J. Crop Sci. Biotech. 2014 (March) 17 (1) : 11~20
DOI No.10.1007/s12892-013-0084-8

RESEARCH ARTICLE

Growth, Physiological, and Biochemical Responses in Relation to Salinity Tolerance for *In Vitro* Selection in Oil Seed Crop *Guizotia abyssinica* Cass.

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Received: July 13, 2013 / Accepted: March 5, 2014

Abstract

The calli cultures of *Guizotia abyssinica* (niger) cultivars IGP 76 and GA 10 were exposed to different levels of salt treatments (0, 30, 60, and 90 mM NaCl), in order to evaluate growth, physiological, and biochemical responses. A significant decrease in relative growth rate and tissue water content of GA 10 calli than IGP 76 under salt-stress conditions was associated with higher sodium ion accumulation. Osmotic adjustment revealed by the osmolytes (proline, glycine betaine, and total soluble sugars) accumulation was significantly higher in IGP 76 salt-stressed calli as compared to GA 10. The sustained growth and better survival of IGP 76 calli was correlated with lower malondialdehyde content and increased superoxide dismutase, ascorbate peroxidase, and catalase activities and higher α-tocopherol content in comparison to GA 10. The higher osmolytes accumulation and presence of better antioxidant system suggested superior adaptation of IGP 76 calli on salt-containing medium for prolonged periods in comparison to GA 10. The regeneration frequency, organogenesis, and acclimatization response of the plants derived from salt-adapted calli was comparatively lower than the plants derived from control calli of IGP 76. The growth, physiological, and biochemical characterization of the salt-tolerant regenerated plants exposed to stepwise long-term 90 mM NaCl treatment revealed no significant changes in comparison to the control. Thus, our results suggests the development of an efficient protocol for *in vitro* selection and production of salt-tolerant plants in self-incompatible crop, niger, and an alternative to traditional breeding programs to increase the abiotic stress tolerance.

Key words: antioxidant enzymes, callus culture, *Guizotia abyssinica*, *in vitro* selection, osmolytes, salt tolerant plant

Introduction

Salinity stress is one of the major abiotic constraints in arid and semi-arid regions of the world producing crop yield below its original potential. Increased urbanization and problems associated with good quality irrigation resources are continuously involved in producing the arable land unfertile. As a consequence, more than 800 million hectares of land throughout the world became saline which is accounting more than 6% of the world's total land area (Munns and Tester 2008). With this rate, the arable land affected by direct and indirect salinization is expected to reach alarming levels by 2050 (Joshi et al. 2011). Unfortunately, most of the crop relatives are glycophytes, able to tolerate the salt concentrations ranging from 40 to 100 mM NaCl (Munns and Tester 2008). However, excess salt concentrations in the soil influence various physiological processes of the plant and differs from plant to plant species (Sheekh et al. 2002). Therefore, in last few decades, managing salt stress and understanding the mechanism of tolerance for the improvement of this trait and development of tolerant crop varieties remains a challenge to ever-growing research. In this context, screening plant species for stress tolerance at the whole plant level has found limitations for the identification of tolerant genotypes; however screening, selection, and regeneration of salt-tolerant lines using

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various biotechnological approaches have contributed to improving the salt tolerance character of the glycophytes and development of tolerant cultivars (Rai et al. 2011).

Guizotia abyssinica Cass., (Family: Asteraceae) commonly called niger, is an important oilseed crop extensively cultivated on the Indian subcontinent and in East African countries. The plant has been widely used for its edible seed oil content (30 - 50%) which has shown its role in nutrition and protection from cardiovascular disorders and cancer due to the presence of a variety of substances such as tocopherols, phospholipids, and sterols (Ramadan and Morsel 2002). Besides the use of niger as a source of nutrition and medicine, the plant has not been studied extensively for the improvement of its yield through conventional plant breeding techniques because of the problem of self-incompatibility, the most serious barrier for the development and maintenance of inbred lines (Getinet and Sharma 1996). In such types of plant species, traditional plant breeding strategies could be supplemented with the application of tissue culture techniques for the production of plants with improved salt tolerance through selection of salt-tolerant cell lines and its successive regeneration into complete plants (Rai et al. 2011). Further, the growth performance of these regenerants under the influence of salt stress conditions followed by its physiological and biochemical characterization will provide the elite seed material to traditional breeding programs for the production of salt-tolerant genotypes. The selection of the regenerants can be made using either stepwise long-term treatment wherein cultures are exposed to stress with a gradual increase in concentration of selecting agents or shock methods in which cultures are directly subjected to a shock of high concentration and only those which would tolerate that level will survive (Purohit et al. 1998).

In plants, the series of events leading to the perturbation of cellular metabolism under NaCl stress are suggested to be as follows: less water availability, stomata closure, altered gaseous exchange, inhibition of photosynthesis, effect on electron flow in electron transport chain (ETC) in chloroplast and mitochondria, increased production of reactive oxygen species (ROS), and imposition of oxidative stress ultimately affecting the entire growth and gross yield of the crop plants (Miller et al. 2009; Munns andTester 2008). In response to alteration in the various metabolic processes of the plants due to salt imposition, the behavior of the regenerants produced through tissue culture under the influence of salinity could be assessed using a variety of growth, physiological, and biochemical parameters. The organic osmolytes like proline, glycine betaine, and total soluble sugars have been known to be involved in maintaining the osmotic balance of the cell due to ionic imbalance and hyper-osmotic stress. The enzymatic antioxidants like superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX) along with the non-enzymatic antioxidants viz., carotenoids, tocopherols, phenolics, ascorbate, and glutathione have shown their role in the fine-tuning of overproduced ROS and in signaling processes of the plant (Ashraf and Foolad 2007; Mittler 2002).

