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Full Length Research Paper

Comparative study for salt stress among seed, root stock and direct regenerated violet (*Viola odorata* L.) seedlings in relation to growth, ion contents and enzyme activities

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The experiments were carried out to evaluate the comparative study for salt stress among seed, root stock and direct regenerated violet (*Viola odorata* L.) seedlings. Violet seedlings propagated through tissue culture (direct regeneration) had significantly higher salicylic acid (SA) concentrations from seed and rootstock propagated plants. Random amplified polymorphic DNA (RAPD) studies prior to the salt treatments revealed that genetic similarity at the molecular level among seed, root stock and direct regenerated violet seedlings was 50.9 to 70.5%. NaCl applications (50 mol m^{-3}) reduced plant and root lengths, plant fresh and dry weights in plants obtained through seeds and rootstock as compared to direct regenerated seedlings. Direct regenerated violet showed better plant growth significantly both in saline and non-saline conditions. Seedlings raised through direct regeneration strongly inhibited accumulation of Na^+, K^+, Ca^{2+} and Cl^- and organic solute accumulations as glycinebetaine (GB) and root total soluble carbohydrates (TSC) but stimulated N and relative water contents (RWC). Direct regenerated seedlings showed an enhanced catalase (CAT), ascorbate peroxidase (APX) and guaiacol dependent peroxidase (GDP) activities as compared to seed and root stock propagated plants. It was concluded that direct regenerated plants had better performance under salt stress in relation to growth and ion accumulations as compared to seed and root stock propagated violet seedlings. This might be due to higher SA concentrations in direct regenerated seedlings which resulted from somaclonal variations or growth media applied during tissue culture technique.

Key words: Propagation, somaclones, salicylic acid, salt tolerance, enzymes, ions.

INTRODUCTION

The violet (*Viola odorata* L.) is a low growing perennial herb. It is propagated mainly through seed and rootstocks. The leaves and flowers of violet have antiseptic and expectorant properties (Kowalchik and Hylton, 1998). Internally, violet is used to cure bronchitis, mucus, cough, asthma, breast cancer, stomach, lungs and digestive tract (Ghani et al., 1997). Direct regeneration is one of the important techniques of plant tissue culture for commercial plant propagations. Direct regeneration involves morphogenesis without the intermediate callus phase (Jawaharlal et al., 1984). The plants derived by direct regeneration usually have higher concentrations of chemical constituent and better quality from parent plants as Vasil (1987) described in cereals and grasses. Direct regeneration is a useful tool for rapid propagation and for induction of somaclonal variations in plants (Snyman et al., 2000).

Salinity stress disturbs the uptake and accumulation of essential nutrients from the soil (Greenway and Munns, 1980; Shannon and Grieve, 1999; Zhu, 2001). Generally,

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Ca^{2+} and K^+ are decreased in plants grown under saline conditions (Khan, 1993; Al-Harbi, 1995). In contrast, Ashraf and Rauf (2001) primed maize seeds with NaCl and reported significantly higher concentrations of Na^+, K^+ and Ca^{2+} in leaves, sheath and root of maize seedlings as compared to untreated plants. Alam and Naqvi (1991) observed that plant height and dry matter yield decreased in pearl millet with increase in salinity levels on 1.95, 4.69, 9.38 and 14.06 dS m^{-1} NaCl. Salinity also caused an increase in N, P, Ca^{++}, Na^+, Fe^{++} and Mn^{++} and decrease in K^+ contents in the leaves.

Several studies also supported a major role of SA in modulating the plant response to several abiotic stresses including salt and water stress (Yalpani et al., 1994; Senaratna et al., 2000). Treating mustard seedlings with SA improved their thermotolerance and heat acclimation (Dat et al., 1998). In maize plants, pre treatment with SA induced the production of antioxidant enzymes, which in turn increased chilling and salt tolerance (Janda et al., 1999).

Molecular markers are useful tools for precise assessment of diversity and phylogenetic relationships among different species and related genera. A major breakthrough with regard to DNA markers is the development of the random amplified polymorphic DNA (RAPD) technique, based on the use of polymerase chain reaction (PCR) to amplify random sequence (Williams et al., 1990). An advantage of the use of RAPD markers is that with a low technical input, a large number of markers can be employed. Therefore, DNA markers are often used by scientists to reduce labor and cost for the determination of genetic relationships among various cultivars (Leon et al., 2001; Pan et al., 2004).

The objective of the present study is to evaluate the comparison for salt stress among seed, root stock and direct regenerated violet seedlings in relation to growth, ion accumulations and enzyme activities with protocol for tissue culturing of violet.

MATERIALS AND METHODS

Seeds and rootstocks of violet plants were sown in 36 earthen pots described by 12 pots of each propagated type and maintained at 3 plants per pot. After 20 days of sowing time, the leaf tips were collected from the violet plants and used for this study.

