Annals of Applied Biology ISSN 0003-4746

RESEARCH ARTICLE

In vitro selection of mung bean and tomato for improving tolerance to NaCl

N.M. Hassan, M.S. Serag, F.M. El-Feky & M.M. Nemat Alla

Botany Department, Faculty of Science at Damietta, Mansoura University, Damietta, Egypt

Keywords
CI; *in vitro* selection; *Lycopersicon esculentum*; PAL; salt resistance; TAL; *Vigna radiata*.

Correspondence
M.M. Nemat Alla, Botany Department, Faculty of Science at Damietta, Mansoura University, Damietta, P.O. Box 34517, Egypt.
Email: mamnematalla@mans.edu.eg

Received: 8 September 2007; revised version accepted: 12 December 2007.

doi:10.1111/j.1744-7348.2008.00221.x

Abstract

Mung bean and tomato were *in vitro* selected from cotyledons on MS medium for improved tolerance to NaCl. The growth responses; the Na, K, proline and anthocyanin contents; and activities of phenylalanine ammonia lyase (PAL, EC 4.3.1.5), tyrosine ammonia lyase (TAL, EC 4.3.1) and chalcone isomerase (CI, EC 5.5.1.5) of the selected plants were characterised and compared with those of the original plants in relation to treatment with NaCl. The treatments significantly reduced fresh and dry weights of shoots and roots; the reduction was least pronounced in selected plants. Meanwhile, Na content was significantly increased; however, K was decreased, a trend that was obvious in original plants but withdrawn following *in vitro* selection with a consequent lowering in Na/K ratio. In addition, proline was greatly induced by NaCl; the induction was most pronounced in selected plants. Moreover, NaCl significantly increased anthocyanin and activities of PAL, TAL and CI in shoots and roots of both species; the increase was lesser in the selected than in the original plants. These findings indicated that selection of mung bean and tomato resulted in a recovery of growth, overproduction of proline and K and withdrawal of Na and secondary metabolism parameters relative to original plants pointing out to an improved tolerance to NaCl following *in vitro* selection.

Introduction

Water stress is one of the most important environmental factors that regulate plant growth and development and limit plant production. Plants can respond and adapt to water stress by altering their cellular metabolism and invoking various defence mechanisms (Jiang & Zhang, 2002). Salinity is one of the most limiting components directly related to the decrease in crop yield. Gorai & Neffati (2007) confirmed that increases in salinity decreased both the germination percentage and the germination speed. The need for salt-tolerant crops increased as the growing world population seeks to feed itself. It has been shown that the expression of several transgenes promotes a higher level of salt tolerance in some species. Despite this promising result, the development of a salt-tolerant cultivar by way of transgenesis has still not been achieved (Cuartero *et al.*, 2006).

Attempts to enhance tolerance have involved the use of *in vitro* selection (Flowers, 2004; Pareek, 2005). Plant cell and tissue culture has become a major tool in the study of an increasing number of fundamental and applied programmes in plant science (Pareek, 2005). *In vitro* culture may offer potential for quick evaluation of germplasm against salt stress (Cano *et al.*, 1998). Micropropagation is one of the innovative methods of asexual propagation that proved to be effective for *in vitro* propagation of valuable plants (Rout, 2002; Faisal *et al.*, 2005). Mohan Jain (2001) reported that tissue culture generates a wide range of genetic variation in plant species. Gulati & Jaiwal (1996) indicated that calli derived from *Vigna* grew better under NaCl stress than those from cultivated species. The tolerant callus lines of *Cymbopogon martinii* that was obtained by exposure to increasing concentration of NaCl (0–350 mM) grew better than the wild-type lines in NaCl (Pattnaik & Debata, 1997).

More attention has been paid to the role of proline as a compatible osmolyte and osmoprotectant (Samaras *et al.*, 1995). A significant accumulation of proline was found in several plant species following salinity exposure (Eraslan *et al.*, 2007; Wang *et al.*, 2007; Ghars *et al.*, 2008). The accumulation of proline might function as osmolyte for intracellular osmotic adjustment and might be playing a critical role in protecting plants under salt stress (Silva-Ortega *et al.*, 2008). Yazici *et al.* (2007) suggested that salinity tolerance might be closely related with the accumulation of osmoprotectant proline under salinity conditions. However, Nandwal *et al.* (2007) confirmed that salinity significantly enhanced Cl and Na/K ratio. Salt-tolerant plants are able to maintain lower Na/K ratio than the unselected plants (Chaudhary *et al.*, 1996). A progressive increase in leaf Na^+ and Cl^- contents and Na^+/K^+ ratio with increasing salinity level was observed in sunflower (Di Caterina *et al.*, 2007). Tolerance to stress could be also mediated by secondary metabolites as defence responses (Nemat Alla & Younis, 1995; Dorey *et al.*, 1999). Secondary metabolism enzymes phenylalanine ammonia lyase (PAL), tyrosine ammonia lyase (TAL) and chalcone isomerase (CI) catalyse production of these metabolites to act as a natural defence in response to biotic and abiotic stress (Hoagland & Duke, 1981; Nemat Alla & Younis, 1995; Nemat Alla *et al.*, 2002). Such metabolites were increased by salinity (Mehta *et al.*, 1993; Nemat Alla *et al.*, 2002).

The present work was aimed to improve plant tolerance to NaCl through *in vitro* selection using the model species mung bean and tomato, two of the economically important crops in Egypt. Therefore, growth responses and contents of Na, K, proline and anthocyanin as well as activities of secondary metabolism enzymes were investigated.

Materials and methods

Plant materials and growth conditions

For original plant study, mung bean (*Vigna radiata* L. Wilczeck Kawmy-1) and tomato (*Lycopersicon esculentum* Super Strain B) seeds were surface sterilised. Sterilisation of mung bean seeds was carried out by immersing in 70% ethyl alcohol for a minute followed by 5 min in 0.1% (w/v) mercuric chloride solution, whereas sterilisation of tomato seeds was performed by immersing in 25% (v/v) commercial Clorox solution (5.25% sodium hypochlorite) for 25 min. The sterilised seeds were thoroughly washed several times (mung bean seeds were soaked in water for 4 h), germinated in sand : clay soil (2:1, v/v) in plastic pots (30 cm diameter) and kept at 32°C with a 14-h photoperiod (580 μmol m^{-2} s^{-1}) and 75% relative humidity. Water was applied daily for 3 days and substituted by Hoagland solution, thereafter. At the two true leaves stage (7 days for mung bean and 21 days for tomato), the seedlings were thinned to five per pot. Then, 50 mL of half-strength Hoagland solution containing 0, 25, 50, 100 or 150 mM NaCl was applied every 2 days. Each concentration was replicated with four pots, and the experiment was repeated twice so that each plant species was represented by 40 pots. After 21 days from treatments, plants were harvested once and separated into shoots and roots.