Since, meager information is available on the screening and selection of the salt-tolerant lines in niger using traditional plant breeding or biotechnological approaches, the present study was aimed with the objectives (1) to study the growth, physiological, and biochemical responses of niger cultivars at the cellular level under the influence of salt stress and maintenance of salt-tolerant cell lines, and (2) development of plants from salt-tolerant cell lines and its characterization for stress tolerance.

Materials and Methods

Source of explant and callus culture establishment

Certified seeds of niger (*G. abyssinica*) cultivars viz., IGP 76 and GA 10 procured from the Zonal Agricultural Research Station, Igatpuri, Nashik, Maharashtra (India) were surface sterilized with 0.1% (w/v) $HgCl_2$ solution for 4 min followed by washing five times with distilled water. The surface sterilized seeds were inoculated on sucrose (1%, w/v), -agar (0.8%, w/v) medium and incubated in dark at 25 ± 2°C for germination. After 7 days, the cotyledons were excised from *in vitro*-germinated seedlings and used as a source of explants for callus induction.

The explants of both the cultivars were aseptically cultured on callus induction MS (Murashige and Skoog 1962) medium containing 3.0% (w/v) sucrose, 6.66 µM BA (6-benzyladenine) and 2.69 µM NAA (α-napthaleneacetic acid) as described earlier by Nikam and Shitole (1997). The pH of the medium was adjusted to 5.8 ± 0.2 and solidified with 0.8% agar-agar (Himedia Pvt. Ltd, India) prior to autoclaving at 121°C for 15 min. The cultures were incubated under control conditions including 25 ± 2°C temperatures, 16-h photoperiod with a light intensity 40 µmol m^{-1} s^{-1}, and 70% relative humidity. At the end of 3 weeks of culture incubation, the calli induced from the cut end of cotyledon explants were excised and sub-cultured regularly for 6 months at an interval of 21 days on the callus induction medium. The proliferated calli were then used for the establishment of salt-tolerant cell lines.

Salt stress treatment to calli

Approximately 500 mg calli of both cultivars were cultured separately on callus induction medium containing various concentrations of NaCl (0, 30, 60, and 90 mM).The cultures were incubated under control conditions as mentioned earlier. After 15 days of salt stress treatment, the calli harvested from control and NaCl treatments were subjected to growth, water content, and various physiological and biochemical analyses.

Growth, physiological, and biochemical analyses

The percent relative growth rate (RGR), tissue water content (TWC), and accumulation of mineral ions (Na^+ and K^+) of treated and non-treated calli were measured according to the protocol described by Lokhande et al. (2010). The total

chlorophyll content was measured according to the protocol described by Lichtenthaler and Buschmann (2001). Accumulation of organic osmolytes such as proline, glycine betaine, total soluble sugars (TSS), and lipid peroxidation in terms of malondialdehyde (MDA) content were estimated following Bates et al. (1973), Grieve and Grattan (1983), Watanabe et al. (2000), and Heath and Packer (1968), respectively, as described previously (Lokhande et al. 2010).

Plant material was homogenized in buffers specific for each enzyme under chilled conditions. Homogenate was squeezed through four layers of cold cheese cloth and centrifuged at 12,000 g for 15 min at 4°C. Protein content of the supernatant was measured following Lowry et al. (1951). The catalase (CAT, EC 1.11.1.6), ascorbate peroxidase (APX, EC 1.11.1.11), and superoxide dismutase (SOD, EC 1.15.1.1) were assayed following Cakmak and Marschner (1992), Nakano and Asada (1981), and Becana et al. (1998), respectively, as described previously (Lokhande et al. 2010).

Extraction and estimation of α-tocopherol content

The α-tocopherol was extracted from the plant material (fresh biomass) according to the protocol described by Panfili et al. (1994). In brief, the calli (1.0 g FW) were hydrolyzed in the solution containing 12% (w/v) potassium hydroxide, 20% (v/v) ethanol, 0.1% (w/v) sodium chloride at 70°C in hot water bath for 30 min. The homogenate was cooled to room temperature and then extracted twice with the mixture of n-hexane-ethyl acetate (9 : 1) for 10 min. The collected organic phase was evaporated to dryness using rotavapour at 55°C, the residue remained was dissolved in 1.0 mL 98% (v/v) HPLC grade methanol and the sample was provided to HPLC analysis.

HPLC analysis

The isocratic HPLC system (DIONEX 170 AU, Germany) consisted of a P-680 solvent delivery pump, a BDS Hypersil C18 (250 × 4.6 mm, 5 µm) column (Thermo scientific, Part No. 28105-254630), and a UV detector (170 U). The mobile phase involved methanol:water (98 : 2, v/v) with a flow rate of 1.0 mL min^{-1} at 25°C. Samples of α-tocopherol (20 µL) were injected and scanned at 220 nm. The content in the samples was quantified by comparing the peak area with that of α-tocopherol standard (Cat. No. 10191, Sigma-Aldrich, USA). The α-tocopherol content was calculated and expressed in terms of mg g^{-1} FW.

Maintenance of salt tolerant calli and development of plantlets

For the production of salt-tolerant calli lines, the calli of both the cultivars grown without (control) or with 90 mM NaCl were sub-cultured regularly on the fresh callus induction medium in the absence or presence of 90 mM NaCl at an interval of 21 days for a period of 13 months and maintained under the control conditions as described earlier.

