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The effect of somaclonal variation on salt tolerance and glycoalkaloid content of potato tubers

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Abstract

Salinity has been a major constraint to the growth and production of important crop plants on a global scale. In addition to conventional breeding and the modern genetic engineering approaches, simple and cost effective tissue culture based methods may prove effective to cope with the salt stress induced crop losses. In the present study, we analysed *in vitro* somaclonal variation on plantlets of potato cv. Desiree and investigated the effect of somaclonal variation on the salt tolerance and tuber glycoalkaloid content. Around 38 regenerated plants were selected from tissue culture-induced calli based on their morphological status. These regenerants were subjected to *in vitro* salt stress evaluation and finally six regenerants were selected based on their salt tolerance performance. Somaclonal variation was confirmed through four RAPD primers. The somaclones and the parental control lines were further evaluated under greenhouse condition for salt tolerance and potato tuber glycoalkaloid content. Out of 38 somaclones, 6 somaclones revealed high salt tolerance evident from maintaining significantly high chlorophyll content. Interestingly, two of the somaclones also exhibited significantly reduced ($P \leq 0.05$) glycoalkaloid content (TGA below 100 mg/100 g dry weight) compared to that of parental control (180 mg TGA/100 g dry weight) indicating low effect of high salt stress on tuber quality. These results suggest that somaclonal variation may be a useful tool to develop salt tolerance and achieve tuber quality in terms of reduced tuber glycoalkaloid content.

Key words: Salt tolerance, potato, tissue culture, somaclonal variation, glycoalkaloid.
Abbreviations: AFLPs_Amplified fragment length polymorphisms; RAPD_Randomly amplified polymorphic DNA; TGA_Total glycoalkaloids.

Introduction

Somaclonal or culture induced variation has long been considered a source of useful unintended effects in *in vitro* cultured plants (Veilleux and Johnson, 1998). Some of these unintended effects include morphological traits, environmental stress tolerance, production of secondary metabolites and other important traits of plant breeder's interest (Veilleux and Johnson, 1998). In the literature many factors have been described as potentially responsible for somaclonal variation, which include desiccation, wounding, improper nutrient supply and osmotic stress (Filipecki and Malepszy, 2006). During *in vitro* conditions the explants are subjected to these stress conditions, which bring both genetic and epigenetic changes in the resultant regenerated plants (Evans and Sharp, 1988). Genetic changes remain permanently in the subsequent generations in the form of ploidy variation, chromosome rearrangements, somatic recombination, gene amplifications, point mutations, and insertions of transposons. Epigenetic changes are comprised of DNA methylation and histone modifications. A number of molecular techniques have been used to detect sequence variation between source material and the resultant somaclones in various crop plants. These molecular techniques include random amplified polymorphic DNAs (RAPDs), amplified fragment length polymorphisms (AFLPs) and representational difference analysis (RDA) (Labra et al., 2001; Linacero et al., 2000; Oha et al., 2007; Aversano et al., 2011). Potato, an economically important crop has been extensively studied for its responses to *in vitro* growth conditions and induction of somaclonal variation as a source of useful traits. In several studies, the use of molecular markers revealed the occurrence of somaclonal variation in regenerated potato plants. For example, in one study, RAPD analysis of *in vitro* regenerated plants from stem and leaf explants of potato cv (Desiree) revealed somaclonal variation (Bordallo et al., 2004). Ehsanpour et al. (2007) used RAPD markers for detection of somaclonal variation in UV treated calli from potato stem and leaf explants. Further evidence came from the study of Sharma et al. (2007), when they used AFLP markers for detection of genetic stability of regenerated plants from several potato plant parts. All the above studies were mainly focused on somaclonal variation as a source of useful variation. However, research has been limited on investigation of unintended effects of somaclonal variation in terms of changed response to plant salt tolerance and impact on key plant nutrients, allergens and toxins. The occurrence of useful unintended effects in *in vitro* regenerated potato plants investigated through morphological, biochemical and molecular markers indicate that such conditions may also bring compositional changes in plants allergens and toxins (Shepherd et al., 2006). Members of the family *Solanaceae* synthesize a variety of secondary metabolites, including alkaloids. Potato, an important member of this family contains several types of alkaloids. The most important group of alkaloids in commercial

potato varieties is the glycoalkaloids (GA), a sugar molecule (usually a trisaccharide) linked to the steroidal alkaloid solanidine (Van Gelder et al., 1988; Matthews et al., 2005). In potatoes, 95% of total glycoalkaloids are comprised of α-solanine and α-chaconine. For plants, glycoalkaloids have been proved very effective to guard off bacteria, viruses and certain foliage feeding insects (Friedman, 2004). But glycoalkaloids are highly toxic to humans and animals, and exposure to high doses can cause severe illness and even death. Keeping in mind the highly toxic nature of glycoalkaloids, the food biosafty regulatory authorities recommend the upper safe limit of glycoalkaloids to be 20 mg/100 g fresh weight of potato tubers (Matthews et al., 2005). Nowadays potato varieties developed either through conventional or transgenic approaches are strictly observed for any compositional changes in glycoalkaloids. Such biosafety evaluation tests are based on the strategy of substantial equivalance, in which the modified variety is compared with a wild control, which has a safe history of use. In addition to tissue culture condition, changes in environmental attributes such as drought, heat and cold temperatures may also have effects on tuber glycoalkaloid content (Levy et al., 1993; Papathanasiou et al., 1999; Bejarano et al., 2000). Tissue culture is a prerequisite for plant genetic transformation. In the presence of hormones and antibiotics, individual cells carrying tDNA molecules are induced to regenerate into whole plants expressing the transgene. Recently, several authors have attributed the occurrence of unintended effects in transgenic plants during the process of transformation and tissue culture. In several studies on transgenic potato varieties, compositional analysis revealed significant changes in the levels of individual and total glycoalkaloids (Birch et al., 2002; Bianco et al., 2003; Stobiecki et al., 2003). However, it is still not clear, how soamclonal variation contributes to compositional changes in potato glycoalkaloids. The present study focuses on the analysis of induced somaclonal variation in potato in terms of changes in the salt tolerance and the resultant effect on potato tuber glycoalakloid content. Further, it has been investigated, whether the plant salt tolerance due to somaclonal variation has any relationship to tuber glycoalkaloid content.