In vitro selection

Mung bean seeds were rinsed in 70% (v/v) ethyl alcohol for 1 min, sterilised by 0.1% (w/v) mercuric chloride solution for 5 min, washed by sterile distilled water several times, rinsed in sterile distilled water for 4 h and germinated in dark at 25°C with 60% humidity on hormone-free medium, MS basal medium (MS salts + B5 vitamins) containing 3% (w/v) sucrose and 0.8% (w/v) agar in 70 × 115 mm glass jars. Media were adjusted to pH 5.8 and autoclaved. Cotyledons of 2-day-old germinated seeds were excised under aseptic conditions and cultured on hormone-free medium, MS basal medium. Each cotyledon was positioned with the proximal surface and embedded in the medium. NaCl was added to the medium to give the same concentrations. Media were adjusted to pH 5.8, autoclaved and the cultures were incubated at 25°C for 28 days in 16-h photoperiod (80 μmol m^{-2} s^{-1}). Each concentration was replicated with three jars and the experiment was repeated twice so that each plant species was represented by 30 jars.

Selection of tomato followed the procedures of Iler *et al.* (1993). Seeds were surface sterilised by 25% (v/v) commercial Clorox solution for 25 min, rinsed in sterile distilled water and germinated on MS basal medium in 70 × 115 mm glass jars. The jars were kept in the dark at 25°C with 60% humidity for 3 days and then transferred to light (16-h photoperiod, 80 μmol m^{-2} s^{-1}). Cotyledons of 7-day-old seedlings were cultured on MS solid medium (pH 5.8) containing 3% (w/v) sucrose, 0.8% (w/v) agar, 1.5×10^{-5} M indole acetic acid (IAA) and 1.8×10^{-5} M kinetin. Each cotyledon was positioned with its adaxial surface facing upwards. The media were adjusted to pH 5.8 and autoclaved. The cultures (six jars from two independent experiments for each concentration) were maintained in a growth cabinet (growth chamber Conviron, CMP 3244; controlled environments, Winnipeg, Manitoba, Canada) at 25°C with 16-h day and 600 μmol m^{-2} s^{-1} for 32 days.

Ann Appl Biol **152** (2008) 319–330

Root formation, plant acclimatisation and greenhouse cultivation

Well-developed shoots were transferred to rooting medium, MS basal medium supplemented with 5×10^{-6} M IAA, containing the concentrations of NaCl. Cultures were kept in a growth cabinet as previously mentioned. The *in vitro* selected plantlets were washed under running tap water and kept in Hoagland solution for 7 days until they formed a good rooting system, then transplanted in plastic pots (25 cm in diameter) containing a mixture of peat moss and sand (2:1, v/v) covered with transparent polyethylene bags and irrigated with Hoagland solution. The pots were uncovered after 7 days, transferred to greenhouse and treated exactly as the original plants (Hoagland solution supplemented with the same concentrations of NaCl was added every 2 days for 21 days). Each concentration was replicated with eight pots from two independent experiments.

The plant material was rapidly dried in an oven at 80°C for 48 h to constant weight using a desiccator. Fresh and dry weights were calculated on plant number basis. Dried plant materials were ground to fine powders and used for the determination of Na, K and proline.

Determination of Na and K

The ground plant material (about 0.5 g) was digested using sulphuric acid–peroxide method (Allen *et al.*, 1986). After cooling, the extract was made up to a known volume. Na and K in the digest were determined using a flame photometer.

Determination of free proline

Proline was extracted in 0.5 g of the dried powder using 10 mL of 3% (w/v) aqueous sulphosalicylic acid (Bates, 1973). After centrifugation at 12 000 *g* for 20 min, 1 mL of the supernatant was allowed to react with 2 mL of acid ninhydrin reagent and 2 mL acetic acid for 1 h at 100°C. The reaction was terminated in an ice bath. The reaction mixture was extracted with toluene, the chromophore containing toluene was warmed to room temperature and the absorbance at 520 nm was measured.

Determination of anthocyanin

Fresh tissue (about 5 g) was homogenised in 20 mL acidic methanol (HCl, 1% v/v) for 5 min (Hoagland, 1980) followed by centrifugation at 5000 *g* for 20 min. Anthocyanin was quantitated by the difference in absorbency at 525 and 585 nm ($\Delta_{525-585}$).

Enzyme extraction and determination

Plant fresh tissues were homogenised in Tris–HCl (50 mM, pH 8.4) containing 15 mM β-mercaptoethanol at 4°C. The homogenates were centrifuged at 16 000 *g* for 20 min. Assays of PAL and TAL were run in reaction mixtures containing 500 μmol of Tris–HCl (pH 8) and either 6 μmol of L-phenylalanine (for PAL) or 5.5 μmol of L-tyrosine (for TAL) (Beaudoin-Eagan & Thorpe, 1985). Activity of CI was measured by determining the decrease in the absorbency at 375 nm in reaction mixture containing 500 μmol of Tris–HCl buffer (pH 8) and 10 μg of chalcone (dissolved in 10 μL of ethylene glycol monomethyl ether) (Hahlbrock *et al.*, 1970).

The experimental design was a randomised experiment or a true experiment based on the random assignment. It was a simple factorial combination for each plant species involving five levels of NaCl for each of the original plants, cultures and selected plantlets. All values are means of at least six replications (±SD) from two independent experiments. The full data were first subjected to analysis of variance (ANOVA) followed thereafter by least significant differences (LSD) method at 5% level (Snedecor & Cochran, 1980).

Results

As shown in Fig. 1, NaCl generally reduced fresh and dry weights of the original mung bean and tomato, either shoots or roots. The magnitude of reduction increased with increasing NaCl concentrations. The *in vitro* selected plants (mung bean and tomato) showed the same response to NaCl concentration. Although NaCl induced significant reduction in growth parameters of the regenerated plantlets, the magnitude of reduction was greater in the original plants. Fresh and dry weights of original mung bean plants were reduced by 50 mM onwards in shoots and by all concentrations in roots. Similarly, all concentrations reduced shoot and root fresh weight of the original tomato plants, while 50 mM onwards reduced dry weight. The same trend was also detected in the selected plants; however, the reduction was significant in shoots and roots of mung bean by 100 and 150 mM and of tomato by 50 mM onwards.

Fig. 2 shows that *in vitro* selection of both species from cotyledons on MS media supplemented with different concentrations of NaCl resulted in good and healthy plantlets, particularly using low concentrations of the salt. These plantlets performed feasible rooting even with the presence of NaCl. The greenhouse growing of these plantlets in pots after adaptation and acclimatisation showed that they are slightly affected by NaCl.

