Pak. J. Bot., 42(3): 1685-1694, 2010.

CRITICAL OSMOTIC, IONIC AND PHYSIOLOGICAL INDICATORS OF SALINITY TOLERANCE IN COTTON (*GOSSYPIUM HIRSUTUM* L.) FOR CULTIVAR SELECTION

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Abstract

Salinity affects the germination, growth and ultimately the yield of cotton (*Gossypium hirsutum* L.) which demands reliable traits for the evaluation and selection of salt tolerant cultivars. Here, ten major osmotic, ionic and physiological parameters have been studied to distinguish the effect of salinity in two different cultivars of cotton. Plants were grown in hydroponic system and exposed to different salinity levels of NaCl followed by its recovery under non saline conditions. Data was recorded at three different stages i.e., before stress, after stress and after recovery for comparative study. Recovery assay proved to be very helpful in extracting reliable results. Both cultivars showed significantly different response to Na^+ and K^+ accumulation and phenotypically salt tolerant cultivar (Coker 312) accumulated less Na^+ and more K^+ in comparison with susceptible (Simian 3). Decrease in leaf area, seed germination and seedling growth were also conclusive to differentiate these cultivars. We also found other physiological parameters like relative leaf water content (RLWC), plant fresh-weight (PFW), plant dry-weight (PDW), relative growth rate (RGR) and stomatal behavior as good indicators of salinity but could not find their significant role to differentiate two closely relevant cultivars regarding salinity tolerance. Our studies revealed that proline accumulation and chlorophyll concentration are not significant to be used as accurate indicators to characterize the sensitivity of cotton cultivars to salinity. We found post-recovery analysis to be very useful in understanding the role and behavior of different indicators of salinity.

Introduction

Cotton is a major crop widely grown in more than 80 countries for fiber and oil purposes. It is placed in moderately salt tolerant group of plant species (Ashraf, 2002). Under field conditions, saline soils can induce a stunted growth of cotton. Leaves of salt affected cotton plants are smaller and dark blue green in color than the normal leaves and the plants exhibit appearances similar to moisture stress conditions. According to a current survey, more than 800 million hectares of land throughout the world are salt affected (Anon., 2008). Salinity affects plant metabolism by disturbing physiological and biochemical processes of plants due to ionic and osmotic imbalances which results in the reduction of plant growth and productivity (Munns, 2005). Plants also have adapted several mechanisms to cope with these stress conditions. For salt tolerance, certain inorganic ions like K^+ and Na^+ and organic osmolytes like proline play key roles. Plants can protect themselves from salt toxicity by maintaining higher K^+ content and K^+/Na^+ ratio (Khan *et al*., 2009) and/or organic solutes (Rodriguez *et al*., 1997). Salt tolerance has also been reported to be directly related with the accumulation of proline (Munns and Tester, 2007).

Like other osmolytes, proline accumulation is proposed to be a response of Na^+ compartmentation in vacuoles by tonoplast Na^+/H^+ antiporters activity. If Na^+ and Cl^- are sequestered in the vacuole of a cell, organic solutes that are compatible with metabolic activity (like proline) accumulate in the cytosol and organelles to balance the osmotic pressure of the ions in the vacuole (Munns, 2005). Germination and earliest developmental stages are very critical which makes seed germination assay necessary for the assessment of the response of a cultivar under stress environment. The impact of salt stress has also been correlated with some morphological and physiological traits like reduction in fresh and dry weight (Chartzoulakis & Klapaki, 2000), chlorophyll contents (Ziaf *et al*., 2009) and stomatal closure (Zhu, 2001). The need to specify distinctive reliable indicators for selecting salt tolerant genotypes (lines/varities) of commercially important crops (Ashraf & Harris, 2004) provoked us for this study. The aim was to (1) sort out reliable osmotic, ionic and physiological indicators of salinity in cotton, (2) to study recovery of cotton crop after salt stress, and (3) to differentiate two cultivars regarding salt sensitivity.

