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Effect of Salt Stress on Growth, Anthocyanins, Membrane Permeability and Chlorophyll Fluorescence of Okra (*Abelmoschus esculentus* L.) Seedlings

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ABSTRACT

To study the response of okra (*Abelmoschus esculentus* cv. Marsaouia) to salinity, the effect on seedling growth, water content, anthocyanins content and chlorophyll fluorescence was investigated. Okra seeds were germinated in Petri dishes and sown in peat culture under controlled conditions and submitted during 2 weeks to saline stress ranging from 0 (distilled water) to 20, 40, 60, 80 and 100 mmol of NaCl. Results showed that salt has no significant effect on aerial part dry weight and it showed no changes in chlorophyll fluorescence parameters (F_o and F_v/F_m). By contrast, root dry weight decreased with increasing NaCl concentration with a subsequent increase in root water content and cotyledons+hypocotyls/root ratio. In addition, anthocyanins content and electrolyte leakage were highly increased by exposure to high levels of salt especially 80 and 100 mmol NaCl.

Key words: Anthocyanins, dry mass, electrolyte leakage, okra, salinity, water content

INTRODUCTION

Among abiotic stresses, salinity is one of the most severe problems in worldwide agricultural production. Salinity changed plant morphological traits (Zadeh and Naeini, 2007) decreased plant dry matter, leaf area (Amirjani, 2010) and crop yield (Bybordi and Ebrahimian, 2011). Around 1/5 of the 2.8 billion hectares of arable land on earth are affected by salt and the problem is becoming an increasing threat to agriculture globally (Pessarackli and Szabolcs, 1999). Soil salinity, one of the most serious problems on planting areas, has the most obstructive impact on crop production in the world. This crisis problem attracts many scientists to overcome this obstruction by improving salt-tolerant lines (Gholizadeh and Navabpour, 2011). In addition, there is an increasing trend for using saline water in agriculture (Alem *et al.*, 2002) because of water scarcity, especially for vegetable crop production in many Mediterranean countries including Tunisia.

Most of cultivated plants are sensitive to salt stress, in which NaCl-salinity involves modification of morphological, physiological and biochemical processes and anatomical changes (Tester and Davenport, 2003). These changes usually result in reduction of shoot (Misra *et al.*, 1995) and restricted rooting (Lopez and Satti, 1996). It has also been reported that the effect of excess salinity reduced transpiration rates (Massai *et al.*, 2004), photosynthesis and pigment contents (Renault *et al.*, 2001; Stoeva and Kaymakanova, 2008). Anthocyanins are a large class of water soluble pigments in the flavonoid group. Many works relate anthocyanin synthesis to the induction

of some type of stress in plants but only few reports dealt with the increases of anthocyanins as a result of response to salt stress (KaliAMOorthy and Rao, 1994; Chalker-Scott, 1999).

The conductivity test, based on solute leakage, has been proposed as a good indicator of salt tolerance in plants (Ghoulam *et al.*, 2002). The cell membrane often suffered injury associated with the increases in permeability and loss of integrity (Blokhina *et al.*, 2003). The increase in electrolyte leakage was due to the loss of ability to reorganize cellular membranes rapidly and completely (McDonald, 1980).

On the other hand, chlorophyll fluorescence has been used by physiologists to evaluate response of various crop species to determine the influences of abiotic stresses at various stages of plant growth. It has proved particularly useful in salinity tolerance screening programs (Jimenez *et al.*, 1997; Pak *et al.*, 2009). Analysis of fluorescence characteristics such as the nature and intensity of the emission bands, quantum yield and induction kinetics, reflects the properties of the chlorophyll molecules and their interaction with the external environment and also with associated physiological processes (Hall and Rao, 1999). The photochemical yield Fv/Fm (where, Fv: variable fluorescence and Fm: maximal fluorescence) which reflects potential quantum yield of PSII indicates the physiological state of the photosynthetic apparatus in intact plant leaves (Pereira *et al.*, 2004). Pospisil *et al.* (1998) mentioned that environmental stresses affect PSII efficiency and lower the Fv/Fm values.

