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Full Length Research Paper

Salt stress induced changes in germination, sugars, starch and enzyme of carbohydrate metabolism in *Abelmoschus esculentus* L. (Moench.) seeds

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During germination stage, the influence of NaCl on cumulative germination percentage, starch, total soluble sugar and phenol content in okra (*Abelmoschus esculentus* (L.) Moench, cultivar Marsaouia) seeds and growth seedlings components (cotyledons and embryonic axes) were studied. Seeds were sown in Petri dishes with varying concentrations of saline solutions (0, 20, 40, 60, 80 and 100 mM NaCl) at 25°C. Seed germination decreased significantly with the increase in NaCl concentration, optimal germination percentage occurred in non saline solution reaching 100%. There was more accumulation of sugar and phenol, starch level decreased markedly especially in cotyledons along with lower amylase activity. Amounts of sodium increased but amounts of potassium did not change significantly with increasing stress.

Key words: *Abelmoschus esculentus*, germination, seeds, salinity, starch, total soluble sugars, phenols, amylase, ion uptake.

INTRODUCTION

Seed germination is one of the most important phases in the life cycle of plant and is highly responsive to existing environment (Saritha et al., 2007). Salinity is one of the major physical parameter of an environment, which determines the success or failure of plants establishment. Consequently, the study of salt tolerance during germination early and late growth of plants is important for determining saline limits at each developmental phase (Zapata et al., 2004). In addition, it has been reported that salinity decreased as well as delayed germination of most crops such as melon (Botia et al., 1998) and tomato (Cuartero and Fernandez-Munoz, 1999). Lower levels of salinity delayed germination, whereas higher levels reduced the final percentage of seed germination (Ghoulam and Fares, 2001). Salt induced inhibition of seed germination could be attributed to osmotic stress or to specific ion toxicity (Huang and Redmann, 1995).

Alternatively, it is assumed that germination rate and the final seed germination decrease with the decrease of the water movement into the seeds during imbibitions (Hadas, 1977). Younis et al. (1991) reported that low moisture content under salt stress caused cessation of metabolism or inhibition of certain steps in metabolic sequences of germination. Evenly, during germination, salt stress increased the intake of toxic ions which may alter certain enzymatic or hormonal activities of seeds (Smith and Comb, 1991). Moreover, several reports suggest that hyper-saline environments cause delayed germination (Prado et al., 1995) by reducing hydrolytic enzyme activities and retarding the mobilization rate of metabolites (Ashraf et al., 2002).

It is assumed that during germination and early seedling growth, cell division and enlargement require transportation of respiratory substrates, in the form of soluble sugars and low molecular weight protein from seeds storage organs to the site of growth (Bewley and Black, 1994). It is suggested that saline stress limited hydrolysis of food reserves from storage tissues as wall as it impaired their translocation from storage tissue to developing embryo axes (Dubey, 1985; Lacerda et al., 2003). In this study, we are interested in understanding the physiological basis of salt tolerance in okra during germination, to detect the variation that occurs in carbohydrate levels, phenols content and ion uptake in germinating embryos and cotyledons in correlation with

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carbohydrate metabolizing enzymes.

In fact, the hard seed coat of okra (*Abelmoschus esculentus* (L.) Moench) seeds interferes with water uptake and is a major physiological constraint to uniform stand establishment and performance (Marsh, 1993). Marsaouia is the most cultivated cultivar in Tunisia, its germinating characteristics under NaCl stress was involved in this study.

MATERIALS AND METHODS

Seed material and germination

Seeds were collected from local plants of okra (*A. esculentus* L., cv. Marsaouia) and were used for the germination test. Okra 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 filter paper, saturated with distilled water (control) or NaCl solutions (20, 40, 60, 80 and 100 mM NaCl) at 25°C in the dark. In each treatment, 8 Petri dish (each one contained 25 seeds) was used. The seeds were considered germinated when the radicle reached 2 mm, cumulative germination percentage was determined. The germinated seeds were counted every 2 days during the 10 days-experimental period.