The field collected materials were washed several times with tap water with a few drops of liquid soap in tissue culture lab. Murashige and Skoog (MS) medium supplemented with different plant growth regulators and additives was used to compare 3-different combinations, M_1, M_2 and M_3, with concentrations of benzyl amino purine (BAP, 0.05 - 3mg/L), indole acetic acid (IAA, 1 - 2 mg/L), kinetin (0.1- 0.3mg/L and sucrose (30000 -50000 mg/L). The pH of the medium was adjusted to 5.7 -5.8 and autoclaved at 121°C for 18 min on 15psi. The cultures were maintained initially in the dark for 3 to 6 weeks and thereafter all the cultures were incubated in the growth room at 26-28°C and 2500-3000 lux light. Regeneration was observed on cultured explants after 3 to 6 week culture period. Regenerated plants were transferred to rooting media (half MS media with different combination (R_1, R_2 and R_3) of NAA (1.5-3mg/L), IBA (2-4mg/L), IAA (1-2mg/L) and sucrose (40000-70000 mg/L) placed in a 12 h PAR light (photo synthetically active radiation) cycle at 25°C. After 4 to 6 weeks in culture, plants were acclimatized. Phytogel was washed-off carefully under running tap water and plants with good shoot and root were allowed to grow.

Pre NaCl applications studies

Isolation of salicylic acid (SA)

Salicylic acid was measured before NaCl treatments in all three propagated types (seed, roots and direct regenerated) of violet plants by the method described by Meuwly and Métraux (1993). Violet cells were collected by filtration under vacuum through four layers of Miracloth, snap frozen in liquid nitrogen and kept at -80°C until the extraction was performed. SA was extracted from cells (0.5 g fresh weight of frozen tissue), separated by high performance liquid chromatography (HPLC) and quantified by spectrofluorescence monitoring at 407 nm emissions.

DNA extraction and PCR amplification

DNA was extracted from 100 mg fresh tissues of violet leaves collected from three propagated types (seed, roots and direct regenerated) using cetyltrimethyl ammonium bromide (CTAB) method (Doyle and Doyle, 1987). Concentration of DNA was determined by spectrophotometer as well as by visualization on 1% agarose gel with ethidium bromide staining.

PCR amplification conditions were optimized in a GeneAmp 2700 thermocycler (Applied Biosystems, 850 Lincoln Centre Drive, Foster City, C.A., USA). Amplifications were performed in a 25 µl reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% (w/v) gelatin, 50 µM each of dATP, dTTP, dGTP and dCTP (Fermentas Inc. 7520 Connelley Drive, Maryland 21076, USA), 50 p moles of each primer, 0.5 µg of template DNA and 2.5 U of *Taq* DNA polymerase (Fermentas). The reactions were subjected to 40 amplification cycles. Initial denaturation was at 92°C for 4 min. Each cycle consisted of denaturation for 45 s at 92°C, annealing for 1.5 min at 28°C and extension for 1.5 min at 72°C, with a final extension at 72°C for 5 min. Eight primers obtained from Bangalore Genei (Pvt) Ltd, India were employed.

Data analyses

Intensively stained DNA bands on agarose gel ranging from 0.2 to 2.5 kb were recorded. The data collected after scoring Minitab (Minitab 13.0. USA) computer software program was used for RAPD coefficients dendrogram by following the method of Nei and Li (1979). The following equation was used for similarity and genetic distance calculation analysis: Coefficient analysis = (2 x common number of bands between genotype A and B) / Total number of bands genotype A and B.

A homology tree of genotypes was constructed for understanding of the genetic diversity at molecular level.

Post NaCl applications studies

Salt treatments and experiment layouts

Plants developed by direct regeneration, seed and rootstock were exposed to 0 (control) and 50 mol m^{-3} NaCl salinity. Each pot contained 7000 g river sand (particle size 1.4 - 2.0 mm). To avoid leaching down of salts, polyethylene bags were laid down in pots before filling up with sand. The sand was washed thoroughly with tap water, distilled water and finally with Hoagland solution

(Epstein, 1972). Two salt treatments 0 (control) or 50 mol m^{-3} NaCl were applied. NaCl (2.925 g NaCl/pot) was dissolved in 1 liter of distilled water to develop NaCl level of 50 mol m^{-3} per pot (pot size 28 cm diameter and 30 cm deep) and for control, only 1 liter of distilled water was applied. Hoagland solution was applied every week regularly for 6 weeks both for control and treated plants. During the week, distilled water (300 - 350 cm^3) was applied to each pot daily to keep the sand moist and hence maintain the salt level. The experiment was laid out in a completely randomized block design (CRBD) with six replicates. Plants were harvested 42 days after NaCl treatments for data analysis.

Morphological attributes and ion contents

Plants were uprooted carefully and washed in distilled water. Plant and root length was measured with the help of scale meter. Shoot fresh weight (g) was noted by electric balance. Plant samples were placed in oven at 75°C. After 4 days, shoot and root dry weight (g/pot) was calculated with the help of electric balance.

Dried plant material was finely ground and digested with a nitric perchloric mixture. In leaves and roots, ion contents of Na^+ and K^+ were determined by emission spectrophotometry and Ca^{2+} by atomic absorption spectrophotometry (Allan, 1969). Total nitrogen was estimated by Kjeldhal procedure (Bremner 1965). Chloride was extracted by stirring ground dried samples with 0.1 M $NaNO_3$ for 30 min. After extract clarification with activated coal, 13.2 mM HG $(SCN)_2$ in methanol and 20.2% (w/v) Fe(NO3)3 (4 + 1) was added and the absorbance was determined at 460 nm (Gaines et al., 1984).

