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EMBRYOGENIC CALLUS INDUCTION OF SOME SUNFLOWER (*Helianthus annuus, L.*) GENOTYPES UNDER *In vitro* SALT STRESS

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ABSTRACT

Response of three genotypes of sunflower (Helianthus *annuus, L.*) to callus induction and embryogenic callus production under *in vitro* salinity conditions were studied. For callus induction, hypocotyls were subjected to *in vitro* culture on Murashige and Skooge (MS) medium supplemented with 1.0 mg Naa + 0.3 mgBa /L. The aims of this study was design to evaluate salt tolerance of the sunflower genotypes, growing calli were exposed after two subsequent subcultures (4weeks each) to different concentrations of Nacl (0, 50,75, 100, 125, 150 and 175 mM/L) added to the culture medium for 4 weeks. Comparison of genotypes was based on callus induction percentage, embriogenic callus production percent and relative fresh weight growth (RFWG). The necrosis percentage and relative fresh weight growth of callus were studied to evaluate callus salinity tolerance. The responses of genotypes to callus induction were 78, 89 and 60% for Sakha 53, Giza 102 and Par- 1617-1 genotypes, respectively. The high percentage of embryogenic callus obtained for the three varieties indicated that sunflower genotypes have a high capacity for embryogenic callus production. Nacl effect resulted in calli necrosis and a reduction of their growth. However, growing calli derived from varieties Giza 102 and Par-1671-1 showed less percent of necrosis and less relative fresh weight growth reduction under salt stress up to 100 m M/L, but they appeared to be more salt tolerant *in vitro* than Sakha 53 , Par-1671-1 was the most salt tolerant under 125 and 150 mM/L Nacl. The study proved that callus growth and salinity tolerance were genotype independent.

Keywords: Callus induction, embryogenic callus, necrosis, in vitro salt tolerance, sunflower genotypes.

INTRODUCTION

Salinity is a major factor in limiting crop productivity in semi-arid areas of the world (Ashraf *et al*, 1994). Different strategies have been listed by various plant scientists to improve crop salt tolerance that will result into enhanced productivity on salt affected soils. Traditional plant breeding methods, requiring long term selection and testing, while *In vitro* culture besides its use as a tool for obtaining salt tolerant plants, may offer potential for quick and early evaluation of germplasm against salt stress.

Tissue and cell culture techniques have been recognized as potentially valuable tools crop improvement program (Carlson ,1975).The successful utilization of *in vitro* methods is dependent upon the development of reliable culture protocol which are applicable to commercially desirable genotypes (Colieman, 1990).Tissue culture as a technique offers a useful tool to plant breeders, because it can markedly reduce time space and

economic requirements involved in selecting new useful plant types (Abdel Hady, 2001).

It is important to study the effect of genotype, explants type, hormone and culture conditions on sunflower (*Helianthus annuus* L.) callus induction and indirect plant regeneration. Ozyigit *et al*, (2002) and Burner,(1992) revealed a high differences among varieties in their callus production ability and plant regeneration rate. Some genotypes showed high regeneration response while others showed lower on the same media. Studies concerning sugarcane *in vitro* salt tolerance ,Gonzalez *et al.(*1995) reported that NaCl stress reduce cell survival rate in four genotypes using cell suspensions and found that genotypes respond differently when confronted with NaCl in their culture medium. Other studies showed that the early *in vitro* selection for salinity resistant could save times for producing tolerant plants which could be used in producing salt tolerant varieties or in conventional breeding (Chaudhary, *et al* 1996 and Zhu, 2001). Gandonou *et a*l (2005) studied the effect of salt on calluses growth and the salinity tolerance. They mentioned that the high necrosis percent is the low salt tolerance. In addition, the Relative Fresh Weight Growth (RFWG) of calluses study is also a good indicator for salinity tolerance. The aim objectives of this *In vitro* study was aimed to produce healthy calluses under high salinity level in the medium during the sub culturing steps using cotyledons explants and to evaluate salt tolerance in the early stage of three sunflower genotypes (Sakha 53, G102 and Par -1617-1) in order to identify those which could be used for *in vitro* salt tolerance selection program.

