THE EFFECT OF SOWING DATE AND DENSITY ON CALLUS INDUCTION AND SHOOT REGENERATION FROM SUNFLOWER ANTHERS

Dragana MILADINOVIĆ[1]*, Branislav KOVAČEVIĆ[2], Siniša JOCIĆ[1], Ivana IMEROVSKI[1], Aleksandra DIMITRIJEVIĆ[1], Sandra CVEJIĆ[1], Igor BALALIĆ[1]

[1]Institute of Field and Vegetable Crops, Maksima Gorkog 30, 21000 Novi Sad, Serbia
[2]University of Novi Sad, Institute of Lowland Forestry and Environment, Antona Čehova 13, 21000 Novi Sad, Serbia

**dragana.miladinovic@ifvcns.ns.ac.rs*

ABSTRACT

The success of anther culture depends on numerous factors such as genotype, donor plant growing conditions, anther pre-treatment and development stage, as well as incubation conditions. We have investigated the effect of sowing date and sowing density of donor plants on callus induction and shoot regeneration from cultivated sunflower anthers. Anthers were collected from of three commercial sunflower hybrids that were sown in four different sowing dates, and at three different sowing densities. Anthers were surface sterilized and placed on MS-medium based solid regeneration media. The appearance of organogenesis or somatic embryogenesis was observed and obtained data statistically analysed. The experiment was set as completely randomised, with two factors. Callus, somatic embryo, shoot and root regeneration on the anthers of the tested genotypes was observed. Data were analysed by ANOVA. Statistical analysis enabled us to determine effect of sowing date and density on anther culture and shoot regeneration induction. Sowing date had a significant effect on all observed parameters, with earlier sowing dates having significant positive effect on shoot regeneration. Sowing density had no effect on either of observed parameters in all tested genotypes. The obtained results will contribute to the better understanding of the conditions needed for haploid production in sunflower and its introduction in sunflower breeding programs.

Key words: Anther culture, Dihaploid, Donor plant, Regeneration, Sunflower

INTRODUCTION

Anther culture results in sunflower (*Helianthus annuus* L.) have been rather unsatisfactory up to now (Marinković et al., 2003). As in other species, anther culture response of sunflower is strongly affected by numerous factors such as genotype, donor plant growing conditions, anther pre-treatment and development stage, as well as incubation conditions (Gurel et al, 1991; Miladinović et al, 2012). By testing a number of different parameters, that is, donor plant growing conditions and stages, as well as culture media and conditions, appropriate protocol could be worked out for the successful regeneration of shoots - at least for a number of genotypes.

Various environmental factors that the donor plants are exposed to may affect haploid plant production. Light intensity, photoperiod, and temperature have been investigated, and at least for some species, these are found to influence the number of plants produced from anther cultures (Reed, 2005). Seasonal variations have been reported to influence anther response in *Triticum aestivum* (Ouyang et al., 1987) and *Solanum tuberosum* (Tiainen, 1992), while different temperature regimes were found to affect anther response in wheat hybrid plants

(Orshinsky and Sadasivaiah, 1997). Growing season and conditions, as well as donor plant age had an effect on anther culture of *Capsicum annuum* (Ercan et al., 2006; Buyukalaca et al, 2004).

Up to our knowledge, there are no reports on effect of donor plant growing conditions on sunflower anther culture. There are only reports on influence of medium composition variation on the frequency of anther callusing and/or somatic embryogenesis and subsequent plant regeneration (Marinković et al., 2003; Miladinović et al., 2012). Different compositions of media used for establishing anther culture were extensively reviewed by Friedt et al. (1997), and variation of other culture parameters by Nichterlein and Horn (2005).

We have investigated the effect of sowing date and sowing density of donor plants on callus induction and shoot regeneration from cultivated sunflower anthers.

MATERIAL AND METHODS

Anthers were collected from three commercial sunflower hybrids (NS Oskar, NS Fantazija, and Orfej). The hybrids were sown in four different sowing dates and at three different showing densities (30,000; 50,000 and 70,000 plants per ha).