Results

In vitro studies and screening of somaclones

Somaclonal variation has been investigated in a number of studies and is considered as a source of useful and required traits such as disease resistance, better plant growth and productivity and tolerance to abiotic stresses. Abiotic stress such as salt stress has been a serious threat to plant productivity. In addition to other methods, somaclonal variation through tissue culture may be a good choice to develop salt tolerant lines. In the present study, stem explants of potato cv. Desiree were used for callus development and regeneration of somaclones (Fig. 1). A total number of 120 somaclonal lines were generated, which were divided into four groups based on their morphological status. Around 40 somaclones were selected out of the total 120 lines. These *in vitro* lines were multiplied through further cuttings and culturing. The *in vitro* plants of both somaclones and the parental lines were evaluated for salinity stress tolerance. Generally, *in vitro* screening has been practiced as the most reliable method to evaluate differences in salt tolerance among different plant lines and varieties. For *in vitro* screening, total number of roots and length of roots are the two important parameters for evaluation. For salinity tolerance screening of somaclones and the parental line, a salt (NaCl) concentration of 100 mM was used. In our early study, we used *in vitro* plants of potato cv. Desiree for salt stress tolerance under four different salt concentrations i.e 0, 25, 50, and 100 mM NaCl. Under 100 mM salt concentration, the plants showed clear and visible differences in terms of root number and root length compared to those cultured under 0 mM salt concentration. Therefore, we selected 100 mM as the salt stress for evaluation of the somaclones and parental lines.

In *in vitro* screening test, a total number of 38 somaclones and one parental line were used for salinity evaluation under 100 mM NaCl concentration. The data on average root number and root length on the somaclones and parental line are shown (Figs. 2 and 3). Somaclonal lines, designated as TC6, TC24, TC30, TC34, and TC36 showed significantly higher ($P \leq 0.05$) average root number as compared to that of control parental line (Fig. 2). On the other hand, somaclonal lines TC6, TC10, TC24, TC29, TC30, TC34, and TC36 exhibited significantly higher ($P \leq 0.05$) average root length compared to that of control line (Fig. 3). These results indicate the usefulness of somclonal variation for required traits such as salt tolerance. Based on the overall comparative performance, somaclonal lines TC6, TC10, TC24, TC30, TC34, and TC36 were selected for further salinity evaluation and impact on potato tuber glycoalkaloid content under greenhouse condition.

Molecular analysis of somaclonal variation

Molecular techniques such as RAPD may provide the best picture of any change at the DNA level between the somaclones and the parental line. In the present study we used 20 RAPD primers to detect polymorphism in the somaclonal lines compared to the parental control. Out of these, four primers detected variation in the regenerated lines. An example of the patterns of DNA amplification using different RAPD primers is shown in Fig. 5. In addition to the OPAA-03, we also detected polymorphic bands in somaclonal lines with other RAPD primers, such as OPAA-01 (AGACGGCTCC), OPAA-05 (GGCTTTAGCC), and OPA-08 (GTGACGTAGG). Some previous studies conducted on induction of somaclonal variation in *in vitro* potato plants revealed similar results (Bordallo et al., 2004; Ehsanpour et al., 2007). Somaclonal variation in the regenerated plants depends on the age of the callus formation process. The longer callus and regeneration process may produce more chances of somaclonal variation in the regenerants. In one study, Soniya et al. (2001) found that more than 90% of regenerated plants did not show somaclonal variation as detected by RAPD primers. They attributed this low rate of somaclonal variation to the short period of callus formation and regeneration. In our case, 4 out of 20 primers detected polymorphism. This might be due to the longer time, the calli remained on media and produced regenerants. This may also increase the chances to obtain somaclones with useful traits such as salt, drought tolerance and lower tuber glycoalkaloid content. The potential impact of somaclonal variation on salinity tolerance and glycoalkaloid content of the regenerants and the parental line was further investigated under greenhouse conditions.

Salt stress evaluation under greenhouse condition

The effect of salt stress was evaluated on plant growth of parental and somaclonal lines grown under non saline and saline conditions. Chlorophyll data was taken after four and eight weeks of the start of salt stress evaluation. The data was expressed as SPAD values. It was observed that after four weeks of salt stress application, all somaclones and the parental

Table 1. Average chlorophyll data of parent and somaclones after four weeks of salt stress application in the greenhouse.

Lines	Average chlorophyll content (SPAD values)		
	0 mM NaCl	50 mM NaCl	100 mM NaCl
Parent	38.4 ± 0.9[a]	36.3 ± 1.3[a]	41.1 ± 1.9[a]
TC6	50.0 ± 6.1[b]	54.5 ± 2.9[b]	57.1 ± 1.9[b]
TC10	46.6 ± 3.0[b]	43.6 ± 3.2[a]	53.0 ± 2.0[b]
TC24	45.7 ± 1.6[b]	43.2 ± 1.0[a]	47.5 ± 1.7[b]
TC30	46.6 ± 2.7[b]	47.7 ± 1.1[b]	49.2 ± 2.4[b]
TC34	48.1 ± 1.6[b]	44.2 ± 2.0[a]	51.0 ± 0.7[b]
TC36	43.6 ± 1.6[a]	41.2 ± 0.9[a]	46.9 ± 1.9[b]

Within each salt concentration, SPAD values with different letters are significantly (P≤ 0.05) different from each other as revealed by Tukey's test.