MATERIALS AND METHODS

Plant materials:

The two Egyptian open pollinated varieties of sunflower Sakha 53, G102 and the American line Par-1671-1 were used.

Seed germination:

Seed of the three selected genotypes were surface sterilized in 70% ethanol (Sigma Chemical Co.) for 3 min. The surface sterilized seeds were rinsed 3 times with sterile distilled water for 5 min and they were dried onto sterile filter papers. Seeds were germinated in glass jars on hormone free MS medium (Murashige and Skoog 1962). The medium contained 1 mL MS vitamin solution, 4.3 g basal salt mixture, 30 g sucrose and 9 gm agar /L (Sigma Chemical Co.). The pH of the media was adjusted to 5.7 with 1 M NaOh (Merck) before autoclaving. Seeds were kept at growth chamber with photoperiod of 16 h light (7500 lx) , 8 h dark at 25^{o}C and 70% humidity.

Callus Induction:

After 10 days, the jars of seedlings were moved to a laminar flow working bench. Under sterilized environments; hypocotyls were dissected out from seedlings and were cut into 0.5 cm long pieces then cultured on Murashige and Skoog (MS) supplemented with 1.0 mg Naa + 0.3 mgBa /L for callus induction in jars (at least 5 explants pieces with 50mm medium).

About 80 explants were used from each of the three genotypes for each treatment and they were kept in darkness at 25°C.

Embryogenic callus evaluation:

Distinction between embryogenic and no-embryogenic callus (plate 1 and plate 2) was carried out on the basis of callus external aspect. Embryogenic calli are of glossed aspect, compact, characterized by their tallow color and their globular structure, while no-embryogenic callus are of wet aspect, translucent and of color more brownish (Gandono *et al*, 2005). After 4 weeks of culture, the number of embryogenic calli was recorded for each genotype. These data were transformed into percentages expressed as percentages of embryogenic calli per total number of calli obtained.

Embryonic callus% = Number of produced calluses / number of explants cultured x 100

***In vitro* salt stress treatments:**

Callus growth:

After two subcultures of 4 weeks, normal callus for the three Genotypes G 102, Sakha 53 and Par-16710-1 were washed under running tap water to remove agar traces for 2 minutes, then they were dried and weighted before their transfer to fresh callus induction medium (W_0) to MS media supplemented with 1.0 mg Naa + 0.3mgBa /L containing 7 levels of Nacl concentrations (0, 50, 75, 100, 125, 150 and 175 mM /L.). After regeneration calli obtained were used for callus growth study. Calli were weighed again after 4 weeks of culture (W_1) Relative fresh weigh growth (RFWG) of callus was calculated as the following formula:

$RFWG = (W_0 - W_1) / W_0$

Necrosis percent: After 4 weeks of sub-culturing on salty medium, the necrotic callus were calculated under each Nacl concentrations and evaluated as a percent of all sub-cultured callus.

RESULTS AND DISCUSSION

Callus Induction Percentage:

After seed germination, 10 days old, hypocotyls cultured on the media and became larger after 1 week of culturing, and then callus were formed in 2 – 3 weeks (Plate 2). For genotypes, Sakha 53 recorded a frequency of 78%, but Giza 102 was the best (Table1) which recorded highest values of callus induction percentage under all treatments (89%). On the other hand Par 1671-1 recorded the lowest frequencies (60%).

Table 1: Callus induction %, Embryogenic callus % and Callus RFWG of three different sunflower genotypes.

Genotypes	Callus Induction (%)	Embryogenic callus (%)
Sakha 53	78	96.2
G102	89	100 .0
Para 671-	60	93.7
LSD	4.2	3.1

Embryogenic Calluses Percentage:.

Distinction between embryogenic and non embryogenic callus was carried out on the basis of callus external aspects. Embryogenic calluses percent of that ones which were able to produce embryos, the results in Table 1 showed that the three genotypes Sakha 53, G102 and Para 1671-1 genotypes showed high embryogenic calluses percentage (96.2, 100 and 93.7%). G102 genotype recorded the highest frequency of embryogenic calluses percentage.