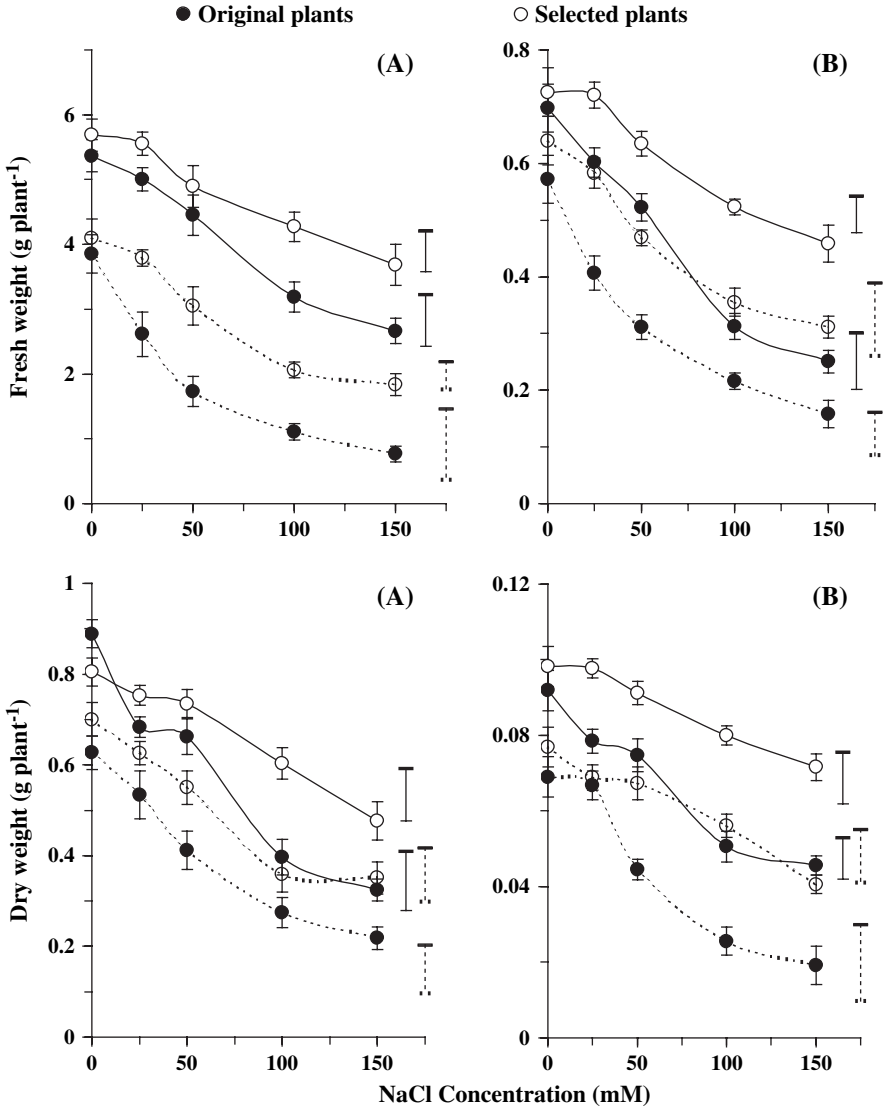

Figure 1 Effect of NaCl on fresh and dry weights of shoots (——) and roots (- - - -) of original and *in vitro* selected mung bean (A) and tomato (B) from cotyledons on MS media. Data are means (±SD) of at least six replications from two independent experiments. Vertical bars (solid for shoots and dotted for roots) represent least significant differences values at 5% level.

Contents of Na and K as well as their ratio in shoots and roots of the original and the *in vitro* selected mung bean and tomato are shown in Fig. 3. All concentrations significantly increased Na in shoots and roots of the original mung bean plants. Similarly, Na of the original tomato was increased in shoots by concentrations over 25 mM and in roots by all concentrations. In the same pattern, NaCl over 25 mM significantly increased Na in shoots or roots of the selected mung bean. However, Na was significantly increased in shoots of the selected tomato by NaCl over 50 mM. Conversely, all NaCl concentrations significantly decreased K of the original mung bean. In the *in vitro* selected mung bean, K in shoots and roots was restored following *in vitro* selection so that NaCl appeared with no significant effects. However, the *in vitro* selected tomato showed slight, if any, changes in K. About 25 and 50 mM NaCl insignificantly changed K in shoots and roots, respectively, of the selected plants. However, higher concentrations (100 and 150 mM) decreased K in shoots of the regenerated tomato. It is clear that Na/K ratio was high in the original plants but decreased in the plantlets regenerated from *in vitro* selection. These ratios increased with increasing NaCl concentrations; the increase was great and progressive in the original plants. Nevertheless, *in vitro* selection delayed these increases.

Ann Appl Biol **152** (2008) 319–330

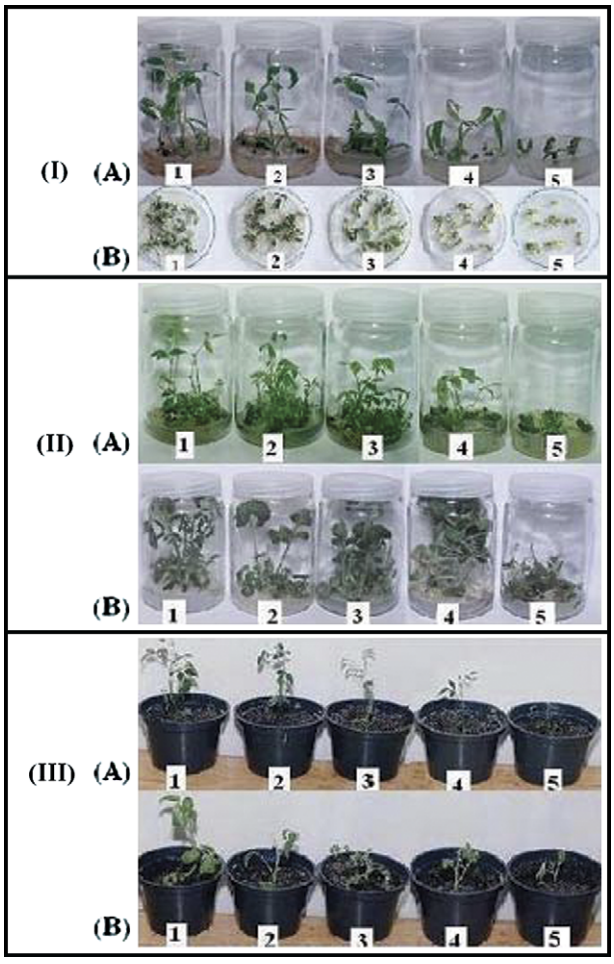

Figure 2 *In vitro* selection of mung bean (A) and tomato (B). (I) Regeneration using cotyledons on MS media supplemented with NaCl. (II) Rooting of the regenerated shoots on MS media supplemented with NaCl. (III) Greenhouse growth of the regenerated plantlets transferred to peat-moss after adaptation and acclimatisation in presence of NaCl. 1, 0 mM; 2, 25 mM; 3, 50 mM; 4, 100 mM; and 5, 150 mM NaCl.

Proline content of shoots and roots of both species, either original or *in vitro* selected plants, was significantly increased by NaCl, the magnitude of increase augmented with increasing NaCl concentrations (Fig. 4). The induction was most pronounced in the *in vitro* selected than the respective original plants. Proline in shoots and roots of original mung bean was increased by NaCl concentrations from 50 and 100 mM, respectively. In a similar manner, 50 mM onwards resulted in increases of proline in shoots and roots of the original tomato plants. Concentrations from 50 mM increased proline in shoots and roots of the regenerated mung bean. Similarly, the accumulation of proline in tomato shoots and roots by NaCl increased following *in vitro* selection; all concentrations led to great accumulations in roots, while 50 mM onwards caused accumulations in shoots.

