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Induction Somaclonal Variation in Pear Plants *(Pyrus Communis* var. *Betulifolia*) for Salinaty Tolerance Through Tissue Culture Technique.

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SALINITY is one of the major environmental stresses that challenge plant growth and crop productivity worldwide. In the present investigation, an attempt to introduce tolerant pear rootstock was achieved. Callus of *betulifolia* were subjected to different concentration of sodium chloride as a source of salinity (1000 mg.L-1, 2000 mg.L^{-1}, 3000 mg.L^{-1}, 4000 mg.L^{-1} and 5000 mg.L^{-1}) via *in vitro* culture technique. Survival calluses decreased in all of the tested concentrations, however, no survival calluses were obtained at 5000 mg.L^{-1}. Moreover, regenerated plants were also decreased with the increasing of salt concentration. On morphological level, the 1000 mg.L^{-1} concentration revealed high measurement in compare to the control. The opposite was true for the 2000 mg.L^{-1}, 3000 mg.L^{-1} and 4000 mg.L^{-1}. Increment of salt concentration reduced both of the fresh weight and relative growth of fresh weight gradually from the 1000 mg.L^{-1} to 4000 mg.L^{-1}. Chemically, proline, sodium and chloride content were gradually increased with the increasing of sodium chloride concentrations. Meanwhile, salinity stress recorded drastic effect on chlorophyll content especially at 4000 mg.L^{-1}, total chlorophyll content were decreased by the increasing of salt concentration. It could be recommended to examine the resistant plants with higher concentrations of salinity and grafted these plants by different cultivars of pear.

Pear (*Pyrus communis*) is the third most important temperate fruit species after grape and apple (Chevreau *et al*.,1997). The genus *Pyrus* belongs to the sub-family *Maloideae* of the Rosaceae. Over the years, plant breeders have developed very productive cultivars of high quality, resistant to diseases and adapted to the demands of the market and of industry (Grandillo *et al*., 1999).

Salinity affects almost every aspect of the physiology and biochemistry of plants and significantly reduces yield (Cuartero *et al*., 2006). As saline soils and saline waters are common around the world (Sengupta and Majumder, 2009), and becoming a serious agricultural problem, especially in irrigated lands located in semiarid zones, where 20-30% of the land is seriously damaged by salt (FAO, 2002). Moreover, salinity is one of the major environmental stresses that challenge plant growth and crop productivity world wide (Sen and Mohammed, 1994). Producing sustainable and profitable crops under these conditions needs technological and biological approaches, including selection of

new, more salt tolerant cultivars of named plants using conventional breeding programs or tissue culture techniques (Al Mansoori *et al.*, 2007). Great effort has been devoted to understanding physiological aspects of tolerance to salinity in plants, as a basis for plant breeders to develop salinity-tolerant genotypes. In spite of this great effort, only a small number of cultivars, partially tolerant to salinity, have been developed. Further effort is necessary if the exploitation of saline soils and saline waters that are not currently usable is to be achieved.

Conventional breeding by hybridization is often inefficient towards salinity tolerance because of difficulty using the pre-existing variability *i.e.*, quantitative nature of resistance, high degree of heterozygosity etc. (Yacoubi *et al*., 2010). The success achieved by past attempts to generate salt-tolerant genotypes, both through *in vitro* culture selection and conventional breeding programmes, (Flowers, 2004) have fuelled hopes that the problems might be solved. Tissue culture is a useful method for increasing the stress tolerance of plants chiefly because of totpotency, which refers to the fact that most plant cells contain a complete genome of the species and thus are at least potentially capable of becoming entire plants. Providing that individual cells can be convinced to produce entire plants or at lest shoots, large population of cell can be maintained and in many cases selected to obtain agriculturally useful mutant (Chandler *et al*., 1987 and Abd El-Rahman *et al.*, 2007).

New cultivars bred for salt tolerance has not only to be salt tolerant, but also achieve the same attributes of productivity and quality seen in modern cultivars. Thus, *in vitro* culture techniques introduce an alternative method to select cell lines tolerant to salinity.

