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Factors affecting somatic embryogenesis from cotyledonary explants of safflower

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Abstract

Frequency of safflower (*Carthamus tinctorius* L.) somatic embryogenesis, number of somatic embryos per responding explant and somatic embryo maturation and germination were affected by genotype, explant age, carbon source, and ethylene. Among 8 cultivars tested, 7 were embryogenic with varying frequencies. The best response was obtained with cv. Girna. Whole cotyledonary explant from 10-d-old plants was best responding compared to 5- or 15-d-old ones. Among different carbon sources, sucrose at 87.6 mM concentration was most suitable for embryo induction, maturation and germination. Of the different ethylene inhibitors, silver nitrate at 50 μM concentration significantly increased the embryogenic frequency and also the number of embryos per responding explant. Silver nitrate has pronounced effect on embryo maturation but had no effect on germination.

Additional key words: *Carthamus tinctorius*, ethylene, phytohormones, somatic embryos, sugars.

Introduction

The basic requirements for application of *in vitro* techniques for improvement of crop plants are successful regeneration of plants, preferably from single cells and stable insertion, integration and expression of agronomically important alien genes into the host genome (Fraley *et al.* 1986). In this respect, plant regeneration via somatic embryogenesis is preferred because it provides high rate of multiplication and often results in true-to-type plants.

Safflower (*Carthamus tinctorius* L.) is an important oilseed crop of semi-arid regions. Plant regeneration by direct organogenesis from cotyledons (George and Rao 1982, Orlikowska and Dyer 1993), seedling leaves and mature embryo explants (Orlikowska and Dyer 1993), callus mediated organogenesis from hypocotyls (George and Rao 1982), and direct somatic embryogenesis from cotyledons in this crop (Mandal *et al.* 1995) have been reported. The embryos were formed directly on adaxial surface of cotyledons within 2 weeks of culture. Subsequently the embryos were matured and germinated in the same cultural medium. A number of factors are known to influence somatic embryogenesis considerably in several other crops. The present study is aimed to describe the role of genotype, explant age, carbon source and ethylene on somatic embryogenesis of safflower which would help in developing a reproducible protocol of somatic embryogenesis in this crop.

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Abbreviations: ACC - 1-aminocyclopropane-1-carboxylic acid; BAP - 6-benzylaminopurine; CEPA - chloroethylphosphonic acid; DNP - 2,4-dinitrophenol; MS medium - Murashige and Skoog medium; NAA - 1-naphthaleneacetic acid.
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Materials and methods

Seeds of safflower (*Carthamus tinctorius* L.) cultivars were aseptically germinated and whole cotyledons from 10-d-old plants were used as explants. Explants were cultured with the abaxial side in contact with the Murashige and Skoog (MS; 1962) medium containing sucrose (3 %), bactoagar (0.8 %), 1-naphthaleneacetic acid (NAA, 0.2, 0.5, 1.0, 2.0 or 4.0 mg dm^{-3}) and 6-benzylaminopurine (BAP, 0.5 mg dm^{-3}). Any change of these conditions was described in the respective experiment. The pH of the medium was adjusted to 5.6 before autoclaving at 121 °C for 15 min. All the cultures were kept at 16-h photoperiod (irradiance of 36 μmol m^{-2} s^{-1}), temperature of 25 ± 1 °C, and relative humidity (inside culture room) of 55 - 60 %.

The cotyledonary explants of 8 cultivars (Table 1) were cultured on medium supplemented with NAA (0.2, 0.5, 1.0, 2.0 or 4.0 mg dm^{-3}) and BAP (0.5 mg dm^{-3}). Since cv. Girna was found to be the best responding (at 2 mg dm^{-3} NAA), it was used in all other experiments.

