphological and physiological parameters including photosynthesis in wheat. In previous studies, proline accumulation was proposed to be associated with osmotic and salt stress (Aziz and Lerher, 1995; Aziz *et al*., 1995; Mansour, 2005). It has also been shown that tolerant plants accumulate proline under salt stress to protect the vital cellular organelles and physiological processes (Khatkar and Kuhad, 2000; Wang *et al*., 2007). Khan *et al*. (2009) investigated the role of proline on chlorophyll contents on the salt tolerance of five wheat varieties. They reported that the high salt tolerance and the resultant better growth of the tolerant genotypes was due to the high proline accumulation, less chlorophyll degradation and high Na^+/K^+ ratio.

In the present study, the Siran variety showed better growth, morphological and biochemical parameters as compared to the other two varieties. In light of the above mentioned studies, the acme protective mechanisms might be responsible for the high salt tolerance and improved growth parameters in the Siran variety.

Conclusion

Based on the *in vitro* and greenhouse experiments, Siran variety is more salt tolerant than Atta-Habib and Pir-Sabaq. The Siran variety may have good suitability for moderate saline areas for growth and productivity.

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