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EFFECTS OF CULTIVAR AND AGAR CONCENTRATION ON *IN VITRO* SHOOT ORGANOGENESIS AND HYPERHYDRICITY IN SUNFLOWER (*HELIANTHUS ANNUUS* L.)

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Abstract

This study was aimed a improves *in vitro* shoot regeneration efficiency without inducing hyperhydricity in sunflower *Helianthus annuus* L. Cotyledons of sunflower were regenerated on Murashige & Skoog medium (MS) containing 4.4 μM of BAP, 5.4 μM of NAA and various concentration of agar-agar. The experimental design was factorial on the basis of randomized complete blocks design with two cultivars and three agar concentrations and three replications. Each replication consisted of 10 plastic Petri dishes with four explants. Statistical analysis showed a significant difference among cultivars and agar concentrations for percentage of explants forming shoots, the average number of shoots per explant plated, the average number of shoots per regenerant explant and the percentage of hyperhydrated shoots. Increasing the agar concentration affected various organogenesis parameters. The cultivar $\times$ agar concentration interaction was significant for all traits 'Gabor' $\times$ 0.6% agar interaction showed the highest value for average number of shoots per explant plated and average number of shoots per regenerant explant (7.1 and 19.3, respectively). Increasing the agar concentration from 0.4 to 0.8% in cv. 'Progres' reduced the number of hyperhydrated shoots from 54.7 to 3%. The addition of agar concentration showed to be useful in improving the quality of sunflower regenerated shoots by reducing hyperhydricity.

Introduction

Sunflower (*Helianthus annuus* L.) is one of the most important oil seed crops and valuable source of protein in the world. Biotechnology involving tissue culture for crop improvement requires the ability to regenerate a great number of plants (Berrios *et al.*, 1999). The application of biotechnology for the improvement of sunflower has been limited due to difficulties associated with the lack of an efficient and reproducible method for plant regeneration (Ceriani *et al.*, 1992). Sunflower has been regenerated by organogenesis (Chraibi *et al.*, 1991; Sarrafi *et al.*, 1996; Azadi *et al.*, 2002) or somatic embryogenesis (Pelissier *et al.*, 1990; Jeannin *et al.*, 1995; Sujatha & Prabakaran, 2001). Cotyledons of mature seeds are a frequent source of explants for organogenesis regeneration (Baker *et al.*, 1999). Sunflower regeneration by organogenesis presents some difficulties such as hyperhydricity of shoots (Knittel *et al.*, 1991; Baker *et al.*, 1999; Mayor *et al.*, 2003), precocious flowering (Lupi *et al.*, 1987; Alibert *et al.*, 1994). Organogenesis response is influenced by the nature and developmental stage of explants (Espinasse *et al.*, 1989; Chraibi *et al.*, 1992a, b), growth regulators (Azadi *et al.*, 2002). Regeneration frequency depends on genotype and interaction with culture conditions (Sarrafi *et al.*, 1996). The choice of gelling agent is also very important for plant *in vitro* regeneration (Debergh *et al.*, 1992). The medium must be firm enough to support explants, but if the rigidity is too high it may prevent adequate contact between the medium and the tissue (Berrios *et al.*, 1999). In other, the phenomenon known as

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hyperhydricity is considered as a striking agar related problem (Paques & Boxus, 1987; Debergh *et al*., 1992). Hyperhydiricity is a frequent problem in tissue culture limiting the growth and multiplication *in vitro* and establishment *ex vitro* (Debergh *et al*., 1992; Ziv, 1991a, b). Losses up to 60% of cultured shoots or explants have been reported due to hyperhydricity in commercial plant micropropagation (Piqueras *et al*., 2002). Shoot hyperhydricity, resulting in failure to root and/or survive transplanting is a frequent problem in sunflower (Baker *et al*., 1999). Hyperhydricity can be controlled in various ways including improved vessel aeration (Rossetto *et al*., 1992), reducing cytokinin levels (William & Taji, 1991), increasing agar concentration (Brand, 1993) and changing the concentration of medium constituents (Ziv, 1991).

The objective of this study was to evaluate the organogenesis capacity and to reduce the occurrence of hyperhydric shoots in two cultivars of sunflower by using three agar concentrations.

Materials and Methods

Sunflower seeds of two cultivars 'Progres' and 'Gabor' (Provided by Seed and Plant Improvement Institute, Karaj, Iran) were used in this experiment. The experimental design was factorial on the basis of randomized complete blocks design with two cultivars and three agar concentrations and three replications. Each replication consisted of 10 plastic Petri dishes with four explants. Shoots of 4 mm or longer were excised and counted 4 weeks after cotyledon culture. The regeneration ability and hyperhydricity were scored by assessing the percentage of explants forming shoots (ES %), the average number of shoots per explant plated (S/E), the average number of shoots per regenerant explant (S/RE) and the percentage of hyperhydrated shoots (HS%).

