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In Vitro Mutagenesis and Somaclonal Variation Assisted Salt Tolerance in 'Rough Lemon' (*Citrus jambhiri* Lush.)

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Summary

In vitro mutagenesis and somaclonal variation are the important tissue culture techniques for fruit crop improvement. We carried out a study to induce salt tolerance in a commercial citrus rootstock, 'Rough Lemon' (*Citrus jambhiri* Lush.) using these techniques. For *in vitro* mutation induction, physical mutagen (gamma rays) and chemical mutagens *viz.*, ethyl methane sulphonate (EMS) and methyl methane sulphonate (MMS) were used. For physical mutagenesis, 40 and 60 days old calli (DOC) were irradiated with gamma rays (0, 10, 20, 30, 40, 50 and 60 Gy). The chemical mutagenesis was carried out on 45 and 60 DOC by culturing them directly (control I) or prior to culturing, shaked in liquid medium without (control II) or with mutagens (EMS and MMS) treatment at different concentrations (0.1, 0.2, 0.3 and 0.4 %). Based on the survival and the regeneration potential, 10–20 and 20–30 Gy were observed to be the optimum mutagenic doses of gamma rays for 40 and 60 DOC, respectively. For chemical (EMS and MMS) mutagenesis, 0.1 % each was the most suitable dose for 45 DOC, whereas 60 DOC did not regenerate after mutagens treatment. Salt concentration of ≥ 25 mM (NaCl) was found to be optimum for *in vitro* salt screening of the somaclones and mutants. Four somaclones at 25 mM and one each at 50 and 100 mM were obtained after exposing 170 DOC to different concentrations of NaCl (0, 25, 50, 75 and 100 mM).

Key words. *Citrus* – rootstock – *in vitro* mutation breeding – tissue culture induced variations – salt tolerance

Introduction

'Rough Lemon' (*Citrus jambhiri* Lush.) is one of the commercial citrus rootstock. This is a deep rooted rootstock and is well adapted to the diverse agroclimatic conditions. This contributes to the production of large sized fruits but with poor quality *viz.*, thicker peel, low TSS, low TSS/acid ratio, low reducing sugars and low total sugars in most of the scion cultivars (Dhatt and Singh 1993). However, it ensures high yields in most of the scion cultivars and is resistant to several biotic stresses (Nafees et al. 2009) but at the same time, 'Rough Lemon' is also susceptible to soil salinity (Ferguson 2002). Soil salinity is a global problem as 22 % of the global arable area is saline (Fao 2004). With the phenomenal rise in temperature, the extent of salinization of soils is likely to be aggravated further, possibly by increased evapotranspiration and ultimately bringing up the salts from deeper layers. Thus, to establish citrus in saline areas, there is an urgent need to induce salt tolerance in this rootstock.

The genetic improvement of 'Rough Lemon' for salt tolerance through conventional breeding is hindered primarily due to high heterozygosity and nucellar embryony (Soost and Cameron 1975). Moreover, in conventional breeding, along with the desirable trait, some undesirable traits also get fixed in the hybrid progeny. Keeping these shortcomings in view, alternate means are required to improve salt tolerance in this elite rootstock. *In vitro* mutagenesis and somaclonal variation are the two important tissue culture based techniques that facilitate improvement in one or few traits of a genotype without disturbing rest of the genome (Predieri 2001). The genetic improvement through these techniques requires an efficient tissue culture based regeneration system and preliminary information on the threshold doses of the mutagens and selective agent against which tolerance is desired. We have developed a reliable regeneration system based on callus cultures of this rootstock (Kumar 2009).

In mutagenesis, mutagens *viz.*, physical (gamma rays) and chemicals such as ethyl methane sulphonate (EMS) and methyl methane sulphonate (MMS) are most frequently used (Jain 2005). These mutagens have different mechanisms of action. Use of all these mutagens can give rise to an array of mutants that can be screened against salt stress. Therefore, the present study was directed to standardize the optimum (LD_{50}) doses of the mutagens (gamma rays, EMS and MMS) and selective agent (NaCl) and use them along with somaclonal variation in order to produce salt tolerant 'Rough Lemon'.