Germination and seedling establishment constitute the most critical periods in the life cycle of plants (Sosebee and Wan, 1987). There are many criteria for determining the levels of salt tolerance of varieties and species. Ideally, they should be simple to measure and permit the identification of salinity tolerance during seed germination or at the seedling stage (Akinci *et al.*, 2004). Adverse effects of salinity on seed germination and seedling growth as well some physiological activities of cultivated plant species have been extensively investigated (Bayuelo-Jimenez *et al.*, 2002; Zafar *et al.*, 2005, Hameed *et al.*, 2008, Kaymakanova *et al.*, 2009).

Okra belongs to family Malvaceae, originated in tropical Africa and was grown in Mediterranean region. In many areas of Tunisia, the productivity of this crop is limited because of the water and soil salinization. The improvement of this productivity requires understanding of the mechanisms of tolerance of this species to salinity. On the basis of previous experiments, Ben Dkhil and Denden (2010) have reported that okra seeds were less sensitive to salinity (below 100 mmol NaCl) at germination stage. Salt tolerance of plants varies during their successive growth stages; the present investigation was carried out to evaluate the morphological and physiological behavior of okra seedlings in saline conditions. For this purpose, we compiled the measurements of seedling growth with measurements of chlorophyll fluorescence, anthocyanins content and membrane permeability. Besides investigating the possible tolerance mechanisms during seedling stage under conditions of moderate and severe salt stress (20, 40, 60, 80 and 100 mmol NaCl).

MATERIALS AND METHODS

Seed material and germination: Seeds were collected from local plants of okra (*Abelmoschus esculentus* L., cv. Marsaouia); they have been provided by Baddar Company (Tunisia). Marsaouia is the most cultivated cultivar in Tunisia. Seeds were sterilized with 15% sodium hypochlorite for 15 min and washed thoroughly with distilled water. They were then placed to germinate in Petri dishes (9 mm diameter) containing two sheets of Whatman No.1 filter paper, saturated with distilled water (control) or NaCl solutions (20, 40, 60, 80 and 100 mmol NaCl) at 25°C in the dark.

Growth conditions: The emergence of radicle from seed was taken as an index of germination. Thus, germinated seeds in distilled water and in saline solutions were transferred in a plastic disposable containers filled with peat/perlite mixture (2:1 v/v) to emerge. Containers, where incubated in a growth chamber with controlled temperature adjusted at 25°C day/night (25°C was determined by Ben Dkhil and Denden (2010) as optimal temperature for germination of okra seeds under saline condition) and relative humidity maintained between 75-80% with 12 h photoperiod. The containers were irrigated continuously with distilled water or salt solution during two weeks. Treatments were replicated 4 times in a factorial experiment laid out in a completely randomized design.

Growth parameters: On day 15, the seedling stage was achieved with cotyledonary expansion and data were recorded. Twenty seedlings, representing each treatment, were harvested and washed with deionised water. Washed seedlings were separated into root, hypocotyls and cotyledons for the determination of Fresh Weight (FW), Dry Weight (DW) after drying the samples to a constant weight in a 80°C oven. The (hypocotyls+cotyledons) dry weight/root dry weight ratio were calculated out of these data. Further, cotyledons and hypocotyls (aerial part) water content and root water content was obtained from the (FW-DW)/DW ratio (Prado *et al.*, 2000).

Anthocyanin determination: The method taken from Mancinelli (1990) was applied for the determination of anthocyanin content of okra seedlings. After being thoroughly extracted in 3 mL methanol-HCl (1% HCl, v/v), the samples were left at 4°C in the refrigerator for 2 days. Later on, the extract were vortexed, filtered and the total anthocyanin content was measured by an UV-visible spectrophotometer as the difference between the absorbance at 530 and 657 nm wavelength and placed in the $A_{530}-A_{657}$ formula to eliminate the chlorophyll content in the extract, defined quantitatively as $OD_{530} g^{-1}$ fresh weight.

Electrolyte leakage (membrane permeability): Electrolyte leakage was used to assess membrane permeability according to Lutts *et al.* (1995). Cotyledon samples were washed with distilled water to remove surface adhered electrolytes and cut into discs of uniform size. Cotyledon discs were put in closed test tubes containing 10 mL of deionized water and incubated at room temperature (25°C) for 24 h and subsequently electrical conductivity of the solution (EC_1) was recorded. Samples were then autoclaved at 120°C for 20 min and the final electrical conductivity (EC_2) was obtained after cooling the solution to room temperature. The Electrolyte Leakage (EL) was calculated as EC_1/EC_2 and expressed as percentage.