Cotyledons from different developmental stages (5-, 10- and 15-d-old) were either taken as a whole or transversely cut into two (proximal and distal) or three (proximal, middle and distal) equal segments and cultured on MS medium containing 2 mg dm^{-3} NAA and 0.5 mg dm^{-3} BAP (hereafter referred to as NB). All the explants were placed abaxially and adaxially with reference to the medium. Sucrose, glucose and maltose were added to the NB medium at various concentrations (Table 3) in order to study their effects on somatic embryogenesis.

Ethylene inhibitors silver nitrate ($AgNO_3$), nickel chloride ($NiCl_2$) and 2,4-dinitrophenol (DNP) and promoters 1-aminocyclopropane-1-carboxylic acid (ACC) and chloroethylphosphonic acid (CEPA) were incorporated at different concentrations (0.0, 0.5, 5, 25, 50 and 75 μM) into NB medium. $AgNO_3$ and $NiCl_2$ were added to the medium before autoclaving while DNP, ACC and CEPA were filter sterilized and incorporated into the autoclaved medium.

Each treatment had 20 replications (culture tubes), each replication consisting of 3 explants, arranged in a completely randomized design. Percent somatic embryogenesis and number of somatic embryos per responding explant were evaluated after 3 weeks of culture. Somatic embryos matured under the same cultural conditions and germinate after 3 weeks of culture. Percent germination was evaluated after 4 weeks of culture. Data were subjected to analysis of variance (*ANOVA*) and means were separated by least significant difference (LSD) at 5 % probability level.

Results and discussion

Genotype had a pronounced effect on the ability of cotyledons to undergo NAA stimulated somatic embryogenesis (Table 1). Out of the 8 genotypes evaluated, cv. A-300 failed to exhibit embryogenic response. The embryogenic frequency was highest in cv. Girna (51.7 %) and APR-3 gave comparable response (50.0 %). S-144 and JLSF-1 showed moderate response with 41.7 % and 40.0 frequency. Tara was ranked next followed by Bhima. The genotype A-1 was least responsive (16.7 %).

The percent of explanted cotyledon that formed somatic embryos is not related with the number of embryos formed on each responding cotyledon. The highest number of somatic embryos per responding explant (16.7) was obtained in cv. S-144 with 1 mg dm^{-3} NAA + 0.5 mg dm^{-3} BAP. Influence of genotype on

Table 1. Effect of genotype and NAA concentrations (0.5 - 4.0 mg dm^{-3}) on somatic embryogenesis percentage and number of somatic embryos at the end of 3 weeks of culture. Data are means ± SE. Means in each column followed by the same letters are not significantly different at $P = 0.05$ according to Fisher's Least Significant Difference (LSD) test.

Genotype	Explants with somatic embryos [%]				Number of somatic embryos [explant^{-1}]			
	0.5	1.0	2.0	4.0	0.5	1.0	2.0	4.0
JLSF-1	13.3 ± 2.0bc	23.3 ± 2.4c	40.0 ± 2.2cd	36.7 ± 1.8d	5.7 ± 0.2bc	7.0 ± 0.2bc	12.0 ± 0.5fg	16.4 ± 0.5f
APR-3	30.0 ± 2.0d	50.0 ± 2.0f	33.3 ± 2.0c	23.3 ± 1.8bc	7.0 ± 0.3c	10.0 ± 0.4d	6.3 ± 0.2bc	3.0 ± 0.1b
A-1	0.0a	10.0 ± 1.7ab	16.7 ± 2.1b	13.3 ± 2.0b	0.0a	5.0 ± 0.2b	7.2 ± 0.2cd	4.5 ± 0.2bc
Bhima	13.3 ± 2.0bc	25.0 ± 2.2cd	18.3 ± 2.3b	13.3 ± 2.0b	5.5 ± 0.2b	6.4 ± 0.2b	4.5 ± 0.2b	6.0 ± 0.5cd
Girna	21.7 ± 2.3cd	36.7 ± 1.8de	51.7 ± 2.0d	38.3 ± 2.3d	7.5 ± 0.2c	9.2 ± 0.3cd	11.2 ± 0.3ef	9.4 ± 0.3e
Tara	10.0 ± 1.7ab	15.0 ± 1.9bc	20.7 ± 2.1b	30.0 ± 2.6cd	4.7 ± 0.2b	9.8 ± 0.5d	9.6 ± 0.3de	9.2 ± 0.3e
S-144	26.7 ± 2.8d	41.7 ± 2.3ef	35.0 ± 1.9c	30.0 ± 2.0cd	10.2 ± 0.5d	16.7 ± 0.6e	14.2 ± 0.4g	7.6 ± 0.2de
A-300	0.0a	0.0a	0.0a	0.0a	0.0a	0.0a	0.0a	0.0a