Materials and Methods

Plant material

The study was carried out in the Tissue Culture Laboratory of Department of Horticulture, Punjab Agricultural University, Ludhiana, Punjab, India. Callus for *in vitro* mutagenesis and somaclonal variation was induced from epicotyl segments. Callus was induced on MS medium supplemented with growth regulators *viz.*, NAA (10.0 mg l^{-1}), BA (1.0 mg l^{-1}), kinetin (0.5 mg l^{-1}) and carbohydrates in combination of sucrose (6 %) + maltose (2 %). Shoot regeneration from the epicotylar callus was performed on MS medium supplemented with NAA (0.5 mg l^{-1}), BA (3.0 mg l^{-1}), kinetin (0.5 mg l^{-1}) and sucrose (3 %) (KUMAR 2009).

In vitro mutagenesis

For mutation induction, physical (gamma rays) and chemical (EMS and MMS) mutagens were used. The 40 and 60 days old calli (DOC) were irradiated with ^{60}CO gamma rays source (3.45 KGy h^{-1}) at different doses (0, 10, 20, 30, 40, 50 and 60 Gy) at Nuclear Research Laboratory, IARI, New Delhi, India. For chemical mutagen treatments, 45 and 60 DOC were cultured directly on the regeneration medium (control I) or prior to culturing, shaked in liquid medium without (control II) or with mutagens (EMS and MMS) at different concentrations (0.1, 0.2, 0.3 and 0.4 %). Control II was used to differentiate the effect of liquid medium shaking from mutagen. The calli were shaken on a rotary shaker (70 rpm) at 25±2°C under dark conditions for 3 h. The calli after treatment with mutagens were washed with sterile distilled water to remove excess chemical. The criteria of 50 % reduction in survival and regeneration relative to control were used for the estimation of optimum mutagenic doses. The calli turning brown from green after culturing were considered as dead.

Establishment of threshold dose of NaCl

For *in vitro* screening of mutants and somaclones against salt stress, NaCl was used as selective agent. The threshold dose of NaCl was established by culturing 40 DOC on regeneration medium containing different concentrations of NaCl (0, 25, 50, 75, and 100 mM). The concentration, beyond which regeneration terminated, was considered as the threshold dose.

Selection of mutants and somaclonal variants

The calli treated with LD_{50} dose of the mutagen were subjected to the threshold dose of NaCl in the regeneration medium. For obtaining somaclones tolerant to salt, 170 DOC were regenerated on medium containing different concentrations of NaCl (0, 25, 50, 75 and 100 mM).

Observations

Thirty calli were used for each treatment and the treatments were replicated thrice. The data on survival and regeneration of the calli were recorded after 30 and 60 d of culturing, respectively.

Statistics

The data were analyzed using CPCS I software (Punjab Agricultural University, Ludhiana, Punjab, India).

Results

Estimation of LD_{50} dose of gamma rays

The data on the impact of gamma rays on survival and regeneration percentage in 40 and 60 DOC are presented in Table 1. The irradiated calli turned brown in colour from green, the frequency of which was influenced by the age of calli and the dose of gamma rays (Table 1). The

Table 1. Effect of gamma rays on the survival, regeneration and number of shoots per callus in 40 and 60 days old calli[1)] (DOC) of 'Rough Lemon'.

Dose (Gy)	40 DOC			60 DOC		
	Survival (%)	Regeneration[2)] (%)	Number of shoots per callus (range)	Survival (%)	Regeneration[2)] (%)	Number of shoots per callus (range)
0	100.00 (89.96)[3)]	57.14 (49.08)	8–14	100.00 (89.96)	51.84 (46.04)	6–10
10	61.00 (51.33)	30.00 (33.16)	2–3	70.00 (56.76)	30.00 (32.56)	3–4
20	39.00 (38.62)	12.50 (20.65)	1	66.66 (54.71)	21.42 (27.55)	3
30	31.00 (33.81)	0.00 (0.00)	–	40.00 (39.21)	10.00 (18.41)	1
40	25.00 (29.98)	0.00 (0.00)	–	38.00 (38.04)	0.00 (0.00)	–
50	18.00 (25.09)	0.00 (0.00)	–	33.33 (35.24)	0.00 (0.00)	–
60	0.00 (0.00)	0.00 (0.00)	–	20.83 (27.13)	0.00 (0.00)	–
CD	(1.45**)	(1.03**)		(2.05**)	(1.29**)	

[1)]Calli after treatment were cultured on regeneration medium. **Significant at P = 0.05.
[2)]Regeneration out of surviving calli
[3)]Figures in parenthesis indicate arc sine transformed values. CD = critical difference.

unirradiated calli on the other hand retained their green colour. The survival percentage of 40 DOC was reduced significantly with increase in the dose of gamma rays and none of the irradiated calli survived at 60 Gy.