Materials and Methods

Plant material: We selected two cultivars of cotton, (Coker 312 and Simian 3) to see the effect of salinity on seed germination and plant growth. Both cultivars are planted under different soil conditions because they are key cultivars for somatic embryogenesis (Zhang *et al*., 2001; Khan *et al*., 2006) with American and Chinese origin, respectively. Seeds of each cultivar were decoated and surface sterilized with 0.1% (w/v) aqueous mercuric chloride ($HgCl_2$) solution for 10 minutes and then washed four times with sterilized distilled water. These sterilized seeds were cultured in 100 ml flasks (four seeds per flask) each containing 15 ml of half-strength MS (Murashige & Skoog, 1962) medium for germination and kept at 28°C with 14/10 h (light/dark) photoperiod for 8 days.

Hydroponic system for salt stress: Eight days after germination, seedlings were transferred to the Styrofoam supports and placed on hydroponic tanks (2×2.5×0.5 feet) containing half strength Hoagland's Solution (Hoagland & Arnon, 1950) with constant aeration. Plants were grown on half strength Hoagland's solution without stress untill the emergence of 3-4 true leaves and then exposed to 0, 50, 100, 150 and 200 mM concentration of NaCl in the Hoagland's solution by adding NaCl to the nutrient solution in 50 mM increments every 24 h, until the final concentrations were reached. Nutrient solution was changed weekly. After 21 days stress, all plants were provided with half strength Hoagland's solution (without salt) for three weeks for recovery assay.

Seed germination and seedling growth: To check seed germination percentage and seedling length under salt stress condition, 20 seeds of each variety were sown in half-strength MS media with 0, 50, 100, 150 and 200 mM NaCl concentration. Germination percentage was calculated 10 days after sowing. A seed was considered germinated at the emergence of the radicle (Chartzoulakis & Klapaki, 2000). Average length of seedlings was measured in centimeters after taking and washing seedling out of media.

Proline estimation: To analyze the accumulation of proline under salt stress, different plant tissues including seedling roots, plant roots and plant leaves were weighed and free proline content was determined according to a standard protocol (Bates *et al*., 1973).

K^+, Na^+ analysis: The dried root and leaf samples were ground to powder and their K^+ and Na^+ contents were determined according to Xu *et al*., (2006).

Chlorophyll concentration/estimation: The chlorophyll contents were determined according to Arnon (1949). Fresh leaves were extracted with 80% acetone, centrifuged and absorbance of the supernatant was read at 645 and 663 nm using a spectrophotometer (DU® 800 UV/Visible Spectrophotometer, Beckman Coulter, Inc. USA).

Leaf relative water content: Leaves were collected from the plants and weighed as fresh mass (FM). These were floated in distilled water for 24 hours until fully imbibed, and their turgid mass (TM) was recorded. Samples were then placed in vacuum oven at 80°C for 48 hours to obtain dry mass (DM). Leaf relative water content was calculated as:

$$LRWC\ (\%) = [(FM-DM)/(TM-DM)] \times 100.$$

Plant biomass and relative growth rate: Four plants of each cultivar were selected randomly and tagged. Fresh weights of these selected plants before and after stress and after recovery were recorded. Relative growth rate was calculated according to Evans (1972). For dry weight, randomly three plants were selected after stress and recovery stages, oven-dried at 65°C for 1 week and dry weight was recorded.

Leaf area: After stress, 5 plants under each stress treatment were selected randomly and leaf area of the biggest leaf from each plant was determined by using LI-3100C Area Meter (LI-COR® Biosciences, LI-COR, inc. Lincoln, Nebraska, USA) and averaged.

Stomatal behavior: Size and condition of stomata on the leaves, after 21 days of stress was observed on transparent nail varnish leaf impression under microscope (DM2500, Leica, Wetzlar, Germany) at ×400 magnification and images were photographed.

Experimental lay out and statistical analysis: The experiment was laid out in completely randomized design with 3 replicates, each having at least 4 plants. The data were subjected to two way analysis of variance (salinity × cultivar) using Statistica (version 5.5 a) and means were compared by DMR test ($p<0.05$).

Results

Germination and seedling length is affected by salinity: Both two cultivars of cotton showed variation in seed germination percentage at 100 mM and higher salinity levels. Considerable loss in germination percentage was recorded at 100 mM NaCl concentration in Simian 3 (25% decrease) while for Coker 312 it was at 150 mM NaCl (20% decrease). At highest salinity level (200 mM NaCl), germination was 70% in Coker 312 and 60% in Simian 3. Increasing salt concentration imposed a gradual decline in seedling length in both cultivars (Fig. 1 and 2A). Average seedling length of Simian 3 was more than Coker 312 in the absence of salt stress but at each salinity level, it was observed less than Coker 312 (Fig. 1 and 2A).