Leaf relative water contents (%)

The leaf relative water contents (RWC) were calculated according to Beadle et al. (1993) using the equation:

RWC (%) = [(FW - DW)/(TW - DW)] x 100

Where, FW is fresh weight, DW is dry weight and TW is turgid weight.

Glycinebetaine, proline and total soluble carbohydrates contents (µg g^{-1} DW)

Glycinebetaine was extracted by stirring finely ground dried samples with demineralized water at 100°C for 1 h. Glycinebetaine contents were determined spectrophotometrically after reaction with KI-I_2 at 365 nm (Grieve and Grattan, 1983). Proline was also determined spectrophotometrically following the ninhydrin method described by Bates et al. (1973) using L-proline as a standard. Approximately, 300 mg of dry tissue was homogenized in 10 mL of 3% (w/v) aqueous sulphosalicylic acid and filtered. In 2 mL of the filtrate, 2 mL of acid ninhydrin was added, followed by the addition of 2 mL of glacial acetic acid and boiled for 60 min. The mixture was extracted with toluene and the free proline was quantified spectrophotometrically at 520 nm from the organic phase using a Shimadzu spectrophotometer (Duisburg, Germany).

Total soluble carbohydrates (TSC) concentrations were determined according to Brun (1978). Samples of 100 mg were homogenized with 10 mL of extracting solution (glacial acetic acid: methanol: water, 1: 4: 5, v/v/v). The homogenate was centrifuged for 10 min at 3,000 rpm and the supernatant was decanted. The residue was resuspended in 10 mL of extracting solution and centrifuged another 5 min at 3,000 rpm. The supernatant was decanted, combined with the original extract and made up to 50 mL with water. For measurement of TSC, a phenolsulfuric acid assay was used as described by Dubois et al. (1956). A volume of 0.5 mL of 5% (v/v) phenol solution and 2.5 mL of concentrated sulfuric acid were added to 0.5 mL aliquots. The mixture was shaken, heated in a boiling water bath for 20 min and cooled to room temperature. The absorption was then determined by spectrophotometry at 490 nm (Shimadzu spectrophotometer, Duisburg, Germany).

Antioxidant and defense enzymes

Roots equivalent to 100 mg fresh weights of violet were homogenized in 1 mL of HEPES/KOH buffer (pH 7.5) using mortar and pestle. The homogenate was spun at 10,000*g* at 4°C for 10 min. The supernatant was used for the enzyme assays. Catalase (CAT) activity was determined by measuring the rate of H_2O_2 conversion to O_2 at room temperature using an O_2 electrode (Dat et al., 1998). Ascorbate peroxidase (APX) activity was measured in the presence of 0.25 mM ascorbic acid and 0.5 mM H_2O_2 by monitoring the decrease in absorption at 290 nm (Janda et al., 1999). Peroxidase activity was determined according to Adam et al. (1995). The assay contained 1.5 mL of 100 mM sodium acetate buffer (pH 5.5), 1 mL of 1 mM guaiacol, 10 µL of tissue extract and 190 µL of water. The reaction was started by addition of 300 µL of 1.3 mM H_2O_2. The increase in absorption was recorded at 470 nm. Chitinase activity was measured using the substrate carboxy methyl chitin remazol brilliant violet (CM-chitin RBV, Blue Substrates, Göttingen, Germany) according to the method described by Wirth and Wolf (1990).

Statistical analysis

Analysis of variance (ANOVA) was employed for carrying out statistical analysis of data collected (Steel and Torie, 1980). The means values were compared with least significant difference (LSD) test following Snedecor and Cochran (1980).

RESUTS AND DISCUSSION

Pre NaCl applications studies

Development of plant through direct regeneration

Composition of different media used for regeneration in violet is given in Table 1. The explants placed on M_2 media showed good shoot formation and on R_2 media showed good root formation.

Salicylic acid (µg g^{-1} FW)

Salicylic acid (SA) was isolated before NaCl applications from three propagated types of violet seedlings (Figure 1). It was noted that direct regenerated plants had significantly higher SA contents in roots and leaves as compared to seed and rootstock propagated plants. Both seed and rootstock had almost equal concentrations of SA, while plants propagated through direct regeneration had higher concentration of SA. The increased content of SA might be due to somaclonal variations or impact of hormones used during regeneration. Khanum et al. (2006) stated that direct regeneration could be a useful tool to induce somaclonal variations in plants especially

Table 1. Composition of different media used for regeneration and root formation in violet.