***In vitro* salt treatments:**

Callus regeneration induction % of the three genotypes were sub cultured on mediums supplemented with different Nacl concentrations (Table 2). The results showed that the increases of Nacl concentration decreases callus induction percent. Sakha 53 and G102 genotypes calluses surpassed Para1671-1 under the low concentrations (0, 50 and 75 mM/L). There are no significant differences between these concentrations on callus production within genotypes, but G102 overcome the other two genotypes. Under the higher concentrations (100 mM/L), G102 overcome the others, but there is insignificant difference with that of Para -1671-1. On the other hand, when salt concentration was increased up to 125, and 150 mM/L), the callus induction % decreased. The highest reduction was in Sakha 53 and the lowest was in Para-1671-1. Under the highest concentration (175mM/L), there were no regenerated calluses produced.

Table 2. Callus regeneration induction % of three different sunflower genotypes under different Nacl concentrations.

Nacl concentrations (mM/L)	Callus regeneration induction %		
	Sakha 53	G102	Para 1671-1
0	78 ± 1.5	89 ± 1.5	60 ± 2.1
50	76 ± 2.6	90± 1.6	56 ± 1.9
75	68 ± 3.7	74± 2.3	55 ± 2.6
100	32 ± 3.1	38± 2.8	37 ± 1.5
125	09 ± 4.3	13± 3.1	17 ± 2.8
150	01 ±0.6	04 ±1.0	08 ±1.6
175	00	00	00
LSD at 5%	6.6	7.1	8.3

Necrosis percent:

In the absence of NaCl or 50 mM/L, none of the calli from G 102 and Par-1671-1 showed necrosis, but Sakha 53 recorded insignificant reduction. The addition of NaCl up to 75mM/L cased an increase in calli necrosis for all genotypes, and significant difference in calli necrosis was observed among genotypes. While G102 under salt concentration up to 100 mM/L recorded the lowest necrosis percent Par-1671-1 has an opposite change under the higher concentrations which recorded the lower values. The increases of salt concentrations up to 175 mM/L, necrotic percent were 100 in all genotypes. These results revealed significant ($p < 0.05$) among genotypes for callus necrosis percent (Table 3).

Table 3. Necrosis percent of regenerated callus obtained from three sunflower genotypes as affected by different concentrations of NaCl.

Nacl concentrations (mM/L)	Genotypes		
	Sakha 53	G 102	Par-1671-1
0	1.86	0	0
50	1.53	0	0
75	19.42	6.84	7.23
100	48.71	37.17	39.13
125	91.34	61.34	56.84
150	99.50	88.47	83.70
175	100.00	100.00	100.00
LSD at 5%	9.7	13.2	11.5

Callus Relative Fresh Weigh Growth (RFWG):

Callus Relative fresh growth weight is an indicator of salt tolerance and the behavior of callus growth, callus relative fresh weight growth (RFWG) decreased as the concentrations of Nacl increased in the culture medium (Table 4).This decreases under low concentration (0 and 50 mM/l) has no significant difference between and within the three genotypes. Under the higher concentrations, Sakha 53 recorded the highest decreases in comparison with the other genotypes and the values of RFWG were rapidly decreased. On the other hand the values of Giza 102 under the low concentrations up to 100 mM were higher than Para - 1671-1. The opposite was right under the higher NaCl concentrations (125 and 150 mM/L) when Para -1671-1 surpassed the other two genotypes. The results clearly indicated that callus relative fresh weight (RFWG) growth significantly affected by studied sunflower genotypes and NaCl concentration. All studied genotypes recorded highest callus relative fresh weight growth under control. The callus relative fresh weight growth significantly reduced at higher NaCl concentration and it become zero under 175mM/L NaCl (mM/L). In the callus induction medium until 50 mM/L, there are little influenced compared with 75mM, 100mM, 125mM and on 150 mM/L. Callus (RFWG) decreased as the concentrations of Nacl increased in the culture medium. This decreases under low concentrations (0 and 50 mM/l) has no significant difference between and within the three genotypes.

Table 4. Callus Relative fresh weigh growth (RFWG) of three sunflower Genotypes (Sakha 53, G 102 and Para -1671-1) under different Nacl concentrations.