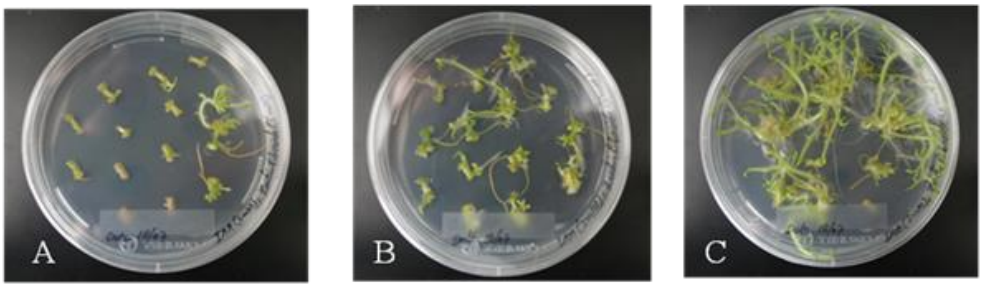

Fig 1. Development of regenerants from potato stem explants. (A) Callai and shoot formation after five weeks of culturing. (B) Shoot formation after eight weeks of culturing. (C) Individual shoots were excised from callai after twelve weeks of culturing. The chances of somaclonal variation increase, as the callus is retained for longer time on regeneration media. Therefore, some shoots were excised from stem explants after sixteen and twenty weeks from the date of callus formation.

Table 2. Average chlorophyll data of parent and somaclones after eight weeks of salt stress application in the greenhouse.

Lines	Average chlorophyll content (SPAD values)		
	0 mM NaCl	50 mM NaCl	100 mM NaCl
Parent	21.1 ± 3.8[a]	22.1 ± 6.7[a]	15.6 ± 3.1[a]
TC6	29.9 ±3.3[b]	33.8 ± 4.6[b]	32.5 ± 4.5[b]
TC10	23.1 ± 4.3[a]	25.4 ± 3.9[a]	24.4 ± 6.0[b]
TC24	18.5 ± 0.5[a]	20.2 ± 1.2[a]	23.1 ± 1.7[b]
TC30	25.9 ± 7.1[a]	27.6 ± 3.1[b]	26.1 ± 2.7[b]
TC34	32.0 ± 2.1[b]	35.4 ± 1.2[b]	34.9 ± 6.8[b]
TC36	24.0 ± 4.0[a]	28.1 ± 2.4[b]	26.2 ± 5.3[b]

Within each salt concentration SPAD values with different letters are significantly (P≤ 0.05) different from each other as revealed by Tukey's test.

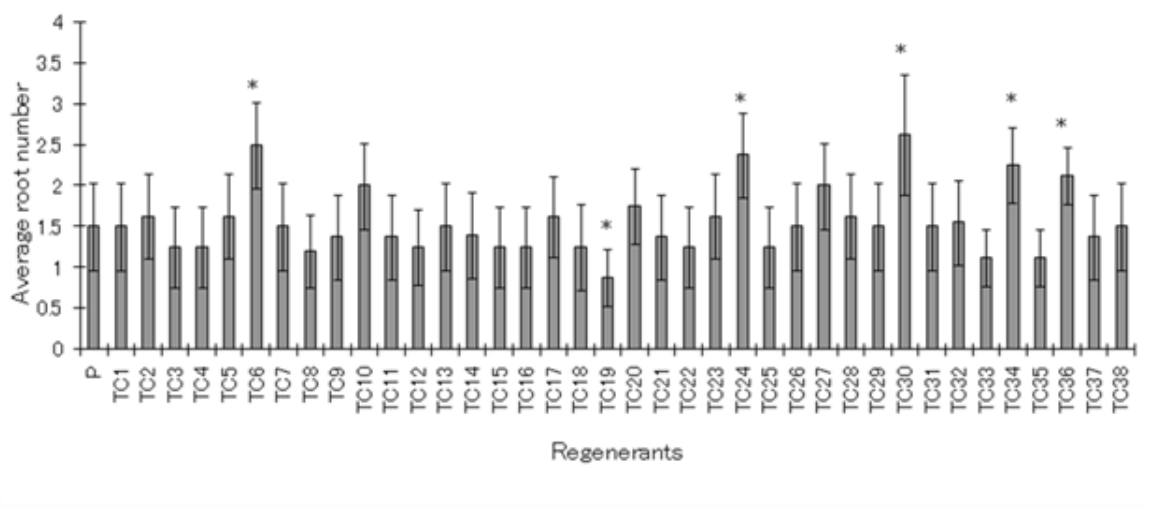

Fig 2. Average root number of *in vitro* plants of parental and tissue culture derived regenerants of potato cv. (Desiree) subjected to NaCl stress (100 mM). Bars represent means ±SD (n=8). Statistical significance is indicated by asterisks when *p*-value was $p \leq 0.05$.

control line did not show significant ($P \leq 0.05$) change in the chlorophyll content with increasing salt concentrations (0, 50 and 100 mM NaCl) (Table 1). However, significant ($P \leq 0.05$) differences were found in the chlorophyll content between the parental control and most of the somaclonal lines under all salt concentrations. The data taken after eight weeks of salt application showed significant reduction ($P \leq 0.05$) in the chlorophyll content in all lines compared to the data taken after four weeks (Table 2). Interestingly, it was observed that compared to the parental control, some of the somaclonal lines such as TC6 and TC34 exhibited enhanced salt tolerance in terms of improved vegetative growth. This was evident from the high chlorophyll content in these two lines as compared to that of parental control line (Table 2). Overall the prolonged application of salt stress coupled with the aging process tended to decrease the leaf chlorophyll content as observed in our results. Despite the effects of salt stress on all lines, the somaclonal variation on chlorophyll content seems to be more evident. Not all but a few somaclonal lines such as TC6 and TC34 showed less reduction in the chlorophyll content under high salt stress as compared to that of parental control. As a result of this high chlorophyll content, these lines showed low effects of high salt concentration and the aging process.