Accumulation of organic and inorganic solutes: Salinity increased Na^+ content and decreased K^+ content as well as K^+/Na^+ ratio in the roots of seedlings, 10 days after

sowing in both cultivars (Table 1; Fig. 2B-C). Comparative studies revealed high Na^+ content, low K^+ content and low K^+/Na^+ ratio in Simian 3 in comparison with Coker 312 during seedling growth. At 50 mM NaCl stress, 5.79 and 6.49 fold increase in Na^+ content while 29.79% and 21.47% decrease in K^+ content was observed in Coker 312 and Simian 3, respectively. At 200 mM NaCl concentration in nutrient solution, Simian 3 had 1.18 times higher Na^+ contents than Coker 312, in the seedling roots while K^+ contents of Coker 312 were 1.61 times more than Simian 3 (Fig. 2B-C). Same kind of behavior was observed in the leaves and roots of both cultivars after treating them at different salinity levels for 21 days. Maximum Na^+ levels (24.87 mg/g DW and 28.19 mg/g DW) were observed at 200 mM NaCl in Coker 312 and Simian 3, respectively. In roots, there was an abrupt increase in Na^+ in Coker 312 and Simian 3 at 50 mM NaCl level of salinity (Fig. 3A2). There was a slow decrease in K^+ content in the leaves and roots of both cultivars and they showed significant differences in K^+ accumulation in roots at 200 mM NaCl with Coker 312 containing 1.41 times more K^+ content than Simian 3 (Fig. 3B2). In the leaves, 44.1% and 34.85% decrease in K^+ content was recorded in Coker 312 and Simian 3, respectively at 200mM NaCl (Fig. 3B1). In both cultivars, a steep fall in K^+/Na^+ ratio was observed with the increase of salinity (Table 1). Minimum K^+/Na^+ ratio in both cultivars was recorded at 200 mM NaCl in all three tissues (seedling roots, plant leaves and plant roots). Significant differences in K^+/Na^+ ratio between Coker 312 and Simian 3 were also observed in all plant parts (Table 1). Recovery assay proved useful in increasing K^+/Na^+ ratio in the leaves of both cultivars treated at 50 and 100 mM NaCl. Roots of Coker 312 showed a significant increase in K^+/Na^+ ratio after recovery from 50, 100 and 150 mM NaCl while Simian 3 just managed significant increase in K^+/Na^+ ratio in the plants treated at 50 and 100 mM NaCl levels of salinity (Table 1).

Endogenous level of proline increased in both cultivars under increased salinity levels. Proline accumulation was more in Coker 312 (282.45 µg/g FW) than Simian 3 (234.27 µg/g FW) at 200 mM NaCl during seedling growth (Fig. 2D). A sharp increase in proline contents was observed at 50 mM NaCl in the leaves of both cultivars while there was comparatively a gradual increase in proline contents in roots (Fig. 3C1-C2). In Coker 312, maximum proline was measured (211.91 µg/g FW) at 150 mM NaCl in roots while leaves accumulated maximum proline at 100 mM (178.84 µg/g FW) at par with 200 mM NaCl (180.9 µg/g FW). In Simian 3, proline piled up more in leaves at 50 mM NaCl (164.25 µg/g FW) with a significant fluctuation in the subsequent salinity levels. On the other hand, maximum proline in roots (170.27 µg/g FW) was observed at 200 mM NaCl. Post-stress plant recovery proline estimation revealed significant decrease in proline