The aim of this study is to develop *in vitro*, salt tolerant cells of pear rootstock namely, *Pyrus communis var. betulifolia*.

Material and Methods

The investigations of the present study was achieved at the tissue culture and Germplasm Conservation Research lab. of the Horticulture Research Institute Giza during 2011 and 2012 seasons.

Plant Material

Selected meristem tips form *Pyrus communis var. betulifolia* seedlings were sterilized by 70% ethanol for 10 min. followed by sterilization with 15% sodium hypochlorite for 15 min. and finally rinsed in sterile distilled water 3 times.

Culture Medium

Explants were dissected into small pieces (0.3 cm) and cultured on Murashige and Skoog media (1962) supplemented with 2.8mg/l May-inositol + 2.5 mg/l thiamine Hcl +1mg/l (BAP). After six weeks, formed calluses were transfer into the same media which supplemented $mg.L^{-1}$ with different concentrations of NaCl for Salinity (0.00, 1000 $mg.L^{1}$, 2000 $mg.L^{-1}$, 3000 $mg.L^{-1}$, 4000 $mg.L^{-1}$ and 5000 $mg.L^{-1}$) and kept in this media for three weeks. Calluses were enhanced for regeneration after

two weeks of salinity treatment, shoots were generated after 30 days using full strength of MS media supplemented with 2.8mg/L May-Inositol + 2.5 mg/L thiamine Hcl +1mg/L (BAP)+ 30gm/L sucrose +7gm/L agar and adjusted to PH 5.6. Regenerated shoots were cultured on multiplication medium (full strength MS media+ 2.8mg/L May-Inositol + 2.5 mg/L thiamine HCl +2mg/L (BAP)+ 30gm/L sucrose +7gm agar/L and adjusted to PH 5.6)

Multipled shoots were separated into individual shoot and transferred onto rooting medium (half strength MS media+ 2.8mg/L May-Inositol + 2.5 mg/L thiamine Hcl +0.5mg/L (IBA)+ 20gm/L sucrose +7gm agar/L and adjusted to PH 5.6) for another 4 weeks.

For acclimization, the plants were transplanted into small plastic pots containing of peatmoos and sand 2:1 (v/v) in greenhouse.

The following data were recorded

Vegetative growth/Plant

- Number of callus survival, number of regenerated plants, number of survival, plants, shoot length (cm), number of leaves, leave length (cm), leave area (cm), root length (cm), number of roots and number of acclimized plants.
- Fresh weight of regenerated plantlets were measured and expressed as growth rate (GR), relative to initial weight (GR= (final weight – initial weight) / initial weight) and as relative growth rate (RGR), relative to GR of control [RGR = 100 x GR stress /GR control] according to Yacoubi *et al*.(2010).

Chemical analysis

Sodium determination: Sample of 0.2 gm was grounded and digested using the procedure suggested by Jackson (1958). The digested solution was then used for sodium determination by flame photometer according to method recommended by Brown and Lilleland (1946).

- *Chloride determination:* A dry sample (0.2) extracted under hot water then titrated with silver nitrate as mentioned by Higinbothan *et al*. (1967).

Total chlorophyll: Total chlorophyll was determined using chlorophyll meter (Model SPAD-502). Total chlorophyll was estimated as µg/cm^2.

Proline determination: The proline content was determined according to Bates *et al*. (1973).

Statistical analysis

Each treatment was performed in six jars containing five explants and each experiment was replicated three times. Data were subjected to analysis of variance by MSTAT-C (1990) Computer statistical analysis program. LSD, test at the 5% level of significance (P=0.05)

Results and Discussions

Effect of different concentrations of sodium chloride on morphological parameters

The phenotype and vegetative growth characteristics were affected with salinity stress treatments (Table 1). It was clear that increasing of salt concentration lead to decrease of number of survival callus (Fig.1). No calluses were survived at 5000ppm salt concentration. The same trend was showed with number of regenerated plantlets and number of survival plantlets. These results are in harmony with Yacoubi *et al.* (2010) who stated that with increasing salinity concentration (0 to 9 gm/L NaCl) in *Troyer citrange*, a gradual decrease in Callus growth was observed.