somatic embryogenesis has earlier been reported in a number of plants (Bingham *et al.* 1975, Brown and Atanassov 1985, Chen *et al.* 1987, Ozias-Akins *et al.* 1992). Our results confirmed a strong interaction between genotype and growth regulators for somatic embryogenesis.

Age and position of the explant significantly affected somatic embryogenesis. 10-d-old cotyledons were more responsive than 5- or 15-d-old ones (Table 2). This may reflect the differences in endogenous hormonal level with age. Alternatively, they might produce some inhibitory factors or there could be the loss of some of the promoting factors as well (Mroginski and Kartha 1984). Particularly in Gramineae, it has been reported that with increase in leaf age, there is either a decrease in the amount of DNA per nucleus (Hasemann and Schroder 1982), or fragmentation of the DNA which may be related to the loss of morphogenic competence (Halperin 1986).

Significant differences were also observed among the different segments of cotyledons of the same age. Whole cotyledons were more embryogenic than cut ones. However, the embryogenic frequency of the whole cotyledons were comparable to the proximal parts when the cotyledons were cut into two equal segments (Table 2). Proximal parts of 3 equal pieces of the cotyledons always resulted in poorer response compared to that of two equal halves or whole cotyledon. Middle and distal segments were less responsive than proximal parts.

No significant difference was observed for somatic embryogenesis between abaxial and adaxial orientation of explants on the medium (data not presented). Somatic embryos always developed from adaxial surface of the cotyledon irrespective of orientation on the medium.

Experiments were conducted to assess the effect of various sugars, such as sucrose, glucose and maltose at different concentrations (54.8, 87.6 and 175.3 mM) on somatic embryogenesis. Among them, sucrose was most favourable. The highest frequency (55.0 %) and number of somatic embryos (12.5) per responding explant were

Table 2. Effect of explant age (5, 10 and 15 d) and position of abaxially oriented segments on somatic embryogenesis from excised cotyledons of *C. tinctorius* L. cv. Girna at the end of 3 weeks of culture. WC - whole cotyledon; 2P, 2D - proximal and distal segments of cotyledon which was cut transversely into two equal halves; 3P, 3M, 3D - proximal, middle and distal segments of cotyledon which was cut transversely into three pieces. Data are means ± SE. Means in each column followed by the same letters are not significantly different at $P = 0.05$ according to Fisher's LSD test.

Explant position	Cotyledon with somatic embryos [%]			Number of somatic embryos [cotyledon^{-1}]		
	5 d	10 d	15 d	5 d	10 d	15 d
WC	33.3 ± 2.2b	56.7 ± 1.8c	26.7 ± 1.6b	9.4 ± 0.3d	14.7 ± 0.5d	4.4 ± 0.2b
2P	31.7 ± 2.3b	48.3 ± 2.3bc	26.7 ± 2.7b	8.5 ± 0.2cd	14.5 ± 0.4d	4.1 ± 0.1ab
2D	23.3 ± 1.8b	48.3 ± 2.0bc	18.3 ± 2.0ab	6.8 ± 0.3c	11.2 ± 0.4c	3.1 ± 0.2ab
3P	10.0 ± 1.7a	43.3 ± 2.1b	20.0 ± 1.9ab	3.7 ± 0.2b	12.4 ± 0.5cd	3.1 ± 0.1ab
3M	0.0a	28.3 ± 2.8a	16.7 ± 1.8ab	0.0a	8.2 ± 0.3b	2.4 ± 0.2ab
3D	0.0a	16.7 ± 1.3a	11.7 ± 1.7a	0.0a	4.6 ± 0.3a	1.8 ± 0.1a

