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Response of Sugarbeet Tissue Cultures to Salinity and Identification of Tolerants Using RAPD Analysis.

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Abstract: Sugarbeet is of significant commercial importance as the major sucrose producer in temperate climates. Salinity considered one of important abiotic challenges faced sugarbeet cultivation in Egypt. In this work, we studied the morphogenic response of sugarbeet tissue cultures to salinity stress and identification of selected lines using molecular markers. Callus initiated from aseptic seedlings was exposed to different levels of salt mixture. Cultures which appear salt tolerance were regenerated on salt-free medium and considered primary selected lines. For *in vitro* confirmation of the salt tolerance, regenerated shoot buds derived from the tolerant callus cultures were exposed to same levels of salts mixture. Survival as well as the proliferated shoot buds were depressed upon increasing of salts in medium. The best results of salt tolerance presented as survival and shoot proliferation were recorded at 3000 ppm of salts. Differences between tolerant and non-tolerant clones have genetically investigated using DNA marker since Random Amplified Polymorphic DNA (RAPD) technique was used to distinguish tolerant cultures. Although there were number of humongous band of the two primers used, only one primer gave genetic variations presented as new bands with the tolerant lines. The tolerant shoot buds were *in vitro* rooted and successfully adapted to grow in the free-living conditions.

Key words: Sugarbeet, tissue culture, salt stress, RAPD analysis.

INTRODUCTION

About 7 % of the world's total land area is affected by salt, as is a similar percentage of its arable land[14]. In developing countries, the limited supply of good quality water in many arid and semi-arid regions necessitates the use of saline water where available for crop production. Salinity inhibits the growth of plants by affecting both water absorption and biochemical processes, such as nitrogen assimilation and protein biosynthesis[6]. This, in turn, requires the screening of crop plant varieties for their tolerance to salinity. Attempts to improve the salt tolerance of crops through conventional breeding programs have met with very limited success, due to the complexity of the trait: salt tolerance is complex genetically and physiologically[8]. The gene transfer approach gaining recently considerable results is still leaving the main question unanswered-how introduction of stress tolerance genes will affect crop productivity. It appears that *in vitro* selection of stress tolerance will continue to have its significant place in the strategy of establishing plant system with optimal stress reaction and productivity.

Sugarbeet is of great importance in Egypt as sucrose producing crop. About 26% of Egyptian sugar production comes from sugarbeet which is grown mainly in North Delta. In cultivated sugarbeet, the development of new and improved crop genotypes is of vital importance for its growing in various ecological areas. However, in view of the fact that sugarbeet is biennial plant and the modern cultivars are highly heterozygous, being naturally cross-pollinated, the generation of new varieties by conventional breeding is difficult[1]. A biotechnology strategy for sugarbeet therefore would be expected to aid the breeder in introducing specific traits such as salt tolerance into commercially valuable genotypes. The present work was planned to *in vitro* select and molecular characterize clones of sugarbeet tolerant to salinity.

MATERIALS AND METHODS

Establishment of Aseptic Culture: Seeds of sugarbeet (cv. Pleno) were washed with distilled water and then immersed in 70 % ethanol for 1 min followed by 30 % commercial Clorox (containing 5.25 % sodium hypochlorite) for 20 min and finally washed three times with distilled sterilized water. The disinfected seeds were placed in jars containing MS-basal medium[13]. Forty-day old seedlings were taken and recultured aseptically on 1 mg/l BA containing medium to obtain stock materials (Fig. 1-A). For callus induction, leaf segments (5 mm in length) were plated on MS medium supplemented 0.5 mg/l kin + 0.5 mg/l 2,4-D according to Bekheet *et al*.[4] (Fig. 1-B).

