Effects of genotype and cotyledon section on organogenesis in sunflower

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Abstract
The present study was conducted to identify the organogenetic potential of cotyledon sections from different genotypes of sunflower. Seeds were surface sterilized and germinated on hormone free half strength MS basal medium. Cotyledons from 2-day-old seedling were split in half and cultivated on MS medium supplemented with 4.4 μM BAP and 5.4 μM NAA. The experiment was designed in a randomized complete block with 3 replications. There were differences among the genotypes and cotyledon sections for all studied organogenesis parameters. Inbred line CMS60/52 and proximal explants presented the highest values for the percentage of explants producing shoots, 57.1% and 58.5%, respectively. CMS60/52 × proximal explants' interactions showed the highest values for average number of shoots per explants plated as well (13.3) as regenerant explant (21.3 shoots) respectively. Shoot organogenesis was optimized on proximal explants.
Keywords*: Cotyledon, Genotype, Helianthus annuus L., Plant regeneration, Sunflower.*

INTRODUCTION

Sunflower (*Helianthus annuus* L.) is the fourth most important oil crop in the world. In order to extend its cultivation area worldwide, genetic improvement of the cultivated sunflower for several important agronomic traits is necessary (Fiore *et al*., 1997). Sunflower improvement by conventional breeding is severely restricted by the availability of a rather limited gene pool owing to natural incompatibilities, even between related species, and by the time scale of most breeding programs. Therefore, much attention has been directed recently to the newly emerging and novel technologies of plant cell and molecular biology that provide a powerful means to supplement and complement the traditional methods of plant improvement (Durante *et al*., 2002). It is important to develop an efficient manipulation system to transfer novel traits into the crop. The main obstacle in the development of this technique is the regeneration of adventitious shoots (Mayor *et al*., 2003). Over the last few years, regeneration methods have been developed for sunflower. There are two main types of regeneration methods: organogenesis (Chraibi *et al*., 1992 b; Sarrafi *et al*., 1996 a, b; Azadi *et al*., 2002) and somatic embryogenesis (Bronner *et al*., 1993; Jeannin *et al*., 1995). Organogenic regeneration has been obtained from cotyledons of mature seeds (Ceriani *et al*., 1992; Chraibi *et al*., 1991; Knittel *et al*., 1991 and Nestares *et al*., 1996), shoot tips or embryonic axes (Knittel *et al*., 1994), hypocotyls (Bolandi *et al*., 1999) immature zygotic embryos (Sujatha and Prabakaran, 2001), leaf, rhizome and stem explants (Laparra *et al*., 1997). Cotyledons of mature seeds are a frequent source of explants for organogenesis regeneration, in part because of their year-round availability, ease of culture initiation and applicability to a number of genotypes (Baker *et al*., 1999). Organogenesis is influenced by the nature and developmental stage of the explants (Chraibi *et al*., 1992 a). Other factors, which can influence organogenesis, are the growth regulators, particularly cytokinins (Azadi *et al*., 2002) and specific

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medium components such as amino acids (Wirtzens *et al*., 1988), myo-inositol, casein hydrolysate and KNO_3 (Paterson and Everett, 1985). Furthermore, regeneration frequency depends on genotype and its interaction with culture conditions (Sarrafi *et al*., 2000).

In the present study we have investigated the regeneration potential of cotyledon sections in four genotypes of sunflower.

MATERIALS AND METHODS

Plant material: Three cytoplasmic male sterile inbred lines (CMS19, CMS31, and CMS60/52) and one F_1 hybrid (CMS31×R43) (obtained from Seed and Plant Improvement Institute, Karaj, Iran) were used in the experiment.

Seeds without pericarps were surface-sterilized for 3 min in 70% ethanol, soaked 20 min in 5% sodium hypochlorite solution with 0.01% (v/v) tween-20, and rinsed three-times in sterile distilled water. The sterilized seeds were then germinated in culture tubes on hormone free half strength MS medium (Murashige and Skoog, 1962) containing 2% sucrose and 0.7% agar-agar (pH 5.6). Cultures were maintained at 25±1ºC under 16 h light/8 h dark cycle with a light flux of 80 μmol m^{-2} s^{-1}. Cotyledons were excised from two-day-old seedlings and care was taken to avoid the inclusion of the apical meristem. Each seed was cut twice perpendicular to its long axis. The first cut was made approximately 2 mm from the proximal end of cotyledon. This removed the entire embryo, which was discarded. The second cut was made across the middle of the cotyledons. Explants of proximal or distal sections were placed abaxial side down onto the regeneration medium in 55 mm diameter Petri dishes. Regeneration medium consisted of full strength MS medium supplemented with 50 μM KNO_3, 1 μM myo-inositol, 5 μM casein hydrolysate, 3% sucrose, 4.4 μM BAP and 5.4 μM NAA (0.7 % agar, pH 5.8). The culture conditions were the same as for germination.