Table 3. Effect of different sugars on somatic embryogenesis, number of somatic embryos (recorded after 3 weeks of culture) and germination of somatic embryos (recorded after 4 weeks of culture). Data are means ± SE.

Sugar	Concentration [mM]	Cotyledon with somatic embryos [%]	Number of somatic embryos [cotyledon^{-1}]	Somatic embryo germination [%]
Sucrose	58.4	38.3 ± 2.5	7.4 ± 0.2	13.2 ± 0.5
	87.6	55.0 ± 1.9	12.5 ± 0.4	17.3 ± 0.6
	175.3	26.7 ± 2.0	7.4 ± 0.3	7.1 ± 0.9
Glucose	58.4	26.7 ± 2.0	6.2 ± 0.2	6.8 ± 1.0
	87.6	18.3 ± 2.0	4.9 ± 0.2	4.1 ± 0.9
	175.3	0.0	0.0	0.0
Maltose	58.4	28.3 ± 2.3	5.9 ± 0.2	0.0
	87.6	23.3 ± 1.8	4.1 ± 0.2	0.0
	175.3	0.0	0.0	0.0

noted at 87.6 mM sucrose (Table 3). Glucose and maltose were also effective but only at lower concentrations. The concentration 175.3 mM completely inhibited somatic embryogenesis.

Sucrose (87.6 mM) was also suitable for embryo germination, while the presence of glucose reduced the germination frequency. Maltose ' was ineffective for somatic embryo germination. The different responses may be due to different osmotic potentials of the sugars (Lazzeri *et al.* 1987) or tissue preference for sucrose (Eapen and George 1993). In most of the somatic embryogenic systems sucrose has been used at concentration range from 58.4 to 116.8 mM. However, in several instances manipulation of carbon supply affected the embryo induction and development (Amirato 1983).

Among the three ethylene inhibitors used, only $AgNO_3$ has significant beneficial effect on somatic embryogenesis. Embryogenic frequency as well as number of embryos per responding explant were found to increase with the increased $AgNO_3$ concentration in the medium. A maximum of 83.3 % embryogenic response with 22.1 number of somatic embryos per responding explant were obtained at 50 μM concentration (Table 4). Further increase in concentration, however, did not produce any beneficial effect. Similarly, inhibitory role of ethylene in somatic embryogenesis and its negation by $AgNO_3$ was demonstrated in *Hevea brasiliensis* (Auboiron *et al.* 1990), *Daucus carota* (Rosutan *et al.* 1990) and *Zea mays* (Vain *et al.* 1989). The other two ethylene biosynthetic inhibitors ($NiCl_2$ and DNP) significantly decreased the embryogenic frequency as well as number of somatic embryos. In other studies also ethylene biosynthetic inhibitor $CoCl_2$ failed to promote embryogenesis (Hatanaka *et al.* 1995).

Table 4. Effect of ethylene inhibitors ($AgNO_3$, $NiCl_2$, DNP) and promoters (ACC, CEPA) on somatic embryogenesis from excised cotyledonary explants of *C. tinctorius* L. cv. Girna. Data are means ± SE. Means in each column followed by the same letters are not significantly different at $P = 0.05$ according to Fisher's LSD test.