After the prolonged period of calli maintenance on the medium without or with 90 mM NaCl supplementation, the calli were subjected for shoot regeneration and elongation on shoot induction medium (MS containing 4.44 µM BA) as reported previously by Nikam and Shitole (1997). The elongation of regenerated shoots was achieved on the same medium. The elongated shoots (4 - 5 cm) were transferred to MS medium containing 0.5 mg L^{-1} NAA for rooting (Nikam and Shitole 1997). The cultures were incubated under control conditions as mentioned earlier. The vigorously grown, well-rooted *in vitro*-raised plants were transferred to *ex vitro* conditions for acclimatization wherein the survival percentage was measured and then the plants were subjected for salt-stress tolerance potential.

Stepwise long-term salt stress treatment and characterization of regenerated plantlets

The regenerated plantlets derived from salt-tolerant calli were gradually exposed to salt stress treatment initially at 40 mM and 70 mM NaCl treatments twice at an interval of 4 days to build up the stress condition and then maintained at 90 mM NaCl stress condition up to 30 days. The plantlets derived from calli grown on callus induction medium without supplementation of 90 mM NaCl were used as control. At the end of the salt stress treatment, the third leaf from the plants was harvested and analyzed for growth and various physiological and biochemical parameters as mentioned earlier.

Statistical analyses

The experiments were laid out in a completely randomized design (CRD). The analyses were repeated with minimum three independent biological samples. The data were analyzed by one-way analysis of variance (ANOVA) using the statistical software SPSS 10.0 and the treatment means were compared by using Duncan's multiple range test (DMRT) at $P \leq 0.05$. Data were expressed as mean ± standard error (SE).

Results

Effect of salt stress treatment on calli growth and water status

The calli of both the cultivars exposed to different salt stress treatments showed significant decline in percent relative growth rate (RGR) and tissue water content (TWC) in comparison to their respective control (Figs. 1A and B). At 90 mM NaCl stress, the RGR of cultivar IGP 76 calli was comparatively higher than GA 10, wherein it decreased by 74 and 85%, respectively, in comparison to their respective control (Fig. 1A). Similarly, the TWC of both the cultivars showed significant reduction (50 and 60%, respectively) at 90 mM NaCl stress over the control (Fig. 1B).

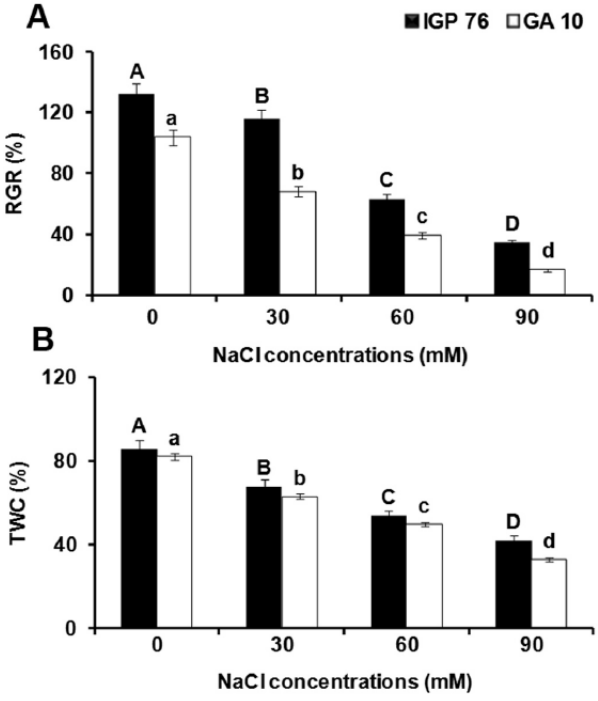

Fig. 1. The relative growth rate (RGR) and tissue water content (TWC) of niger cultivars IGP 76 and GA 10 calli under different salt stress treatments. Callus (~ 0.5 g) was transferred to media containing 0 - 90 mM NaCl, and (A) RGR, and (B) TWC was measured after 15 days of culture. Error bars SE (n = 3). Within each set of experiments, means with different letters are significantly different at $P \leq 0.05$

Influence of salt stress treatment on accumulation of Na^+ and K^+ ions

Mineral ions accumulation affected significantly in response to salt stress treatment to the calli of IGP 76 and GA 10 cultivars. Na^+ ion accumulation increased significantly in both the cultivars with an increase in the salt concentrations, wherein IGP 76 and GA 10 revealed 28.48- and 16.50-fold higher Na^+ content at 90 mM NaCl in comparison to their respective control (Fig. 2A). However, K^+ content decreased significantly in both the cultivar calli with an increase in the salt concentrations in comparison to their respective control, the decreasing trend was found almost similar in both cultivars (Fig. 2B). The change in Na^+ and K^+ content of both the cultivar calli resulted in a significant increase in the Na^+/K^+ ratio at higher levels of NaCl (90 mM) in comparison to their respective control; the increase was significantly higher in IGP 76 (48.46-fold) than GA 10 (26.65-fold) (Fig. 2C).

Osmolytes accumulation in salt-stressed calli

The accumulation of proline, glycine betaine, and total soluble sugars (TSS) content increased significantly in salt-stressed calli in comparison to non-stressed calli of cultivars IGP 76 and GA 10, however, these osmolytes accumulation showed significant variations among the cultivars exposed to different salt concentrations (Figs. 3A, B, and C). Although

