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BRIEF COMMUNICATION

Proliferation and differentiation from endosperms of *Carthamus tinctorius*

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Abstract

The endosperms of *Carthamus tinctorius* cv. HUS-305, excised at globular to heart-shaped stages of zygotic embryo development, were cultured on Murashige and Skoog's medium (MS) supplemented with different concentrations of 6-benzylaminopurine (BAP), kinetin, thidiazuron (TDZ), 2,4-dichlorophenoxyacetic acid (2,4-D) or α-naphthaleneacetic acid (NAA). The highest incidence of callusing was on 2,4-D supplemented media. However, embryos differentiated only from the calli developed on media supplemented with BAP, kinetin or TDZ with the last eliciting maximum embryogenic response. The addition of a reduced nitrogen source, casein hydrolysate to MS medium supplemented with BAP and/or NAA, did not stimulate the response. However, adenine sulphate (100 mg dm^{-3}) promoted the induction of somatic embryos. Upon transfer to MS basal medium or the same supplemented with 0.61 μM gibberellic acid (GA_3), plumular poles of few embryos elongated resulting in the development of shoots.

Additional key words: *Asteraceae*, endosperm culture, growth regulators, safflower, triploids.

Polyploids are reported to have some advantage over diploids. In case of oil crops they show better grain filling, high oil content, enhanced photosynthetic ability, delayed maturity and increased biomass (Li *et al.* 1999). Conventionally, triploids are raised by crossing tetraploid with a diploid parent or alternatively, triploids can be regenerated from endosperms. These are then either doubled to raise hexaploids, or further crossed with a diploid or a tetraploid parent to obtain plants of different ploidies. For instance, the tetraploids and pentaploids of *Tripascum*, have been produced by hybridizing a diploid and a tetraploid male parent, respectively, with a triploid female parent (Li *et al.* 1999).

A similar approach can be adopted for improving *Carthamus tinctorius*, an oil-yielding crop. Besides polyploids, the importance of triploids of *Carthamus* as a source of trisomics, which are used for linkage mapping studies (Tsuchiya 1969, Fastnaught and Ramage 1974, Wijsman and Berg 1982), has also been greatly realized (Schank and Knowles 1961, Kumar 1991). The attempts to produce triploids of *Carthamus tinctorius* by crossing diploid and tetraploid parents did not succeed because of the production of abortive embryos (Schank and Knowles 1961). Thus, endosperm culture of *Carthamus tinctorius* w*as* attempted. The present communication reports, for the first time, proliferation and differentiation from the endosperms of *Carthamus tinctorious*. Incidentally, this would be the first such report in the family *Asteraceae*.

The *Carthamus tinctorius* L. cv. HUS-305 plants were raised in October in the garden of Department of Botany, University of Delhi, Delhi. During May and June, capitula containing immature achenes were picked 7 - 8 d after pollination (DAP). Achenes, which were 6 - 8 mm long and cream-coloured were selected and washed with 5 % (v/v) *Teepol* (*Qualigens*, Mumbai, India) for 15 min, followed by a treatment with 0.2 % (m/v) $HgCl_2$ for 15 min. After rinsing thrice with sterilized distilled water, achenes were treated with 0.01 % (m/v) streptomycin for 10 min and thereafter, rinsed twice with sterile water.

Endosperms along with embryos were dissected aseptically. While dissecting, care was taken to avoid puncturing of the endosperms. The isolated endosperms were cultured, with their micropylar ends (containing embryos) facing towards the base of the slant of the medium.

All media were gelled with 0.8 % (m/v) agar (*Qualigens*), supplemented with 3 % (m/v) sucrose, pH was adjusted to 5.8 with 1 M NaOH or HCl and sterilized at 103 kPa and 121 ºC for 15 min. However, certain chemicals such as gibberellic acid (GA_3), abscisic acid

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Abbreviations: ABA - abscisic acid; BAP - 6-benzylaminopurine; 2,4-D - 2,4-dichlorophenoxyacetic acid; GA_3 - gibberellic acid; NAA - α-naphthaleneacetic acid; TDZ - thidiazuron.
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(ABA), thidiazuron (TDZ), adenine sulfate and glutamine were filter-sterilized. For all experiments, except the one involving culture of endosperms in liquid medium and for sub-culture of shoots, culture tubes (25 × 150 mm, *Borosil*, Mumbai, India) each containing 20 cm^3 of culture medium and plugged with non-adsorbent cotton wrapped in cheese cloth were used. The growth regulators and adjuvants were procured from *Sigma Chemicals Co*., St. Louis, USA. In all experiments, cultures were initially incubated in dark, and after induction of calli, transferred to continuous light (irradiance of 17 µmol m^{-2} s^{-1}) provided by cool daylight fluorescent tubes (40 W, *Philips*) at 25 ± 2 ºC.