Glycoalkaloid analysis in potato tubers

The total glycoalkaloid (TGA) content is comprised of α-solanine and α-chaconine. The TGA content in parental control and somaclonal lines was analyzed. Fig. 6, shows peaks of α-solanine and α-chaconine standards. The TGA content was determined in the peel samples of potato tubers under non saline (0 mM NaCl) and saline conditions (50 mM and 100 mM NaCl) based on the curves generated from the standards. Analysis of variance (ANOVA) showed a significant effect ($P \leq 0.05$) of salt stress on the TGA content in the parental and somaclonal lines (Fig. 7). Under non-saline condition, the parental line accumulated TGA around 65 mg $(100\ g)^{-1}$ of freeze-dried matter. With salt stress application of 50 mM NaCl, the TGA content in parental control jumped to about 85 mg $(100\ g)^{-1}$ dw added an increase of 10 mg. The increase was even more pronounced under 100 mM NaCl concentration that increased the TGA content to 180 mg $(100\ g)^{-1}$ of freeze-dried matter. This trend in the TGA content increase with increasing salt concentration was also observed for all somaclonal lines (Fig. 7). However, in comparison to the TGA content in parental control line under different salt concentrations, two somaclonal lines i.e. TC6 and TC34 accumulated significantly lower ($P \leq 0.05$) TGA content. Both of these lines accumulated TGA around 100 mg $(100\ g)^{-1}$ dw, under 100 mM salt concentration. This was a significant reduction ($P \leq 0.05$) in the TGA content as compared to the parental control, which accumulated TGA around 180 mg $(100\ g)^{-1}$ dw. These results showed a visible effect of the genotype and the salt stress application on the potato tuber TGA content.

Discussion

In this study, our main objective was to investigate the effect of *in vitro* condition and the potential somaclonal variation on the overall salt tolerance of plants and the TGA content of tubers of potato cv. Desiree. Somaclonal variation has been considered as a useful source of desired plant characteristics. Somaclonal variation has been investigated in potato *in vitro* plants in a number of previous studies (Rietveld et al., 1991; Polgar et al., 1999; Bordallo et al., 2004). Environmental stresses such as salt, drought and temperature extremes are serious constraints to crop production. In addition to other available strategies, somaclonal variation offers an easy and cost effective means to develop crop varieties with desired traits such as salt tolerance and lower tuber glycoalkaloid content. In this study, we produced somaclonal lines of potato cv. Desiree. The somaclonal variation was confirmed by DNA based RAPD analysis. Molecular markers such as RAPDs have been in consistent use for genetic polymorphism detection in *in vitro* cultured explants and calli of potato and other plants (Soniya et al., 2001; Bordallo et al., 2004; Ehsanpour et al., 2007). We detected somaclonal variation by polymorphic bands with four RAPD primers. These twenty primers may not cover the whole potato genome. However, despite the low number of primers used in this study, somaclonal variation was detected with four primers. Our RAPD results are in complete agreement with the previously detected polymorphism in potato *in vitro* explants and calli, using low number of RAPD primers. Bordallo et al. (2004) used RAPD primers to detect somaclonal variation in potato calli, treated with different concentrations of plant growth regulators. They reported polymorphism based on the additional or missing bands in the pattern of DNA using OPD-01, OPS-11, OPB-07, OPB-08. Similar to our results, Ehsanpour et al. (2007) detected somaclonal variation in potato calli exposed to UV-C radiation. They used 28 different RAPD primers and detected polymorphic bands with 4 primers. In some other studies, somaclonal variation was detected with low number of RAPD primers in potato (Khatab and El-Banna, 2011; Munir et al., 2011; Afrasiab and Iqbal., 2012; Ahmad et al., 2013) and tobacco (Ghartavol et al., 2010). Somaclonal variation may also be a useful source of salt tolerance in regenerated plants. To investigate the occurrence of salt tolerance in the somaclona lines, we did *in vitro* screening of the parental control and the somaclonal lines under 100 mM NaCl concentration. Based on the root and shoot data, some somaclonal lines, which performed better, were selected for greenhouse tests. Salt tolerance of parental and somaclonal lines was evaluated under greenhouse conditions. A few somclonal lines i.e. TC6 and TC34 exhibited enhanced salt stress tolerance than the parental control. This was evident from the high chlorophyll content that these lines maintained throughout their growth under all salt stress conditions. In contrast, the parental control line showed reduced chlorophyll content and high salt stress further caused reduction that ultimately led to early leaf senescence and wilting of plants. These results are consistent with those reported in the literature. Fidalgo et al. (2004) reported negative effects of salt stress on relative water content, leaf stomatal conductance and transpiration rate of the cultivar Desiree. These effects tend to bring changes at the ultrastructural level that ultimately affect chloroplast structure, resulting loss of chlorophyll and reduced photosynthesis. Similar effects of salt stress have been observed in several studies (Bruns and Hechtbuchholz, 1990; Ghosh et al., 2001; Fidalgo et al., 2004). In this study the effect of salt stress on tuber glycoalkaloid content was determined in parental control and the somaclonal lines. Under non saline condition, the parental control tubers accumulated TGA up to 65 mg $(100\ g)^{-1}$ dw. In most of the cultivated potato varieties, the glycoalkaloid content ranged from 20-60 mg $(100\ g)^{-1}$ dw, (Peksa et al., 2002; Bianco et al., 2003). In the present study, the slightly high glycoalkaloid content under non saline condition may be explained by two factors. First, the glycoalkaloid content was determined in the peel part. Peel contains the highest TGA concentration compared to flesh (Maga, 1994; Kozukue et al., 1999; Sotelo and Serrano, 2000). Second, tubers for TGA analysis were grown in small 10 cm pots and therefore had relatively smaller sizes. Small size