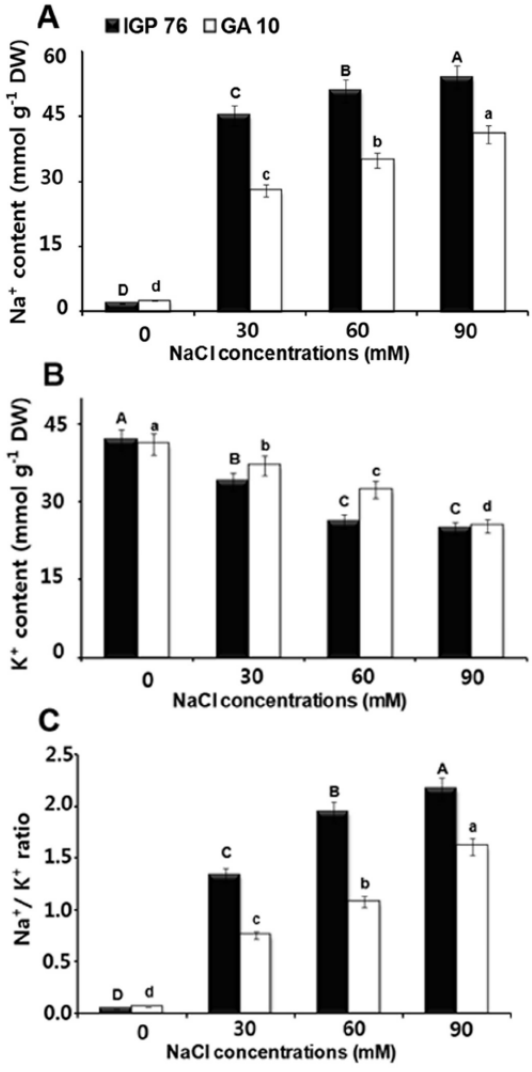

Fig. 2. Mineral ions (sodium- Na^+ and potassium- K^+) accumulation and sodium/potassium- Na^+/K^+ ratio of niger cultivars IGP 76 and GA 10 calli under different salt stress treatments. Callus (~ 0.5 g) was transferred to media containing 0 - 90 mM NaCl, and (A) Na^+, (B) K^+, and (C) Na^+/K^+ ratio was measured after 15 days of culture. Error bars SE (n = 3).Within each set of experiments, means with different letters are significantly different at $P \leq 0.05$

the proline accumulation in cultivars IGP 76 and GA 10 increased significantly at higher concentrations of NaCl (90 mM) in comparison to their control, the trend of accumulation was found to be similar (3.66-fold over control) in both the cultivars (Fig. 3A). However, glycine betaine accumulation revealed significantly the highest content in cultivar IGP 76 (2.32-fold) in comparison to GA 10 (1.30-fold) at 90 mM NaCl stress (Fig. 3B). Likewise, TSS accumulation also increased significantly in both the cultivars with an increase in the salt concentrations from 30 to 90 mM NaCl in comparison to their respective control; however, the content was significantly higher in IGP 76 than GA 10. As like proline, the trend of TSS accumulation was found comparatively similar in both the cultivars (Fig. 3C).

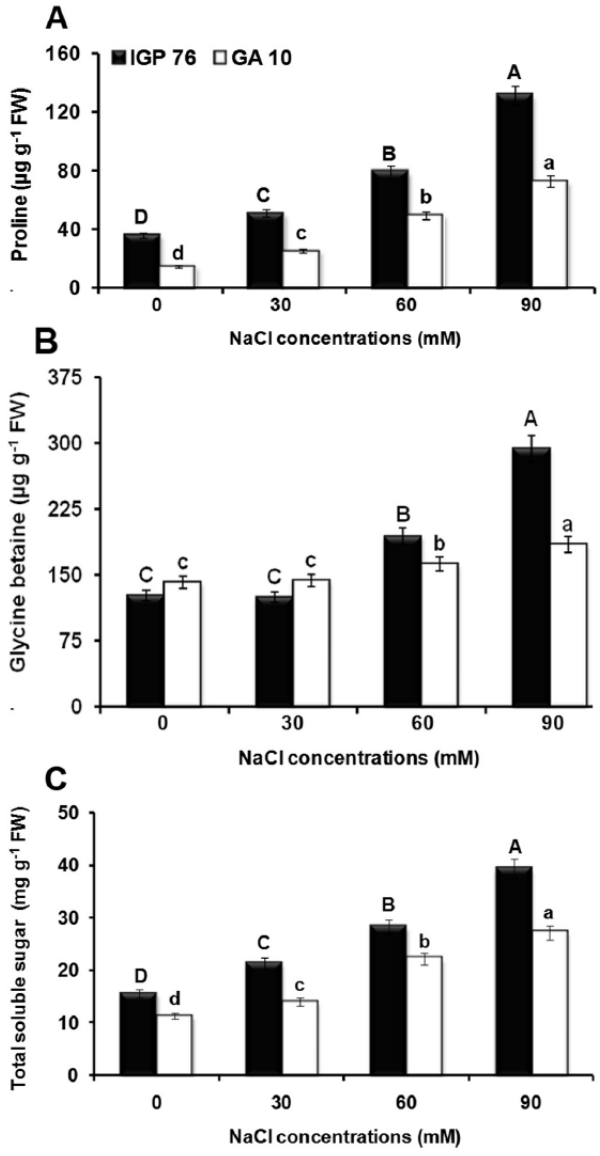

Fig. 3. Osmolytes (proline, glycine betaine, and total soluble sugars- TSS) accumulation of niger cultivars IGP 76 and GA 10 calli under different salt stress treatments. Callus (~ 0.5 g) was transferred to media containing 0 - 90 mM NaCl, and (A) proline, (B) glycine betaine, and (C) TSS contents were measured after 15 days of culture. Error bars SE (n = 3).Within each set of experiments, means with different letters are significantly different at $P \leq 0.05$

Effect of salt stress treatment on oxidative damage and antioxidant enzyme activities