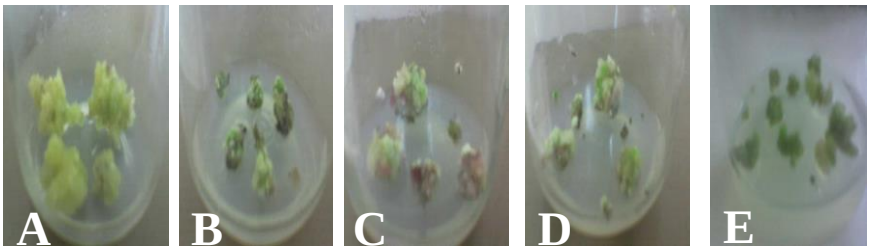

Fig. 1. Callus formation resulted from the control(A), 1000 mg.L^{-1}(B), 2000 mg.L^{-1}(C), 3000 mg.L^{-1}(D) and 4000 mg.L^{-1}(E) NaCl.

Retardant effect of salt concentrations (2000 mg.L^{-1}, 3000 mg.L^{-1} and 4000 mg.L^{-1}) was observed on vegetative growth parameters (plantlet length, number of leaves, leaf area, number of roots and length of roots). The opposite was true for 1000 mg.L^{-1}of sodium chloride; morphological measurements of vegetative growth parameters were increased. This could be interpreted by Na^+, Cl^- or both together play a chemical role that may enhanced growth of pear plantlets at this concentration. In this respect, yacoubi *et al*. (2010) found that 57 out of 1400 explants cultured for a month of stress on 4 gm/L of sodium chloride showed fast growth and equivalent size to that of control.

TABLE 1. Effect of different concentrations of sodium chloride on callus Survival, regenerated plantlets and vegetative parameters of plantlets resulted from using different concentrations.

Salt Concentrations	No. of callus cultures	No. of callus Survival	No. of regenerated plantlet	No. of Survival plantlet	Plantlet length (cm)	No. of leaves/ plant	Leave area (cm)	Root length (cm)	No. of roots /plant
Control	60	60	53.00	51.75	6.52	5.67	2.62	5.64	5.90
1000 mg L^{-1}	60	43.67	37.47	25.00	7.33	6.33	3.02	6.39	6.93
2000 mg L^{-1}	60	34.33	26.33	12.67	6.24	4.67	2.22	5.00	5.29
3000 mg L^{-1}	60	20.00	9.50	7.00	6.17	4.33	2.37	4.00	5.16
4000 mg L^{-1}	60	10.67	4.00	1.00	5.22	4.00	2.12	3.33	4.23
5000 mg L^{-1}	60	—	—	—	—	—	—	—	—
L.S.D at 0.5		3.41	2.49	2.81	0.43	0.69	0.41	1.26	1.01

Effect of different concentrations of sodium chloride on growth rate development

Increment of salt concentration reduced the fresh weight gradually from the 1000 mg.$L^{-1}$to 4000 mg.L^{-1} (Fig.2 &Table 2). The growth rate of fresh weight decreased in 2000 mg.L^{-1}, 3000 mg.L^{-1}, and 4000 mg.L^{-1} salt concentration in comparison with the control; meanwhile, it was increase by 1.28 times for 1000ppm. Furthermore, the relative growth rate of fresh weight also decreased with all of the examined concentrations except for 1000ppm which folded by 1.1 in comparison with the control.

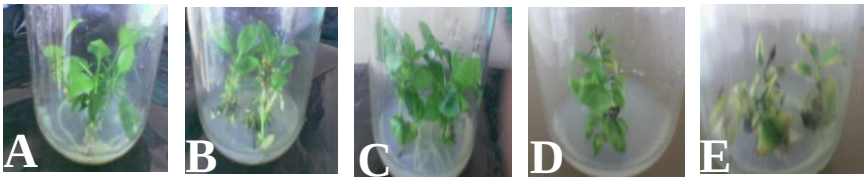

Fig. 2. Regenerated plantlet resulted from the control(A), 1000 mg.L^{-1}(B), 2000 mg.L^{-1}(C), 3000 mg.L^{-1}(D) and 4000 mg.L^{-1}(E) NaCl.