Chlorophyll fluorescence measurements: Chlorophyll fluorescence emission from the upper surface of the cotyledons of intact seedlings was measured by a portable fluorescence monitoring system (F.I.M, 1500, ADC, Fluorescence Induction Monitor 1550, Analytical Development Company Limited). Prior to fluorescence measurements, a circular surface of the upper face of the cotyledons were dark adapted for 20 min using the dark adaptation clips. The initial fluorescence (F_o), the maximum fluorescence value (F_m) were assessed. The variable fluorescence (F_v) was calculated as $F_v = F_m - F_o$ and the maximum quantum efficiency of PSII photochemistry (F_v/F_m) was determined.

Statistical analysis: Data were analysed with SPSS for windows: version 13.0 (standard version) using one-way analysis of variance (ANOVA) procedure. Differences among the means were compared by Duncan's test.

RESULTS

Seedling growth response: The physical growth parameters such as hypocotyls+cotyledons and root dry weights were measured under varying NaCl salinity stress levels (Table 1). Present results proved that aerial part (hypocotyls and cotyledons) dry weight did not vary whatever the NaCl treatments; it was in the range of 0.035 and 0.043 g, under (80 and 100 mmol NaCl) and (20, 40, 60 mmol NaCl), respectively. By contrast, the root dry weight was significantly reduced with increasing salt concentration, resulting in an increase on the shoot/root ratio. Indeed, salt treatment significantly affected shoot/root ratio ($p < 0.05$), the 60 and 80 mmol treatments increased this ratio to 7.4 and 7.5, respectively. Moreover, the highest value of shoot/root ratio was registered in seedlings treated with 100 mmol NaCl, it was around 10. The magnitude of root growth reduction was highly dependent upon NaCl concentrations. The root dry weight was reduced by around 67% at the highest salt concentration (100 mmol NaCl) compared to control (Table 1).

In hypocotyls and cotyledons, water content gradually decreased with increasing salt concentration. As stress became more severe (60, 80 and 100 mmol NaCl) aerial part water content was considerably limited (respectively -35 and -39% with respect to control).

However, root water content significantly increased with increasing NaCl concentration, reaching values of 15.83 and 19.08 under 60 and 100 mmol NaCl, respectively (Table 2).

Anthocyanins content: Changes in anthocyanin amounts by NaCl concentrations of 15 days old seedlings are presented in Table 3. As can be shown by statistical analyses ($p < 0.05$), anthocyanins content was significantly induced by salinity. With the increase in NaCl concentrations, there was a marked increase in anthocyanin amounts. For 100 mmol treatment, the value of anthocyanins content was 25 times higher than control one.

Membrane permeability: The extent of membrane damage was assessed indirectly by conductometric measurements of solute leakage from cells. The amount of electrolyte leakage

Table 1: Variation of the aerial part (cotyledons+hypocotyls) dry weight (DW), root dry weight (RDW) and the aerial part/root ratio of okra variety subjected to different NaCl concentrations (0, 20, 40, 60, 80 and 100 mmol) during early seedling stage

| NaCl (mmol) | Aerial part DW (g) | Root DW (g) | Aerial part /root ratio |
|-------------|--------------------|--------------------|-------------------------|
| 0 | 0.043 ^a | 0.012 ^a | 4.22 ^a |
| 20 | 0.042 ^a | 0.007 ^b | 6.93 ^{ab} |
| 40 | 0.038 ^a | 0.007 ^c | 5.42 ^{ab} |
| 60 | 0.037 ^a | 0.006 ^d | 7.45 ^{bc} |
| 80 | 0.035 ^a | 0.005 ^e | 7.52 ^{bc} |
| 100 | 0.035 ^a | 0.004 ^f | 9.96 ^c |

Values of each column followed by the same letter indicate no significant difference ($p < 0.05$) according to Duncan's test