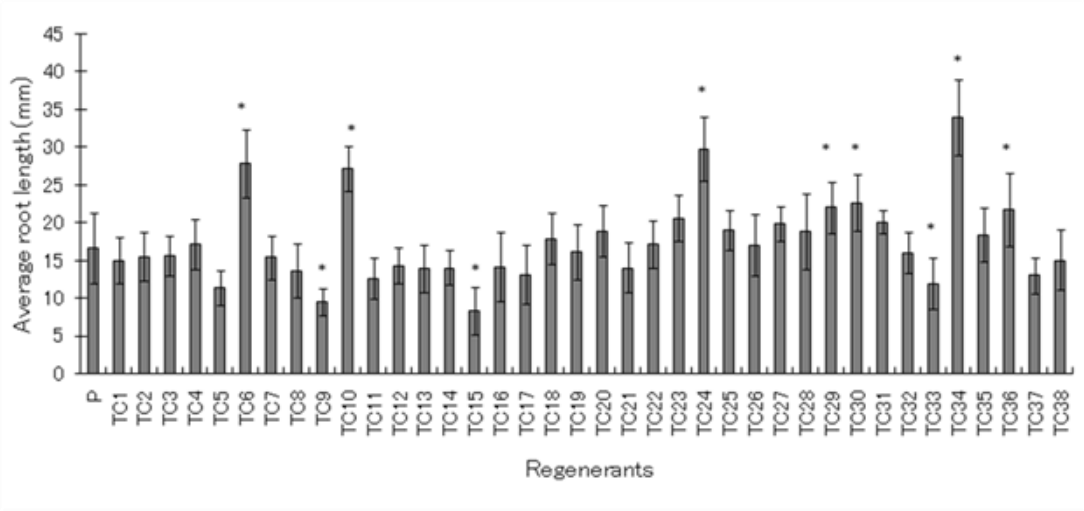

Fig 3. Average root length of *in vitro* plants of parental and tissue culture derived regenerants of potato cv. (Desiree) subjected to NaCl stress (100 mM). Bars represent means ±SD (n=8). Statistical significance is indicated by asterisks when *p*-value was $p \leq 0.05$.

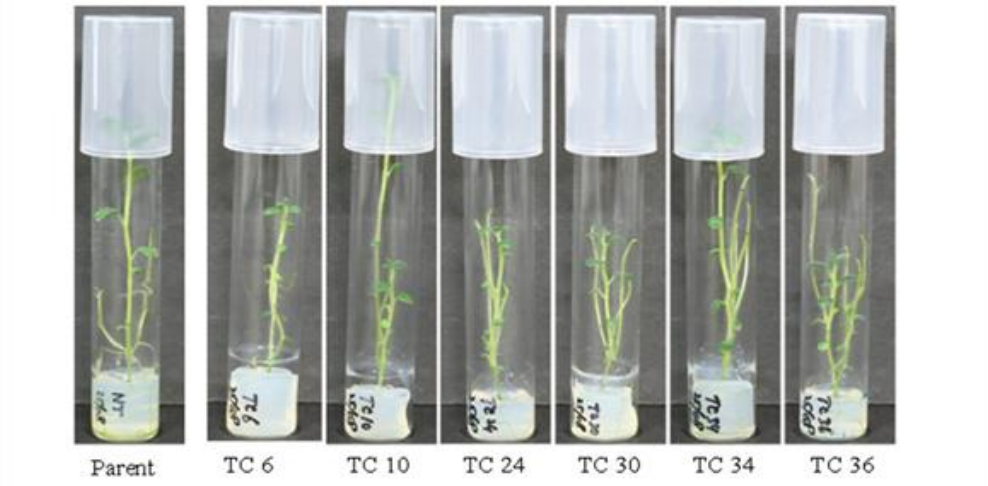

Fig 4. These somaclonal lines showed improved salt stress tolerance at 100 mM NaCl compared to parental line.

tubers have increased surface area/volume ratio and thus contain high TGA content (Esposito et al., 2002). On the contrary, tubers of somaclona lines TC6 and TC34 contained very low TGA content under non saline condition. Both these lines accumulated TGA content ranging from 35-45 mg (100 g)$^{-1}$ dw, significantly lower than that of parental control. The reason of lower TGA content in somaclonal lines under non stress condition might be due to the effect of somaclonal variation. Even under non saline condition, plants may experience some stress effects due to minor changes in temperature, light and moisture content. Under such conditions, the potential somaclonal variation may confer stress tolerance by alleviating the negative effects on plant growth and tuber yield. The high chlorophyll content in somaclonal lines under non saline condition reflects the same stress tolerance. The effect of high salt stress (100 mM NaCl) on TGA in parental control tubers was significant. TGA reached about 180 mg (100 g)$^{-1}$ dw, almost triple the content accumulated under non saline condition. However, the high salt stress had comparatively small effect on some somaclonal lines such as TC6, TC30 and TC34. These lines accumulated TGA in the range of 90-140 mg (100 g)$^{-1}$ dw. Although limited work is available that could explain the effect of salt stress on potato TGA content, our results are consistent with those found by Ahmad and Abdullah (1979). They conducted screening of seven potato varieties for salt tolerance under 0.2 to 1.5% salt and identified cvs Patrones, Norland and Red Lasoda as salt tolerant. They also reported a decrease in the glycoalkaloid content. In the present study, some of the somaclonal lines also exhibited enhanced salt stress tolerance as measured by the high chlorophyll content that suppressed the negative effects of salt stress on tuber quality by decreasing TGA content. Apart from this, in several studies, the effect of drought and heat stress was investigated on the tuber TGA content. Bejarano et al. (2000) determined the effect of drought stress on α-solanine, α-chaconine and TGA content of three drought tolerant and three susceptible potato varieties. A significant increase in TGA concentration was observed under drought stress in most varieties. Drought tolerant varieties