Conc. [μM]	$AgNO_3$ response [%]	number of embryos [explant^{-1}]	$NiCl_2$ response [%]	number of embryos [explant^{-1}]	DNP response [%]	number of embryos [explant^{-1}]	ACC response [%]	number of embryos [explant^{-1}]	CEPA response [%]	number of embryos [explant^{-1}]
0.0	53.3±2.0a	12.3±0.4a	55.0±1.9e	12.9±0.3c	51.7±1.7d	10.1±0.4d	48.3±1.7d	9.3±0.3c	51.1±2.0e	9.2±0.4d
0.5	55.0±1.9a	15.8±0.3bc	41.7±1.7d	11.2±0.5c	33.3±2.0c	6.0±0.3c	41.7±1.7d	9.4±0.3c	36.7±1.8d	7.7±0.1cd
5.0	68.3±1.7b	15.1±0.4ab	35.0±1.9cd	6.0±0.3b	18.3±0.6b	4.3±0.2bc	26.7±2.3c	5.9±0.3b	26.7±2.5cd	6.2±0.4c
25.0	78.3±2.0c	17.9±0.4c	28.3±1.7bc	6.5±0.3b	0.0a	0.0a	20.0±2.5bc	4.9±0.3ab	16.7±1.8bc	3.9±0.3b
50.0	83.3±1.8c	22.1±0.6d	18.3±2.0ab	6.4±0.4b	0.0a	0.0a	13.3±2.0ab	3.9±0.3a	11.7±1.7ab	3.1±0.2ab
75.0	68.3±2.6b	13.6±0.4ab	11.7±1.7a	3.0±0.2a	0.0a	0.0a	8.3±1.7a	3.2±0.2a	5.0±1.2a	1.7±0.1a

Table 5. Effect of $AgNO_3$ on somatic embryo maturation (after 3 weeks of culture) and germination (after 4 weeks of culture) from excised cotyledonary explants of *C. tinctorius* L. cv. Girna.

$AgNO_3$ [μM]	Globular [%]	Heart-shaped [%]	Torpedo-shaped [%]	Cotyledonary [%]	Germination [%]
0.0	25.2	22.1	26.9	25.7	17.2
0.5	42.0	24.1	17.4	16.7	16.0
5.0	37.6	25.3	19.3	17.7	13.5
25.0	35.0	23.6	22.3	19.2	15.9
50.0	30.0	23.6	23.0	23.0	15.0
75.0	28.9	27.7	22.7	20.7	14.5

Embryo maturation and germination were also influenced by ethylene inhibitors. The highest number of mature embryos at cotyledonary stage (23 %) was observed at 50 μM concentration of $AgNO_3$ (Table 5). A decrease in percent germination was observed with increasing concentration of $AgNO_3$. $NiCl_2$ and DNP inhibited both embryo development and germination (data not presented). Enhancement of embryo maturation by $AgNO_3$ in white spruce was thought to be due to an increase in endogenous abscisic acid content (Kong and Yeung 1994).

Addition of ethylene promoters - ACC (ethylene biosynthesis precursor) and CEPA (exogenous source of ethylene) to the medium significantly reduced the somatic

embryogenesis and number of somatic embryos per explant (Table 4). The inhibition was also noticed on embryo maturation and germination (data not presented). This result shows that ethylene has an inhibitory effect on somatic embryogenesis in safflower as has been demonstrated in other plants (Songstad *et al.* 1989, Auboiron *et al.* 1990, Rosutan *et al.* 1990).

References

Amirato, P.V.: Embryogenesis. - In: Evans, D.A., Sharp, W.R., Amirato, P.V., Yamada, Y. (ed.): Handbook of Plant Cell Culture. Vol. I. Pp. 82-123. MacMillan, New York 1983.

Auboiron, E., Carron, M.P., Michaux Ferriere, N.: Influence of atmospheric gases, particularly ethylene, on somatic embryogenesis of *Hevea brasiliensis*. - Plant Cell Tissue Organ Cult. **21**: 31-37, 1990.