After collection, anthers were surface sterilized and placed on solid regeneration media, supplemented with basic MS macro and micro salts (Murashige and Skoog, 1962), 0.3% gelrite, pH 5.7, while composition of hormones varied (Vasić et al., 2000; Miladinović et al., 2012). Anthers were cultured in the dark at 30^{o}C.

Two experiments were set as completely randomized, with two factors. In the first experiment factors were sowing date and genotype of donor plants, while in the second experiment factors were sowing density and genotype. Callus, somatic embryo, shoot, root and plant regeneration on the anthers of the tested genotypes was observed. The data were transformed by *arc sine* transformation in order to obtain normal distribution of their frequencies, which is required for further statistical analysis. Analysis of variance and Fisher's least significant difference test were performed in statistical program STATISTICA 12.0 (StatSoft Inc., 2013) in order to establish the significance of factor effects and their interaction, and significance of difference among treatments. Based on results of ANOVA, in order to estimate the relative importance of examined sources of variance, expected variances and their contribution to the total variance were calculated.

RESULTS AN DISCUSSION

Sowing date

Regarding contribution of components of variance to the total variance, sowing date had the highest effect on root regeneration since its variance contributed to over 50% of total variance (Figure 1). It also had the strongest effect on callus formation, as well as shoot and plant regeneration, contributing to 45%, 20% and 15% of the total variance, respectively. The effect of sowing date and interaction was similar for embryo formation, as their variances contributed approximately to 25% of total variance, each.

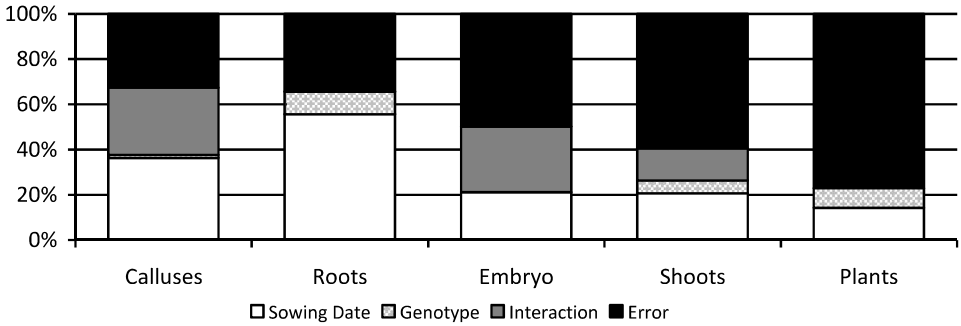

Figure 1. Contribution of expected variances of tested variation sources to the total variance (%)

Although sowing date significantly affected only plant regeneration, LSD test showed that earlier sowing dates had more positive effect on all observed parameters (Table 1). This especially stands for shoot and plant regeneration, as there were no regenerants from anthers collected at later sowing dates. Anthers collected from the plants sown at the earliest planting date had the best androgenic response, as they formed the highest number of calluses and embryos, and had the highest percentage of regeneration of roots, shoots and plants. Genotype had significant effect on shoot and plant regeneration. LSD test indicated that there was no significant difference among tested hybrids for embryo formation.

Table 1. LSD test for percentage of callusogenesis, somatic embryogenesis, root, shoot and plant regeneration at different sowing dates

		Calluses	**Roots**	**Embryos**	**Shoots**	**Plants**
		p	p	p	p	p
Date		0.000	0.000	0.000	0.009	0.071
Genotype		0.027	0.025	0.025	0.606	0.119
Date*Genotype		0.009	0.562	0.562	0.035	0.448
Variant	**Genotype**					
DATE I		86.809a	27.898a	1.612a	0.974a	0.374a
DATE II		83.351a	32.995a	0.784a	0.244ab	0.042ab
DATE III		48.322b	8.441b	1.008a	0.000b	0.000b
DATE IV		52.103b	2.950b	0.014b	0.000b	0.000b
	OSKAR	79.006a	24.313a	0.747a	0.000b	0.000b
	FANTAZIJA	70.243ab	10.526b	0.868a	0.457a	0.211a
	ORFEJ	56.924b	13.811b	0.442a	0.189ab	0.023ab

The values within the same column marked with different letters differed significantly at $\alpha_{0.05}$

Sowing density

Sowing density did not have any effect on the observed parameters (Figure 2). Genotype had the strongest effect on callus formation, as it contributed to 55% of total variance. Embryo formation was equally influenced by genotype and interaction (25% of total variance, each) while interaction had the highest effect on root regeneration (20% of total variance).