Before culturing, pericarps were removed and the seeds were washed under running tap water. The seeds were surface-sterilized with 70% (v/v) ethanol for 3 min and were rinsed 1 time with sterilized distilled water. Also, 20 min in sodium hypochlorite (4.6% available chlorine) solution, containing 2-3 drops of Tween-20 and were rinsed 3 times with sterilized distilled water. The sterilized seeds were then germinated in culture tubes on hormone free half strength Murashige & Skoog (1962) medium containing 2% sucrose and 0.7% agar-agar. The pH was adjusted to 5.6 before autoclaving at 121ºC for 20 min. 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}. Two days after culturing, cotyledons from seedlings were excised, taking great care to avoid the inclusion the axillary meristem. Each seed was cut twice perpendicular to the long axis of the seed. 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. There were thus 4 cotyledonary explants derived from each seed. Explants were placed adaxial side down onto the regeneration medium in 55 mm diameter plastic Petri dishes. Each Petri dish, sealed with a Parafilm strip to prevent desiccation, contained four explants of cotyledons. Regeneration medium consisted of full strength MS medium supplemented with 50 μM KNO_3, 1μM myo-inositol, 5 μM casein hydrolysate, 3% sucrose, 4.4 μM BAP and 5.4 μM NAA. This medium was solidified with 0.4, 0.6 and 0.8% agar-agar and pH was adjusted to 5.8 before autoclaving.

Statistical analysis was performed using the program MSTATC and the means separated using a Duncan's Multiple Range Test (p = 0.01).

Results and Discussion

Results of analysis of variance show that the effect of cultivar on organogenesis ability and hyperhydricity was highly significant for studied organogenesis and hyperhydricity traits (Table 1). The effect of agar concentration as well as the interactions between cultivar and agar concentration were also significant for all the studied parameters.

Cultivars and agar concentrations mean performances are summarized in Table 2. 'Progres' cultivar presented higher value for the percentage of explants producing shoots (49.6%) and lower value for the percentage of shoots were hyperhydric (24.2%). 'Gabor' cultivar showed higher values for the average number of shoots per explant (6.1 shoots) and per regenerant explants (16.1 shoots). The main effect of agar concentration presented significant changes on organogenesis and hyperhydricity parameters (Table 2). The interaction between cultivar and agar concentration was also significant for all studied traits. 'Progres' × 0.8% agar and 'Progres' × 0.6% agar interactions showed the highest percentage of explants forming shoots (55 and 50.3% respectively). 'Gabor' × 0.6% agar interaction showed the highest values for average number of shoots per explants plated and average number of shoots per explants shooting (7.1 and 19.3, respectively). 'Progres' × 0.4% and 'Progres' × 0.8% agar interactions showed the highest and lowest values for percentage of hyperhydrated shoots (54.7 and 3% respectively).

A significant genetic variation for organogenesis parameters was observed. In sunflower, regeneration potential from cotyledon is strongly dependent on the genotype used. Genetic variability for cotyledon culture responses in sunflower has been reported previously by Power (1987); Espinasse *et al*., (1989); Sarrafi *et al*., (1996); Berrios *et al*., 1999; Azadi *et al*., (2002); Mayor *et al*., (2003) and for percentage of hyperhydrated shoot (Mayor *et al*., 2003). The percentage of explants forming shoots and the percentage of hyperhydric shoots increased with decreasing agar concentration. Agar concentration is important in determining culture response. The percentage of explants forming shoots increased at low concentration of agar, such as 0.4% which clearly increased the percentage of hyperhydric shoots (Table 2). When the agar concentration had increased (0.4 to 0.8%), the percentage of hyperhydric shoots was significantly reduced from 53.3 to 7.8%. Reduction of hyperhydric shoots in response to increased agar concentration concurs with the finding of studies with the same species (Mayor *et al*., 2003) or others (Debergh, 1983; Brand, 1993; Marga *et al*., 1997). According to Gaspar *et al*., (1987), the uptake of water from plant growth medium is the most important cause of hyperhydricity.

Soft consistency gels permit tissue cultures to extract more water from the medium (Debergh, 1983). The causes for the inverse correlation between agar concentration and degree of hyperhydricity of the tissues in culture having been previously discussed, it has been suggested that the physical state of the medium could affect the diffusion of plant growth regulators and nutrients (Bornman & Vogelman, 1984). Moreover, agar could modify the availability of soluble substances by means of chemical interactions (Brand, 1993).

This study indicates the occurrence of significant genetic variability for organogenesis and hyperhydricity response and indicates that increasing agar concentration can significantly reduce hyperhydricity of sunflower organogenesis. Besides, it was necessary to use the highest concentration of agar to produce no symptoms of hyperhydricity.