Regeneration media (mg/L)	Composition		
	M_1	M_2	M_3
Benzyl amino purine (BAP)	0.05	1.5	3
Indoleaceticacid (IAA)	1	1	2
Kinetin	0.2	0.1	0.3
Sucrose	40000	30000	50000
Rooting media (mg/L)	R_1	R_2	R_3
Naphthalene acetic acid (NAA)	2	3	1.5
Indole butyric acid (IBA)	3	4	2
Indole acetic acid (IAA)	1	1.5	2
Sucrose	40000	60000	70000

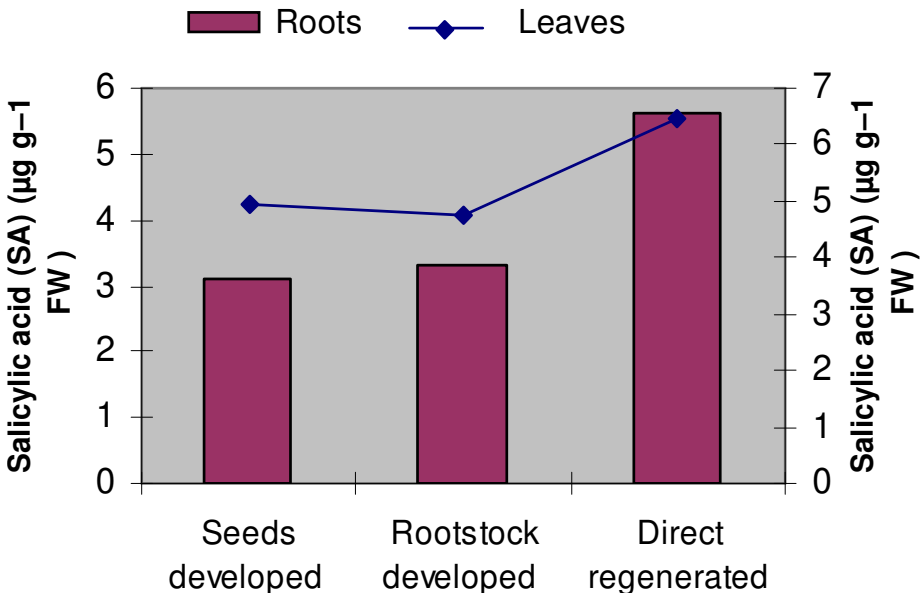

Figure 1. Salicylic acid (SA) (µg g^{-1} FW) in violet in three types of plants before NaCl treatments.

for concentrations of hormones. A number of similar studies showed that adventitious regeneration caused somaclonal variations in plants, in garlic (Sata et al., 2000), minor millet (Vikrant and Rashid, 2001) and rice (Sahasrabudhe et al., 2000).

Detection of genetic diversity

Primer wise detail of DNA polymorphism detected in violet is elaborated in Table 2. Each of the random primers produced distinct polymorphic banding patterns in all three types of plants in violet. The level of polymorphism was different with different primers among different propagated seedlings. Results obtained from 10 experiments with 5 fragments through gel electrophoresis system are shown in Figure 2A. A homology tree was constructed by an unweighted pair group method with arithmetic averages clustering algorithm from the pair wise matrix of genetic similarity among sugarcane genotypes (Figure 2B). The results indicated that plants regenerated by direct regeneration had higher genetic heterogeneity as compared to seed and root stock propagated seedlings. Genetic similarity among the violet plants at molecular level was 50.9 to 70.5% among seed, root and direct regenerated violet plants. Trend of genetic diversity among violet plants suggested that differences observed in morphological traits might be the result of somaclonal variations or growth media applied during tissue culture technique.

Similarly, genetic homology coefficient ranging from 60.5 to 88.5% has earlier been reported by Pan et al. (2004).

Table 2. Primer used for polymorphism in violet.

S/N	Primer code	Sequence	Total bases
1.	BT61.F	5′--GGTGAGAACTCTCGAGGGTCGGGA--3′	24
2.	BT61.R	5′--GCCCCGATCCGACACCCGAGC--3′	21
3	BT101.F	5′--CCCTCACTCCTCGAGAATATG--3′	21
4.	BT91.F	5′--CTATTTACTTCTCTCACCGCGG--3′	22
5.	BT811.F	5′--TTTGTAAACACGGAGGGGGC--3′	20
6.	BT811.R	5′--ACAAACCCACGATTGGATTGGGC--3′	23
7.	BT71.F	5′--TTGTAAACACAGAGGAGGG--3′	19
8.	BT71.R	5′--CACGATTGGATTACACGC--3′	18

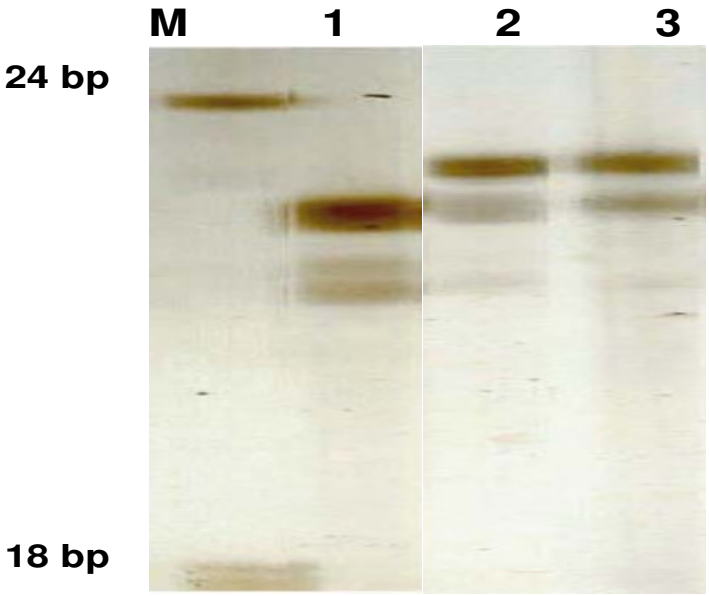

Figure 2A. RAPD DNA fragments of 3-types of violet plants propagated by different methods (M = marker, Lane 1 = direct regenerated, Lane 2 = seed propagated and Lane 3 = rootstock propagated).