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Effect of Salt Stress on Callus Cultures: Equal pieces of the proliferated callus were subcultured on callus growth medium (0.5 mg/l kin + 0.5 mg/l 2,4-D) supplemented with 3000, 4000 and 5000 ppm of salt mixture [3 NaCl:1(3 MgCl : 1CaCl2)] described by Ibrahim and El-Kobbia[11]. After five weeks of culturing, salt tolerance ratio was calculated as follow:

$$\text{-Salt tolerance ratio} = \frac{\text{Fresh weight on saline medium}}{\text{Fresh weight on salt-free medium}}$$

Effect of Salt Stress on Regenerated Shoot Bud Cultures: Calli survived and grown on saline media were taken and then regenerated on salt-free-medium. The regenerated shoot buds were exposed to same salt levels. Survival and the number of the new proliferated shoot buds were scored after five weeks of culturing. Culture media were adjusted to pH 5.8 before autoclaving at 126 ºC and 1.5 Ib/M^2 for 20 min. Cultures were incubated in growth chamber at 25± 2ºC under 16 hr light (2000 Lux) and 8 hr dark. The experiment was designed in complete randomized design and obtained data were statistically analyzed according to Waller and Duncan[6].

***In Vitro* Rooting and Acclimatization:** Single shoots were cultured on MS-medium supplanted with 2 mg/l IBA and amended with 0.03 % of activated charcoal for *in vitro* rooting[3]. For acclimatization of *in vitro* rooted plantlets to the free-living conditions, the healthy complete plantlets were washed with tap water and soaked in Benlate solution (1g /l) for 20 min. Then plantlets were transplanted into plastic pots containing peat moss and vermiculite (1:1). The pots were covered with clear polyethylene bags which were sprayed with water to maintain a high relative humidity. After two weeks, humidity was gradually reduced and covers were completely removed within four weeks.

RAPD Analysis: DNA extraction was carried out using leaf samples of tolerant and non-tolerant lines of sugarbeet. Genomic DNA was extracted and purified using the DNeasy plant Mini Kit following the manual instructions (QIAGEN, Chatsworth, CA). Five–mer oligonucleotide primers (Operon technology, USA) were used (Table 1). PCR reactions were performed in a 0.5 ml microcentrifuge tube containing 5 µl of DNA (5 ng/µl),1 µl of the ten base primer (15 ng/l), 0.5 l of Taq DNA polymerase (AmpliTaq, Perkin Elmer Cetus 5U/l), 11.5 l water and 7 l of a 3.57X buffer solution. The 3.57X buffer solution is freshly made by adding 280 l of a solution of ATP, TTP, CTP, and GTP (2.5 mM each) and 280 l of MgCl (10 mM) to 350 l of the 10X buffer supplied with the taq polymerase. The reaction mixture was vortexed and centrifuged briefly and 50 µl of mineral oil was overlaid on top of the aqueous layer. PCR was initiated by denaturation step at 94 °C for 1 min and then the reaction was subjected at 44 cycles of 94 °C for 30 sec., 36 °C for 1 minute, and 72 °C for 2 minutes. A final elongation step of 2 minutes at 72 °C were performed. In order to select the optimal conditions of the RAPD-PCR different optimization experiments were carried out. Samples were stored at 4 °C after the final step. The amplification products were resolved by electrophoresis on a 1.5% agarose gel with ethidium bromide and visualized under UV.

RESULTS AND DISCUSSION

Influence of Salt Stress on Callus Cultures: This part of study aimed at investigating the effect of different salinity levels on callus cultures of sugarbeet. In order to obtain a callus, MS medium supplemented with 0.5 mg/l kin + 0.5 mg/l 2,4-D was used. Growth value and salt tolerance ratio of sugarbeet calli grown on three concentrations of salt mixture, i.e., 3000, 4000 and 5000 ppm are presented in Table (1). Clear differences between salt levels were observed in the callus growth expressed by its fresh weight. The results showed that growth value decreased as salt mixture increased in culture medium. Moreover, The salt concentration in the medium affected the color of the callus. On a medium containing low salt concentration, the callus was light while it was dark on high concentration of salt mixture used. The best result of salt tolerance ratio was noticed with 3000 ppm of salt mixture (Table 1 and Fig. 1-C). This may be due to presence of cell lines tolerant or adapted to salt at such level. A higher level of salinity in the medium (5000 ppm) led to the necrosis of the callus of sugarbeet. The differentiated conditions for callus growth on a medium containing salt mixture rendered it possible to select in sugarbeet lines cell fragments tolerant to the salt stress. The reduction of callus fresh weight of sugarbeet as a result of salinity stress is in line with the results obtained by Bekheet *et al*.[2] and El-Bahr *et al*.[7] in their studies on *Asparagus officinalis* and Globe artichoke, respectively. They reported that growth parameters of callus gradually depressed as salt level increased in culture medium. Moreover, Dubey[6] mentioned that the marked increase of tolerance in callus cultures grown on saline media may be due to synthesis of new proteins (osmoprotectant protein). In this connection, Uno *et al*.,[15] established salt tolerant cell lines of Sea Aster, which survived and grew under high salinity conditions.