Seedling analysis

Germinated seeds in distilled water and in saline solutions were sampled after 48 h from the beginning of incubation and then placed at -20°C to stop the germination process. Cotyledons and embryonic axes were separated under binocular microscope. A part of these tissues were weighed to obtain the fresh weight. The dry weight was obtained after drying the tissue at 75°C for 48 h; then the tissue water content was calculated as (Prado et al., 2000) based on the (FW-DW/DW) ratio.

Starch determination

Starch content was determined according to Allefrey and Northcote (1977) as cited by Bewley et al. (1993). Batches of 12 cotyledon pairs or axes were homogenized in an ice-cold mortar and pestle in a volume of 16 ml 80% (v/v) ethanol. The homogenates were centrifuged (30000 x*g*, 10 min at 2°C) and then perchloric acid ($HClO_4$; 6 ml, 30%, v/v) was added to solubilize starch from the pellet. The slurry was left at room temperature for 6 h, starch was detected with I_2-KI reagent prepared by diluting 0.1 ml stock solution (0.06 g I_2 and 0.60 g KI in 10 ml deionized water) with 0.05 M HC1 just prior to the assay. Samples of 0.5 ml starch solution were mixed with 0.5 ml I_2-KI reagent, 1 ml 30% (v/v) perchloric acid and then were vortexed and left standing at room temperature. The absorbance (620 nm) of the samples was compared to that of the standard curve of 0 to 5 mg/ml which was obtained using soluble starch dissolved in 30% $HClO_4$ and detected with the same I_2-KI reagent. The assay was conducted in triplicate for each sample.

Determination of soluble sugars

Three replicates of cotyledons or axes samples of germinated seeds were suspended in test tubes with 3 ml of 80% ethanol, the extract was evaporated to dryness and the residue was dissolved in 20 ml of distilled water. Total soluble sugars were determined by the phenol sulfuric acid method (Dubois et al., 1956) using glucose as standard.

Amylase activity

Total amylase preparations were made according to method of (Liu et al., 2005) by grinding cotyledons of germinated seeds in a mortar and pestle with 0.05 M acetate buffer (pH 6.0). All operations were performed at 4°C. The resulting homogenate was strained through cheesecloth and then centrifuged (18000*g, 10 min) and filtered. The substrate for amylase is a 1% solution of soluble starch in 0.1 M acetate buffer (pH 5.6). The enzyme prepared to be tested was diluted to 1 ml with water and then 1.0 ml of the substrate solution was added. After 1 h incubation at 25°C, 2 ml of a 3,5-dinitro-salicylic acid was added and the tubes were placed in a boiling water bath for 5 min (Swain and Dekker, 1966) and then cooled to room temperature and diluted with 20 ml of water. The absorbance values of the resulting colored solutions were determined by a spectrophotometer. The results are the average of three replicates ± SE.

Total phenolics assay

The phenolics assay modified by Shetty et al. (1995) was followed; it was measured as gallic acid equivalents (Kwok and Shetty, 1996). Okra sprouts (50 mg) was soaked in 2.5 ml of 95% ethanol and kept in the freezer for 48 h. The sample was homogenized and centrifuged (13000 rpm, 10 min). One ml of the supernatant was transferred to a test tube, and 1 ml of 95% ethanol, 5 ml of distilled water and 0.5 ml of Folin-Ciocalteu phenol reagent were added. After an incubation period of 5 min, 1 ml of 5% Na_2CO_3 was added, mixed well and kept in the dark for an hour. The samples were vortexed and absorbance was measured at 725 nm using a UV spectrophotometer. Phenolic content was reported as mg/g fresh weight (FW).

RESULTS

Seed germination under different salinity levels

Cumulative germination percentage of seeds was influenced by NaCl concentrations (Figure 1). Seed germination in distilled water reached the maximum (100%) after 8 days, whereas germination rate was adversely affected by salinity treatment. However, salinity caused a slightly decrease in final germination percentage to 80.5% under 100 mM NaCl.

Seedling growth

Table 1 shows the influence of NaCl on the growth and water content of embryonic axes and cotyledons in germinated seeds. In the cotyledons the FW (fresh weight) values did not show significant differences between control and salt solution. However, in embryonic axes, the FW values decreased by 60 mM NaCl treatment.