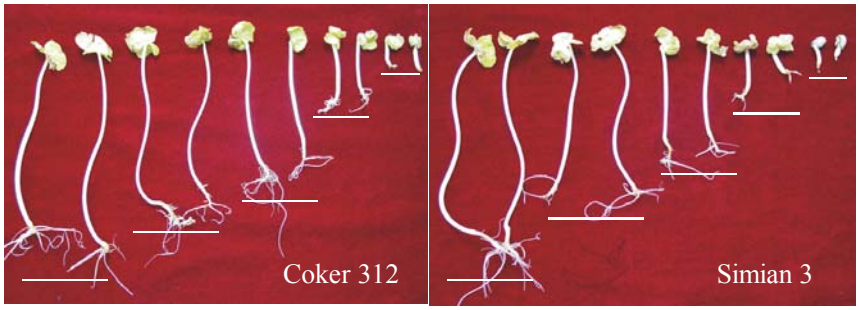

Fig. 1. Seedlings of two cotton cultivars, grown on half-strength MS medium at different salinity levels. Two consecutive seedlings represent one of the five salinity levels, which vary as 0, 50, 100, 150 and 200 mM NaCl concentrations, from left to right.

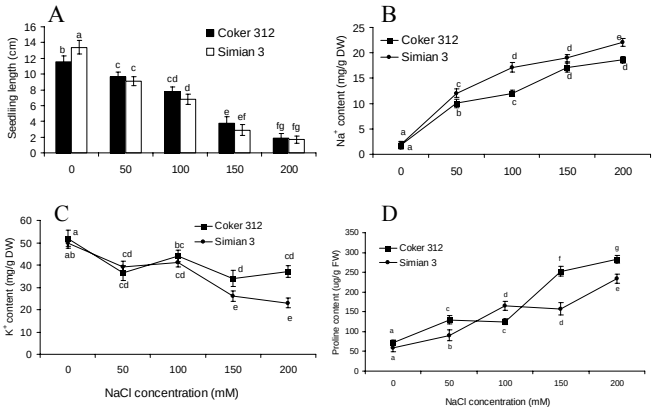

Fig. 2. Seedling lengths (A), Na^+ (B), K^+ (C) and proline content (D) measured in seedling roots, 10 days after seed sowing at different salinity levels. Same letters (a, b, c, d, e, f and g) indicate non significant difference at $p<0.05$ by DMR test. Vertical bars represent standard error of means (n=3).

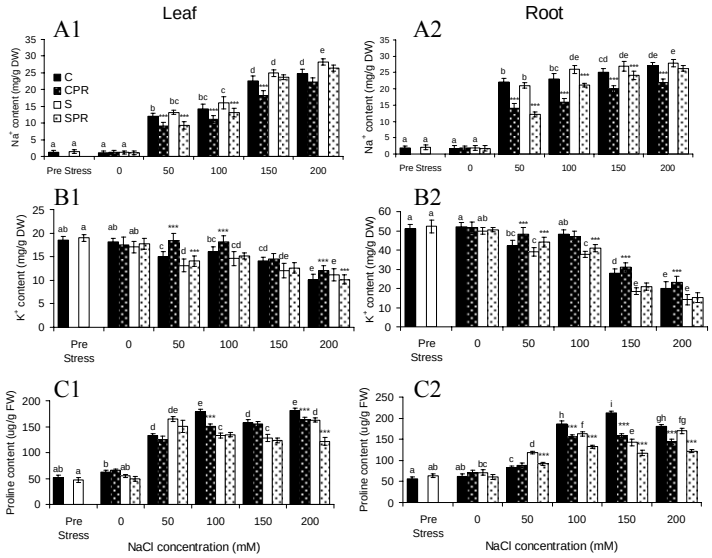

Fig. 3. Na^+ (A1, A2), K^+ (B1, B2) and proline content (C1, C2) in the leaves and roots of two cultivars under different salt stress levels. C: Coker 312, CPR: Coker 312 post recovery, S: Simian 3, SPR: Simian 3 post recovery. Values followed by the same letters (a, b, c, d, e, f and g) are not significantly different from each other while asterisks (***) on CPR and SPR bar indicate significant post recovery difference from the same level of salinity at $p<0.05$ by DMR test. (n=3).

Fig. 4. Effect of different salinity levels on leaf area. Leaves at 0, 50, 100, 150 and 200 mM NaCl salinity are placed from left to right representing two different cultivars of cotton.

Table 1. K^+/Na^+ ratio in the seedling roots, plant leaves and plant roots of Simian 3 and Coker 312 under five salt treatments (n=3).