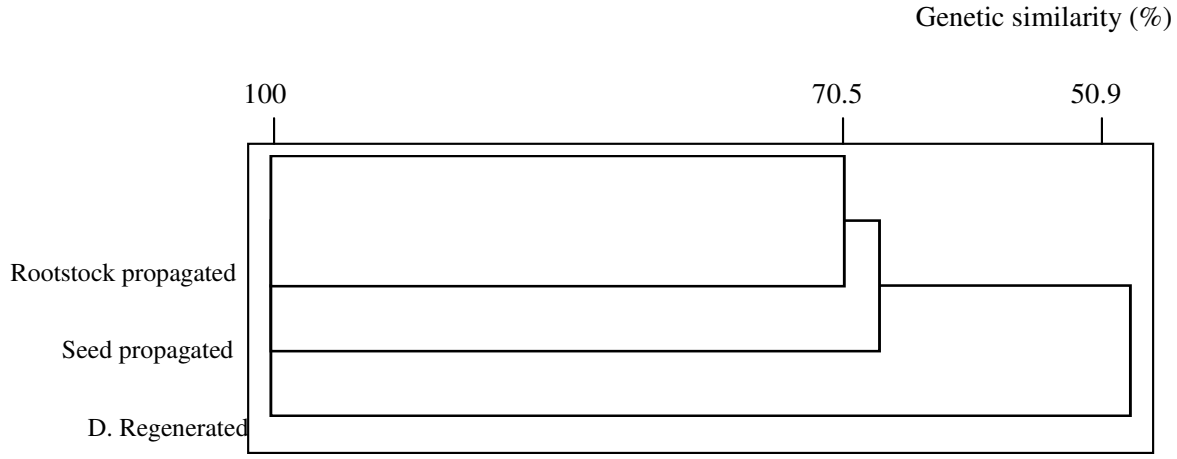

Figure 2B. Homology tree constructed by an un-weighted pair group method with arithmetic averages clustering algorithm from the pair wise matrix of genetic similarity amongst different propagated violet plants.

Table 3. Comparison of means for NaCl and SA effect on different propagated types of violet seedlings for morphological attributes and ions contents.

Attribute	Plants developed through seeds		Plants developed through rootstock		Plant developed through direct regeneration	
	T_1 (0 mol m^{-3})	T_2 (50 mol m^{-3})	T_1 (0 mol m^{-3})	T_2 (50 mol m^{-3})	T_1 (0 mol m^{-3})	T_2 (50 mol m^{-3})
Plant length (cm)	47.4±0.21 aA	35.2 + 0.19 bB	36.7 ± 0.51 bB	25.4 ± 0.66 cC	49.9 ± 0.41 aA	47.1 ± 0.35 aA
Root length (cm)	10.6+0.21 aA	9.5 ± 0.09 abB	11.2 + 0.35 aA	8.9 + 1.1 abB	10.1 ± 0.04 aA	10.4 ± 1.4 aA
Plant fresh weight (g)	10.9±0.33 aA	7.6 ± 0.07 bC	11.1 ± 0.11 aA	8.1 ± 0.07 bB	11.0 + 1.1aA	10.8 ± 0.91 aA
Plant dry weight (g)	5.9±0.19 aB	3.2 ± 0.01bC	6.3 + 0.08 aA	3.9 ± 0.02 bC	6.2 ± 0.05 aA	6.1 ± 0.04 aA
Na^+ (ppm) in roots	12.4±0.12 bC	28.5 ± 0.15aA	14.6 + 0.04 bC	26.6 + 0.66 aA	17.2 ± 1.12aB	16.8 + 1.01vaB
Na^+ (ppm) in leaves	27.2±1.14 aC	87.7 ± 0.19 bA	32.4 ± 0.88 aB	72.2 + 1.22 bA	34.5 ± 0.54aB	32.1 ± 1.15aB
K^+(ppm) in roots	654.6±2.21 bD	965.2 ± 1.15 aB	987.7 ± 1.48 aA	1033.2 ± 2.99bA	866.2 + 4.33aC	845.2 ± 2.10aC
K^+(ppm) in leaves	488.8±0.99 bD	548.6 ± 2.23 aC	525.5 ± 2.33 bC	721.6 ± 2.41 aA	621.1 ± 2.11aB	632.0 ± 2.56aB
Ca^{2+} (ppm) in roots	125.2+0.19 aC	65.5 ± 1.16 bD	132.5 + 0.09 aB	50.0 ± 1.12 bE	140.1 + 0.08aA	137.2 ± 1.06aA
Ca^{2+} (ppm) in leaves	40.0±1.12 bC	54.4 ± 0.06 aA	45.5 ± 1.26 aB	36.6 + 0.94 bD	37.7 ± 0.04aD	41.2 + 2.00aC
Cl^- (ppm) in roots	111.4±1.19 bD	213.5 ± 0.95 aB	125.6 ± 2.66 bCD	276.7 ± 1.33 aA	140.3 ± 1.12aC	136.5 ± 1.65aC
Cl^- (ppm) in leaves	218.6+2.11 bC	372.8 ± 0.66 aA	198.3 ± 0.04 bD	328.2 + 1.68 aB	209.2 ± 2.15aC	202.9 ± 1.42aD
N (%)in roots	1.9±0.01 aA	1.2 ± 0.02 bC	1.7 ± 0.03 aB	1.1 ± 0.01 bC	1.6 + 0.06bB	2.1 ± 0.02aA
N (%)in leaves	2.1±0.02 aB	1.4 ± 0.01 bD	1.9 + 0.56 aBC	1.2 + 0.01 bD	2.4 + 1.02bB	2.9 ± 0.01aA