Likewise survival, regeneration from the calli also decreased significantly with increase in the dose of gamma rays. The number of shoots/callus also decreased with the irradiation treatment. Maximum regeneration (57.14 %) was obtained from unirradiated calli (control) and no regeneration occured beyond the gamma rays dose of 20 Gy. Based on the survival and regeneration pattern, the mean lethal dose (LD_{50}) for 40 DOC was considered between 10 and 20 Gy (Table 1).

Like 40 DOC, the survival percentage in 60 DOC also decreased with gamma rays treatment in a dose dependent manner. However, in contrast to 40 DOC, they exhibited survival even at 60 Gy gamma rays. Regeneration from the surviving calli with respect to control was reduced significantly following the gamma irradiation (Fig. 1 A,B). Similar to 40 DOC, after gamma irradiation, the number of shoots/ callus also decreased in 60 DOC in a dose dependent manner. The calli irradiated with a dose more than 30 Gy gamma rays did not regenerate. Based on the survival and regeneration pattern, the mean lethal dose (LD_{50}) was considered between 20 and 30 Gy.

Determination of LD_{50} doses of EMS and MMS

The data on the effect of EMS and MMS on survival and regeneration in 45 and 60 DOC for the estimation of optimum mutagenic dose is given in Table 2. The survival of calli was influenced by the shaking treatment and the concentration of EMS (Table 2). No mortality was observed in the calli cultured directly on the regeneration medium (control I). The survival and regeneration of both 45 and 60 DOC declined significantly following liquid medium shaking. The number of shoots/ callus also decreased with liquid medium shaking (Table 2). This indicated the detrimental effect of liquid medium shaking on the callus. Therefore, instead of control I, the control II was used as check, relative to which the LD_{50} doses of the mutagens were determined. The addition of EMS in the liquid medium further reduced the survival of the 45 DOC in a concentration dependent manner and none of the treated calli survived at 0.4 %. As for survival, the regeneration in the control II calli of 45 d age was also significantly lower than control I calli (Table 2). Among the various EMS treatments, only 0.1 % EMS treated calli showed some regeneration (5.6 %), which was significantly lower than control II. On the basis of callus survival relative to control II, the optimum mutagenic dose (LD_{50}) was observed to be between 0.3 and 0.4 %. However, at the concentration higher than 0.1 %, the EMS treated calli did not regenerate (Fig. 1C).

For 60 DOC, significant differences for survival among various concentrations of EMS were observed. In contrast to 45 DOC, however, approximately 21 % of the 60 DOC survived even at 0.4 % EMS treatment. The control II and EMS treated calli of 60 d age did not regenerate (data not shown).

The data in Table 2 depicts the behaviour of 45 and 60 DOC for survival and regeneration after treatment with MMS. The control II was used as check, where 65 % of the 45 DOC survived. The survival of the calli decreased significantly with increase in MMS concentration. At 0.4 % MMS, none of the treated calli survived. For regeneration in the surviving calli of 45 d age, there was no significant difference between control II and 0.1 % MMS treatment (Fig. 1D). The calli treated with MMS at a concentration higher than 0.1 % did not regenerate.

The survival of 60 DOC decreased significantly after MMS treatment, but 11 % of the calli survived even after treatment with 0.4 % MMS. Like EMS, control II and MMS treated calli of 60 d age also did not regenerate (data not shown).