The DW (dry weight) displayed significant differences on one hand between control and 80 mM NaCl treatment in embryonic axes, between control and 60 mM NaCl

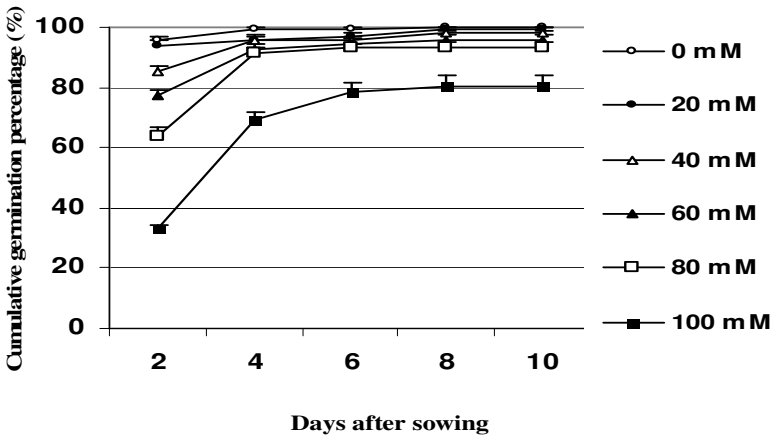

Figure 1. Cumulative germination percentage of okra cv. Marsaouia under increasingly saline conditions. Values are means of 8 replicates. Bars on data points are ± SE of the mean.

Table 1. Fresh weight (FW), dry weight (DW) and water content in embryonic axes and cotyledons of okra seeds grown at 0, 20, 40, 60, 80 and 100 mM NaCl.

	0 mM	20 mM	40 mM	60 mM	80 mM	100 mM
Embryonic axes						
FW (mg)	11.725±1.65^{a}	11.22±1.7^{ab}	10.58±1.19^{ab}	10.31±1.42^{b}	11.42±1.35^{ab}	10.61±1.34^{ab}
DW (mg)	3.52±6.05^{a}	3.63±0.54^{ab}	3.82±0.45^{ab}	3.81±0.53^{ab}	4.03±0.47^{b}	3.92±0.43^{ab}
Water content	2.37±0.44^{a}	2.10±0.36^{b}	1.78±0.28^{c}	1.72±0.29^{c}	1.85±0.4^{c}	1.72±0.34^{c}
Cotyledons						
FW (mg)	44.35±7.03^{a}	46.18±6.1^{a}	47.93±7.05^{a}	49.22±8.2^{a}	43.02±6.0^{a}	43.04±7.8^{a}
DW (mg)	22.5±4.2^{a}	23.86±3.6^{ab}	25.87±5.4^{ab}	27.33±4.3^{b}	22.83±4.0^{a}	25.06±5^{ab}
Water content	1.04±0.54^{a}	0.94±0.17^{ab}	0.90±0.34^{ab}	0.81±0.2^{ab}	0.90±0.18^{ab}	0.73±0.1^{b}

For each tissue, value followed by a different letter are significantly different at the 0.05 confidence level (n = 20).

treatment on the other hand.

Further, a significant decrease in water content of embryonic axes was observed from 40 mM NaCl. The lowest value of this parameter was observed at 60 and 100 mM NaCl (1.72). In cotyledons, the water content varied only slightly at this stage of germination (radicles length 2 mm) water content vary from 1.04 (0 mM NaCl) to 0.73 (100 mM NaCl).

Starch changes during germination

Starch is a food reserve in okra seeds, it was distributed equally between cotyledons and embryonic axes respectively among 0.09 ± 0.01 mg/g FW and 0.11 ± 0.029 mg/g FW. Figure 2 shows the influence of NaCl concentrations on starch level in cotyledons and in embryonic axes of germinating seeds.