All concentrations of NaCl resulted in significant increases in anthocyanin contents of the original mung bean and tomato plants (Fig. 4). The contents in roots of both species, although retracted by the highest concentration, remained higher than control levels. To a lesser extent, anthocyanin contents in both species of the *in vitro* selected plants were significantly increased particularly by higher concentrations; however, lower concentrations had mostly no significant effects. The NaCl-induced anthocyanin seemed to be decreased in both species following *in vitro* selection; however, 50 mM onwards caused significant increases. In spite of the decreased anthocyanin induced in shoots by the highest concentration, contents remained significantly higher than those of controls. In general, anthocyanin contents were higher in the original than in the respective *in vitro* selected plantlets.

In Fig. 5, there were gradual increases in the activities of PAL, TAL and CI in shoots and roots of the original or the *in vitro* selected plants, the increases augmented with increasing NaCl concentration. However, PAL activity decreased in shoots of both selected mung bean and original tomato. Similarly, TAL activity decreased in shoots of selected mung bean and in roots of selected tomato. TAL activity of roots of selected and original mung bean remained constantly from 100 to 150 mM NaCl. CI activity decreased in roots of selected mung bean. The magnitude of increase in PAL activity was greater in shoots than in roots of both species. Moreover, the enzyme activity was higher in the original than in the selected plants. Following *in vitro* selection, 25 mM NaCl had no significant effects on PAL activity in shoots and roots of the selected mung bean and tomato except for mung bean shoots. Moreover, 50 mM had a similar insignificant effect on the enzyme activity of tomato shoots. The enzyme activity was significantly enhanced in both tissues by the other high concentrations. In general, TAL activity was more detected in shoots than in roots. In addition, the enzyme activity of the original mung bean and tomato shoots and roots was significantly increased by all concentrations of NaCl. However, the enhanced activity was retracted following *in vitro* selection. About 25 mM appeared without effect on TAL activity of shoots and roots of the selected mung bean and tomato. Moreover, 50 mM non-significantly affected the enzyme activity in tomato shoots only. The activity of CI was lowered following *in vitro* selection. The enzyme activity of the original mung bean and tomato was enhanced by all concentrations. Nevertheless, CI activity in shoots or roots of the selected mung bean was significantly enhanced by only 100 or 50 mM onwards, respectively. On the other hand, NaCl over 25 and

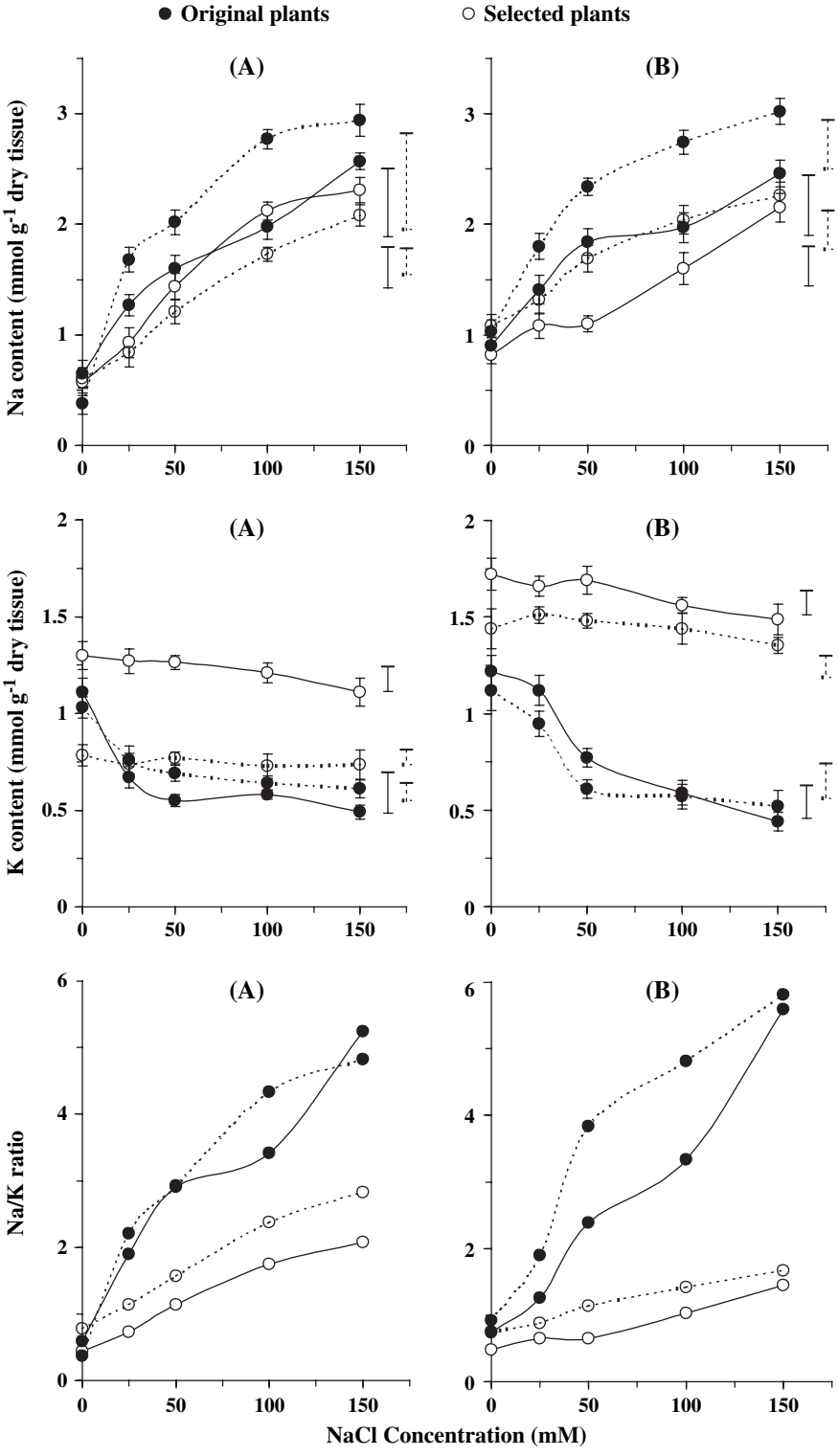

Figure 3 Effect of NaCl on Na, K and Na/K ratio of shoots (——) and roots (- - - -) of original and *in vitro* selected mung bean (A) and tomato (B) from cotyledons on MS media. Data are means (±SD) of at least six replications from two independent experiments. Vertical bars (solid for shoots and dotted for roots) represent least significant differences values at 5% level.