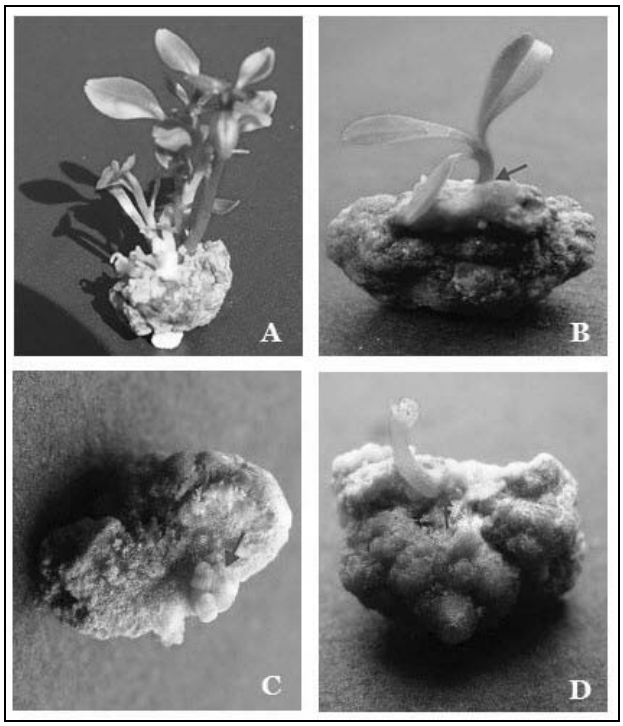


Fig. 1. Regeneration in control and mutagens treated calli of 'Rough Lemon' (*Citrus jambhiri* Lush.). Panel A, regeneration in 60 days old calli (DOC) (control). Panel B, gamma (20 Gy) irradiated 60 DOC. Panel C, 0.1 % EMS treated 45 DOC. Panel D, 0.1 % MMS treated 45 DOC.

Establishment of threshold dose of NaCl

In the control medium devoid of NaCl, 52.63 % shoot regeneration was obtained from the calli and the percentage of differentiating calli was reduced by 10 fold in the presence of 25 mM NaCl (Fig. 2). Further increase in the level of NaCl in the regeneration medium completely inhibited the regeneration.

Survival and regeneration from the mutated calli in salt containing medium

The 45 DOC after treatment with chemical mutagens (EMS and MMS at 0.1 %) and 60 DOC after gamma irradiation (20 and 30 Gy) were cultured in the regeneration medium containing 25 mM NaCl. The MMS treated or gamma irradiated calli did not survive in the presence of salt. In contrast, 10.2 % of EMS treated calli survived in this medium (data not shown). However, the surviving

Table 2. Effect of ethyl methane sulphonate (EMS) and methyl methane sulphonate (MMS) on survival, regeneration and number of shoots per callus in 'Rough Lemon' calli of 45 and 60 days age.

	45 DOC			60 DOC[1)]
EMS (%)	Survival (%)	Regeneration[2)] (%)	Number of shoots per callus (range)	Survival (%)
Control I (0.0)	100.00 (89.96)[3)]	54.45 (47.53)	8–14	100.00 (89.96)
Control II (0.0)	65.00 (53.71)	9.00 (17.43)	2–3	87.00 (68.87)
0.1	62.66 (52.31)	5.60 (13.68)	1	69.00 (56.15)
0.2	33.00 (35.04)	0.00 (0.00)	–	52.00 (46.12)
0.3	24.70 (29.08)	0.00 (0.00)	–	33.33 (35.24)
0.4	0.00 (0.00)	0.00 (0.00)	–	11.00 (19.35)
CD	(1.52**)	(0.73**)		(1.98**)
MMS (%)				
Control I (0.0)	100.00 (89.96)	54.45 (47.53)	8–14	100.00 (89.96)
Control II (0.0)	65.00 (53.71)	9.00 (17.43)	2–3	87.00 (71.01)
0.1	34.30 (35.83)	7.00 (14.76)	1	79.37 (63.02)
0.2	12.03 (20.28)	0.00 (0.00)	–	63.72 (52.95)
0.3	3.26 (10.27)	0.00 (0.00)	–	35.33 (36.42)
0.4	0.00 (0.00)	0.00 (0.00)	–	11.00 (18.68)
CD	(1.75**)	(3.96**)	–	(2.63**)

[1)]The surviving 60 d old control II and EMS treated calli did not regenerate. **Significant at P = 0.05.
[2)]Regeneration out of surviving calli
[3)]Figures in parenthesis indicate arc sine transformed values. CD = critical difference.

calli did not form shoots in the NaCl free regeneration medium even after 90 d of culturing.

Selection of somaclones tolerant to NaCl

After first subculture, no mortality occurred in the calli cultured on control and medium containing 25 mM NaCl (Table 3). The minimum callus survival (57.14 %) was obtained at 100 mM NaCl. After the 2nd subculture, the percentage of surviving calli was reduced to 66.67 % in medium containing 25 mM NaCl.

In control medium, 9 out of 22 cultured calli regenerated, while in 25 mM NaCl treatment, 4 out of the 16 calli regenerated. One somaclone each was also obtained in the medium supplemented with 50 and 100 mM NaCl. The number of shoots/ callus ranged from 4 to 5 in control to 1 in 50 and 100 mM NaCl treatments. These somaclones had stunted growth compared with control and are being maintained on MS medium devoid of growth regulators for further development.

Discussion

Optimum dose of gamma rays

In vitro mutation breeding for salt tolerance in 'Rough Lemon' requires the prior information on optimum doses of mutagens and salt. Physical mutagen (gamma rays) and chemical mutagens *viz.*, ethyl methane sulphonate (EMS) and methyl methane sulphonate (MMS) were employed in this study. The optimum dose (mean lethal dose, LD_{50}) was determined based on callus survival and regeneration.