Table1. Analysis of variance for organogenesis and hyperhydricity parameters in sunflower.

Source of variation	df	ES%	S/E	S/RE	HS%
Cultivar	1	636.06 **	1.16 **	32.83 **	174.22 **
Agar	2	29.56 *	3.05 **	30.09 **	3295.5 **
Cultivar × Agar	2	384.22 **	0.45 **	23.93 **	90.39 *
Replication	2	32.89 *	0.06 *ns*	7.66 **	42.00 *ns*
Residual	10	5.29	0.02	0.41	17.00

* and ** Significant at $p = 0.05$ and 0.01 levels, respectively. ns: Non-significant. df : Degree of freedom.
ES%: Percentage of explants forming shoots. S/E: Average number of shoots per explant plated.
S/RE: Average number of shoots per regenerant explants. HS%: Percentage of hyperhydrated shoots.

Table 2. Mean effects of cultivar and agar concentration for organogenesis and hyperhydricity parameters in sunflower.

Effects	ES%	S/E	S/RE	HS%
Cultivar				
Progres	49.6 ± 5.9 *a*	5.6 ± 0.5 *b*	13.3 ± 2.4 *b*	24.2 ± 23.6 *a*
Gabor	37.7 ± 9.1 *b*	6.1 ± 0.8 *a*	16.1 ± 3.2 *a*	30.5 ± 17.9 *b*
Agar (%)				
0.4	46.2 ± 4.2 *a*	5.7 ± 0.5 *b*	12.1 ± 0.8 *b*	53.3 ± 5.1 *c*
0.6	42.5 ± 9.1 *b*	6.6 ± 0.5 *a*	15.7 ± 4.2 *a*	20.8 ± 7.3 *b*
0.8	42.2 ± 14.3 *b*	5.2 ± 0.2 *c*	16.2 ± 1.3 *a*	7.8 ± 6.4 *a*
Cultivar x Agar (%)				
Progres x 0.4	43.3 ± 3.5 *c*	5.3 ± 0.1 *c*	11.6 ± 0.8 *c*	54.7 ± 4.5 *d*
Progres x 0.6	50.3 ± 3.8 *ab*	6.2 ± 0.1 *b*	12.1 ± 0.9 *c*	15.0 ± 3.0 *bc*
Progres x 0.8	55.0 ± 3.0 *a*	5.3 ± 0.2 *c*	16.3 ± 1.5 *b*	3.0 ± 2.0 *a*
Gabor x 0.4	49.0 ± 2.6 *bc*	6.1 ± 0.1 *b*	12.6 ± 0.6 *c*	52.0 ± 6.2 *d*
Gabor x 0.6	34.7 ± 3.2 *d*	7.1 ± 0.2 *a*	19.3 ± 1.9 *a*	26.7 ± 4.9 *c*
Gabor x 0.8	29.3 ± 2.5 *d*	5.2 ± 0.2 *c*	16.2 ± 1.4 b	12.7 ± 5.5 *ab*

Mean followed by different letters in the same column are significantly different at $p = 0.01$
ES%: Percentage of explants forming shoots. S/E: Average number of shoots per explant plated.
S/RE: Average number of shoots per regenerant explants. HS%: Percentage of hyperhydrated shoots.

References

Alibert, G., J.C. Aslan–Chanab and M. Burns. 1994. Sunflower tissue and cell cultures and their use in biotechnology. *Plant Physiol. And Bioch*., 32: 31-44.

Azadi, P., A. Moieni and M.R. Ahmadi 2002. Shoot organogenesis from cotyledons of sunflower. *Helia*, 25: 19-26

Baker, C.M., N. Munoz-Fernandez and C.D. Carter. 1999. Improved shoot development and rooting from mature cotyledons of sunflower. *Plant Cell, Tiss. and Org. Cult*., 58: 39-49.

Berrios, E.F., L. Gentzbittel, H. Serieys, G. Alibert and A. Sarrafi. 1999. Influence of genotype and gelling agents on *in vitro* regeneration by organogenesis in sunflower. *Plant Cell, Tiss. and Org. Cult*., 59: 65-69.

Bornman C.H. and T.C. Vogelman. 1984. Effect of rigidity of gel medium on benzyl-induced adventitious bud formation and vitrification *in vitro* in *picea abies*. *Physiol. Plant*., 61: 505-512.

Brand, M.H. 1993. Agar and ammonium nitrate influence hyperhydricity, tissue nitrate and total nitrogen content of serviceberry (*Amelanchier arborea*) shoots *in vitro*. Plant Cell, Tiss. and Org. Cult., 35: 203-209.