Plant tissue	Cultivar	NaCl (mM)	K^+/Na^+ (Post Stress)	K^+/Na^+ (Post recovery)
Seedling root	Coker 312	0	29.96 a	
		50	3.63 c	
		100	3.67 c	
		150	1.98 de	
		200	1.99 de	
	Simian 3	0	26.92 b	
		50	3.25 c	
		100	2.39 d	
		150	1.37 ef	
		200	1.04 f	
Plant leaf	Coker 312	0	15.68 a	15.38
		50	1.26 c	2.01**
		100	1.13 d	1.62 **
		150	0.62 f	0.79
		200	0.40 h	0.54
	Simian 3	0	14.13 b	14.31
		50	0.99 e	1.52 **
		100	0.91 e	1.16 **
		150	0.48 g	0.53
		200	0.39 h	0.40
Plant root	Coker 312	0	29.84 a	29.52
		50	1.92 cd	3.44 **
		100	2.09 c	2.94 **
		150	1.12 de	1.56 **
		200	0.74 e	1.06
	Simian 3	0	28.83 b	29.12
		50	1.85 cd	3.64 **
		100	1.45 cd	1.94 **
		150	0.68 e	0.87
		200	0.51 f	0.61

In each of three tissues (seedling root, plant leaf and plant root), the values represented by the same letter are not significantly different from each other while asterisks (**) in K^+/Na^+ post recovery column indicate significant post recovery difference at that particular level of salinity at $p<0.05$ according to the DMR test. Seedling Root: roots of seedlings 10 days after seed germination on MS medium, Plant Leaf and Plant Root: leaves and roots of plants grown in Hoagland's solution for NaCl stress and recovery.

content in the roots of both cultivars subjected to 100, 150 and 200 mM NaCl stress (Fig. 3C1-C2), while after recovery from 50 mM NaCl, Coker 312 had non-significant increase and Simian 3 showed significant decrease in proline contents like other salinity levels (Fig. 3C2). In the leaves, recovery assay caused significant decrease in proline accumulation at 100 mM and 200 mM NaCl in Coker 312 while in Simian 3 there were non significant change in proline content at all salinity levels except significant increase at 200 mM NaCl concentration (Fig. 3C1).

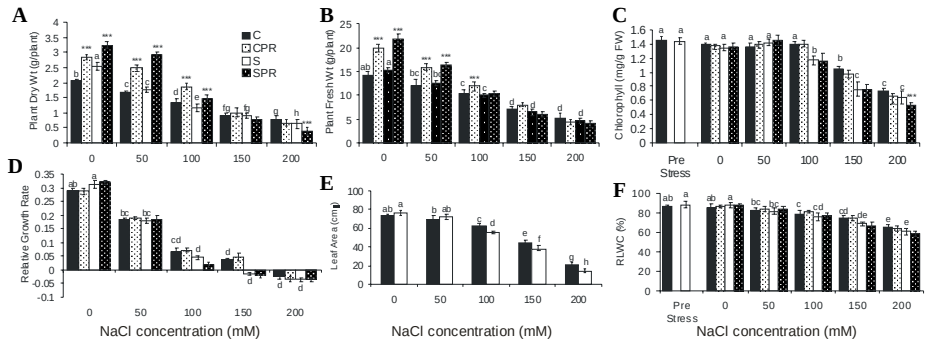

Fig. 5. Physiological changes under salt stress in two cultivars of cotton. C: Coker 312, CPR: Coker 312 post recovery, S: Simian 3, SPR: Simian 3 post recovery. Values followed by the same letters (a, b, c, d, e, f, g and h) are significantly similar while asterisks (***) on CPR and SPR bar indicate significant post recovery difference from the same level of salinity at $P<0.05$ by DMR test. Number of replicates are three (for A, C and F), four (for B and D) and five (for E).