During germination, the amount of starch in embryos increased clearly (seeds treated with distilled water) and reached 0.35 mg/g FW, starch level in cotyledons was less important attaining 0.18 mg/g FW. Both tissues cotyledons and embryonic axes treated with NaCl contained lower levels of starch than that treated with distilled water. No decrease of starch amount was observed in embryonic axes or cotyledons of seeds treated with 20 mM of NaCl. A large decrease of starch was observed in embryonic axes treated with 100 mM NaCl, while cotyledons showed a lower decrease.

Changes in total soluble sugars during germination

The influence of salinity levels on total soluble sugar content of germinated seeds is shown in Table 2. As compared with control, imposition of NaCl treatment resulted in a significant increase in total sugar content in cotyledons and mainly in embryonic axes. The rate of increase under NaCl treatment was more pronounced in embryonic axes, significant differences were observed

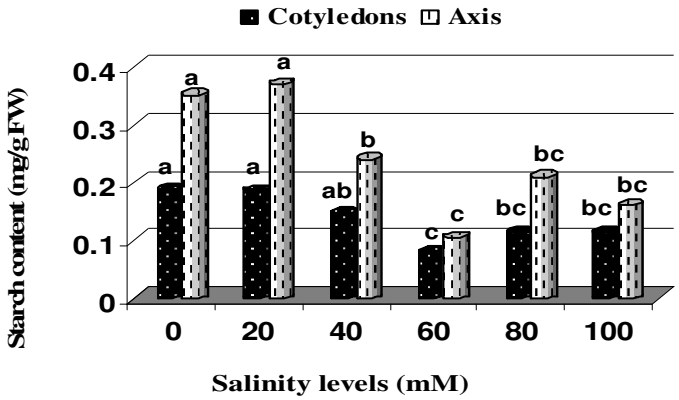

Figure 2. Changes in the levels of starch in cotyledons and embryonic axes of germinating okra seeds treated with distilled water and saline solutions. Values followed by the same letter indicate no significant differences ($p \leq 0.05$) according to Duncan test.

Table 2. Changes of total soluble sugar content (mg/g FW) in cotyledons and embryonic axes of okra seeds germinating in distilled water and saline solutions.

NaCl (mM)	Cotyledons	Embryonic axes
0	0.78[a]	0.71[a]
20	1.04[b]	1.11[b]
40	1.18[b]	1.66[c]
60	1.19[b]	2.24[d]
80	1.23[b]	2.55[e]
100	1.25[b]	3.28[f]

Values of each column followed by the same letter indicate no significant differences ($p \leq 0.05$) according to Duncan test.

between the various NaCl concentrations for total sugars in embryonic axes. Whereas, the level of total soluble sugars increased to a smaller extent over stressed seeds in cotyledons, no significant differences were displayed between different salt solutions.

Under the influence of high concentrations of NaCl (60 to 100 mM) soluble sugars in cotyledons was around 1.2 mg/g FW, while embryonic axes contain an important amount of soluble sugars fluctuated markedly between 2.2 and 3.28 mg/g FW.

Changes in amylase activity in the cotyledons

The activity of total amylase detected in seeds germinated in the control treatment was higher compared to those detected in stressed seeds. Figure 3 shows the influence of the presence of various concentrations of NaCl on the amylase activity in cotyledons of germinated seeds. The activity of this enzyme showed a decreasing trend with the increase in NaCl concentration even at low concentration. Amylase activity decreased particularly by 38% at 80 mM NaCl treatment.

Total phenolics accumulation

The total phenolics content of germinated okra sprouts was different across NaCl treatments (Figure 4); within untreated sprouts contain 18.5 mg/g FW of polyphenols. The amount of total phenols increased with salinity, principally, sprouts treated with 60 and 100 mM NaCl showed the greater accumulation of polyphenols which was around 21 mg/g FW.