Yacoubi *et al*. (2010) observed that, in *Troyer citrange* rootstock calli treated with NaCl at 4 and 6 gm/L were insufficient to cause a relative reduction of 50% compared to the control. However, more significant retarding effect was observed at 8 and 9 gm/L NaCl concentration, and the relative growth rate of stressed calli strongly decreased by 65% and 75% , respectively. On the other hand, Balal *et al*. (2011) demonstrated that, the fresh and dry weight was affected by NaCl treatments, with a greater reduction as NaCl concentration was increased in *Citrus* rootstocks.

TABLE 2. Growth rate development as affected by salinity stress.

Treatments	Initial fresh weight (gm)	Final fresh weight (gm)	Fresh growth rate	Relative growth rate of fresh growth
Control	9.37	20.37	1.17	100.00
1000 mg/L^{1}	8.12	18.56	1.28	109.40
2000 mg/L^{1}	7.60	13.34	0.76	64.95
3000 mg/L^{1}	7.57	10.60	0.40	34.18
4000 mg/L^{1}	5.14	6.67	0.29	24.79

Effect of different salinity concentrations on some chemical constitutes

Accumulation of different solutes due to salt stress has been reported by many workers. The commonly reported solutes are proline and total cholorophyll; therefore, estimation of proline and total chlorophyll content were achieved in the present study. Concerning the prolin, as salinity concentration increase, the proline content increase. However, in 1000 mg.L^{-1}concentration the difference was not significant in compare with the control.

No significant difference was observed again with 3000 mg.L^{-1} and 4000 mg.L^{-1} concentrations. Furthermore, soudium chloride at 2000 mg.L^{-1} concentration recorded a significant differences within all of the tested

concentrations (Fig. 3). It has been reported that proline content increase due to salinity stress in different plants (Ashraf & Bashir, 2003). Inherited difference in the accumulation of proline in various geotypes of plants has also been reported and its osmolyte is more widely accumulated in higher plants, than other amino acids under salt stress conditions (Abraham *et al*., 2003). Moreover, accumulation of proline contributes towards membrane stability (Dondini *et al.*, 2001).

Furthermore, total chlorophyll was decreased when salt concentration increased (Fig. 3). The difference was significant between the control and the tested concentrations of salinity (1000 mg.L^{-1}, 2000 mg.L^{-1}, 3000 mg.L^{-1} and 4000 mg.L^{-1}). These results could be interpreted the shortage in plant fresh weight, decreasing in chlorophyll content will subsequently affect the accumulation of nutrients necessary for plant growth (Gu *et al.,* 2004). However, another study (Evain *et al.,* 2004) reported an increasing in chlorophyll contents in some cultivars of different plant species. In this respect, Balal *et al*. (2011) stated that, different workers gave different reasons for increasing or decreasing of chlorophyll contents. Salinity may be responsible for lower value of stomatal conductance, photosynthesis and relative water content. Meanwhile, another researchers summarized the reduction in chlorophyll may be due to variation in its synthesis between the plant species due to variation in enzymes under saline conditions (Balal *et al*., 2011).

Sodium and chloride content were much higher than the control especially with the 3000 mg.L^{-1} and 4000p mg.L^{-1} concentrations (Fig. 4)**.** Sodium and chloride accumulation were folded by 1.1 and 1.2, respectively for 1000 mg.L^{-1} concentration. However, in the 3000 mg.L^{-1} concentration, the increments were 1.95 and 1.84 for both sodium and chloride, respectively. On the other hand, both sodium and chloride were twice highest at 2000 mg.L^{-1} concentration. These results are in harmony with Yacoubi *et al.* (2010).

It could be concluded that, the resistant plants resulted from this experiment might be subjected to a higher saline concentrations and grafted by some pear cultivars to examine it.