Small letter indicates difference among NaCl treatments (that was 0 and 50 mol m^{-3}) and capital letters show mean difference among plant types propagated (seed, rootstock and direct regeneration).

Post NaCl applications studies

Morphological attributes

Data regarding morphological attributes of plant and root lengths, plant fresh and dry weights are presented in Table 3. Comparison of treatments means for NaCl showed that application of 50 mol m^{-3} NaCl significantly reduced the plant and root lengths, plant fresh and dry weights in plants obtained through seeds and rootstock as compared to control (0 mol m^{-3} NaCl). In contrast, effect of NaCl application was non-significant on seedlings raised through direct regeneration (Table 3). Similarly, Hussain et al. (2009) found the reduction of growth in black seeds under salt stress.

Ion contents

Results obtained regarding ion contents (Na^+, K^+, Ca^{2+}, Cl^- and N) are given in Table 3. Impact of NaCl stress was highly significant on violet plants. Na^+, K^+ and Ca^{2+} concentrations were higher under 50 mol m^{-3} NaCl applications over control in seed and rootstock propagated plants both in roots and leaves. Concentrations of these ions were always higher in leaves than in roots. In contrast, regenerated plants had non-significant effect of salt treatment on these ions. N contents were reduced in seed and rootstock propagated plants under salt stress, while *in vitro* regenerated plants N contents increased under salt stress than control in both roots and leaves. These results are similar with the earlier findings in maize(Izzo et al., 1996). Similar results for accumulations of inorganic ions (Na^+, K^+, Ca^{2+}, Cl^- and N) in salt sensitive and resistant pearl millet lines were described by Hussain et al. (2008).

Relative water contents (%)

Salt stress reduced the relative water contents (RWC) in seed and rootstock propagated violet plants. In contrast, the effect of salt stress was non signification in direct regenerated seedlings