The cultivars IGP 76 and GA 10 calli exposed to different concentrations of salt revealed significant oxidative damage to membrane lipids measured in terms of MDA content (Fig. 4A). The extent of damage was significantly higher in the cultivar GA 10 in comparison to IGP 76 at each concentration of NaCl; however, 90 mM NaCl treatment revealed severe damage to membrane lipids in comparison to the control in both the cultivars. SOD enzyme activity showed a significant increase at 30 and 60 mM NaCl; whereas, it decreased at 90 mM NaCl in both the cultivars in comparison to their respective control. The activity was comparatively better in cultivar IGP 76 than GA 10 at all the concentrations

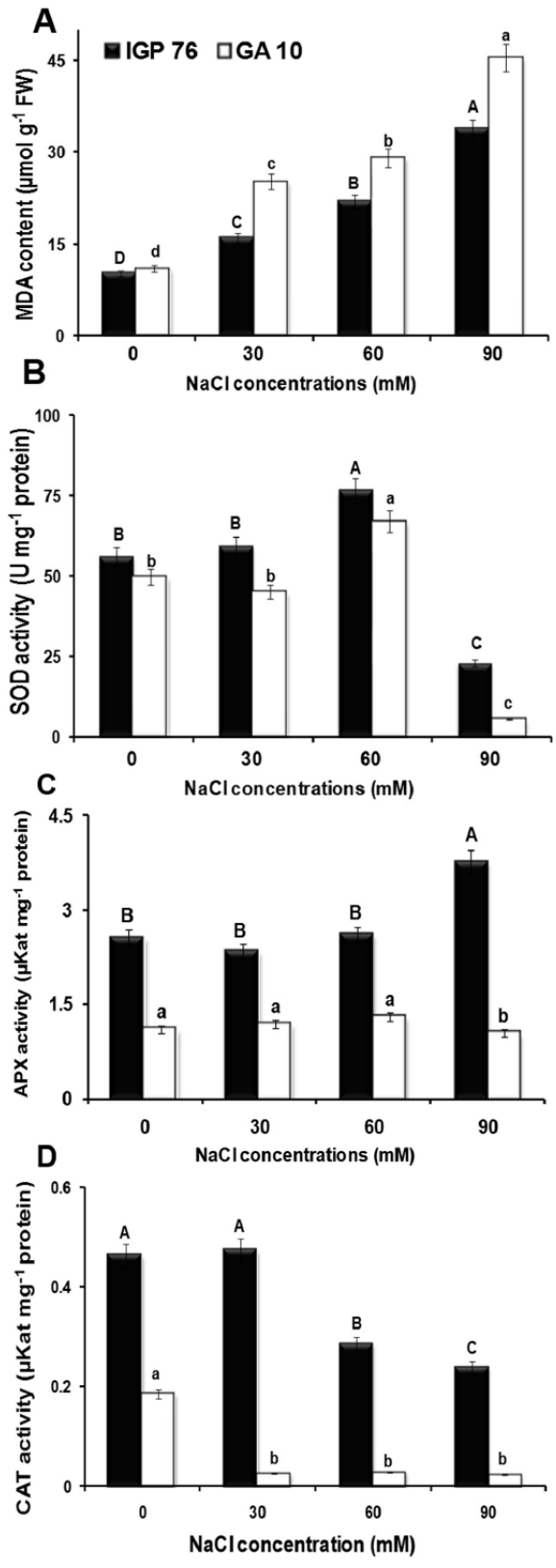

Fig. 4. MDA content and antioxidant enzyme (SOD, APX, and CAT) activities of niger cultivars IGP 76 and GA 10 calli under different salt stress treatments. Callus (~ 0.5 g) was transferred to media containing 0 - 90 mM NaCl, and (A) MDA content, (B) *SOD*, (C) *APX*, and (D) *CAT* activities were measured after 15 days of culture. Error bars SE (n = 3).Within each set of experiments, means with different letters are significantly different at $P \leq 0.05$

of NaCl (Fig. 4B). Similarly, the APX enzyme activity was comparatively higher in IGP 76 than GA 10, wherein the activity showed a significant increase in IGP 76 and decrease in GA 10 at 90 mM NaCl in comparison to their respective control (Fig. 4C). However, the CAT enzyme activity showed a significant decrease in response to different salt concentrations in both the cultivars, except at 30 mM NaCl the activity was at par to the control in IGP 76. In addition, the decrease in activity was comparatively more severe in GA 10 than in IGP 76 (Fig. 4D).

Effect of salt stress treatment on α-tocopherol accumulation

The HPLC chromatogram of the extracts from control and treated fresh calli of both the cultivars harvested at 15 days of treatment showed a single peak for α-tocopherol at a retention time 21.30 min (data not shown).The analysis of α-tocopherol content revealed its accumulation at par to the control in calli of both the cultivars exposed to 30 mM NaCl, whereas it increased significantly at 60 mM NaCl and was found to decrease to a severe extent at 90 mM NaCl stress (Fig. 5).