Ann Appl Biol **152** (2008) 319–330

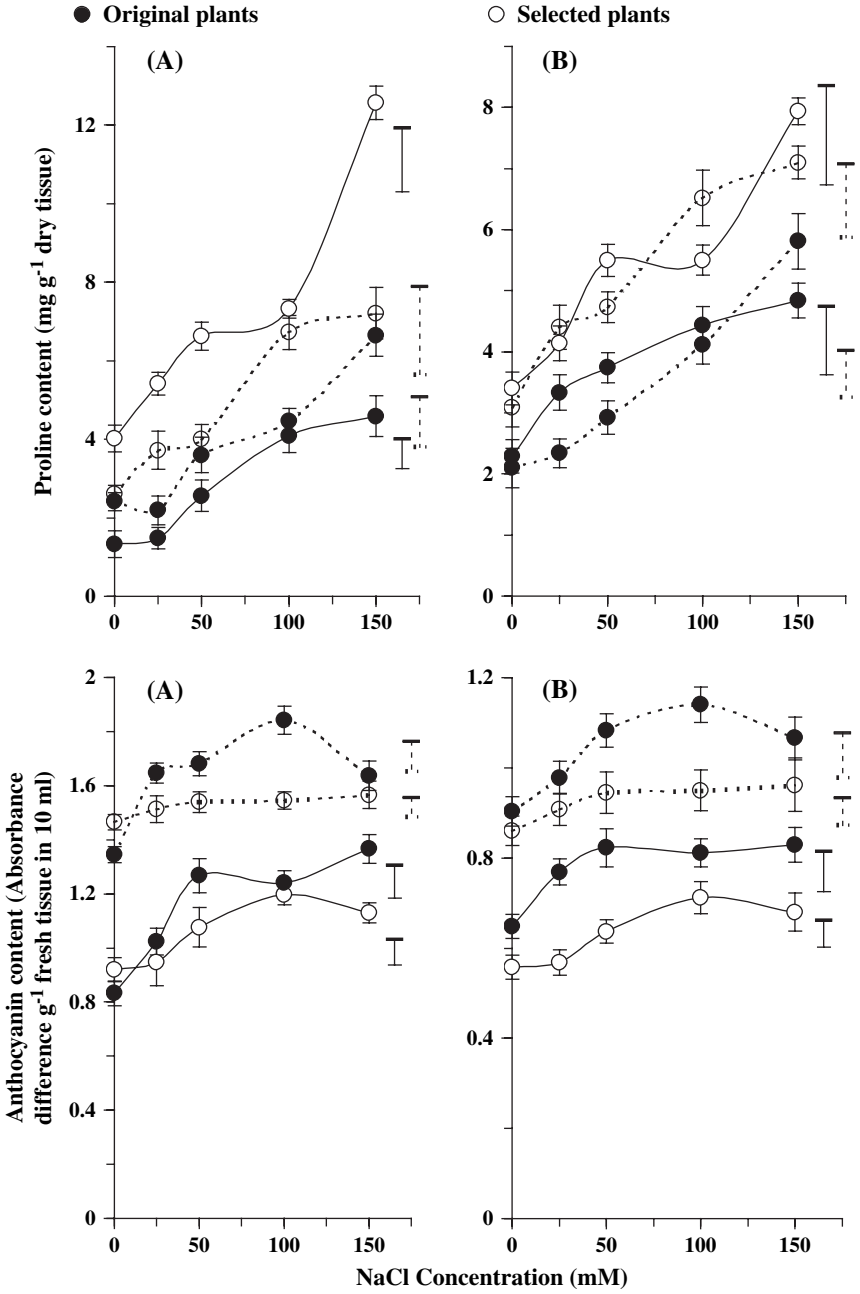

Figure 4 Effect of NaCl on proline and anthocyanin contents of shoots (——) and roots (- - - -) of original and *in vitro* selected mung bean (A) and tomato (B) from cotyledons on MS media. Data are means (±SD) of at least six replications from two independent experiments. Vertical bars (solid for shoots and dotted for roots) represent least significant differences values at 5% level.

50 mM increased CI activity in shoots and roots of the regenerated tomato, respectively.

Discussion

Plant cell and tissue culture has become a major tool in the study of an increasing number of fundamental and applied programmes in plant science. Its increasing use to investigate cell and developmental biology, biochemistry, physiology, genetics and molecular biology is providing new knowledge about fundamental characteristics of plants (Flowers, 2004; Pareek, 2005). The present results showed significant reductions in fresh and dry weights of both the original and the *in vitro* selected mung bean

