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Investigations of *in vitro* Selection for Salt Tolerant Lines in Sour Orange (*Citrus aurantium* L.)

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Abstract: The present study was conducted to create new stable somaclonal variants of sour orange in citrus. Embryogenic calli of *Citrus aurantium* that used widely as a rootstock were successfully used *in vitro* selection for salt tolerance. Calli were cultured on basal MT medium containing three different concentrations of NaCl 100, 200 and 300 mmol. A great number of salt tolerant cell lines were isolated evaluating some morphological aspects of the callus material then, totally 67 plantlets were obtained from embryoids of these selected callus clusters from selective medium containing of 100 mmol NaCl. Further attempts should be made to support the level of salt tolerance through physiological and biochemical analysis.

Key words: *In vitro* selection, salt tolerance, tissue culture, citrus

INTRODUCTION

Environmental stresses from temperature extremes, drought, salinity generally cause to the results in decreased growth and yield in plants. Plants require essential mineral compounds for their growth and development and absorb them from external physical surroundings. Excessive mineral salts in the soil can have detrimental effects on plants due to their interference on plant metabolism and disturbance on plant water relations (Xiong and Zhu, 2002). Salt stress is one of the most serious factor limiting the productivity of crop plants in agriculture. Tissue culture techniques have been widely used for breeding purpose, especially in selection for stress tolerance such as salinity tolerance, cold tolerance. Due to the limitations of classical breeding of citrus, somaclonal variation has been used in several crops including citrus to generate genetic variability. Classical breeding is conducted by conventional crossing in various plants with breeders. Traditional breeding methods are not effective since a number of factors could potentially limit to rapid improvement within species. Citrus that is one of the most important agricultural commodities in the world, has crossing problem. However,

citrus varieties and its close relatives are known to possess to be important agricultural characters (i.e., pest and disease tolerance, tolerance of environmental stresses), there is some problems associated with reproductive biology for example nucellar embryony, sterility, heterozygosity in citrus varieties. Thus, this mentioned factors are the restrictions to citrus breeding using the classical crossing. Traditional breeding methods are also often constrained by the long a time consuming.

Plantlets were regenerated from a selected salt-tolerant cell line of Shamouti orange (*Citrus sinensis* L. Osbeck) then salt-tolerant calli derived from regenerated plantlets indicate acquisition of salt tolerance on the whole plant level (Ben-Hayyim and Yehudit, 1989). Beloualy and Bouharmont (1992) obtained salt-tolerant cell lines of citrus rootstock (*Poncirus trifoliata* cv. Pomeroy) by subculturing embryo-derived calli on media containing sublethal concentrations of NaCl (5 and 10 g L⁻¹), these shoots and plants regenerated from selected cell lines showed improved growth and salt tolerance.

Singh *et al.* (2002) used callus cultures from internodal stem segments of rough lemon, rangpur lime and trifoliolate orange to display for salt tolerance at callus

level and suggested for screening of large citrus germplasm population for salt tolerance via *in vitro* techniques.

Dang and Nuguyen (2003) reported that somaclonal variants for salt tolerance were obtained in rice cell culture in presence 1.5% of NaCl through *in vitro* selection. Likewise the research carried out by Noaman and Ahmad (2004) was useful for development of Alfalfa Tolerant to Salinity Stress Using Tissue Culture Technique. In their experiment, salinity stress in increasing of NaCl concentration in the media caused significant reduction in number of generated shoots from root. Shah *et al.* (2004) screened for salt tolerance clones using tissue culture techniques thus regenerated salt tolerant somaclones from tiller/plant of sugarcane transplanted in saline sodic soil after plantlets regenerated from one month old callus.

Patnaik and Debata (1997) obtained sodium chloride tolerant callus lines of *Cymbopogon martinii* (Roxb.) Wats by exposing the callus to increasing concentrations of NaCl (0-350 mM) in the MS medium. The selected lines of *Cymbopogon martinii* retained their salt tolerance after 3-4 subcultures on salt-free medium indicating the stability of the induced salt tolerance.

Nanakorn *et al.* (2003) regenerated into plants from the calli selected under saline conditions and indicated that the plantlets formed from selected tolerant calli have resisted higher levels of NaCl than plants regenerated from non-selected calli.