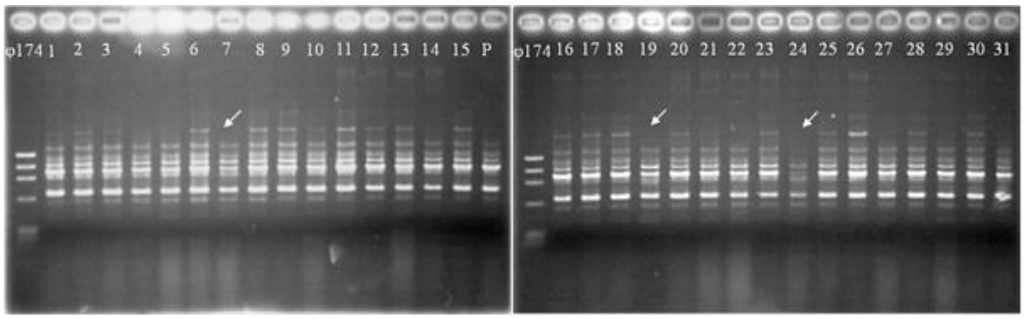

Fig 5. DNA pattern of parental control and somaclonal lines after amplification with RAPD primer OPAA-03. The arrows indicate missing bands in the respective lines.

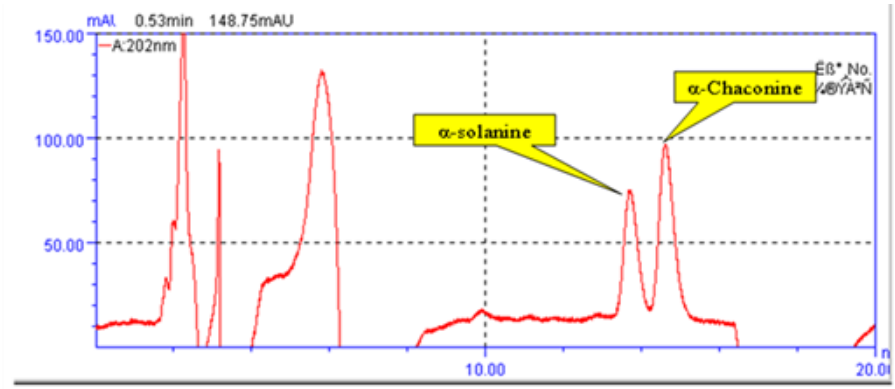

Fig 6. HPLC chromatogram of standards α-solanine and α-chaconine. Column: Inertsil CN-3, φ 0.46×250 mm, GL Sciences, Flow rate: 1.0 ml/min, Gradient: 0-20 min, 50% acetonitrile (in 0.01 M Tris-HCl pH 7.8 buffer).

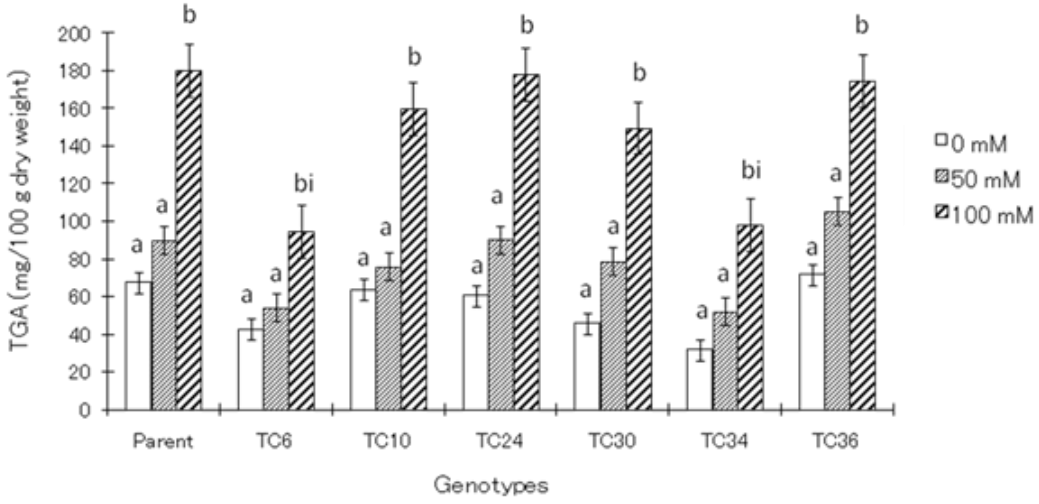

Fig 7. Total glycoalkaloids analysed in control and somaclonal lines of potato cv. Desiree subjected to 0, 50, and 100 mM NaCl concentrations. Bars represent means ± SD (n=3). For each salt concentration, the same letters are not significantly ($P \leq 0.05$) different from each other.

showed comparatively less increase in TGA content than susceptible varieties. They also observed that the potato cv. Desiree was the most affected by drought with an increase of 75% TGA content. In some other studies, the drought and heat stress caused increase in TGA content (Coria et al., 1998; Papathanasiou et al., 1999 ; Andre et al., 2009). At the molecular level, the abiotic stress responsive genes are mostly overlapping and they work in multiple stress-response pathways (Shinozaki and Shinozaki, 2007). The combined effect of heat, drought and salt stress on plant growth and production is nearly same because all these stresses impose osmotic and oxidative stresses. Therefore, it is possible that like the drought and heat stresses, salt stress may also have negative effects on tuber TGA content. Our results are in general agreement with results found in the above mentioned studies.