The survival, regeneration of calli and number of shoots/callus decreased progressively with increase in the dose of the gamma rays, which could be due to the ultra structural damages caused by the radiation to the cells (Hu et al. 1993). Gamma rays are the widely used physical mutagen for crop improvement. They generally affect more than one trait but point mutations are also produced, though in low frequency. They alter more than one trait by inducing nicks in DNA and mutants are formed due to deletion, duplication or inversions (Britt 1996). They liberate hydroxyl (OH^-) ions from the water molecules of the cell due to their ionization behaviour. The free OH^- ions, in turn, interact with nucleic acid to produce point mutations (Britt 1996). When the damage induced by gamma rays exceeds the repairing capability of the cells, it can affect cell division, differentiation or ultimately results into their death.

Based on callus survival and regeneration, the LD_{50} appeared to exist between 10 to 20 and 20 to 30 Gy for 40 and 60 DOC, respectively. It is advocated to use LD_{50} ±10 % dose of gamma rays for mutation induction (Heinze and Schmidt 1995). In that context, 10 and 20 Gy for 40 DOC and 20 and 30 Gy for 60 DOC can be used simultaneously for production of mutants.

The calli of 60 d age showed lesser sensitivity to gamma rays compared to 40 DOC. The age based sensitivity of calli to gamma rays has not been investigated in citrus and some other fruit crops. It was observed that the callus became more compact with age and this might have contributed in tolerating the detrimental/damaging effects

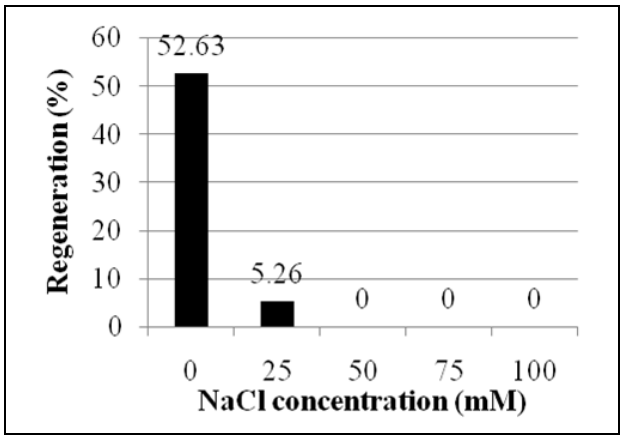


Fig. 2. Effect of different concentrations of NaCl on regeneration in 40 days old calli (DOC) of 'Rough Lemon'.

of gamma rays in a better manner. Tulmann Neto et al. (1994) reported 120 Gy as suitable dose of gamma rays for mutation induction in embryogenic calli of sweet orange cv. 'Pera'. The optimum dose of gamma rays for embryogenic calli reported by them was much higher than the dose obtained in this study, which could be due to genotype difference (Fuentes et al. 2004).

In this study, 60 DOC had a comparative edge over 40 DOC as they showed less mortality and regeneration was obtained at the dose up to 30 Gy. Moreover, when the older calli are irradiated with gamma rays, the somaclonal variation is added to the mutagen effect, which, in turn, can increase the range of variability. Thus, it can be concluded that 60 DOC are more useful for gamma rays induced mutations than 40 DOC.

Optimum dose of EMS and MMS

Our results with chemical mutagens showed that not only mutagen but liquid medium shaking also reduced the survival and regeneration of the calli. During liquid medium shaking, disintegration of the surface cells of the calli takes place. The surface cells are predominantly of parenchymatous type, which differentiate into shoot buds (Grinblat 1972). Thus, loss of these cells contributes to the reduced survival and regeneration of the control II calli. The chemical mutagens mediated reduced survival and regeneration of the calli could be linked to the production of toxic products (Sedgewick and Vaughan 1991). The alkyalating agents induce mutation by adding either methyl or ethyl group to one of the four DNA bases, leading to the altered base formation. Among these bases, beside the mutagenic bases, toxic bases (3-alkyl adenine) are also produced. When such toxic bases are inserted in template DNA, DNA replication is blocked (Britt 1996) and therefore, suppress cell division and differentiation.