Genetic variability could be recovered in regenerated plants. So, somaclonal variation for direct recovery of novel genotypes from cell cultures is an important tool for supporting new varieties in improvement programs of citrus. Generally the salinity of the soil is very high in Çukurova plain and soil salinity of the plain is increasing due to irrigation with saline water, intensive agriculture systems, hot climate, water drainage. Therefore, we have to select the plants of suitable variants from cell lines adopted to extreme environmental conditions of the region. The ultimate goal of present research, it was conducted to create novel variants of Turkey in sour orange callus culture to select salt tolerance plants via *in vitro* selection.

MATERIALS AND METHODS

Establishment of callus cultures: The research was carried out in the Biotechnology-Tissue Culture Lab, Department of Plant Protection, University of Çukurova. Embryogenic calli of *Citrus aurantium* previously obtained was used in this study. The culture medium used was based on MT (Murashige and Tucker, 1969) medium, containing 5% sucrose, solidified with 1% agar (Difco).

The medium pH was adjusted to 5.8 prior to autoclaving at 15 psi for 20 min. Callus pieces (~ 50 mg) were plated on culture media containing three different NaCl concentrations as 100, 200 and 300 mmol and incubated in culture room at 25±1°C temperature, 16 h light (1000 lux)/8 h dark conditions. Each treatment was included 10 replicate of petri dishes containing ~50 calli pieces for each petri dishes. The controls were consisted of non-selective medium basal MT. Fresh weight of the calli was measured after 4-5 weeks then growth index of calli was calculated by the variance between, before and after subcultures in fresh weights of the callus pieces. Calli were transferred on the same concentration of NaCl for 8 passage during 8 selection months. At the end of selection, surviving sectors of callus carefully selected were transferred to the basal MT medium in absence of NaCl and maintained for 2 subcultures then in order to test the continuity of salt tolerance whole callus were cultured on the same media containing NaCl.

Establishment of cell suspension cultures: Cell suspensions were obtained by placing 100 mg of calli in 100 mL flasks containing 25 mL liquid basal MT medium on shaker at 125 rpm and subcultured 2 week intervals. Experiment was done in two different manner:

- **Cells on filter paper:** Cells in suspension containing liquid basal MT in addition to NaCl (100 mmol of NaCl or 200 mmol of NaCl or 300 mmol of NaCl) were incubated for a 15 days then drained cells were spread on filter paper placed to layer of solid MT with inclusion of same NaCl concentration and maintained for 3 months subculturing onto fresh medium every month
- **Cells embedded in agar:** Cell suspension was incorporated to basal MT containing of agar at 1:4 ratio to give 0.4% of final agar concentration in presence of NaCl (100 mmol of NaCl or 200 mmol of NaCl or 300 mmol of NaCl). Embedded cells in agar were poured in sterile petri dishes and incubated for 12 weeks. After callus formation, these cells were transferred onto solidified MT medium+same concentration of NaCl

Induction of somatic embryogenesis and plant regeneration: Selected calli masses were cultured to induce somatic embryogenesis on basal MT medium supplemented with different concentrations of glycerol (1, 2, 3, 4 and 5%) with and without salt. For root induction, normal plantlets developed from somatic embryos were transferred on MT medium containing 0.05 mg L⁻¹ NAA+2.5% sucrose.

RESULTS AND DISCUSSION

Reduction of cell growth on medium containing three different concentrations of NaCl was the apparent after 8 weeks (Fig. 1, 2), then cell growth was completely inhibited with exception a few petri dishes containing 100, 200 and 300 mmol of NaCl.

Developments on medium containing 100 mmol of NaCl: Some of calli colonies on 100 mmol of NaCl medium has relatively slow grown for 8 subcultures although growth of a greater number of colonies ceased. The callus masses that were grown on medium including 100 mmol of NaCl were transferred to the basal MT media without saline for two months. Due to the normal growth of callus, those callus were recultured on the same medium with saline plus one month to confirm stability of salt tolerance. The selected callus clusters, upon indicated normal growth as control after 4 weeks, maintained on MT basal medium without saline. Thus aspects of salt tolerance in a NaCl-selected cultures was stable.

Developments on medium containing 200 mmol of NaCl: The callus growth decreased markedly with increasing NaCl concentration in the medium and almost arrested growth of the several calli colonies on the medium including 200 mmol of NaCl at the end of 8th passages. While at seventh subculture while a few calli clump has maintained healthy and stable growth, nine spontaneous embryos from callus completely inhibited the growth were regenerated and a few numbers of these embryos produced callus at higher frequencies. These grown calli clump and spontaneous embryos were subcultured for two months onto medium MT basal without salt. Calli development was visually normal as control lines there upon the calli clumps were transferred to MT basal with saline and without saline. All calli lines were observed to develop to be similar to each other as control, persistence of salt tolerance after passages on salt free media may be as a consequence of salt adaptation. Spiegel-Roy and Thorpe (1986) has displayed the increased level of salt tolerance in the medium in *Citrus sinensis*.