The exact mechanism of TGA increase under salt stress is unknown. Salt stress imposes oxidative stress on plants that in turn generate reactive oxygen species (ROS). Also salt stress affects plants ability to up take nutrients from the soil and their supply to photosynthetically active parts. Excessive sodium ions in saline water cause sodium carbonate formation that raises the pH. These alkaline conditions restrict availability of essential nutrients to the plants (Levy and Veilleux, 2007). Richardson et al. (2001) observed that in soils, rich with calcium carbonate, the process of nutrient uptake by plants is restored. Abdullah and Ahmad (1982) investigated the effect of gypsum application to saline soil on tuber yield and glycoalkaloid content. They found that application of 2% gypsum to saline soil improved tuber yield and increased protein, potassium and calcium content and decreased glycoalkaloid content. It reveals that tuber glycoalkaloid content is associated with better tuber yield and nutrient uptake. In the present study, the somaclonal lines TC6 and TC34 accumulated lower TGA content under high salt stress. The lower TGA content might be due to the efficient mitigation of salt stress effects by up-regulation of several protective mechanisms induced by *in vitro* culturing and the resultant somaclonal variation. The improved response to salinity stress and lower TGA content under high salt concentration in somaclonal lines TC6 and TC34 might be due to the improved biochemical and physiological mechanisms such as ROS scavenging, nutrient uptake from the soil and efficient photosynthesis.

Materials and Methods

Plant materials

Potato tubers cv. Desiree were grown in soil pots under greenhouse condition. After attaining a suitable vegetative growth (three weeks after sowing), individual shoots with nodes were excised. The stem parts with nodes were cultured in MS media in tissue culture tubes. The cultured *in vitro* plants were then multiplied by re-culturing from time to time. The intermodal parts of the *in vitro* plants were then used for callus induction and regeneration.

Callus formation and shoot regeneration

In vitro potato stem explants were cultured on MS media, supplied with auxin (3mM IAA) and three different concentrations of cytokinin (1mM, 3mM and 5mM zeatin riboside) (ten plates/zeatin riboside concentration). Approximately 6-8 explants were placed on each plate with MS media. The purpose of using three different concentrations of cytokinin was to check the optimal concentration best suited for callus formation and shoot regeneration. Explants on MS media containing all three concentrations of zeatin riboside developed calli. However, only on MS media containing 5 mM zeatin riboside, explants developed better shooting. Therefore, this concentration was used for shoot regeneration from stem explants in future experiments. Explants were cultured on around 30 plates with MS media containing 5 mM zeatin riboside. Explants were shifted to fresh media after every two weeks. After four weeks of culturing, explants started callus formation. On each plate, explants produced 4-6 calli. After eight weeks, shoot formation started from calli. Upon attaining a suitable growth (two weeks after shoot emergence), shoots were excised and cultured on hormone free MS media in tissue culture tubes. The frequency of somaclonal variation in regenerated shoots increases with longer time for callus induction. Therefore, we took regenerated shoots on two different time intervals. At first, some 23-25 individual shoots were taken from calli after twelve weeks of culturing on regeneration media. The remaining calli were kept on regeneration media for another four weeks and second batch of shoots were taken after almost sixteen weeks on regeneration media. While in test tubes, the plantlets were divided into three groups depending upon their root and shoots development. Plantlets in group I and II showed normal growth; however group I plants were more vigorous and healthy. Group III plantlets showed abnormal and stunted growth and therefore, these plantlets were discarded. Around 38 regenerated plantlets from group I were tested for salt stress tolerance under in vitro condition in the test tubes.

DNA extraction and RAPD analysis

DNA was extracted from leaves using the CTAB method (Doyle and Doyle, 1990). For DNA extraction, approximately 1 g fresh leaves from 38 regenerants were ground in liquid nitrogen and then extracted using CTAB method. PCR reactions were carried out in a total volume of 20 ul at a final concentration of 1 mM $MgCl_2$, 2 mM dNTP, *Taq* DNA polymerase enzyme (1u/20 µ), with approximately 100 ng DNA as a template and a single random primer (0.2 mM). Conditions were 94 ℃ for 2 min, one cycle, 94 ℃ for 15 sec, 35℃ for 15 sec, 72 ℃ for 30 sec which were repeated in 40 cycles followed by 5 min-extension at 72 ℃. Then 9 µl of each PCR product was revealed on 1% agarose gel subjected to electrophoresis at 100 V for 40 minutes.

Random Primer Sequence

For detection of somaclonal variation, we used 20 randomly selected RAPD primers. Only the following primers were able to detect polymorphism in the somaclonal lines.
OPAA-01 (AGACGGCTCC)
OPAA-03 (TTAGCGCCCC)
OPAA-05 (GGCTTTAGCC)
OPA-08 (GTGACGTAGG)

Optimization of salt (NaCl) concentration for regenerants screening

For screening of regenerants at a specific salt concentration, we used *in vitro* plants of control (Desiree) and cultured them on MS media containing various salt concentrations such as 0, 25, 50, 75 and 100 mM NaCl. For every salt concentration, we used five *in vitro* plants. Data of average root number and average root length were taken 14 days after salt stress application. The experiment was repeated three times.