Genotypes	Nacl (mM/l) in the callus induction medium						
	0 (mM)	50 (mM)	75 (mM)	100 (mM)	125(mM)	150mM/L	175mM/L
Sakha 53	1.32	1.26	0.65	0.22	0.16	0.08	0.00
G 102	1.49	1.37	0.93	0.81	0.48	0.17	0.00
Para -1671-1	1.43	1.36	0.95	0.72	0.55	0.19	0.00
Mean	1.41	1.33	0.84	0.58	0.33	0.14	0.00
LSD at 5%	0.31	0.22	0.21	0.18	0.21	0.09	00.0

Plate 1: Emrbryogecic and non emmbryogenic calluses.

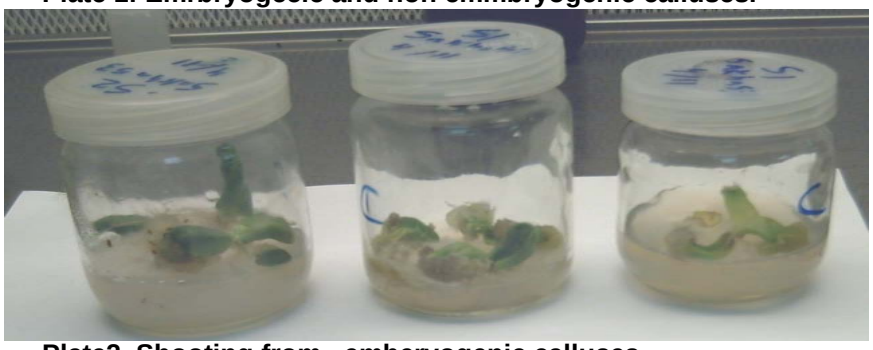

Plate2. Shooting from emberyogenic calluses

The results of this study showed the increases of NaCl level over 50 mMl/L decrease the induction of callus and sub sequence, the RFWG was decreased and the necrotic callus was increased. But the behavior within the genotypes was different, while Sakha 53 genotype was the most effected under all levels of salt concentrations. Giza 102 genotype showed higher RFWG values and lower necrosis percent under the lower salt concentrations (0 up to 100 mM/L) but these values changes under the higher concentrations. On the other hand, Par -1671-1 line was the most tolerant under the highest concentration, this probably goes to it's wild parent (*Helianthus paradoxis*) habit. Thus, callus growth was genotype independent which is in agreement with the data reported by Ozigit *et al* (2002) in sunflower, Gandonou *et al* (2005) in sugarcane and in bread wheat by Hess and Carman (1998) when a high effect of genotype on callus growth under salinity was observed. Although all genotypes were differently affected, this probably goes to the toxic influence of Na^+ and Cl^- ions; the behavior of each genotype was different.

CONCLUSION

Callus induction and embryiogenic callus percentage were genotypic independent and salt tolerance was variable within calluses and between

genotypes. This study concluded that salt tolerant embryos could be obtained via *in vitro* culture for the production of salt tolerant lines.

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استحداث كلس جنينى لبعض طرز عباد الشمس تحت تاثير الملوحة معمليا

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لقد اختير صنفين مفتوحين التلقيح من عباد لشمس وهما سخا 53 وجيزة 102 والسلالة الأمريكية بارا 1617 -1 لهذه الدراسة القائمة على طريقة زراعة الأنسجة حيث تم إنبات البذور فى المعمل على بيئة معقمة .و بعد عشرة أيام تم تقطيع السويقات بطول0.5 سم وزراعتها فى البيئة الغذائية لموراشيج و سكوج (1962) مضافا اليها 1مجم نفتاليك اسيتك اسيد + 0.3 مجم من حمض البوريك / اللتر 0 بعد أربعة أسابيع نمى الكلس وتم اعادة زراعته على بيئة جديدة معقمة مثل السابقة مضافا اليها سبعة تركيزات مختلفة من ملح كلوريد الصوديوم (صفر، 50، 75، 100، 125، 150 و175 مللى مكافئ) . بعد اربعة اسابيع اخرى تم مقارنة السلالات الثلاثة طبقا لنسبة الكلس الكلى الناتج و نسبة الكلس الجنيني الى الكلس.

قام بتحكيم البحث

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