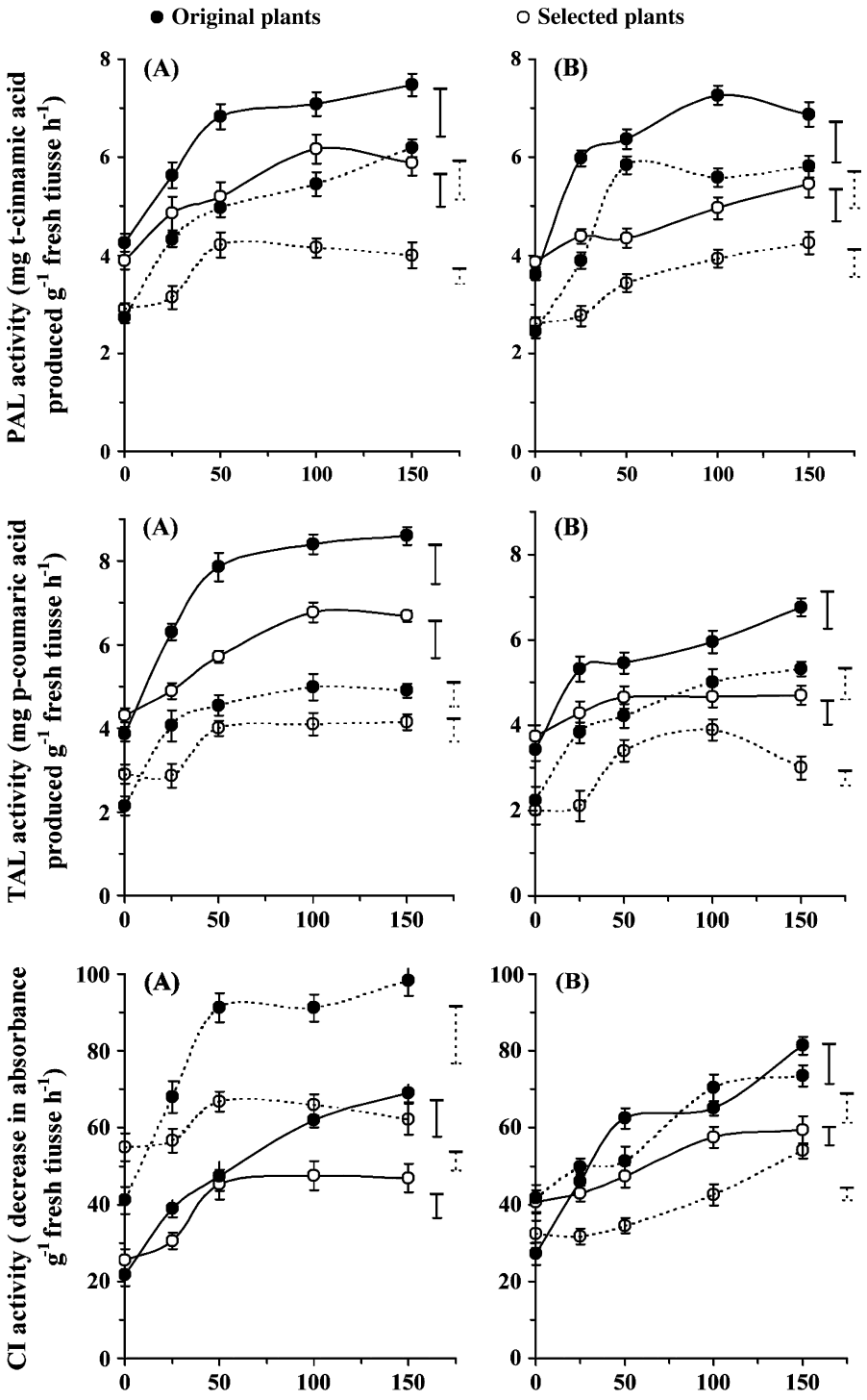

Figure 5 Effect of NaCl on phenylalanine ammonia lyase (PAL), tyrosine ammonia lyase (TAL) and chalcone isomerase (CI) activities of shoots (——) and roots (- - - -) of original and *in vitro* selected mung bean (A) and tomato (B) from cotyledons on MS media. Data are means (±SD) of at least six replications from two independent experiments. Vertical bars (solid for shoots and dotted for roots) represent least significant differences values at 5% level.

and tomato by salinity. NaCl significantly reduced fresh and dry weights of both original and *in vitro* selected plants. The salinity-induced growth reduction was observed in many plant species (Nemat Alla *et al.*, 2002; Hassan *et al.*, 2003; Younis *et al.*, 2003; Agong *et al.*, 2004; Murillo-Amador *et al.*, 2007; Ghars *et al.*, 2008).

Ann Appl Biol **152** (2008) 319–330

Murillo-Amador *et al.* (2006*b*) concluded that salt-tolerant cowpea genotypes showed higher biomass than genotypes classified as salt sensitive. Gorai & Neffati (2007) confirmed that highest germination percentages of *Reaumuria vermiculata*, a xerohalophytic perennial dwarf shrub, were obtained under non-saline conditions and increases in salinity inhibited seed germination. Salt stress decreased both the germination percentage and the germination speed. Seed germination decreased with an increase in NaCl concentrations. Fresh weight of *Chenopodium quinoa* was reduced by salinity in both embryonic axes and cotyledons (Prado *et al.*, 2000).

However, the magnitude of reduction decreased following *in vitro* selection. Such mitigation of NaCl effect might point to an improvement or development of tolerance in the selected plantlets to overcome, to some extent, the effects of NaCl. NaCl-tolerant callus lines of *C. martinii* were obtained by exposing the callus to increasing concentrations of NaCl (0–350 mM) in the MS medium (Pattnaik & Debata, 1997). The tolerant lines grew better than the sensitive wild-type lines in all concentrations of NaCl tested up to 300 mM. The selected lines retained their salt tolerance after three to four subcultures on salt-free medium.

The depression of growth may be attributed to a decrease in water absorption, meristematic activity, photosynthetic capacity, chlorophyll content, stomatal conductance, transpiration and cell enlargement; to an increase in respiration rate; and to changes in hormone imbalance, nitrogen metabolism, oxidative stress, secondary metabolism and excessive accumulation of toxic ions such as Na and Cl in plant cells (Nemat Alla *et al.*, 2002; Younis *et al.*, 2003; Agong *et al.*, 2004; Murillo-Amador *et al.*, 2006*a*,*b*, 2007; Eraslan *et al.*, 2007; Nandwal *et al.*, 2007; Yazici *et al.*, 2007; Tuna *et al.*, 2008). Na may have direct toxic effects through interference with enzyme structure and function or interfere with function of K as a cofactor in various reactions (Ayala & Oleary, 1995). Na and Cl in shoots and roots in cowpea and kidney bean increased in the presence of NaCl, while K decreased (Murillo-Amador *et al.*, 2006*a*,*b*, 2007). Tester & Davenport (2003) confirmed that metabolic toxicity of Na is largely a result of its ability to compete with K for binding sites essential for cellular function. More than 50 enzymes are activated by K, and Na cannot substitute in this role (Bhandal & Malik, 1988). Thus, high levels of Na or Na/K ratios can disrupt various enzymatic processes in the cytoplasm. K was reduced in shoot and root of salt-treated cowpea and kidney bean plants (Murillo-Amador *et al.*, 2007).

In the present results, there was a retraction in the accumulated Na in mung bean and tomato following *in vitro* selection, while K was increased with a consequent low in Na/K ratio. These findings are in accordance with Chaudhary *et al.* (1996) who found that the salt-tolerant plants are able to maintain lower Na/K ratio than the unselected plants. Moreover, Ghars *et al.* (2008) confirmed that the better NaCl tolerance in *Thellungiella halophila* was associated with a better K supply resulting higher K/Na ratios. Therefore, the present result could conclude that an improved tolerance to NaCl might be developed in the regenerated plantlets following *in vitro* selection. The decrease in K may be attributed to the antagonism at the site of uptake at plasmalemma between K and Na cations, which increased considerably as salinity increased (Mozafer & Dertli, 1990). Di Caterina *et al.* (2007) detected a progressive increase in leaf Na and Cl contents and Na/K ratio with increasing salinity level in sunflower. Na increased sharply in NaCl-sensitive *C. martinii* callus, while K declined continuously with the corresponding increase in external NaCl concentrations but the NaCl-tolerant callus lines always maintained higher Na and K levels than that of the sensitive lines (Pattnaik & Debata, 1997). They concluded that the degree of NaCl tolerance of the selected lines was in negative correlation with the K/Na ratio.

Under stress conditions imposed by salinity, the contents of proline are expected to be greatly enhanced. Proline was accumulated in roots and shoots of salt-stressed plants (Pattnaik & Debata, 1997; Eraslan *et al.*, 2007; Wang *et al.*, 2007; Yazici *et al.*, 2007; Ghars *et al.*, 2008). Martinez *et al.* (1996) found a positive relationship between proline accumulation and salt tolerance in potato. In accordance, the increases of proline in the present study were higher in the selected relative to the original plants. Most attention has been concerned with the role of proline as a compatible osmolyte and osmoprotectant (Samaras *et al.*, 1995). Accumulation of proline is one of the most remarkable metabolic consequences of salt stress in higher plants. The accumulated proline mediates tolerance by serving as a source of cytoplasmic osmoticum and protecting cytoplasmic enzymes and cellular structures (Nemat Alla *et al.*, 2002; Hassan *et al.*, 2004; Yazici *et al.*, 2007; Silva-Ortega *et al.*, 2008). Proline affects the solubility of various proteins and could protect them against denaturation. Proline may act as non-toxic osmotic solute, preferentially located in the cytoplasm and as enzyme protectant, stabilising the structures of macromolecules and organelles. The NaCl-selected callus lines of *C. martinii* accumulated high level of proline under salt stress (Pattnaik & Debata, 1997). It seems that osmoregulating substances such as proline affect the conformation of enzymes, thereby stabilising their active conformation and in this way protect enzymes against conformational perturbations caused by mineral ions. Therefore, the enhanced

proline accumulation following *in vitro* selection might aid in overcoming the effect of NaCl and hence, might be considered as a signal for improving tolerance to NaCl. The greater production of proline in the selected mung bean and tomato might conclude that *in vitro* selection regenerated plantlets capable of counterbalancing, to a great extent, the deleterious effects of NaCl.