Developments on medium containing 300 mmol of NaCl: At the end of ninth month, only 14 out of 200-250 calli colonies successfully survived was selected and this calli masses were transferred to MT basal medium in absence of the salt then maintained for 2 subcultures on to this medium. While colour of some callus turned from white-creamy to brown during the two subculture, several spontaneous somatic embryo formation was resulted in

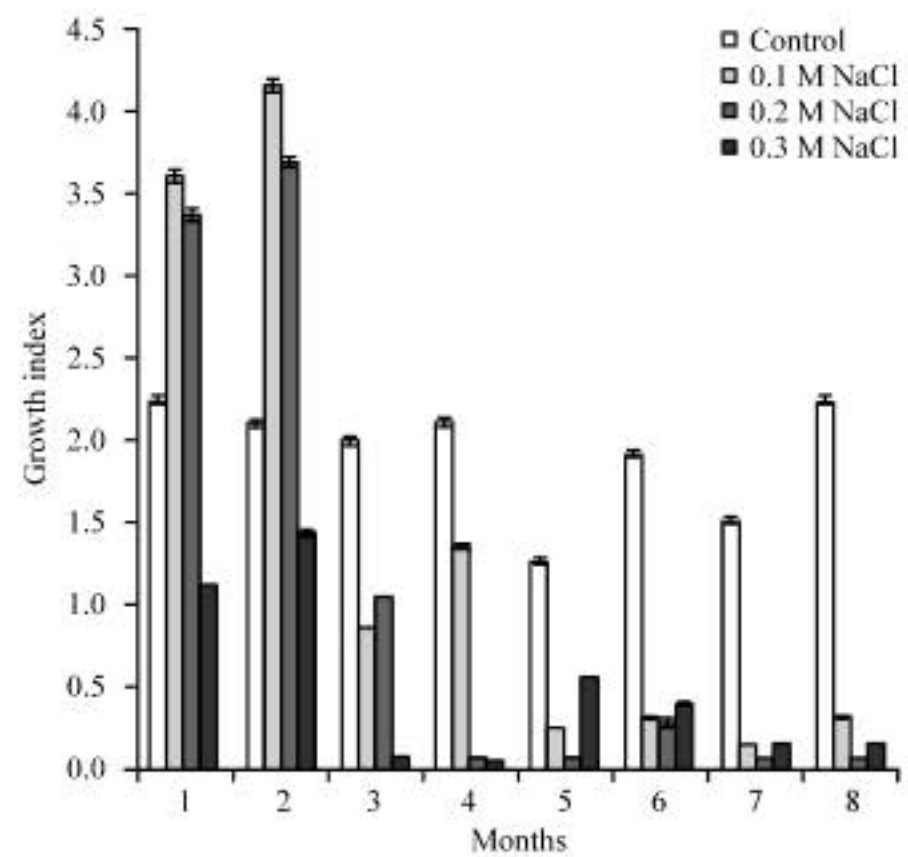


Fig. 1: Growth index of the callus on the medium modified with NaCl and without NaCl. Mean separation was performed with General Linear Models (GLM) procedure of SAS 6.12