Table 1: Effect of different concentrations of salt mixture i.e. 3000, 4000 and 5000 ppm on growth and tolerance ratio of callus cultures of sugarbeet.

Salt level (ppm)	Growth value	Salt tolerance ratio
3000	3.20	0.80
4000	2.80	0.61
5000	2.10	0.49

Influence of Salt Stress on Regenerated Shoot Cultures: The effect of salt stress on the growth of selected cultures of sugarbeet was investigated. Regenerated shoot buds of sugarbeet derived from callus exposed to salt mixture were individually cultured on MS medium contained the different levels of salt mixture. As shown in Table (2), vegetative growth presented as survival and number of proliferated shoots significantly depressed as salt mixture increased in culture medium. The reduction in the growth under salinity condition may be as a result of reduction in net assimilation rate. Otherwise, the highest values of survival and shoot proliferation were registered at 3000 ppm of salt mixture. Although shoot buds grown on medium contained 5000 ppm scored the lowest growth parameters, some shoots remained viable and proliferated few new buds. Fragments of shoots from tolerant clones formed new leafy shoots and roots from clones of partially tolerance only shoots, while those from non-tolerant clones did not initiate organogenesis (Fig. 1-D). In this respect, the successful adaptation of cell lines to salinity[5] suggested that a genetic potential for salt tolerance is present in cells of plants from which the lines were derived and that exposure of the cells to salt triggers the expression of this information. The present results are accordance with those of Freytag *et al*.[10]. They mentioned that lines of sugarbeet (*Beta vulgaris* L.) tolerant of multiple salts was accomplished by an *in vitro* multiple salt challenge. In this context, Mills[12] reported that salinity results in a decline in the metabolic activity of plant cells which is reflected in an inhibition of their growth.

Table 2: Effect of different concentrations of salt mixture i.e. 3000, 4000 and 5000 ppm on survival and number of proliferated shoots of sugarbeet.

Salt level (ppm)	Survival (%)	Proliferated shoots no.
3000	77.7 b	2.55 b
4000	55.5 c	2.10 c
5000	33.3 e	1.55 d

*Means having the same letter(s) are not significantly different (p<o.o5).

***In Vitro* Root Formation and Acclimatization:** In this part of work, sugarbeet shoots were cultured on 2 mg/l IBA and 0.03 % of activated charcoal was added to culture medium. Our finding indicate that the root were thin and weak but they were enough to obtain successful adaptation to the free-living conditions. The success of *in vitro* methods in plant propagation depends not only on the number of plantlets produced but also on their survival rate upon transfer to nursery and field conditions. In our study, high percentage of survival was obtained after five weeks of transplanting. Three weeks after transfer, new leaves were produced. At the end of the fifth week, the plantlets grew into plants of normal appearance (Fig.1-E).

RAPD Analysis: The aim of marker-assisted breeding is to speed up selection and to directly select for traits. Molecular markers have been used for a particular DNA segment which is linked to a trait of interest. In this study, assessment of variation between tolerant and non-tolerant lines by RAPD was performed using two–mer oligonucleotide primers (Operon technology, USA (Table 3). The reproducibility of RAPD appears to be highly influenced by the experimental conditions. It was therefore essential to optimize the PCR conditions to obtain reproducible results before going on routine analysis. Results of PCR banding indicated that the two primers gave sufficient and reproducible amplification products. Primer A amplified a total number of 18 bands in all lines, however primer B amplified a total number of 22 bands. The smallest size of the amplified products was 850 bp and the largest size of the amplified products was 1650 bp in all accessions (Fig. 2). The tolerant and non-tolerant clones gave typical polymorphic bands with primers A. However, some variations were noticed between the two types of cultures with primer B since new band appeared with the tolerant lines (Fig. 2). The correlation between the increasing level of salt tolerance and the expression level suggests that the altered expression of tolerance genes may be functionally involved in the ability of cells to survive and grow in salt containing medium. In this respect, Flowers and Yeo[9] suggested that one of the possible ways of develop salt tolerance crop plants is generate variation within existing crops by using recurrent selection, mutagenesis or tissue cultures.