Bingham, E.T., Hurley, L.V., Kaatz, D.M., Saunders, J.W.: Breeding alfalfa which regenerates from callus tissue in culture. - Crop Sci. **15**: 719-721, 1975.

Brown, D.C.W., Atanassov, A.: Role of genetic background in somatic embryogenesis in *Medicago*. - Plant Cell Tissue Organ Cult. **4**: 111-122, 1985.

Chen, T.H.H., Marowitch, J., Thompson, B.G.: Genotypic effects on somatic embryogenesis and plant regeneration from callus cultures of alfalfa. - Plant Cell Tissue Organ Cult. **8**: 73-81, 1987.

Eapen, S., George, L.: Somatic embryogenesis in peanut: Influence of growth regulators and sugars - Plant Cell Tissue Organ Cult. **35**: 151-156, 1993.

Fraley, R.T., Rogers, S.G., Horsch, R.B.: Genetic transformation in higher plants. - CRC crit. Rev. Plant Sci. **4**: 1-46, 1986.

George, L., Rao, P.S.: *In vitro* multiplication of safflower (*Carthamus tinctorius* L.) through tissue culture - Proc. indian Acad. Sci. **99**: 293-296, 1982.

Halperin, W.: Attainment and retention of morphogenic capacity *in nitro*. - In: Vasil, I.K. (ed.): Cell Culture and Somatic Cell Genetics of Plants. Vol. 3. Plant Regeneration and Genetic Variability. Pp. 3-47. Academic Press, Orlando 1986.

Hasemann, C.U., Schroder, G.: Loss of nuclear DNA in leaves of rye. - Theor. appl. Genet. **62**: 128-131, 1982.

Hatanaka, T., Sawabe, E., Azuma, T., Uchida, N., Yusuda, T.: The role of ethylene in somatic embryogenesis from leaf discs of *Coffea canephora*. - Plant Sci. **107**: 199-204, 1995.

Kong, L., Yeung, E.C.: Effects of ethylene and ethylene inhibitors on white spruce somatic embryo maturation. - Plant Sci. **104**: 71-80, 1994.

Lazzeri, P.A., Hildebrand, D.F., Collins, G.B.: Soybean somatic embryogenesis. Effects of nutritional, physical and chemical factors. - Plant Cell Tissue Organ Cult. **10**: 209-220, 1987.

Mandal, A.K.A., Chatterji, A.K., Dutta Gupta, S.: Direct somatic embryogenesis and plantlet regeneration from cotyledonary leaves of safflower. - Plant Cell Tissue Organ Cult. **43**: 287-289, 1995.

Mroginski, L.A., Kartha, K.K.: Tissue culture of legumes for crop improvement. - Plant Breed. Rev. **2**: 215-264, 1984.

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

Orlikowska, T.K., Dyer, W.E.: *In vitro* regeneration and multiplication of safflower (*Carthamus tinctorius* L.). - Plant Sci. **93**: 151-157, 1993.

Ozias-Akins, P., Anderson, W.F., Holbrook, C.C.: Somatic embryogenesis in *Arachis hypogaea* L.: genotype comparison. - Plant Sci. **83**: 103-111, 1992.

Rosutan, J.P., Latche, A., Fallot, J.: Control of carrot somatic embryogenesis by $AgNO_3$, an inhibitor of ethylene action: effect on arginine decarboxylase. - Plant Sci. **67**: 89-95, 1990.

Songstad, D.D., Petracek, P.D., Sams, C.E., Conger, B.V.: Effect of 1-aminocyclopropane-1-carboxylic acid and aminoethoxyvinyl-glycine on ethylene emanation and somatic embryogenesis from orchard grass leaf cultures. - Plant Cell Rep. **7**: 677-679, 1989.

Vain, P., Flament, P., Soudan, P.: Role of ethylene in embryogenic callus initiation in *Zea mays* L. - J. Plant Physiol. **135**: 537-540, 1989.