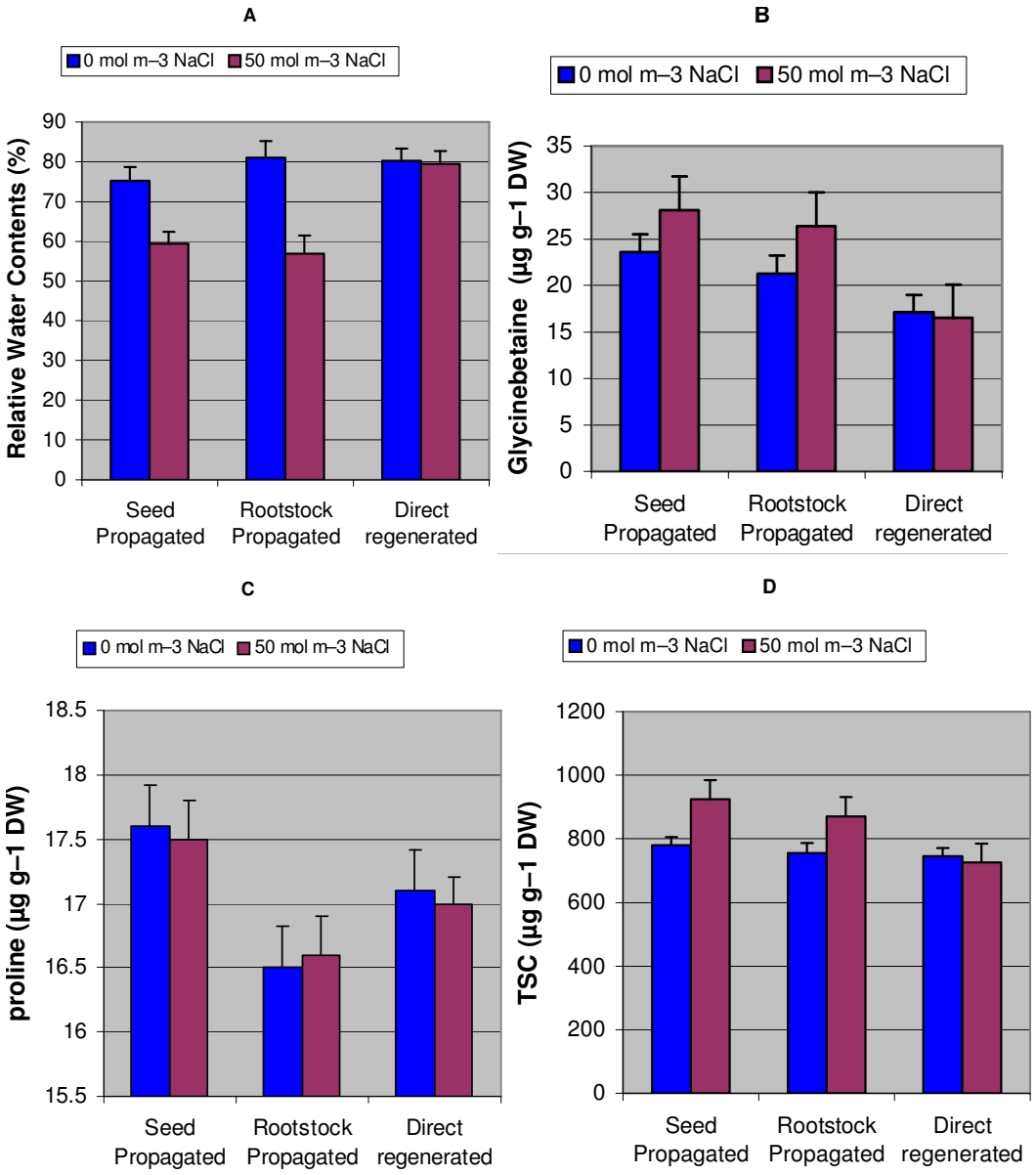

Figure 3. Effect of NaCl on: (A) Relative water contents (%); (B) glycinebetaine (µg g^{-1} DW); (C) proline (µg g^{-1} DW); (D) total soluble carbohydrates concentration in violet.

regarding RWC (Figure 3A).

Glycinebetaine (µg g^{-1} DW)

Glycinebetaine (GB) accumulations increased in response to salinity among seed and rootstock propagated plants. On the other hand, GB accumulation deceased in direct regenerated plants under salt stress (Figure 3B). Accumulation of GB represents a major biochemical adaptation in several bacteria and plants under stresses (Rhodes and Hanson, 1993). It has been demonstrated that the biosynthesis of GB is stress inducible (Sakamoto and Murata, 2002) and that the level of accumulated GB is correlated with the degree of salt tolerance (Saneoka et al., 1995). Moreover, an exogenous supply of GB also increases the salt tolerance of some plants that are otherwise unable to accumulate GB (Hayashi et al., 1998).

Proline (µg g^{-1} DW)

Effect of salt stress was non-significant on proline accumulation among all three propagated types of violet plants (Figure 3C). Ashraf (1989), Lutts et al. (1996) and Meloni et al. (2001) reported that proline was not involved in the osmotic adjustment of black gram, sorghum and cotton cultivars, respectively.