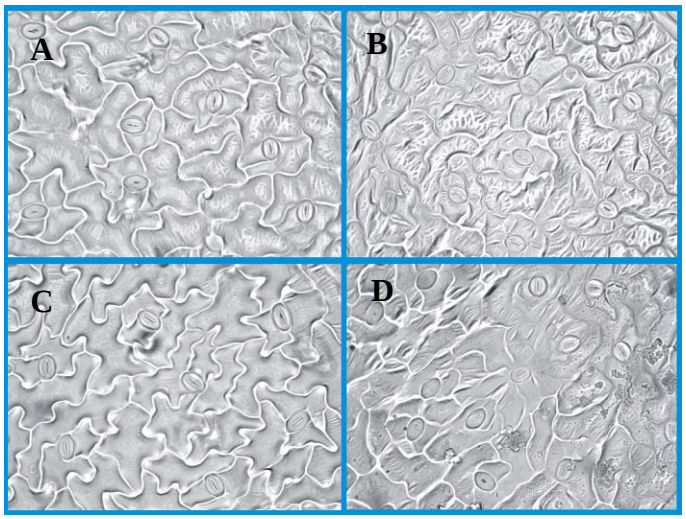

Fig. 6. Visual observation of increase in number and decrease in size of stomata at ×400 magnification. A and C represent stomata in the plants at 0 mM NaCl while B and D show stomata at 200 mM NaCl in Coker 312 and Simian 3, respectively.

Modulation of morphological and physiological traits: Increase in salinity reduced leaf area in both cultivars (Fig. 4). In comparison with control (0 mM NaCl), there was a significant reduction in leaf size at 100 mM NaCl in both cultivars but it was more prominent in Simian 3 than Coker 312. At 200 mM NaCl stress, 70.94% and 81.17% reduction in leaf area was found in Coker 312 and Simian 3, respectively (Fig. 5E).

Increased salinity also caused reduction in plant fresh weight (PFW) and plant dry weight (PDW), which consequently affected relative growth rate (Fig. 5A-C). At 200 mM NaCl concentration, cultivar Coker 312 showed 62.8% and 61.4% while Simian 3 exhibited 69.3% and 75.1% reduction in PFW and PDW, respectively, in comparison with control (Fig. 5A-B). During recovery period, Coker 312 managed to increase PFW

significantly in the plants previously exposed to 50 mM and 100 mM NaCl salinity but Simian 3 just displayed significant PFW increase at 50 mM NaCl stress (Fig. 5B). During recovery assay, both cultivars showed same behavior of increase in PDW in the plants treated at 50 and 100 mM NaCl concentration but a significant decrease in PDW was observed in the plants of Simian 3 subjected to 200 mM NaCl stress (Fig. 5A). Relative growth rate (RGR) decreased significantly and Simian 3 exhibited negative growth rate at 150 mM and 200 mM NaCl. In Coker 312, RGR was also negative at 200 mM NaCl indicating a decrease in PFW at these salinity levels (Fig. 5C). Chlorophyll contents fluctuated at different levels of salinity in both cultivars (Fig. 5D). Maximum chlorophyll contents (1.39 mg/g FW and 1.42 mg/g FW) were achieved at 50 mM and 100 mM NaCl salinity in Simian 3 and Coker 312, respectively. Coker 312 exhibited significantly higher chlorophyll contents than Simian 3 at 100 and 150 mM NaCl. In both cultivars, recovery assay had no significant influence on chlorophyll contents. A gradual decrease in relative leaf water contents (RLWC) was observed with increase in salinity (Fig. 5F). There was 23.8% and 30.8% decrease in RLWC in Coker 312 and Simian 3, respectively at 200 mM NaCl salinity level. Recovery treatment did not affect RLWC in both cultivars. We also observed decrease in stomatal size and increase in its frequency in both cultivars at different levels of salinity which was more prominent at 200 mM NaCl (Fig. 6).

Discussion

Salinity is one of the major yield limiting factors in cotton. With a salinity threshold level of 7.7 dS m^{-1}, cotton is classified as a salt-tolerant crop but its tolerance varies greatly among genotypes (Ashraf, 2002). In this study, we evaluated different ionic, osmotic and physiological indicators of salinity to check their role in the selection of cotton cultivars under salt stress conditions.