Table 3: Sequence of amplified products of two arbitrary primers (Operon Technologies) to generate RAPD markers.

Code	Primer	Sequence
A	OPC-09	5` CTCACCGTCC 3`
B	OPC-01	5` TTCGAGCCAG 3`

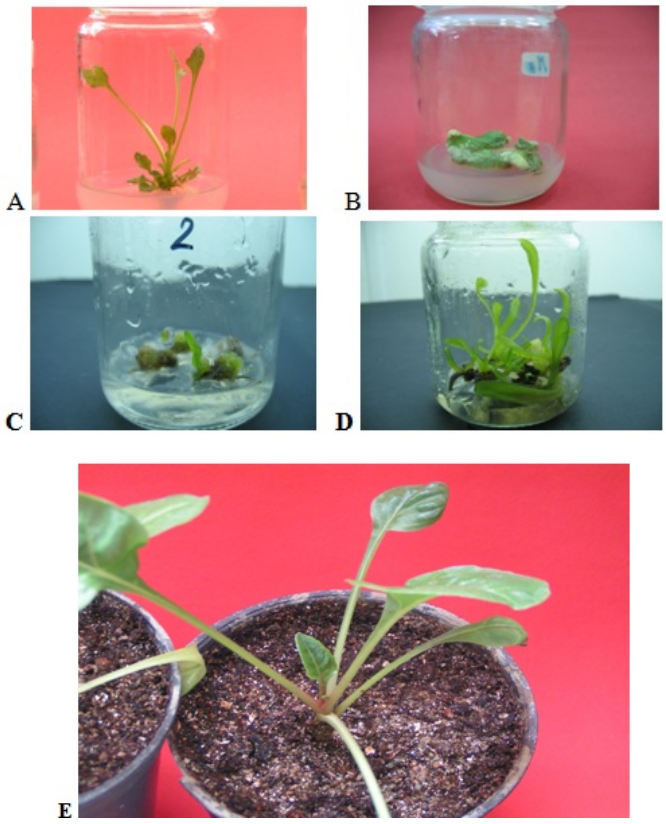

Fig. 1: A: Reculturing of aseptically grown seedlings of sugarbeet on 1 mg/l BA containing medium; B: Callus induction from leaf segments of sugarbeet using medium contained 0.5 mg/l kin + 0.5 mg/l 2,4-D; C: Callus of sugarbeet growing on medium contained 3000 ppm of salt mixture; D: Regenerated shoot buds of sugarbeet derived from callus exposed to salt mixture and E: The tolerant sugarbeet plantlets which successfully adapted to the free-living conditions.

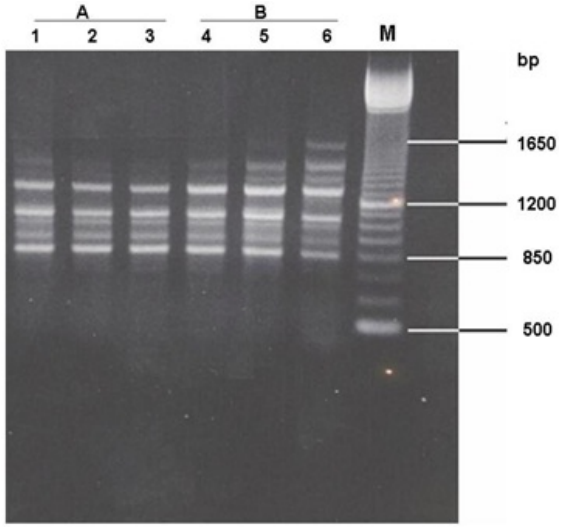

Fig. 2: Amplification products of salt tolerant lines of sugarbeet (2, 3, 5, 6) and control (1 , 4) with two primers (A and B).

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