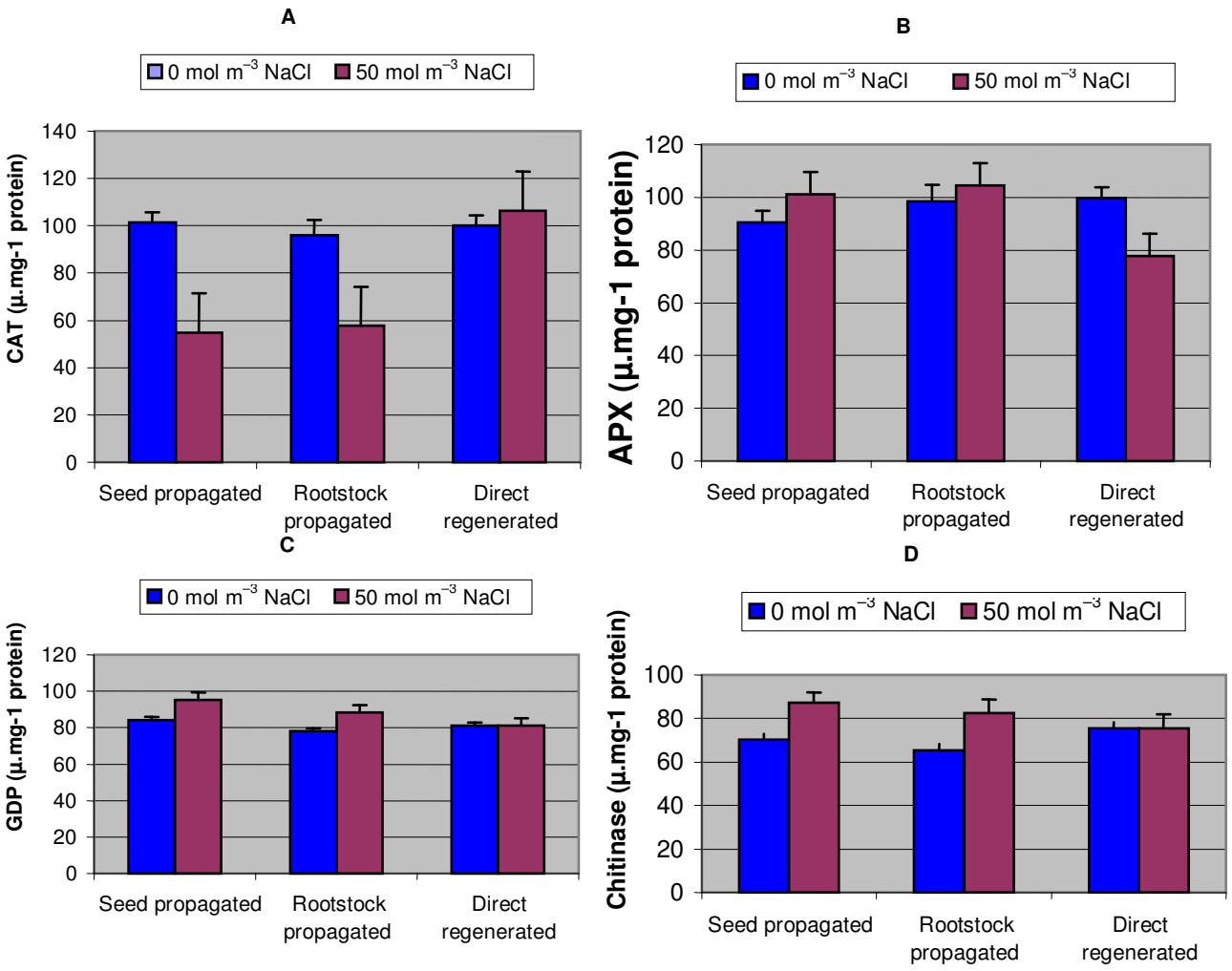

Figure 4. Effect of NaCl on: (A) CAT (µ mg^{-1} protein); (B) APX (µ mg^{-1} protein); (C) GDP (µ mg^{-1} protein); (D) Chitinase µ mg^{-1} protein in violet.

Root total soluble carbohydrates (µg g^{-1} DW)

Root total soluble carbohydrates (TSC) concentrations increased sharply under salt stress intensity in seed and rootstock propagated plants, while TSC concentrations were unchanged in direct regenerated plants (Figure 3D). This probably reflects the maintenance or even induction of root elongation at low water potentials, which can be considered as an adaptive response to salinity (Balibrea et al., 2000).

Antioxidant and defense enzymes

CAT and APX detoxify H_2O_2 in peroxisomes, cytosol and chloroplasts, respectively. Their activities were measured as representative enzymes involved in antioxidant metabolism. CAT activity of direct regenerated plants increased under NaCl stress. In a converse manner, APX activity was decreased in direct regenerated plants (Figures 4A and B). The response pattern of seed and rootstock propagated plants was in opposite for both enzymes as compared to direct regenerated plants. Whereas CAT activity dropped in seed and rootstock propagated plants while APX activity increased in these plants, guaiacol dependent peroxidase (GDP) and chitinase activities were slightly increased in seed and rootstock propagated plants and were stimulated under NaCl treatments, but unaffected in direct regenerated plants (Figures 4C and D).

Salt (NaCl) stress is among the factors most limiting to plant productivity. Plants exposed to salt stress adapt their metabolism in order to cope with the changed environment. Survival under these stressful conditions depends on the plant's ability to perceive the stimulus, generate and transmit signals and instigate biochemical changes that adjust the metabolism accordingly (Hasegawa et al., 2000). It was clear from results of the present experiments that the only difference among the three propagated types of seedlings was the accumulations of

SA that might helped violet seedlings to cop with the effect of salt stress. Snyman et al. (2000) described that direct regeneration is a useful tool for rapid propagation and for induction of somaclonal variations in plants. Salicylic acid (SA) plays an important role in the defense response to stresses (salts, water) in many plant species (Shirasu et al., 1997). SA constituted the principle mechanisms employed to avoid salt stress by controlling the activities of guaiacol dependent peroxidase and chitinase. In maize plants, pre treatment with SA induced the production of antioxidant enzymes, which in turn increased chilling tolerance (Janda et al., 1999). Activities of antioxidant enzymes confirmed the positive SA effect under salt stress. SA constituted the principle mechanisms employed to avoid salt stress by controlling the activities of guaiacol-dependent peroxidase and chitinase in direct regenerated plants (Stevens et al., 2006).

Conclusion

It was concluded that direct regenerated plants had better performance under salt stress in relation to growth and ion accumulations as compared to seed and root stock propagated violet seedlings. It might be due to higher SA concentrations in direct regenerated seedlings resulting from somaclonal variations or growth media applied during tissue culture technique.

We suggest that further studies for somaclonal variations with different levels of NaCl should be carried out. It could be that 50 mol m^{-3} NaCl is low level of salt stress for violet.

Abbreviations

SA, Salicylic acid; **RAPD,** random amplified polymorphic DNA; **GB,** glycinebetaine; **TCS,** root total soluble carbohydrates; **RWC,** relative water contents; **GDP,** guaiacol dependent peroxidase; **MS,** Murashige and Skoog; **BAP,** benzyl amino purine; **IAA,** indole acetic acid; **PAR,** photo active radiation; **CTAB,** cetyltrimethyl ammonium bromide; **HPLC,** high performance liquid chromatography; **CRBD,** completely randomized block design; **TSC,** total soluble carbohydrates; **ANOVA,** analysis of variance; **LSD,** least significant difference; **CAT,** catalase; **APX,** ascorbate peroxidase; **PCR,** polymerase chain reaction.

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