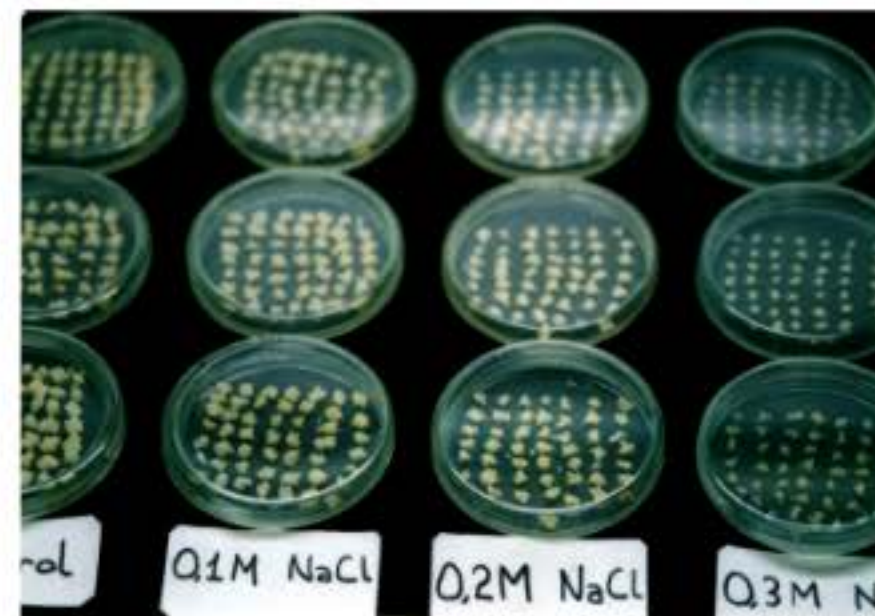


Fig. 2: Physiological aspects of callus at three different NaCl levels at the end of two months after first subculturing

grown cells of one colony. It may be evident that callus grown on media without NaCl altered colour as consequence of salinity adoption. Then those callus were resubjected to MT basal media supplemented with 300 mmol of NaCl. Upon a few clumps of callus was the browning in 15-20 days, calli were subcultured on MT basal media supplemented with or without NaCl. Regeneration of callus occurred on MT basal media without salt, colour of calli on media containing salt did not change but their development was slower. Also callus browning can be an indication of the production of stress response compounds such as phenolic compounds (Jinglan *et al.*, 2005). Biotic or abiotic factors may induce