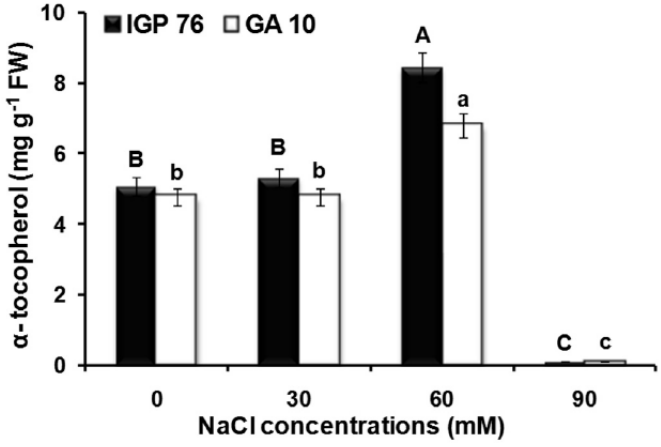

Fig. 5. The α–tocopherol accumulation of niger cultivars IGP 76 and GA 10 calli under different salt stress treatments. Callus (~ 0.5 g) was transferred to media containing 0 - 90 mM NaCl, and α–tocopherol accumulation was measured after 15 days of culture. Error bars SE ($n = 3$). Within each set of experiments, means with different letters are significantly different at $P \leq 0.05$

Organogenesis of salt-adapted calli lines

The calli of the IGP 76 and GA 10 cultivars maintained on the medium containing 90 mM NaCl for a prolonged period (13 months) revealed significant blackening in the cultivar GA 10 and inhibited the growth to a severe extent in comparison to its control (data not shown); however, IGP 76 calli revealed growth (FW and DW 3.45 and 0.18 g culture^{-1}, respectively) comparable to its control (FW and DW 4.47 and 0.21 g culture^{-1}, respectively) and showed a better capacity of survival and adaptation to such a high salt stress. Therefore, control calli (Fig. 6A) and the green part of the IGP 76 salt-tolerant calli (Fig. 6B) maintained on the medium without or with 90 mM NaCl for a prolonged period were used for further regeneration studies. The IGP 76 calli upon transfer to shoot bud regeneration medium supplemented with 90 mM NaCl did not show a regeneration response, therefore, the shoot regeneration and rooting to the shoots was carried out on the medium without supplementation of salt.

The green pieces of IGP 76 control and salt-tolerant calli revealed shoot bud regeneration (Fig. 6C) frequency of 52 and 43% with 20 and 12 shoot buds regenerated per culture, respectively. The elongated shoots (Fig. 6D) derived from the control and salt-tolerant calli produced an average number of roots in the range of 12 to 14 per shoot (Fig. 6E). The well-grown rooted plants showed acclimatization to *ex vitro* conditions (Fig. 6F) with a survival rate 56.6% in the control and 46.6% in the salt-tolerant plants. These plants developed from the control and salt-adapted calli were subjected for growth, physiological, and biochemical characterization in response to salt stress.

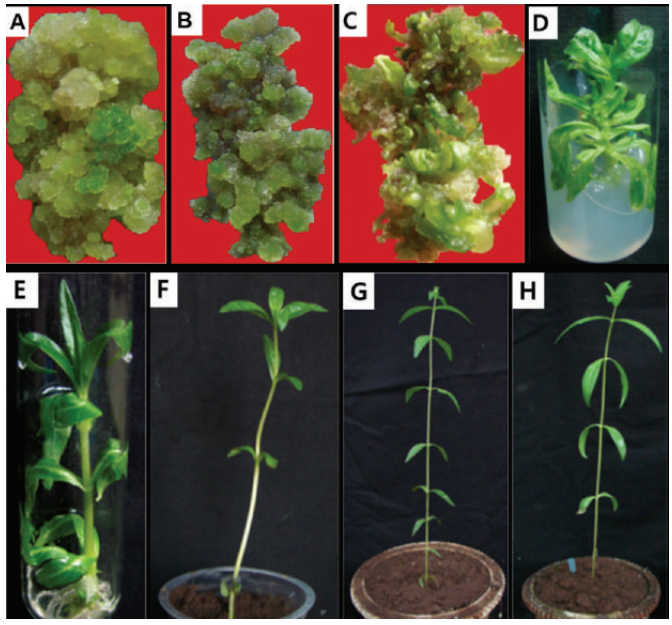

Fig. 6. Establishment of callus culture, selection of salt-tolerant calli lines, and development of salt-tolerant plants of niger cultivar IGP 76. (A) Calli grown on callus induction medium without NaCl, (B) calli grown and maintained on callus induction medium in presence of 90 mM NaCl, (C) regeneration of salt-adapted calli, (D) regenerated single shoot, (E) rooting to the regenerated shoot on root induction medium, and (F) acclimatization of *in vitro*-regenerated salt-tolerant plantlets in small plastic pots. (G) The phenotype of regenerated control and (H) salt-tolerant plants after 30 days of step-wise long-term salt stress treatment

Effect of stepwise long-term salt stress treatment on growth, physiological, and biochemical parameters of regenerated plants

The plants of the cultivar IGP 76 derived from the control and salt-adapted calli upon exposure to control and NaCl stress (gradually from 40 to 90 mM) for 30 days revealed variable growth, physiological, and biochemical responses. The growth (shoot and root length, number of leaves, and capitula per plant) and relative water content of the leaf of the control and salt-stressed plants did not show significant differences; however, total chlorophyll content decreased significantly in salt-stressed plants as compared to the control

(Table 1; Figs. 6G and H). Similarly, the osmolyte accumulation such as proline, glycine betaine, and total soluble sugar content increased significantly by 2.84-, 1.40-, and 3.46-fold, respectively, in comparison to the control (Table 1). The lipid peroxidation measured in terms of MDA content revealed significant damage to the membrane in the plants exposed to salt stress in comparison to the control (Table 1).