Table 2: Variation of the water content in aerial part (cotyledons+hypocotyls) and root of okra variety subjected to different NaCl concentrations (0, 20, 40, 60, 80 and 100 mmol) during early seedling stage

| NaCl (mmol) | Aerial part water content (FW-DW)/DW | Root water content (FW-DW)/DW |
|-------------|--------------------------------------|-------------------------------|
| 0 | 14.34 ^a | 10.83 ^d |
| 20 | 11.60 ^{ab} | 12.39 ^d |
| 40 | 12.48 ^{ab} | 12.31 ^d |
| 60 | 9.35 ^b | 15.83 ^b |
| 80 | 8.80 ^b | 14.10 ^{bc} |
| 100 | 8.79 ^b | 19.08 ^a |

Values of each column followed by the same letter indicate no significant difference ($p < 0.05$) according to Duncan's test

Table 3: Effects of NaCl treatments (0, 20, 40, 60, 80 and 100 mmol) on anthocyanins content (OD 530 g⁻¹ fresh weight) and electrolyte leakage (%) at early seedling stage of okra variety

| NaCl (mmol) | Anthocyanins (OD 530 g ⁻¹ FW) | Electrolyte leakage (%) |
|-------------|--|-------------------------|
| 0 | 0.019 ^a | 20.89 ^a |
| 20 | 0.058 ^b | 20.59 ^a |
| 40 | 0.16 ^c | 21.76 ^{ab} |
| 60 | 0.18 ^d | 23.40 ^{ab} |
| 80 | 0.47 ^e | 25.34 ^b |
| 100 | 0.48 ^e | 25.99 ^b |

Values of each column followed by the same letter indicate no significant difference (p<0.05) according to Duncan's test

Table 4: Effect of increasing NaCl concentration on fluorescence parameters in cotyledons of okra seedlings

| NaCl (mmol) | Fo | Fm | Fv/Fm |
|-------------|------------------|-------------------|-------------------|
| 0 | 520 ^a | 2993 ^a | 0.82 ^a |
| 20 | 517 ^a | 2996 ^a | 0.82 ^a |
| 40 | 514 ^a | 2998 ^a | 0.82 ^a |
| 60 | 481 ^a | 2428 ^b | 0.79 ^a |
| 80 | 497 ^a | 2508 ^b | 0.79 ^a |
| 100 | 518 ^a | 2479 ^b | 0.79 ^a |

Values of each column followed by the same letter indicate no significant difference (p<0.05) according to Duncan's test

increased under salt stress (Table 3). The magnitude of this increase was significant at 80 and 100 mmol NaCl. Electrolyte leakage was slightly modified by moderate salinity; it was ranged between 20.59 and 21.76% at 20 and 40 mmol NaCl, respectively.

Chlorophyll fluorescence: The effect of six levels of NaCl on chlorophyll fluorescence parameters were examined on dark-adapted cotyledons (Table 4). The initial level of fluorescence (Fo) didn't change with NaCl level, it was not significantly affected by salinity stress in developing cotyledons. It was around 520 and 481, respectively at 0 and 60 mmol NaCl. Maximum level of fluorescence (F_m) was different in different salinity treatments than in control, 20 and 40 mmol NaCl, F_m being lower in salt treated seedlings with 60, 80 and 100 mmol NaCl. The Fv/Fm ratio was ranged between 0.79 and 0.82, demonstrating no significant effect of NaCl treatments on PSII photochemical activity.

DISCUSSION

In this work, salt stress inhibited significantly root growth, although, hypocotyl and cotyledons growth was not affected by the increasing NaCl concentrations. Our results were similar with the findings of Jamil *et al.* (2006) who suggested that salt stress inhibited the growth of root more than shoot in four vegetables species (cabbage, sugar beet, *Paniculate amaranth* and pak-choi). Similarly, Saha *et al.* (2010) found that roots of mungbean seedlings were more sensitive to salinity than shoots. Nevertheless, Maghsoudi and Maghsoudi (2008) reported that salt stress inhibited coleoptile growth more than root growth of wheat seedlings. Afzal *et al.* (2005) suggested that salinity affect seedling stage of wheat range from reduction in germination percentage, fresh and dry weight of shoots and roots to the uptake of various nutrient ions. Moreover, Cicek and Cakirlar (2002) reported that salinity reduced shoot length, fresh and dry weight of maize seedlings.