In the first experiment, isolated endosperms were cultured on MS (Murashige and Skoog 1962) medium supplemented with various growth regulators, 6-benzyl-aminopurine (BAP, 2.22, 4.44 or 8.88 µM), kinetin (Kn, 2.32, 4.65 or 9.29 µM), α-naphthaleneacetic acid (NAA, 2.69, 5.37 or 10.74 µM), 2,4-dichlorophe-noxyacetic acid (2,4-D, 2.26, 4.52 or 9.05 µM) or TDZ (0.91 or 2.27 µM). In the second experiment, endosperms were cultured on MS medium containing BAP (2.22, 4.44 or 8.88 µM) and/or NAA (2.69, 5.37 or 10.74 µM), and supplemented with casein hydrolysate (CH, *Hi Media Laboratories*, Mumbai, India) at 1.0 g dm^{-3}. The embryos and the calli that developed in these experiments were transferred after three weeks to MS, ½ MS, MS + GA_3 (0.61 µM), ½ MS + GA_3 (0.61 µM), MS + 12 % sucrose or MS + ABA (1.89 or 4.92 µM).

In the third experiment, endosperms were cultured in 100-cm^3 flasks containing 25 cm^3 liquid MS medium supplemented with BAP (2.22 or 4.44 µM) and/or 2,4-D (2.26 or 4.52 µM). The flasks were incubated on a shaker (*New Brunswick Scientific Co.*, Edison, USA) at 100 rpm. After 10 d, the calli developed in liquid media were transferred to agar-gelled MS medium supplemented with 4.44 µM BAP + glutamine and/or adenine sulphate (*HiMedia Laboratories*) at 50 and 100 mg dm^{-3}.

In the fourth experiment, zygotic embryos were excised from the endosperms cultured on MS medium supplemented with BAP (4.44 µM) for one week and the residual endosperms, which had started callusing by that time, were cultured back on the same medium. In the fifth experiment, prior to culture, embryos were dissected out of the endosperms by gently pressing the latter. These embryo-less endosperms were cultured on MS + BAP 4.44 µM with or without a preceding treatment of 1.52 or 3.03 µM GA_3 in liquid MS medium for 6 or 24 h.

At least 30 endosperms were cultured for each treatment of the five experiments described above. Each experiment was repeated twice. The data were subjected to one-way analysis of variance and means were compared using Tukey's HSD test ($P = 0.05$).

For cytological analysis, peel mounts of leaves from the cotyledon-derived (following the protocol of Walia *et al*. 2005) and endosperm-derived shoots were prepared. The leaves of each shoot were taken separately in individual culture tubes and warmed in nitric acid (40 %) till mesophyll tissue dissolved. Peels were washed 3 - 4 times with water by slow pipetting. Subsequently, the peels were de-acidified with KOH (one pellet in 25 cm^{-3} of water) followed by 3 - 4 washes with water. Thereafter, peels were left overnight in 1 % (m/v) safranin and then washed with water to remove extra stain. Peels from five leaves of each type of shoots, were studied. For each peel, ten microscopic fields were observed under light microscope (*Spencer*, Buffalo, USA). The stomatal dimensions were measured using ocular and stage micrometers. This experiment was repeated twice.

The stage at which intact endosperms could be dissected was 7 - 8 days after pollination (DAP). Therefore, immature achenes (Fig. 1*A*) with embryos at globular to heart shape stages (Fig. 1*B*) of development were used as source of endosperms. At these stages, endosperm is mostly nuclear free.

The endosperms cultured with embryos on MS basal medium or the same supplemented with different growth regulators exhibited different types of response. These were either further growth of the zygotic embryos, callusing and subsequent differentiation of secondary embryos from the zygotic embryos, as reported for *Castanea sativa* (Sauer and Wilhelm 2005), and/or callusing from only the endosperm portion followed by differentiation of embryos (Fig. 1*C*,*D*,*E*). The callusing from the endosperms took place during the dark incubation. The calli had globular shiny structures. However, further development of embryos to heart-shaped and cotyledonary stages and differentiation of secondary embryos from these took place when cultures were transferred to light after two weeks of dark incubation (Fig. 1*F*). In few cultures, upon transfer to light, zygotic embryos turned green and started developing. However, the observations described hereafter refer to the developments (callusing and/or embryogenesis) only from the endosperm portion of the explants and not from the zygotic embryos.