Table 1. Effect of stepwise long-term salt stress treatment on growth, physiological and biochemical characterization of control and salt-tolerant regenerants derived from control and salt-adapted calli of niger cultivar IGP76

Parameters	Regenerated plants	
	Control	Tolerant
Shoot length (cm)	36.32 ± 4.8[a]	34.72 ± 5.6[a]
Root length (cm)	5.4 ± 1.1[a]	5.0 ± 1.2[a]
Number of leaves per plant	14.87 ± 3.2[a]	13.00 ± 1.5[a]
Number of capitula per plant	1.87 ± 0.47[a]	1.65 ± 0.31[a]
Relative water content (%)	88.3 ± 0.6[a]	88.6 ± 0.4[a]
Total chlorophyll content (mg g^{-1} FW)	2.12 ± 1.0[b]	1.80 ± 0.8[a]
Na^+ content (m mol g^{-1} DW)	0.03 ± 0.001[b]	0.04 ± 0.001[a]
K^+ content (m mol g^{-1} DW)	0.005 ± 0.001[a]	0.004 ± 0.001[a]
Proline (μg g^{-1} FW)	75.31 ± 1.7[b]	213.61 ± 2.1[a]
Glycine betaine (μg g^{-1} FW)	126.28 ± 3.8[b]	169.14 ± 16.7[a]
Total soluble sugar (mg g^{-1} FW)	7.05 ± 0.6[b]	24.39 ± 0.7[a]
MDA content (μmol g^{-1} FW)	19.18 ± 0.5[b]	23.61 ± 0.1[a]

Data presented in the table are mean ± SE of 15 replicates per treatments scored after 30 d of stepwise long-term salt stress treatment and repeated thrice. Mean followed by same letters are not significantly different at $P \leq 0.05$ level by Duncan's multiple range test

Discussion

In vitro culture of the plant cell, tissue, or organ on a medium containing selective stress agents offers the opportunity to select and regenerate plants with desirable characteristics and could be more constructive in the improvement of crop yield on contaminated soils which is otherwise not useful for agriculture (Rai et al. 2011). Additionally, *in vitro* selection considerably shortens the time for assortment of desirable traits under selection pressure with minimal environmental interaction and can complement field selection (Jain 2001). With this approach, many reports have been published in last few years on the production of salt-tolerant plants using *in vitro* culture systems such as callus, suspension cultures, somatic embryos, shoot cultures, etc., which were exposed to variable salt concentrations to screen their ability of tolerance (Alvarez et al. 2003; Basu et al. 2002; Davenport et al. 2003; Hossain et al. 2007; Liu and Staden 2000; Patade et al. 2008; Rai et al. 2011; Zhao et al. 2009).

Similarly, in the present investigation attempts have been made in the development of salt-tolerant plants through selection of salt-tolerant calli lines in niger cultivars and growth, physiological, and biochemical characterization of the regenerated plants in response to salt stress. The different salt stress treatments to calli cultures of IGP 76 and GA 10 cultivars revealed a significant decrease in RGR and TWC which was correlated with the significant accumulation of Na^+ ions and nutritional imbalance due to an interference of salt ions (Patade et al. 2008). However, cultivar IGP 76 showed more tolerance to salt stress in terms of higher growth rate and water content apart from significant accumulation of Na^+ ions in its tissue than cultivar GA 10. Furthermore, the presence of comparatively higher Na^+/K^+ ratio in IGP 76 calli than GA 10 also suggested additional tolerance potential of the tissue and better adaptation to a higher salt stress environment. Reduced tissue growth in a stressful medium is a usual phenomenon and it has been interpreted that a certain amount of the total energy available for tissue metabolism is channeled to resist the stress (Cushman et al. 1990). It could be the possible reason that calli of both the cultivars adapted well to stressful conditions and survived at the expense of excess energy utilization in tissue metabolism for osmotic adjustment of the cell, however the differences that occurred between the cultivars might be associated with the variations at the genetic level. Similar results have been observed on reduced growth rate and water content of the calli exposed to salt stress conditions and linked with the excess accumulation of Na^+ ions in their tissues (Alvarez et al. 2003; Hossain et al. 2007; Liu and Staden 2001; Patade et al. 2008; Queiros et al. 2007; Zhao et al. 2009).

Accumulation of osmolytes under salt stress is an indication of oxidative damage which provides protection to cytosol from dehydration through the development of compatible cytoplasmic osmoticum (Lokhande et al. 2010). Proline, glycine betaine, and total soluble sugars act as an osmotica in the presence of low water content and play a major role in maintenance of osmotic balance, which has been reported to be higher in salt-tolerant species than in salt-sensitive species (Ashraf and Foolad 2007; Rai et al. 2011; Zhang et al. 2004). In the present investigation, the significance of excess energy utilization in tissue metabolism of stressed calli of both the cultivars could be associated with significant accumulation of these osmolytes. IGP 76 salt-stressed calli revealed better osmotic adjustment through more accumulation of osmolytes and its reflection in the maintenance of growth and water content in comparison to GA 10. Our results are in concurrence with the significant accumulation of proline, glycine betaine, and total soluble sugar accumulation in salt-stressed calli of other crop plants such as groundnut, sunflower, sugarcane, and *Arabidopsis* (Alvarez et al. 2003; Jain et al. 2001; Patade et al. 2008; Zhao et al. 2009) and the change in content of these osmolytes has been correlated with the capacity of the tissue to tolerate and adapt to salinity conditions. Besides the role of these osmolytes in osmotic adjustment, they have also been actively involved in protecting sub-cellular structures, mitigating oxidative damage caused by free radicals (Attipali et al.2004), and maintenance of enzyme activities under salt stress (Yokoi et al. 2002).