Ceriani, M.F., H.E. Hopp, G. Hane and A.S. Escandon. 1992. Cotyledons: an explant for routine regeneration of sunflower plants. *Plant Cell Physiol*., 33: 157-164.

Chraibi, K.M.B., J.C. Catelle, A. Latche, J.P. Roustan and J. Fallot. 1992a. A genotype-independent system of regeneration from cotyledons of sunflower (*Helianthus annuus* L.). The role of ethylene. *Plant Sci*., 89: 215- 221.

Chraibi, B.K.M., J.C. Castelle, A. Latch, J.P. Roustan and J. Fallot. 1992b. Enhancement of shoot regeneration potential by liquid medium culture from mature cotyledons of sunflower (*Helianthus annuus* L.). *Plant Cell Rep*., 10: 617-620.

Chraibi, K.M.B., A. Latche, J.P. Roustan and J. Fallot. 1991. Stimulation of shoot regeneration from cotyledons of *Helianthus annuus* by the ethylene inhibitors, silver and cobalt. *Plant Cell Rep*., 10: 204-207.

Debergh, P.C, J. Aitken-Christie, D. Cohen, B. Grout, S. Von Arnold, R. Zimmerman and M. Ziv. 1992. Reconsideration of the term vitrification as used in micropropagation . *Plant Cell, Tiss. and Org. Cult*., 30: 135-140.

Debergh, P.C. 1983. Effects of agar brand and concentration on the tissue culture medium. Physiol. Plant. 59: 270-276.

Espinasse, A., C. Lay and J. Volin. 1989. Effect of hormone concentration and explant size on shoot organogenesis from callus derived from zygotic embryos of sunflower (*Helianthus annuus* L.). *Plant Cell, Tiss. and Org. Cult*., 17: 7-8.

Jeannine, G., R. Bronner and G. Hahne. 1995. Somatic embryogenesis and organogenesis induced on the immature zygotic embryo of sunflower (*Helianthus annuus* L.) cultivated *in vitro*: role of the sugar. *Plant Cell Rep*., 15: 200-204.

Knittel, N., A.S. Escandon and G. Hahne. 1991. Plant regeneration at high frequency from mature sunflower cotyledons. *Plant Sci*., 73: 219-229.

Knittel, N., V. Gruber, G. Hahan and P. Lanee. 1994. Transformation of sunflower (*Helianthus annuus* L.): a reliable protocol. *Plant Cell Rep*., 14: 81-86.

Lupi, M.C., A. Bennici, F. Locci and D. Gennai. 1987. Plantlet formation from callus and shoot-tip culture of *Helianthus annuus* L. *Plant Cell, Tiss. and Org. Cult*., 11: 47-55.

Mayor, M.L., G. Nestares, R. Zorzoli and L.A. Picardi. 2003. Reduction of hyperhydricity in sunflower tissue culture. *Plant Cell, Tiss. and Org. Cult*., 72: 99-103.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiol. Planta*., 15: 437-496.

Paques, M. and P. Boxus. 1987. A model to learn 'vitrification', the rootstock apple M. 26- present results, *Acta. Hort*., 212: 193-210.

Pelissier, B., O. Bouchfra, R. Pepin and G. Freyssient. 1990. Production of isolated somatic embryos from sunflower thin layers. *Plant Cell Rep*., 9: 47-50.

Piqueras, A., M. Cortian, M.D. Serna and J.L. Casas. 2002. Polyamines and hyperhydricity in micropropagated carnation plants. *Plant Sci*., 162: 671-678.

Power, C.J. 1987. Organogenesis from *Helianthus annuus* inbreds and hybrids from the cotyledons of zygotic embryos. *Am. J. Bot*., 47: 497- 503.

Rossetto, M., K.W. Dixon and E. Bunn. 1992. Aeration: a simple method to control vitrification and improve *in vitro* culture of rare Australian plants. *In vitro Cell. Dev. Biol*., 28: 192-196.

Sarrafi, A., A.R. Bolandi, H. Serieys, A. Berville and G. Alibert. 1996. Analysis of cotyledon culture to measure genetic variability for organogenesis parameters in sunflower (*Helianthus annuus* L.). *Plant Sci*., 121: 213-219.

Sujatha, M. and A.J. Prabakaran. 2001. High frequency embryogenesis in immature zygotic embryos of sunflower. *Plant Cell, Tiss. and Org. Cult*., 65: 23-29.

Williams, R.R. and A.M. Taji. 1991. Effects of temperature, gel concentration and cytokynins on vitrification of *olearia microdisca* (J.M. Black) *in vitro* shoots cultures. *Plant Cell, Tiss. and Org. Cult*., 26: 1-6.

Ziv, M. 1991a. Quality of micropropagated plants- vitrification. *In vitro Cell. Dev. Biol*., 27: 64-69.

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