The experiment was designed in a randomized complete block with 4 genotypes, two cotyledon sections and with three replications. Each replication consisted of 18 Petri dishes. Shoots of 4 mm or longer were excised and counted 4 weeks after culture initiation. The regeneration ability of genotypes and cotyledon sections was scored by assessing:

- Number of explants shooting per 100 explants plated (ES/100E).
- Average number of shoots per explant plated (S/E).
- Average number of shoots per regenerant explants (S/RE).

Analysis of variance of data has been done based on completely randomized block design and all possible pairs of means were compared by Duncan's multiple range tests using an MSTAT-C computer program (Michigan State University). Data were subjected to *arcsine* $x^{1/2}$ transformation (Snedecor and Cochran, 1967) before statistical analysis.

RESULTS

Genotype effect: Shoot regeneration by direct organogenesis was observed 4 weeks after explant culturing (Figure 1). Results of the analysis of variance indicated the existence of highly significant differences among genotypes and cotyledon sections for all studied organogenesis parameters and among their interactions for the average number of shoots per regenerant explants and the average number of shoots per explant (Table 1). Since the interactions among the average number of shoots per regenerant explants and the average number of shoots per explant are significant, their main effects were invalid (Montgomery, 2001).

Cotyledon section effect: There were significantly differences between cotyledon sections for all studied organogenesis parameters. Proximal explants presented higher values for average number of shoots per explants plated than distal explants (Table 2).

'Proximal explant × CMS60/52' and 'Distal explant

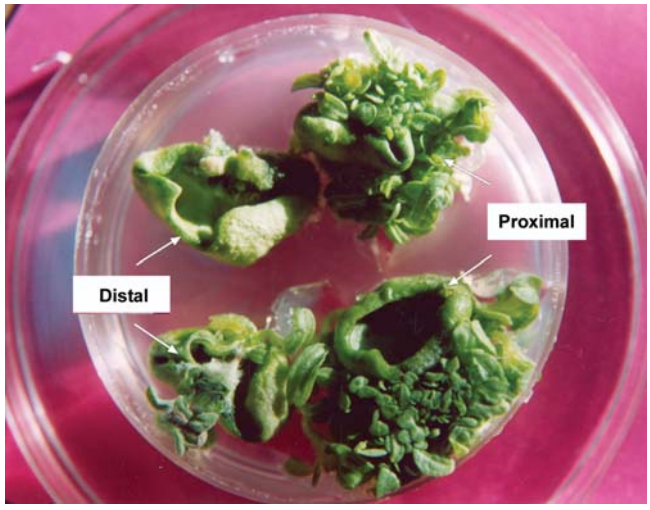

Figure 1. Shoot regeneration of cotyledonary explant, 4 weeks after initiation of culture (A: Proximal section and B: Distal section).

× (CMS31 × R43)' interactions showed the highest and lowest values (13.3 and 0.7) respectively, for the average number of shoots per explants plated. Also, the interaction 'Proximal explant × CMS60/52' showed the highest values (21.3 shoots) for average number of shoots per regenerant explants (Table 3).

DISCUSSION

A significant genetic variation for organogenesis parameters was observed. Among the four genotypes, inbred line CMS60/52 and F_1 hybrid CMS31×R43 presented the highest and lowest values, 57.1% and 30.8%, respectively for the percentage of explants producing shoots (ES/100E). Inbred line CMS60/52 showed higher levels for the organogenesis parameters analyzed. On the other hand, the hybrid 'CMS31×R43' usually showed low values for all of the traits and should be considered as the poorest genotype (Table 2). This study clearly demonstrated that regeneration efficiency in sunflower is strongly dependent on the genotype used. Chraibi *et al*. (1991) using the same technique for organogenesis, obtained

Table 1. Analysis of variance for organogenesis parameters in sunflower.