Secondary metabolites are a natural defence against biotic and abiotic events and so may increase plant adaptation to stress conditions (Dorey *et al.*, 1999). They are produced in plant tissues under the control of the secondary metabolism enzymes PAL, TAL and CI (Hoagland & Duke, 1981; Jez *et al.*, 2000). In the present results, anthocyanin and enzyme activities were increased by NaCl. These increases were high in original but seemed to be withdrawn in the *in vitro* selected plantlets. In accordance, salinity mostly increased the content of secondary metabolites (Mehta *et al.*, 1993) as well as PAL and TAL activities (Nemat Alla *et al.*, 2002). Therefore, the withdrawal of the accumulation of these compounds in the selected plantlets relative to the original plants might be considered as a retraction of the stress status. Consequently, these regenerated plantlets could insist the presence of NaCl, to some extent, and thereby overcome its effects. These compounds might lead to growth inhibition through decrease of protein synthesis as a result of reduction of phenylalanine and tyrosine pool, increasing levels of phenolic compounds that are growth inhibitors and increasing ammonia to toxic levels. Hoagland & Duke (1981) indicated that high PAL activity might divert phenylalanine from protein synthesis or other critical cellular processes.

In conclusion, the *in vitro* selected mung bean and tomato plantlets seemed to overcome, to great extent, the deleterious effect of NaCl relative to their respective original plants. So, these plantlets developed some resistance to NaCl following *in vitro* selection. Most of the *in vitro* selected plantlets set fruits, and their seeds have been checked for germination. However, their progeny should be tested for resistance to NaCl, the point that requires further investigation. The selected plantlets seemed to overcome the effects of NaCl and meanwhile contained relatively lesser amounts of Na and higher amounts of K and proline and exhibited more withdrawal of anthocyanin and secondary metabolism enzymes than the original plants. These findings conclude a development of an improved tolerance to NaCl following *in vitro* selection of mung bean and tomato.

References

Agong S.G., Yoshida Y., Yazawa S., Masuda M. (2004) Tomato response to salt stress. *Acta Horticulturae*, **637**, 93–97.

Allen S., Grimshaw H., Rowland A. (1986) Chemical analysis. In *Methods in Plant Ecology*, pp. 285–344. Eds P.D. Moore and S.B. Chapman. Oxford, UK: Blackwell.

Ayala F., Oleary J.W. (1995) Growth and physiology of *Salicornia bigelovii* Torr. at suboptimal salinity. *International Journal of Plant Science*, **156**, 197–205.

Bates L.E. (1973) Rapid determination of free proline for water stress studies. *Plant and Soil*, **39**, 205–207.

Beaudoin-Eagan L.D., Thorpe T.A. (1985) Tyrosine and phenylalanine ammonia lyase activities during shoot initiation in tobacco callus cultures. *Plant Physiology*, **78**, 438–441.

Bhandal I.S., Malik C.P. (1988) Potassium estimation, uptake, and its role in the physiology and metabolism of flowering plants. *International Review of Cytology*, **110**, 205–254.

Cano E.A., Perez-Alfocea F., Moreno V., Caro M., Bolarin M.C. (1998) Evaluation of salt tolerance in cultivated wild tomato species through *in vitro* shoot apex culture. *Plant Cell, Tissue and Organ Culture*, **53**, 19–26.

Chaudhary M.T., Wainwright S.J., Merrett M.J. (1996) Comparative NaCl tolerance of lucerne plants regenerated from salt-selected suspension cultures. *Plant Science*, **114**, 221–232.

Cuartero J., Bolarin M.C., Asins M.J., Moreno V. (2006) Increasing salt tolerance in the tomato. *Journal of Experimental Botany*, **57**, 1045–1058.

Di Caterina R., Giuliani M.M., Rotunno T., De Caro A., Flagella Z. (2007) Influence of salt stress on seed yield and oil quality of two sunflower hybrids. *Annals of Applied Biology*, **151**, 145–154.

Dorey S., Kopp M., Geoffroy P., Fritig B., Kaufmann S. (1999) Hydrogen peroxide from the oxidative burst is neither necessary nor sufficient for hypersensitive cell death induction, Phenylalanine ammonia lyase stimulation, salicylic acid accumulation or scopoletin consumption in cultured tobacco cells treated with elicitin. *Plant Physiology*, **121**, 163–172.

Eraslan F., Inal A., Savasturk O., Gunes A. (2007) Changes in antioxidative system and membrane damage of lettuce in response to salinity and boron toxicity. *Scientia Horticulturae*, **114**, 5–10.

Faisal M., Ahmad N., Anis M. (2005) Shoot multiplication in *Rauvolfia tetraphylla* L. using thidiazuron. *Plant Cell, Tissue and Organ Culture*, **80**, 187–190.

Flowers T.J. (2004) Improving crop salt tolerance. *Journal of Experimental Botany*, **55**, 307–319.

Ghars M.A., Parre E., Debeza A., Bordenave M., Luc R., Laurent L., Alain B., Arnould S., Chedly A. (2008) Comparative salt tolerance analysis between *Arabidopsis thaliana* and *Thellungiella halophila*, with special emphasis on K^+/Na^+ selectivity and proline accumulation. *Journal of Plant Physiology*, in press.

Gorai M., Neffati M. (2007) Germination responses of *Reaumuria vermiculata* to salinity and temperature. *Annals of Applied Biology*, **151**, 53–59.

Gulati A., Jaiwal P.K. (1996) Effect of NaCl on nitrate reductase, glutamate dehydrogenase and glutamate synthase in *Vigna radiata* calli. *Biologia Plantarum*, **38**, 177–183.

Hahlbrock K., Wong E., Schill L., Grisebach H. (1970) Comparison of chalcone-flavonone isomerase heteroenzymes and isoenzymes. *Phytochemistry*, **9**, 949–958.

Hassan N.M., Serag M.S., Momtaz O.A., El-Feky F.M. (2003) *In vitro* selection of NaCl resistant mung bean and tomato plants. *Egyptian Journal of Agronomy*, **25**, 25–43.

Hassan N.M., Serag M.S., El-Feky F.M. (2004) Changes in nitrogen content and protein profiles following *in vitro* selection of NaCl resistant mung bean and tomato. *Acta Physiologiae Plantarum*, **26**, 165–175.