Lipids play an important role as the structural constituent of most of the cellular membranes (Parida and Das 2005). It is well known that free radical-induced peroxidation of lipid membranes is a sign of stress-induced damage at the cellular level. Therefore, the level of malondialdehyde (MDA), pro-

duced during peroxidation of membrane lipids, is often used as an indicator of oxidative damage (Demiral and Turkan 2005) and the cell, tissue, or organs upon exposure to salt stress showed less MDA content are considered to be salt tolerant. In the present investigation, IGP 76 calli exposed to salt stress treatment revealed less damage to membrane lipids in comparison to GA 10 calli; such increase was more pronounced at 90 mM NaCl stress. Our results indicated that IGP 76 calli were more effective in coping with the salt-induced oxidative damage than GA 10 calli. Similar observations on increased MDA content have also been recorded in the salt-stressed calli of *Arachis hypogea* (Jain et al. 2001), *Helianthus annus* (Davenport et al. 2003), *Catharanthus roseus* (Elkahoui et al. 2005), eggplant (Yasar et al. 2006), and *Solanum tuberosum* (Queiros et al. 2007).

In response to excess generation of toxic reactive oxygen species due to salt-induced oxidative stress, enzymatic and non-enzymatic antioxidants have shown to play a crucial role in detoxification of these species and their fine tuning for the signaling processes involved in various plant metabolism (Miller et al. 2009; Mittler 2002). In the present investigation, significantly higher SOD, CAT, and APX enzymatic activities in IGP 76 calli under the influence of salt stress has suggested their role in efficient detoxification of excessively produced ROS; however, the enzymatic activities in GA 10 calli were not found to be effective for the removal of ROS, which therefore showed more MDA content and loss tolerance to elevated saline conditions in comparison to IGP 76 calli. In addition, α-tocopherol accumulation, a non-enzymatic antioxidant, has shown its induction only at moderate levels of salt stress (60 mM NaCl) and played an important role in mitigation of oxidative stress but no significant differences were observed between calli of these two cultivars. These inductive and elevated levels of antioxidant systems for the removal of toxic ROS under the influence of salt stress has also been shown and correlated with higher stress tolerance in other crop plants (Ashraf 2009; Davenport et al. 2003; Elkahoui et al. 2005; Hossain et al. 2007).

The results of the growth, physiological, and biochemical analyses of salt-stressed IGP 76 and GA 10 calli suggested the presence of comparatively better survival and tolerance in IGP 76 calli than GA 10 which therefore is used for the production of salt-tolerant plants under the influence of a higher concentration of NaCl (90 mM). The IGP 76 calli maintained by regular sub-culturing on salt-containing (90 mM NaCl) medium for 13 months revealed lower growth retention ability in comparison to control calli which might be due to excess accumulation of Na^+ ions during the period of maintenance. This reduced but stable growth in salt-adapted calli after regular passage of sub-cultures indicated the occurrence of new homeostatic equilibrium through alteration in cellular metabolism (Leone et al. 1994). Besides, the gradual adaptation of the tissue due to repeated sub-culture in saline medium has been correlated with the accumulation of excess Na^+ ions for the maintenance of osmotic equilibrium against the external hypertonic milieu which has also been reported in other plant species (Basu et al. 2002; Hossain et al. 2007; Queiros et al. 2007). Once selected, salt-tolerant cell lines could be transferred to a regeneration medium or maintained in salinity conditions to study the biochemical changes induced by NaCl (Davenport et al. 2003; Queiros et al. 2007). Therefore, the salt-adapted and control IGP 76 calli subjected to shoot and root organogenesis revealed optimum shoot bud formation and its elongation on shoot regeneration medium (MS + 4.44 µM BA) devoid of NaCl and root formation to the shoots on rooting medium (MS + 2.69 µM NAA). However, the organogenesis response observed for salt-adapted calli was comparatively lower than in control calli. Our results reconfirmed the organogenesis (shoot and root) capacity of niger callus culture on media supplemented with plant growth regulators (BA and NAA), respectively, as reported earlier by Nikam and Shitole (1997). The acclimatization response was also found to be lower in salt-adapted, calli-derived plants in comparison to the control.

The growth, physiological, and biochemical characterization of salt-adapted and control calli-derived regenerated plants under the influence of stepwise long-term salt stress treatment revealed improved growth characteristics except variation in total chlorophyll content. This has been correlated with significantly higher accumulation of organic osmolytes which could have been actively involved in maintaining the osmotic balance of the cell to the optimum level in close proximity to control conditions. Hossain et al. (2007) similarly developed salt-tolerant plants from the salt-adapted calli of *Chrysanthemum morifolium*. Therefore, the results of the present investigation have more significance for the development of tolerant plants from salt-adapted calli cultures of IGP 76 which could be useful in crop improvement programs of niger in salt contaminated soil. Further, the study suggests that agronomic performance of these salt-tolerant regenerants under saline conditions need to be undertaken to check the genetic stability of induced salt-tolerance.

Conclusion

Self-incompatibility has remained the major limiting factor for the improvement of agronomically important traits through conventional breeding in niger. Although, it is one of the important oilseed crops, not much efforts has been made on the production of abiotic stress tolerant cultivars; therefore, categorizing the plant as a more neglected crop. The results of the present investigation suggest an implication of tissue culture technique for the *in vitro* selection of salt-tolerant cell lines in niger cultivar IGP 76 followed by its maintenance for a long period on saline medium. Following regeneration of plants from the salt-adapted calli, the tolerance to salinity of the regenerated plants was evaluated by measuring growth as well as physiological and biochemical traits. In the present investigation, the protocol developed for the *in vitro* selection and production of salt-tolerant plants could be used as an alternative to traditional breeding programs to increase

the abiotic stress tolerance capacity of the niger cultivars.

Acknowledgments

The senior author is thankful to the University Grant Commission (UGC), New Delhi for financial assistance through the Rajiv Gandhi National Fellowship and Zonal Agricultural Research Institute, Western Ghat, Igatpuri (MS), India for providing the niger cultivars. The help of Department of Botany, University of Pune, Pune and UGC-ASIST and UGC-DRS-SAP programs are duly acknowledged.

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