Reduced seedling growth under salt stress condition has also been reported by Huang and Redmann (1995) on barley, Foolad and Jones (1993) on tomato and Jeannette *et al.* (2002) on

Phaseolus. In reviewing, the effect of salt stress on seedling growth found a reduction in root and shoot development that may be due to toxic effects of NaCl used as well as unbalanced nutrient uptake by the seedlings (Jamil *et al.*, 2006). On the other hand, it was reported that decline in seedling dry weight in response to salinity is a consequence of decline in weight of mobilized seed reserve (Renault *et al.*, 2001). Indeed, Jaleel *et al.* (2009) suggested that the reduction of plant growth under salt stress is the result of the alteration of many physiological activities in the plant, such as photosynthetic activity, mineral uptake and antioxidant activity. Amirjani (2010) reported that increasing salinity level to 100 and 200 mmol NaCl resulted in a reduction of plant height and fresh weight; an increase in proline content and diamine oxidase activity in soybean.

Salt stress also resulted in a significant increase in cotyledons+hypocotyls/root ratio which be considered as a direct effect of the observed roots dry mass reduction of treated seedlings (Table 1). These results contradict with Maghsoudi and Maghsoudi (2008) that salt stress inhibited coleoptile growth more than root growth. Rahman *et al.* (2008) reported that salinity depressed shoot growth more than root growth and increased root/shoot ratio.

According to the data obtained from the experiments, anthocyanin production increased markedly as salt stress level increased (Table 3). Anthocyanin accumulation that is known as a hallmark of plant stress was stimulated by increasing concentrations of NaCl in various parts of tomato and red cabbage seedlings (Eryilmaz, 2006). As reported by Winkel-Shirley (2002) anthocyanin synthesis is one of the subsequent production and its localization in root, stem and especially leaf tissues may allow the plant to develop resistance to a number of environmental stresses. Ali and Abbas (2003) found that saline (NaCl) stress in barley seedlings causes an increase in total phenolic compounds, flavonoids and enhancement of peroxidase and indoleacetic acid oxidase activities and consequent decrease in growth rate.

Therefore, increasing electrolyte leakage was used to assess membrane permeability. Addition of 100 mmol NaCl induced slightly membrane damage (Table 3). Kaya *et al.* (2001a) suggested that increases in membrane permeability at seedling stage were lower than at vegetative stage at high salinity and this also shows a strong link between time of exposure to high salinity and membrane permeability.

Salt stress induced electrolyte leakage has also been previously observed in maize (Hichem *et al.*, 2009). Similar results were obtained in tomato by Kaya *et al.* (2001b) and in cucumber and pepper Kaya *et al.* (2001c).

Measurements of chlorophyll fluorescence parameters were attempted to evaluate the direct effects of salt stress on PSII photochemistry. Results showed that salinity level increased with no significant effect on Fv/Fm ratio, these results can be related to some findings in which it has been observed that salt stress has no effect on PSII photochemical activity, example in wheat (Raza *et al.*, 2006). According to Zribi *et al.* (2009), the Fv/Fm ratio was not affected by any salt treatment of tomato seedlings (cv. Rio Grande). Similar results were reported by Jamil *et al.* (2007). They observed a lack of changes in the maximal efficiency of PSII photochemistry (Fv/Fm) in control and salt-stressed plants suggests that salt stress had no effects on PSII primary photochemistry in cabbage and sugar beet. It seems that the effects of salt stress on PSII photochemistry in plants depend on light intensity.

The results presented in this paper suggested that salt levels applied did not create high membrane damage and did not significantly affect the PSII activity. At seedling stage, NaCl levels ranged from 0 to 100 mmol might be low for okra. On the other hand, Ul-Haq (2009) reported that many genotypes of okra failed to germinate at 80 mmol NaCl, this concentration was found to be suitable for discriminating tolerant and non tolerant okra genotypes.

CONCLUSION

Some important conclusions can be drawn from the results achieved during this experiment. Okra is considered to be a salt tolerant species. Our results indicate its ability to maintain high physiological activities when subjected to relatively high levels of NaCl at early seedling stage. Therefore, comparisons of salinity tolerance mechanisms in different varieties will be fundamental to understand the regulatory points assigning physiological mechanisms that are likely to provide salinity tolerance.

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