In the first experiment, the endosperms cultured on media supplemented with anyone of the growth regulators, started callusing within a week of culture. Though the percentage of callusing endosperms on these varied numerically, the differences were not statistically significant (Table 1). In earlier studies, either an auxin alone (Lakshmi Sita *et al.* 1980, Thomas *et al.* 2000) or in combination with a cytokinin (Bhojwani and Johri 1971, Nag and Johri 1971, Srivastava 1971a,b, Lakshmi Sita *et al.* 1980, Kumar *et al.* 1985, Tulecke *et al.* 1988, Thomas *et al.* 2000) has been reported to support callusing response from the endosperms. In cases where callusing was induced by auxin alone, cultures were transferred to medium supplemented with both auxin and cytokinin for further growth and differentiation. However, in the present study, besides 2,4-D and NAA, callusing was also observed on media supplemented with cytokinin alone (BAP or kinetin, Table 1). The calli, which developed on BAP, kinetin or TDZ supplemented media, had globular shiny structures which grew up into cotyledonary stage embryos, after one week of transfer to light (Fig. 1*E*,*F*,*G*,*H*). The percentages of embryogenic

endosperms in these treatments though varied numerically were not significantly different. However, average number of heart-shaped to cotyledonary stages of embryos per responding explant was significantly higher in medium supplemented with 2.27 µM TDZ than all other treatments (Table 1). The effect of TDZ in embryogenesis has been widely reported in different species (Visser *et al.* 1992, Ravishankar and McComb 2002, Vila *et al.* 2003).

In the second experiment, endosperms (along with zygotic embryos) were cultured on MS basal medium fortified with 1.0 g dm^{-3} casein hydrolysate and the same supplemented with BAP and NAA in different concentrations and combinations. Though first and second experiments were not conducted simultaneously, CH appeared to inhibit the overall response (callusing and embryogenic), when compared with the corresponding media lacking CH (Tables 1, 2).

In the present study, endosperms cultured on solidified medium shriveled probably due to lower osmotic potential of the medium than that of the endosperm. Therefore, in the third experiment, endosperms were pre-cultured on liquid medium supplemented with different concentrations and combinations of BAP and 2, 4-D. After 10 d 8.3 % endosperms on 4.44 µM BAP, 6.6 % on 4.44 µM BAP + 4.52 µM 2,4-D and 3.3 % on 2.26 µM 2,4-D exhibited callusing (detailed data not presented). The calli obtained on these media were transferred to agar-gelled MS media supplemented with 4.44 µM BAP and fortified with glutamine or adenine sulphate at 50 and 100 mg dm^{-3}. Embryos started differentiating after 2 weeks. Among all the media, best embryogenic response, approximately 30 embryos per endosperm, was observed on MS medium supplemented with 100 mg dm^{-3} adenine sulfate followed by 20 in 50 mg dm^{-3}. On glutamine adjuvated media only globular structures were formed. In all the cultures differentiation of secondary embryos was also observed.