We found reduced seed germination % and seedling growth under salt stress in both cultivars. Similar results have already been reported in cotton (Chachar *et al*., 2008). Our comparative studies revealed that Coker 312 accumulated less Na^+ and higher K^+ content than Simian 3 which may be a reason of genotypic differences among cultivars (Meloni *et al*., 2001). This ionic balance is a main reason for stress tolerance because Na^+, Cl^- and K^+ are the principal inorganic solutes under salt stress conditions (Rodriguez *et al*., 1997) and the maintenance of K^+ and Na^+ homeostasis is very crucial under salt stress (Zhu, 2003). This decrease in Na^+ may be due to the compartmentation of Na^+ because vacuolar sequestration of Na^+ not only lowers Na^+ concentration in the cytoplasm but also contributes to osmotic adjustment to maintain water uptake from saline solutions. Other organelles, such as plastids and mitochondria, may also accumulate some Na^+ and thus contribute to the overall subcellular compartmentation of Na^+ (Zhu, 2003). High Na^+ content, low K^+ content and low K^+/Na^+ ratio in Simian 3 in comparison with Coker 312 under salt stress conditions reveals it a salt susceptible variety. This also confirms K^+/Na^+ ratio as a reliable index of salinity in cotton. Salt Stress increased the level of proline in both cultivars which has already been reported in cotton under saline conditions (Meloni *et al*., 2001). Accumulation of organic solutes (like proline) in higher concentrations has been reported to be non-toxic to cytoplasmic functions, allowing turgor maintenance and/or protection of macromolecular structures (Ashraf & Harris, 2004). We found increased proline content under saline conditions but there was a great fluctuation at different salinity levels in both cultivars which may be related to the variation in the concentration of K^+ and other compatible solutes because its increase depends on the concentration of other solutes (Munns & Tester, 2008). On the basis of our results, we concluded that proline is produced in cotton leaves and roots in response to salt stress but this response can not be considered

as a discrete criterion to select a cultivar for salt resistance. Some other studies (Lutts *et al*., 1999; Munis *et al*., 2010) also negate the reliability of proline as an indicator of salinity.

There was a gradual decrease in plant fresh weight, plant dry weight and leaf area in both cultivars with increasing salinity. This decrease in growth might be a reason of too much Na^+ in the solution which results in delayed maturity of the crop (McConnell *et al*., 2008). Decrease in leaf area was significant among these two cultivars which may be helpful in differentiation and selection of better cultivar. This decrease in leaf area may be attributed to the accumulation of Na^+ and other inorganic solutes because the cultivar Coker 312, showing more resistance to decrease in leaf area, accumulated less Na^+ than other one (Simian 3). Reduction in plant fresh weight and plant dry weight has been reported by Ziaf *et al*., (2008), which strengthen our results.

Both cultivars showed significant decrease in RLWC at different salinity levels but we found non-significant differences within cultivars at the same level, which concluded that RLWC is an indicator of salinity but it was not good enough to differentiate cotton cultivars used in our experiment. Leaf chlorophyll contents were increased in both cultivars under stress but this fluctuation in chlorophyll contents was not sequential. Furthermore, recovery assay did not reveal any significant increase in the chlorophyll contents. In conclusion, chlorophyll content was not found as a good parameter, due to uneven variation in chlorophyll concentration at different salinity levels in both cultivars.

Increased number of stomata with decrease in their size under salt stress is possibly due to decrease in sap flow and turgidity of guard cells in response to salt stress in order to maintain their water status (Robinson *et al*., 1997). Thus, on the basis of stomatal closure, cultivars can be differentiated for their salinity tolerance. Though stomatal size and number are also affected by light intensity and position of leaf on the plant, still leaves of different cultivars grown under similar conditions can be used for this study. We could not measure stomatal size and frequency because there was a great variation at different areas of every leaf in both cultivars which needs a separate comprehensive study to sort out the most effective time and area of the leaf for detecting salinity effects.

Conclusion

On the basis of our results, we found Na^+ and K^+ content, decrease in leaf area, seed germination and seedling growth as the best indicators of salinity in cotton for cultivar selection. RLWC, plant fresh weight, plant dry weight, relative growth rate and stomatal behavior are good parameters but not good enough to differentiate cultivars with narrow genotypic differences regarding salt tolerance. Proline and chlorophyll concentration were not useful for accurate assessment of salinity tolerance in cotton cultivars.

Acknowledgments

This work was supported by National Natural Science Foundation of China (Grant No. 30471109) and the National Cotton Production Tech-support Program (Ministry of Agriculture, China).

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(Received for publication 21 July 2009)