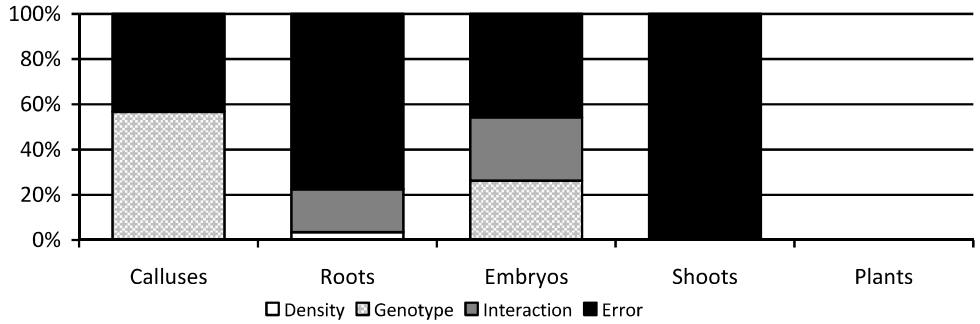

Figure 2. Contribution of expected variances of tested variation sources to the total variance (%)

Analysis of variance showed that sowing density generally had significant effect on tested parameters, but that there were no significant differences among different densities (Table 2). Interaction was significant for all tested traits, while genotype had significant effect on root and shoot formation.

Table 2. LSD test for percentage of callusogenesis, somatic embryogenesis, root, shoot and plant regeneration at different sowing densities

		Calluses	**Roots**	**Embryos**	**Shoots**	**Plants***
		p	p	p	p	p
Density		0.428	0.273	0.863	0.387	-
Genotype		0.000	0.816	0.003	0.387	-
Density*Genotype		0.961	0.186	0.055	0.433	-
Variant	**Genotype**					
30,000		78.057a	19.743a	0.510a	0.000a	-
50,000		70.928a	17.376a	0.694a	0.000a	-
70,000		78.131a	22.920a	0.420a	0.042a	-
	OSKAR	70.408b	19.419a	0.355b	0.000a	-
	FANTAZIJA	63.339b	21.206a	2.182a	0.000a	-
	ORFEJ	90.220a	19.291a	0.014b	0.042a	-

The values within the same column marked with different letters differed significantly at $\alpha_{0.05}$
*This parameter did not vary in some variants, so it was not possible to do variance analysis.

In our study, we have found that sowing date had an effect on establishment and plant regeneration from sunflower anther culture. Higher regeneration frequencies were obtained with plants from earlier sowing dates. The prevailing temperature during the growth of donor plants is reported to play a crucial role in microspore embryogenesis in Crucifers (Pratap et al., 2009). A high frequency of embryogenesis was consistently obtained in donor plants grown at low temperatures (Keller et al., 1987; Dunwell et al., 1985). This could be the reason for better regeneration frequencies in earlier sowing dates in our experiment, as low temperature is thought to increase the number of microspores suitable for embryogenesis due to slow pollen development, and it also prolongs the duration for which suitable microspores are available in a crop (Pratap et al., 2009). The opposite results were observed in wheat anther culture where embryo regeneration was usually greater when anthers were obtained from plants grown at high temperatures than plants grown at lower temperatures (Orshinsky and Sadasivaiah, 1997).

The lack of effect of sowing density on the observed parameters indicates that in our experiment irradiation and the temperature within the canopy were not important for androgenic response in tested hybrids, and that sowing date and temperature conditions during the plant growth have greater effect on this trait.

The results obtained in our study indicate that although genotype plays an important role in sunflower anther culture, the regeneration frequency could be improved by taking care of growing conditions of donor plant. Future studies should be focused on further optimisation of donor plants growing conditions in order to minimize genotype effect and sunflower androgenic potential and enable creation of the environment that will favour haploid plant regeneration.