Source of variation	df	Mean squares		
		ES/100E	S/E	S/RE
Genotype (G)	3	0.081 **	67.571 **	106.405 **
Cotyledon section (C)	1	0.347 **	171.539 **	73.235 *
G × C	3	0.009 ns	8.951 **	44.358 *
Block	2	0.001 ns	10.261 **	9.507 **
Residual	14	0.004	0.797	8.29

* and ** Significant at p = 0.05 and 0.01 levels, respectively. ns: Non-significant. df : Degree of freedom. ES/100E: Number of explants shooting per 100 explants plated. S/E: Average number of shoots per explant plated. S/RE: Average number of shoots per regenerant explants.

Table 2. Effects of genotypes and cotyledon sections for organogenesis parameters in sunflower.

Main effects	ES/100E	S/E	S/RE
Genotype:			
CMS19	45.1 c	7.376 b	16.575 a
CMS31	53.1 b	7.189 b	12.439 a
CMS60/52	57.1 a	10.054 a	17.230 a
CMS31×R43	30.8 d	2.026 c	8.139 b
Cotyledon section:			
Distal	34.5 b	3.988 b	11.849 b
Proximal	58.5 a	9.335 a	15.343 a

Means followed by different letters in the same column for each factor (genotype or cotyledon sections) are significantly different at p = 0.05 (Duncan's Multiple Range Test). ES/100E: Number of explants shooting per 100 explants plated. S/E: Average number of shoots per explant plated. S/RE: Average number of shoots per regenerant explants.

Table 3. Interaction between genotype and type of cotyledon section for organogenesis in sunflower.

Cotyledon section × Genotype	S/E	S/RE
Distal × CMS19	5.4 cd	16.9 ab
Distal × CMS31	3.3 d	8.4 d
Distal × CMS60/52	6.6 c	13.1 bcd
Distal × CMS31 × R43	0.7 e	9 cd
Proximal × CMS19	9.4 b	16.2 abc
Proximal × CMS31	11.1 b	16.5 abc
Proximal × CMS60/52	13.3 a	21.3 a
Proximal × CMS31 × R43	3.3 d	7.3 d

Means followed by different letters in the same column for each organogenesis parameter are significantly different at p = 0.05 (Duncan's Multiple Range Test). S/E: Average number of shoots per explant plated. S/RE: Average number of shoots per regenerant explants.

regeneration frequencies which varied strongly according to genotype: 0% for 15 cultivars among 30, and 70% for one of the F_1 hybrids studied. Moreover, genetic variability in sunflower has been reported previously by Power (1987), Espinasse *et al*. (1989), Knittel *et al*. (1991); Sarrafi *et al*. (1996a, b) and Moieni *et al*. (2001).

Cotyledon sections were critical for high levels of shoot regeneration in the four genotypes studied. Proximal and distal explants differed significantly in terms of the percentage of explants producing shoots, 58.5% and 34.5%, respectively. Proximal section had better regeneration capacity than distal section, as observed by some researchers. Knittel *et al*. (1991) observed that explants derived from distal and proximal regions of cotyledons developed differently and direct shoot regeneration was rare on distal cotyledonary explant. Ceriani *et al*. (1992) observed the development of abundant meristematic centers in discrete regions of the proximal section of cotyledon. In contrast, in the distal region the cells were arranged in compact radial rows devoid of intercellular spaces and contained periclinal divisions. This unusual mitotic activity was probably induced by their culture conditions and no tissue or group of cells resembling a meristematic center were observed in distal regions. Baker *et al*. (1999) observed that shoot production was greater on proximal sections, and distal sections required NAA for shoot regeneration, also distal cotyledonary explants consistently regenerated less frequently and produced smaller shoots than proximal explants. It is well known that the relative concentrations of auxin to cytokinin in the regeneration medium strongly influence shoot organogenesis in plants. In many sunflower explants, the morphogenic response appears to be regulated by the balance between these plant growth regulators (Charriere *et al*., 1999). The balance influence of the endogenous and exogenous plant growth regulators in cotyledon sections on organogenesis parameters can be supposed. Also these results showed that the interactions of genotype and cotyledon section are very important.

It has been concluded that shoot regeneration frequency from cotyledon culture in sunflower depends on genotype, the nature of the explants (cotyledon sections), and their interactions, also shoot production was greater on proximal sections.

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