Effects of salt stress on ion uptake

Analysis of variance revealed significant differences among the NaCl concentrations for sodium uptake. Sodium amount increased markedly in response to increasing stress levels (Figure 5). This increase was more signify-cant at 80 and 100 mM NaCl treatment. However, salinity effects on potassium concentration were less evident than those of Na^+. The concentration of K^+ in germinated seeds was not significantly influenced by salinity except at 80 mM NaCl, the potassium content decreased (345.5 µéq/g DW). This reduction was

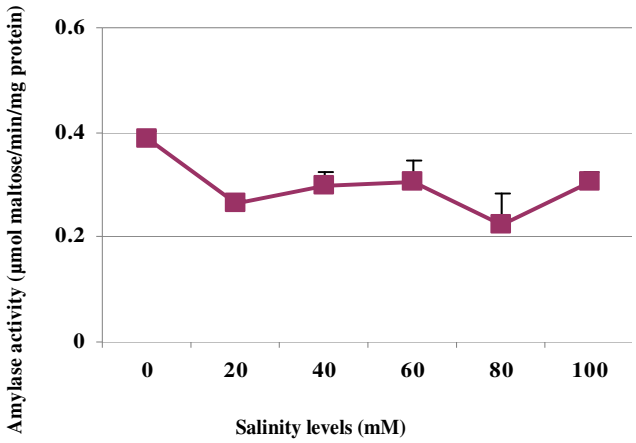

Figure 3. Influence of NaCl on the activity of amylase in cotyledons of germinating okra seeds.
Vertical bars represent standard errors. Only those standard errors larger than the symbol size are shown.

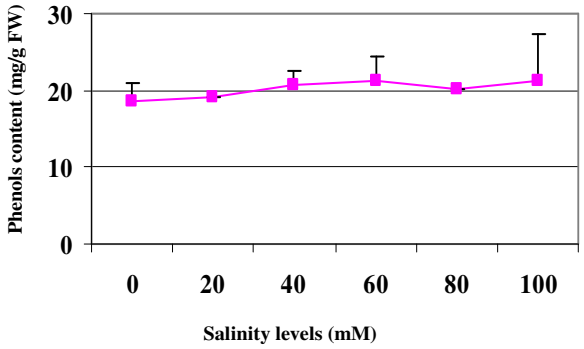

Figure 4. Changes of total phenols concentrations in sprouts of germinating okra seeds treated with distilled water and saline solutions. Vertical bars represent standard errors. Only those standard errors larger than the symbol size are shown.

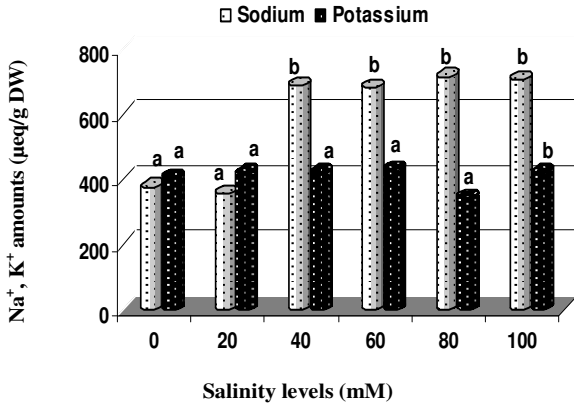

Figure 5. Sodium (Na^+) and potassium (K^+) concentrations (µeq/g DW) of okra seeds soaked in distilled water and salt solutions. Values followed by the same letter indicate no significant differences ($p \leq 0.05$) according to Duncan test.

accompanied with an increase of sodium content (706 µéq/g DW).

DISCUSSION

The completion of germination in okra seeds (in distilled water and under saline conditions) occurs within a 48 h period commencing 24 h from the start of imbibition in darkness at 25°C. Our results showing a decrease in germination of seeds with increasing salinity, Thakur and Sharma, (2005) has reported that decrease in germination particularly under salt stress may be due to the fact that seeds seemingly develop an osmotically enforced "dormancy" under water stress conditions. It has been reported that salinity delays germination of several species but does not appreciably reduce the final germination percentage (Ayers and Westcot, 1985). We did not observe this in the germination of okra seeds, where the final percentage of germination was reduced (-20%) by high levels of salt. Also, we have distinguished that okra seeds were less sensitive to salinity.