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LITERATURE

Buyukalaca, S., Comlekcioglu, N., Abak, K., Ekbic, E. and N. Kilic. 2004. Effects of silver nitrate and donor plant growing conditions on production of pepper (*Capsicum annuum* L.) haploid embryos via anther culture. Europ. J. Hort. Sci. 69: 206-209.

Dunwell, J.M., Cornish, L.M. and A.G.L. Decourcel. 1985. Influence of genotype, plant growth, temperature and anther incubation temperature on microspore embryo production in *Brassica napus* ssp. *Oleifera*. J. Exp. Bot. 36: 679-689.

Ercan N., Funda, A., Sensoy, A. and S. Sensoy. 2006. Influence of growing season and donor plant age on anther culture response of some pepper cultivars (*Capsicum annuum* L.). Sci. Hortic. 110: 16-20.

Friedt, W., Nurhidayah, T., Rocher, T., Kohler, H., Bergmann, R., and R. Horn. 1997. Haploid production and application of molecular methods in sunflower (*Helianthus annuus L.*). p. 17-35. In: S.M. Jain (ed.), In vitro haploid production in higher plants. Kluwer Academic Publishers, Amsterdam, the Netherlands.

Gurel, A., Nichterlein, K., and W. Friedt. 1991. Shoot regeneration from anther culture of sunflower (Helianthus annuus L.) and some interspecific hybrids is affected by genotype and culture procedure. Plant Breed. 106: 68-76.

Keller, W.A., Arnison, P.G. and B.J. Cardy. 1987. Haploids from gametophytic cells: recent developments and future prospects. p. 233-241. In: Plant Tissue and Cell Culture. Allan R. Liss, New York, USA.

Marinković, R., Dozet, B. and D. Vasić. 2003. Sunflower breeding. Školska knjiga, Novi Sad, Serbia.

Miladinović, D., Kovačević, B., Dimitrijević, A., Imerovski, I., Jocić, S., Cvejić, S. and V. Miklič. 2012. Towards dihaploid production in sunflower - Selection of regeneration medium. p. 674-677. In: Proc. 18th Int. Sunfl. Conf., Mar del Plata, Argentina. Int. Sunfl. Assoc., Paris, France.

Murashige, T., and F. Skoog. 1962. A revised medium for growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15:473-497.

Nichterlein, K., and R. Horn. 2005. Haploids in the improvement of *Lineaceae* and *Asteraceae*. p. 277-291. In: C.E. Don Palmer, W.A. Keller, K.J. Kasha (eds.) Haploids in crop improvement II. Springer-Verlag Berlin Heidelberg, Germany.

Orshinsky, B. R. and R. S. Sadasivaiah. 1997. Effect of plant growth conditions, plating density, and genotype on the anther culture response of soft white spring wheat hybrids. Plant Cell Rep. 16: 758-762.

Ouyang, J.W., He, D.G., Feng, S.E. and S.E. Jia. 1987. The response of anther culture to culture temperature varies with growth conditions of anther-donor plants. Plant Sci. 49: 145-148.

Pratap, A., Gupta, S.K. and Y. Takahata. 2009. Microsporogenesis and haploidy breeding. p. 293-303. In: S.K. Gupta (ed.), Biology and breeding of crucifers. CRC Press, Boca Raton, USA.

Reed, S. (2005). Haploid cultures. p. 225-234. In: D.J. Gray and R.N. Trigiano (eds.), Plant Development and Biotechnology. CRC Press, Boca Raton, USA.

Tiainen, T. 1992. The role of ethylene and reducing agents on anther culture response of tetraploid potato (*Solanum tuberosum* L.). Plant Cell Rep. 10: 604-607.

StatSoft, Inc. (2013): STATISTICA (data analysis software system), version 12. www.statsoft.com.

Vasić, D, Škorić, D., and S. Jocić. 2000. Anther culture of sunflower cultivars. p. L-52-55. In: Proc. 15th Int. Sunfl. Conf., Toulouse, France. Int. Sunfl. Assoc., Paris, France.