The survival percentage following chemical mutagens treatment was more in control II calli of 60 d age as compared to 45 DOC. It could be ascribed to their higher degree of compactness due to which, they might have absorbed less amount of mutagen. In the present investigation, 50 % callus survival relative to control was noticed in the range of 0.3–0.4 and 0.1–0.2 % for EMS and MMS, respectively. Wan et al. (1991) also recommended the use of EMS at same concentration for mutagenesis in callus cultures of citrus. But, for the production of mutants, regeneration from the mutated calli is of utmost importance. In this study, only the 45 DOC regenerated after treatment with the chemical mutagens (EMS/MMS). At concentration more than 0.1 %, these completely inhibited the callus regeneration. Therefore, the 45 DOC can be treated with 0.1 % EMS/MMS for mutation induction.

Optimum dose of salt (NaCl) for screening of calli

The addition of salt (NaCl) in the medium reduced the regeneration of calli and at amount more than 25 mM, regeneration was completely terminated. Thus, NaCl at the concentration of ≥ 25 mM can be used for *in vitro* salt screening of somaclones and mutants. The cellular membrane is damaged under salt stress leading to the loss of cellular integrity (Ferreira and Lima Costa 2006). The damaged cells remain no more potent for regeneration.

Fate of mutagen treated calli in salt containing medium

The calli after treatment with optimum doses of mutagens were cultured in salt containing regeneration medium. The treated calli either did not survive in this medium (in case of MMS and gamma rays) or could not regenerate (in case of EMS). Based on callus survival in salt containing medium, out of the two chemical mutagens (MMS and EMS), MMS appeared to be more toxic. It

Table 3. Selection of 'Rough Lemon' somaclonal variants on regeneration medium containing salt (NaCl).

NaCl (mM)	Total number of cultured calli	Survival (%) after first subculture	Survival (%) after second subculture	Regeneration out of the surviving calli (in numbers)	Number of shoots per callus (range)
Control					
0	22	100.00 (22)[1)]	100.00 (22)	9	4–5
25	24	100.00 (24)	66.67 (16)	4	2–3
50	24	83.33 (20)	58.33 (14)	1	1
75	28	60.42 (20)	57.14 (16)	–	–
100	35	57.14 (20)	48.57 (17)	1	1

[1)]Values in parenthesis indicate the number of cultured calli of 170 d age.

could be attributed to its higher potential for inducing chromosomal breaks than EMS (NATARAJAN 2005). When the mutagen treated calli are subjected to salt (NaCl), they are forced to face double stress of ions (Na^+ and Cl^-) and toxic effects of mutagens (LUAN et al. 2007). The survival and regeneration of the putative mutated calli is suppressed under such circumstances. Therefore, we suggest that the putative mutants should only be exposed to the salt stress after the regeneration of the callus on stress free medium.

Somaclonal variation for salt tolerance

After culturing 170 DOC in the medium containing NaCl, salt tolerant somaclones with tolerance in the range of 25–100 mM were obtained. As per previous findings, the threshold concentration of NaCl for 'Rough Lemon' rootstock is 350 mg l^{-1} (GRATTAN and OSTER 1992). Thus, the somaclones obtained in the present investigation are 4–16 fold more NaCl tolerant than the parent 'Rough Lemon'. Under NaCl stress, there is excessive production of free radicals which can cause cellular damage. The salt tolerance in the somaclones could be possibly due to higher inherent amount of antioxidant enzymes such as superoxide dismutase, catalase etc. and amino acids such as proline (FERREIRA and LIMA COSTA 2006). The antioxidant enzymes scavenge the free radicals while proline protects these osmoprotectant enzymes from salt stress and act as stored energy for cell differentiation.

Utilizing this technique, the salt tolerant cell lines in *C. sinensis* cv. 'Shamouti' (BEN HAYYIM and KOCHBA 1982), *C. limonum* (PIQUERAS and HELLIN 1992) and 'Troyer citrange' (EL YACOUBI et al. 2004) were obtained. BEN HAYYIM and KOCHBA (1982) reported sweet orange cv. 'Shamouti' somaclone that could tolerate 200 mM NaCl but in the present investigation, 'Rough Lemon' somaclones with NaCl tolerance in the range of 25–100 mM were obtained. Based on salinity tolerance data of rootstocks from Australia, GRATTAN and OSTER (1992) reported that 'Rough Lemon' is less tolerant to Cl^- salinity than sweet orange. Thus, the salt tolerant somaclones obtained in this study can be of great significance if the salt tolerance is genetically stable and is further expressed at the field level. This will ensure establishment of citrus industry in hitherto unexploited saline areas of the world.

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