In vitro salinity test

The somaclonal lines and parental control were tested at the selected 100 mM salt concentration. The purpose of this experiment was to screen these lines for any variation for salt stress tolerance. Average root number, average root length and shoot color were taken during a period of two weeks from the date of culturing of shoots at 100 mM salt concentration.

Soil acclimatization of the selected somaclones in growth room

The *in vitro* plants of selected somaclonal and parental control lines were acclimatized to soil condition (Fig. 4) in the growth room for two weeks. Individual plants were further multiplied through their shoot cutting and planting in small chambers for three weeks. These small plantlets were then shifted to soil pots and were watered for another two weeks. These plants were then shifted to greenhouse for further salt stress evaluation and tuber glycoalkaloid analysis. In greenhouse experiment, individual plant pots of the selected somaclones and parental line were arranged in a randomized complete block design (RCBD) in three groups. One group of plants was watered with normal tap water. The other two groups were watered with 50 mM and 100 mM salt (NaCl) concentration. The salinity test started two weeks after the plants were shifted from growth room. The experiment was replicated three times.

Measurement of leaf chlorophyll content

Leaf chlorophyll content was measured for all plants growing under tap and saline water using a chlorophyll meter (Konica Minolta SPAD-502, Japan). Three SPAD readings were taken from three fully expanded leaves of upper, middle and lower canopy. Thus chlorophyll content of each plant represented an average of nine SPAD values per plant.

Extraction of glycoalkaloids from potato tubers

To determine whether somaclonal variation has any effect on tuber quality in terms of tuber glycoalkaloid content and salinity tolerance, we analysed total glycoalkaloid content. From four plants of each line under both normal and saline water, tubers of approximately same size, weight and quality were selected for glycoalkaloids extraction. For glycolkaloids extraction and analysis, we followed an HPLC method developed by Edwards and Cobb (1996) with some modifications. Tubers were rinsed with tape water and then peel and flesh were separated with a vegetable peeler. The peel diameter was approximately 1-3 mm in diameter. Peel and flesh were then cut into small pieces and were put into separate falcon tubes filled with liquid nitrogen. The frozen material was lyophilized in a freeze drier for 72 hours and stored at -80^{0}C until glycoalkaloid extraction. The freeze-dried material was ground to fine powder in pestle and mortar. Five hundred milligram of the powdered sample was mixed with 15 ml of the extraction buffer (0.02 M heptanesulfonic acid in 1% aqueous acetic acid (v/v) with 1 mg/mL sodium bisulfite). The extract was centrifuged at 5250 X g in a Beckman centrifuge (Avanti^{TM} HP-25, USA) at 4 ^{o}C for 16 minutes. After centrifugation the clear supernatant was taken in a separate tube. Samples were kept on ice throughout the procedure.

Sample purification

For extract purification, we used SPE Isolute C18 (EC) column (Biotage Japan) with 1 g sorbent weight. The column was activated with 6 ml methanol and equilibrated with 10 ml of extraction medium. After equilibration, 10 ml of the supernatant was applied and passed through the column with a speed of one drop per second. The column was washed with 5 ml acetonitrile: water (20:80 v/v), and the glycoalkaloids were finally eluted with 3 ml acetonitrile: water (80:20 v/v). For concentrating the glycoalkaloids in samples, eluents were evaporated in a nitrogen evaporator and the dried glycoalkaloids were dissolved in 500 ul elution buffer instead of 3 ml. A sample of 50µl from peel samples were injected onto the HPLC for analysis.

HPLC analysis

The purified samples were analyzed through an HPLC system with DP-8020 Pump, SD-8022 degasser, RI-8021 refractive index, PD-8020 photodiode detector (Tosoh, Tokyo, Japan). The mobile phase was acetonitrile-0.01M Tris-HCl buffer (40: 60 v/v) adjusted to pH 7.8 with HCl. The HPLC column, Inertsil CN-3 (GL Sciences) was used for analysis. The HPLC flow rate was maintained at 1.5 ml/min and the detector was set at 202 nm. Standards of α-solanine and α-chaconine (purity >99%, Sigma) were dissolved in methanol-0.5M HCl (60:40 v/v). The HPLC was set to run each sample for 25 min. Glycoalkaloids in the experimental samples were quantified based on standard curves of α-solanine and α-chaconine.

Statistical analysis

Analysis of variance (ANOVA) was used to determine the effect of salt stress on potato glycoalkaloids in somaclones and parental lines. In the greenhouse experiment, soil pots with six somaclonal lines and one parental line were arranged in an RCBD design with 5 replications/each line. Tukey's test was used for multiple comparisons at p-level ($P \leq 0.05$).

Conclusion

Agriculture production of important crop plants has been challenged by a number of constraints including abiotic stresses. A number of strategies are in practice to develop better varieties of crop plants with improved nutrient content and tolerant to environmental stresses. In addition to other advanced methods, *in vitro* induced somaclonal variation has been proven as an important tool to develop crop plants with desirable traits. Salt tolerance in potato and its impact on the overall tuber yield and quality has been an area of research for the last several decades. Somaclonal variation may prove a useful means to achieve both salt tolerance and better quality in terms of lower tuber glycoalkaloid content. This possibility has been successfully investigated in the present study. However, the study must be progressed with more in depth investigation into the molecular level of somaclonal variation and its impact on salt tolerance and the glycoalkaloid content. In addition, further large scale tests of these somaclones are required to fully investigate the desired traits under realistic field conditions.

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