In relation to seedling growth, both germinated embryos and cotyledons were slightly influenced by salinity treatment after radicle protrusion. Some studies have reported that biomass accumulation increased (Jones and Turner, 1980; Munns and Weir, 1981) while others have found that it decreased (Hanson and Hitz, 1982) or remained unchanged (Morgan, 1992) during stress conditions. The DW did not show significant differences in both embryonic axes and cotyledons, thus the DW increases are associated with cell division and new materiel synthesis (Sunderland, 1960). The [(FW-DW/DW)] ratio showed a small variation in embryonic axes, it changes from 2.37 to 1.72.

The radicle protrusion was accompanied with an important increase in the starch level in embryonic axes, correlated with a slightly increase in cotyledons suggesting the transfer of hydrolytic products from the cotyledons to the embryonic axes. The starch content (mg/g of FW) of both embryonic axes and cotyledons decreased under saline conditions. NaCl suppressed significantly starch content in both cotyledons and embryonic axes. It has been reported that salt stress limit the mobilization of starchy endosperm reserves in several species, as a result of inhibition of different enzymatic activities (Lin and Kao, 1995; Almasouri et al., 1999).

Our results shows variations in total soluble sugars content that occured in cotyledons and in embryonic axes in germinated okra seeds under saline conditions. The amount of total soluble sugar/embryonic axes fresh weight increase rapidly answering to the increasing concentrations of NaCl, this result agree with the result of some researchers that indicate that salinity stress induce soluble sugar accumulation (Prado et al.,2000). This is also the case with sorghum seeds, the stress causes a decrease in starch content and an increase in sugar content (Thakur and Sharma, 2005).

As explained by Giorgini and Suda (1990), the higher level of soluble sugars detected is probably necessary for the turgor and growth of embryonic axes during emergence. Nevertheless, in cotyledons, sugar levels were not as large as in embryonic axes possibly as explicated by Prado et al. (2000), it was due of the weak development followed by a diminished metabolic activity in cotyledons. In addition, Singh (2004) proved that a greater accumulation of sugar lowers the osmotic potential of cells and reduces loss of turgidity in tolerant genotypes. The other possible role of sugar may be as a readily available energy source.

The increase in sugar levels accompanied by decrease in starch content in embryos and cotyledons was directly linked to the activity of α-and β-amylases, which is in agreement with the existing reports of Monerri et al., 1986; Gupta et al., 1993). Kameli and Losel (1995) confirmed that this increase might be considered to play an important role in osmotic adjustment, which is widely regarded as an adaptive response to water deficit conditions. In addition, our results showed that total amylase activity was less affected by high salinity concentration. In this study, we found a correlation between amylase activity, soluble sugars and reduction of starch in cotyledons. Thus, the slowly hydrolysis of starch in cotyledons under salinity stress is attributed to the lower amylase activity in cotyledons.

Phenolic compounds are important at post germination process for lignification of seedlings (Mng'omba et al., 2007). Our results showed an increase of total phenols amount in stressed sprouts. It appears that a decrease in germination is related to salinity induced disturbance of metabolic process leading to increase in phenolic compounds (Ayas et al., 2000). Singh (2004), found that tolerant genotypes of chickpea (*Cicer arietinum*) showed a higher level of total phenols, whereas a significant reduction was observed in susceptible genotypes which is the same as the results of Dostanova et al. (1979); Latha et al. (1989). The same author confirmed also that phenol accumulation in tolerant genotypes could be a cellular adaptative mechanism for scavenging the free radicals of oxygen and preventing subcellular damage during stress.

Moreover, in this present study, it has been noted that the amounts of Na^+ increased gradually replying the salt concentration increase, whereas, those of K^+ did not change regularly. Haq et al. (2003) found that sodium concentration increased significantly with an increase in salinity from 1.2 to 15 dSm^{-1}. Na^+ concentration increased also in barley seeds soaked in salt solution (Othman et al., 2006). According to Bhivare and Nimbaker (1984), the reduction of potassium content and the increased of sodium content in plant could be attributed to the effect of competition between Na^+ and K^+ ions on the absorptive sites of plant.

In conclusion, the result of this experiment indicates that NaCl disturbed the mechanism of germination in okra seeds, starch reserve accumulation which was the support of seedling growth decreased in embryonic axes, total soluble sugars and Na^+ amounts increased markedly.

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