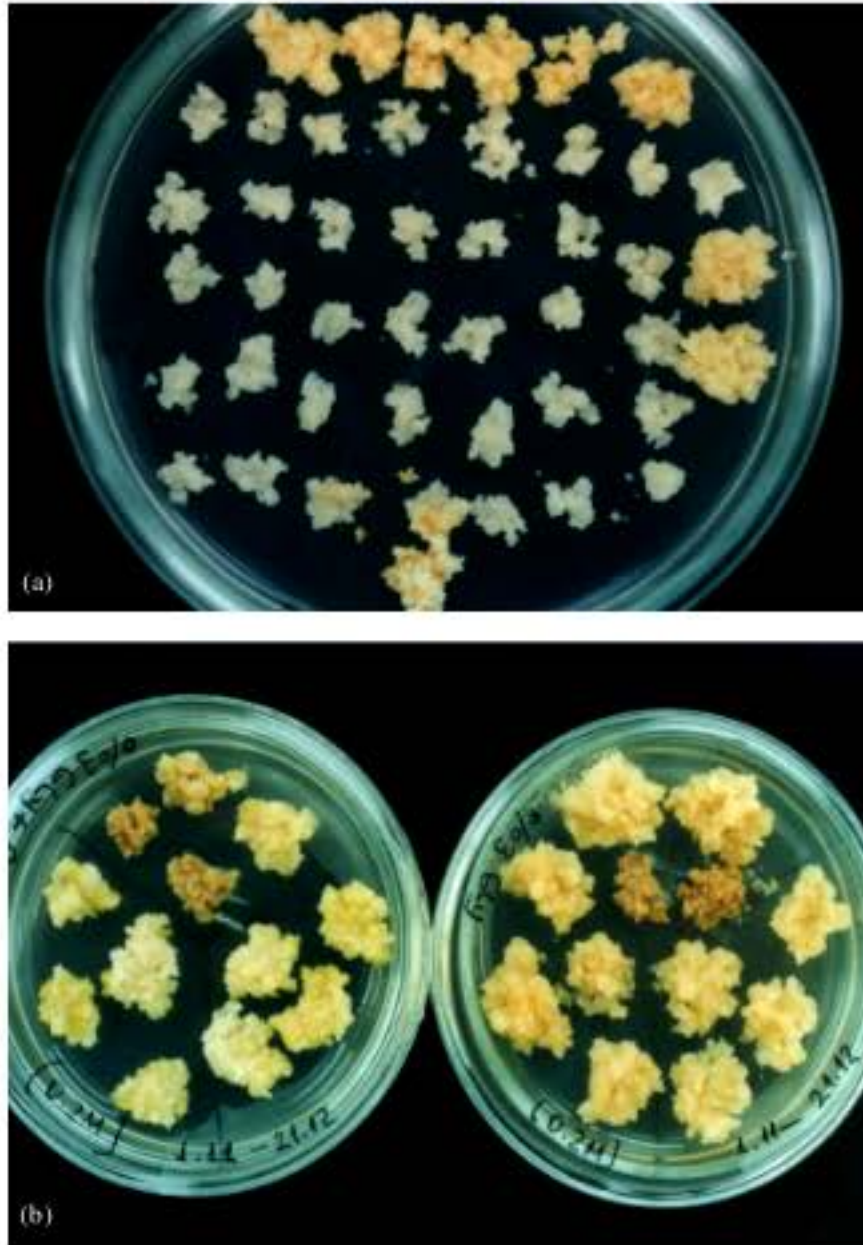


Fig. 3: (a) Selected calli and induction of embryogenesis. (b) The growth of salt-adapted calli in presence of 100 mmol NaCl (left), development of induced embryoids from selected callus on media including 100 mmol NaCl+3% glycerol

the synthesis and accumulation of wide range of phenolic compounds in plants (Dixon and Paiva, 1995). It can be a result that some toxic compounds such as secondary metabolites that may be induced to excess salinity stress in response can interfere with the physical and biochemical processes of the cells to cause coloration. Several spontaneous somatic embryo formed from one calli colony were separated from callus and transferred in the presence of containing basal MT+1500 mg L⁻¹ of Malt Extract (ME). Development performance of embryos on media containing ME was significantly higher. After plantlets formation from these embryos were achieved following maintenance in the same media during 3-4 subcultures, the shoots were transferred to rooting media consisted of basal MT+2.5% sucrose+0.05 mg L⁻¹ NAA.

The callus growth decreased markedly with increasing NaCl concentration in the medium. A prolonged exposure of callus to the salt environment led to discoloration and arrested growth in the majority of the calli. Citrus is one of the unusual woody plants to select for salinity resistance and to regenerate salt tolerant



Fig. 4: Four to eight week plantlets *in vitro* raised plantlets from selected calli of sour orange in the media supplemented with 300 mmol NaCl (Control on left)

plants. However, mechanism of salinity resistance including complex series of a biochemical cascades is still unclear, it may be hopeful possibility to reselect increased salt-resistance cells from valuable plant materials which have successfully obtained on 100 mmol of NaCl.

Mature spontaneous embryoids achieved from callus selected on 300 mmol of NaCl developed into complete plantlets, despite the loss of regeneration potential in callus (Fig. 3). Twenty four and 43 out of totally 67 plantlets, respectively, was cultured on medium containing salt and without salt (Fig. 4). However, differentiation of selected callus from medium incorporated with 200 mmol of NaCl for conversion into plantlets has been still continuing, embryogenesis induction of selected callus was poor on the medium including 300 mmol of NaCl either there was no somatic embryogenesis development or regenerated embryos was turned to brownish.

In presented study, citrus salt-tolerant callus yields salt-tolerant embryos. Regenerated plantlets derived from salt-tolerant calli may acquire of salt tolerance on the whole plant level. Ben-Hayyim and Yehudit (1989) reported that regenerants were selected from salt-tolerant cell line of Shamouti orange and expression of salt tolerance in the whole plant formed from salt tolerant embryos. Spiegel-Roy and Thorpe (1986) has displayed

the increased level of salt tolerance in the medium in *Citrus sinensis*. Also in citrus, damage caused by salinity is mostly due to toxic ion accumulation, since this salt-sensitive crop adjusts osmotically with high efficiency (Vicent *et al.*, 2005), in order to successfully understand salt tolerance in plants, the mechanisms at three different levels: cellular, tissue and the whole plant level must be studied individually (Epstein, 1980). Many of studies have suggested that salt tolerance in plants is a quantitative character (Foolad and Jones, 1993). Promising researches on the salt-stress signal transduction in plants will hopefully reveal direction of management action of salt tolerance in plants through comprehensive models of fundamentals of salt tolerance and relation among these cellular processes and the environment.

Cell suspension was tried in all concentration of NaCl used for callus. Preliminary experiments showed that callus cells indicated higher performance than cell culture selection for salt tolerance. However, regeneration works of plantlets is going, somatic embryos from callus selected in cell suspension cultures containing of NaCl were induced in presence of 4% glycerol+300 mmol NaCl during 3-5 months *in vitro* culture.

CONCLUSION

Due to highly sensitive nature of sour orange to salty environment, we obtained variants the gradual increased of salt tolerance. After these lines should be tested in soil and farmer fields, an essentially derived variety should be obtained individual plants from salt tolerants as the initial variety. We are hopeful, it may be possible to be valuable salt-tolerant plant materials that would be successfully integrated in a plant production scheme.

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