Hoagland R. (1980) Effect of glyphosate on metabolism of phenolic compounds, VI. Effect of glyphosine and glyphosate metabolites on phenylalanine ammonia lyase activity, growth and protein, chlorophyll and anthocyanin level in soybean (*Glycine max*) seedlings. *Weed Science*, **28**, 393–400.

Hoagland R., Duke S.O. (1981) Effect of herbicides on extractable phenylalanine ammonia-lyase activity in light-and dark-grown soybean (*Glycine max*) seedlings. *Weed Science*, **29**, 433–439.

Iler S.E., Swanton C.J., Pauls K. (1993) *In vitro* selection of imazethapyr-tolerant tomato (*Lycopersicon esculentum* Mill.). *Weed Science*, **41**, 12–17.

Jiang M., Zhang J. (2002) Water stress-induced abscisic acid accumulation triggers the increased generation of reactive oxygen species and up-regulates the activities of antioxidant enzymes in maize leaves. *Journal of Experimental Botany*, **53**, 2401–2410.

Jez J.M., Bowman M.E., Dixon R.A., Noel J.P. (2000) Structure and mechanism of the evolutionarily unique plant enzyme chalcone isomerase. *Natural Structural Biology*, **7**, 786–791.

Martinez C.A., Maestri M., Lani E.G. (1996) *In vitro* salt tolerance and proline accumulation in Andean potato (*Solanum* spp.) differing in frost resistance. *Plant Science*, **116**, 177–184.

Mehta N., Bharti S., Datta K.S. (1993) Effect of chloride and sulphate salinities on the concentration of some important metabolites in chickpea. *Haryana Agricultural University Journal of Research*, **23**, 150–155.

Mohan Jain S. (2001) Tissue culture-derived variation in crop improvement. *Euphytica*, **118**, 153–166.

Mozafer A., Dertli J.J. (1990) Multiple effects of salinity stress and temperature shock on growth of Barley. *Plant and Soil*, **128**, 153–216.

Murillo-Amador B., Jones H.G., Kaya C., López-Aguilar R., García-Hernández J.L., Troyo-Diéguez E., Ávila-Serrano N.Y., Rueda-Puente E. (2006*a*) Effects of foliar application of calcium nitrate on growth and physiological attributes of cowpea (*Vigna unguiculata* L. Walp.) grown under salt stress. *Environmental and Experimental Botany*, **58**, 188–196.

Murillo-Amador B., Troyo-Diéguez E., García-Hernández J.L., López-Aguilar R., Ávila-Serrano N.Y., Zamora-Salgado S., Rueda-Puente E.O., Kaya C. (2006*b*) Effect of NaCl salinity in the genotypic variation of cowpea (*Vigna unguiculata*) during early vegetative growth. *Scientia Horticulturae*, **108**, 423–431.

Murillo-Amador B., Yamada S., Yamaguchi T., Rueda-Puente E., Ávila-Serrano N., García-Hernández J.L., López-Aguilar R., Troyo-Diéguez E., Nieto-Garibay A. (2007) Influence of calcium silicate on growth, physiological parameters and mineral nutrition in two legume species under salt stress. *Journal of Agronomy and Crop Science*, **193**, 413–421.

Nandwal A.S., Kukreja S., Kumar N., Sharma P.K., Jain M., Mann A., Singh S. (2007) Plant water status, ethylene evolution, N_2-fixing efficiency, antioxidant activity and lipid peroxidation in *Cicer arietinum* L. nodules as affected by short-term salinization and desalinization. *Journal of Plant Physiology*, **164**, 1161–1169.

Nemat Alla M.M., Younis M.E. (1995) Herbicide effects on phenolic metabolism in maize (*Zea mays* L.) and soybean (*Glycine max* L.) seedlings. *Journal of Experimental Botany*, **46**, 1731–1736.

Nemat Alla M.M., Younis M.E., El-Shihaby O.A., El-Bastawisi Z.M. (2002) Kinetin regulation of growth and secondary treated *Vigna sinensis* and *Zea mays*. *Acta Physiologiae Plantarum*, **24**, 19–27.

Pareek L.K. (2005) *Trends in Plant Tissue Culture and Biotechnology*. Jodhpur, India: Agrobios.

Pattnaik J., Debata B.K. (1997) *In vitro* selection of NaCl tolerant callus lines of *Cymbopogon martinii* (Roxb.) Wats. *Plant Science*, **124**, 203–210.

Prado F.E., Boero C., Gallardo M., González G.A. (2000) Effect of NaCl on germination, growth, and soluble sugar content in *Chenopodium quinoa* Willd. seeds. *Bot. Bull. Acad. Sin*, **41**, 27–34.

Rout G.R. (2002) Direct plant regeneration from leaf explants of Plambago species and its genetic fidelity through RAPD markers. *Annals of Applied Biology*, **140**, 305–313.

Samaras Y., Bressan R.A., Csonka L.N., García-Ríos M.G., Paino D'Urzo M., Rhodes D. (1995) Proline accumulation during drought and salinity. In *Environment and Plant Metabolism: Flexibility and Accumulation*, pp. 161–187. Ed. N. Smirnoff. Oxford, UK: BIOS Scientific Publishers.

Silva-Ortega C.O., Ochoa-Alfaro A.E., Reyes-Agüero J.A., Aguado-Santacruz G.A., Jiménez-Bremont J.F. (2008) Salt stress increases the expression of *p5cs* gene and induces proline accumulation in cactus pear. *Plant Physiology and Biochemistry*, **46**, 82–92.

Snedecor W., Cochran G. (1980) *Statistical Methods*. 7th edn. Ames, IA, USA: The Iowa State University Press.

Tester M., Davenport R. (2003) Na^+ tolerance and Na^+ transport in higher plants. *Annals of Botany*, **91**, 503–527.

Tuna A.L., Kaya C., Higgs D., Murillo-Amador B., Aydemir S., Girgin A.R. (2008) Silicon improves salinity tolerance in wheat plants. *Environmental and Experimental Botany*, **62**, 10–16.

Wang Z., Yuan Y., Ou J., Lin Q., Zhang C. (2007) Glutamine synthetase and glutamate dehydrogenase contribute differentially to proline accumulation in leaves of wheat (*Triticum aestivum*) seedlings exposed to different salinity. *Journal of Plant Physiology*, **164**, 695–701.

Yazici I., Turkan I., Sekmen A.H., Demiral T. (2007) Salinity tolerance of purslane (*Portulaca oleracea* L.) is achieved by enhanced antioxidative system, lower level of lipid peroxidation and proline accumulation. *Environmental and Experimental Botany*, **61**, 49–57.

Younis M.E., El-Shihaby O.A., Nemat Alla M.M., El-Bastawisy Z.M. (2003) Kinetin alleviates the influence of water logging and salinity on growth and affects the production of plant growth regulators in *Vigna sinensis* and *Zea mays*. *Agronomie*, **23**, 277–285.

Ann Appl Biol **152** (2008) 319–330