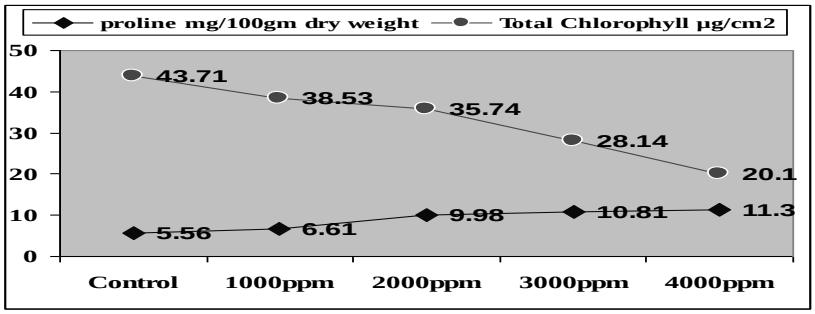

Fig. 3. Effect of different salinity concentrations on proline and total chlorophyll content.

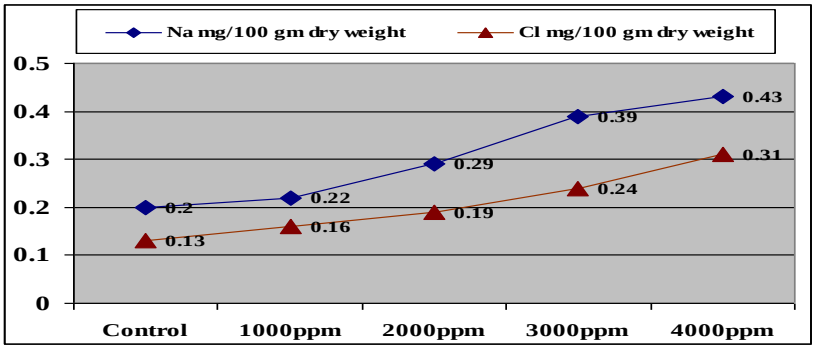

Fig. 4. Effect of different salinity concentrations on sodium and chloride content.

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استحداث اختلافات فى نبات الكمثرى للمقاومة للملوحة من خلال زراعة الانسجة

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تعد الملوحة من أهم المشاكل البيئة التى تمثل تحدى لنمو النباتات وانتاجية المحاصيل على مستوى العالم.

ثمة محاولة من خلال هذة الدراسة لانتاج أصل من الكمثرى (البيتشلفوليا) مقاوم للملوحة وذلك بتعريض الكالوس الناتج من زراعة الانسجة لهذا الاصل على بيئات زراعة انسجة تحتوى على تركيزات مختلفة من كلوريد الصوديوم (١٠٠٠،٢٠٠٠،٣٠٠٠،٤٠٠٠ و ٥٠٠٠ جزء فى المليون). أدت الملوحة الى نقص عدد الكالوسات والنباتات المتبقية الحية، كما لوحظ موت جميع الكالوسات النامية على تركيز ٥٠٠٠ جزء فى المليون.

على المستوى المورفولوجي، ادى التركيز ١٠٠٠ جزء فى المليون الى زيادة نسبية فى القياسات المورفولوجية (عدد الاوراق، ارتفاع النبات، مساحة الورقة، عدد الجذور وكذلك طول الجذور).

سجل الوزن الطازج للنباتات وكذلك معدل النمو الخضرى انخفاضاً ملحوظ بزيادة تركيز الملوحة.

من الناحية الكيميائية، زاد تركيز البرولين و الصوديوم والكلوريد بزيادة تركيز الملوحة. فى حين ادت زيادة تركيزات الملوحة الى نقص حاد فى المحتوى الكلورفيلى خاصة فى التركيز ٤٠٠٠ جزء فى المليون.

من هذة الدراسة يمكن التوصية بتجربة تركيزات أعلى على النباتات المقاومة الناتجة وكذلك تطعيم بعض أصناف الكمثرى لاختبار مدى صلاحية هذا الاصل.