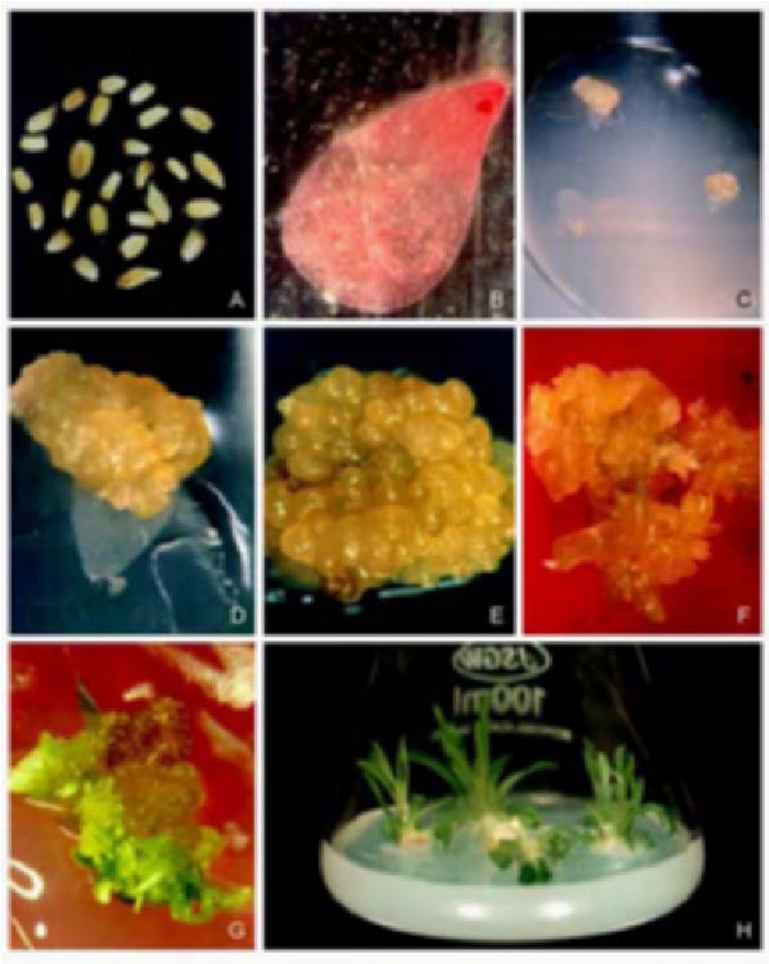

Fig. 1. Proliferation and differentiation from endosperms of *Carthamus tinctorius*: *A* - immature achenes collected from heads, 7 - 10 d after pollination; *B* - a dissected endosperm along with zygotic embryo at heart shape stage of development; *C* and *D* - development of calli from endosperm on MS medium supplemented with 4.44 µM BAP, after 5 weeks of culture; *E* - development of embryos from endosperm on MS medium supplemented with 2.27 µM TDZ after 4 weeks of incubation; *F* - development of embryos from endosperm on MS + 4.44 µM BAP + 100 mg dm^{-3} adenine sulphate after 5 weeks of incubation; *G* - germination of embryos, developed from endosperm, after they were transferred to light after 2 weeks of incubation in dark; *H* - shoots developed from unipolar (plumular) endosperm-derived embryos, on MS basal medium, after 4 weeks of incubation.

Table 1. Response of endosperms of *Carthamus tinctorius* on MS medium supplemented with different concentrations of plant growth regulators. * - only value significantly different from other corresponding values at $P \leq 0.05$.

Concentration [μM]	Callusing [%]	Embryogenesis [%]	Number of embryos [explant^{-1}]
2.22 BAP	7.61	5.43	11.2 ± 2.38
4.44 BAP	7.61	6.52	12.2 ± 2.50
8.88 BAP	3.16	3.16	10.7 ± 5.75
2.32 kinetin	7.77	7.77	6.0 ± 2.33
4.65 kinetin	5.49	4.39	12.0 ± 3.75
9.29 kinetin	3.33	3.33	9.3 ± 0.50
2.26 2,4-D	9.18	0	0
4.52 2,4-D	9.68	0	0
9.05 2,4-D	8.25	0	0
2.69 NAA	4.60	0	0
5.37 NAA	6.52	0	0
10.74 NAA	5.43	0	0
0.91 TDZ	3.30	2.20	10.0 ± 2.00
2.27 TDZ	6.60	5.50	35.0 ± 5.00*

The somatic embryos developed from the endosperm calli on various media did not grow beyond two leaf or cotyledonary stage and started callusing. Therefore these embryos were transferred to different media (MS, ½ MS, MS + 0.61 μM GA_3, ½ MS + 0.61 μM GA_3, MS + 12% sucrose, MS + 1.89 μM ABA and MS +4.92 μM ABA). On MS and ½ MS + 0.61 μM GA_3, 10 and 7.4 % embryos, respectively, exhibited unipolar growth as has been reported for *Clematis integrifolia* × *C. viticella* (Mandegaran and Sieber 2000). The plumular axis of such embryos elongated into shoots (Fig 1*H*), while radicular end callused. Other media did not support any further growth of embryos. The shoots developed on the responsive media were transferred to rooting medium (1/2 MS + 1.07 μM NAA). However, these shoots failed to form roots and turned brown after 4 weeks of transfer. The difficulty in rooting of regenerated shoots of safflower is not only restricted to endosperm-derived shoots, but is also encountered in shoots differentiated from seedling-derived explants (George and Rao 1982, Nikam and Shitole 1999, Walia *et al.* 2005).

The residual endosperm calli, left after excision and sub-culture of embryos, were transferred to media with different concentrations of BAP (0 - 8.88 μM) or kinetin (0 - 9.29 μM). On media supplemented with BAP, these calli kept on differentiating embryos for a month. Subsequently, the calli even if transferred to fresh medium did not differentiate further. The calli transferred to kinetin supplemented media did not exhibit response even during first passage.

As in few cases along with endosperms, embryos also proliferated, in the fourth experiment, to avoid mixing of callus/regenerants from these two sources, embryos were dissected out from the endosperms, after a week of culture on MS + 4.44 μM BAP. However, dissection of embryos mutilated the endosperms, which had started callusing by that time. Such endosperms along with calli turned brown within a week of embryo excision.

Table 2. Effect of media fortified with 1.0 g dm^{-3} casein hydrolysate and different concentrations and combinations of BAP and/or NAA on the response of endosperms of *Carthamus tinctorius*.

Concentration [μM]	Callusing [%]	Embryo-genesis [%]	Number of embryos [explant^{-1}]
4.44 BAP	5.45	3.92	5.5 ± 3.5
2.69 NAA	3.64	0	0
5.37 NAA	3.85	0	0
10.74 NAA	2.13	0	0
2.22 BAP + 2.69 NAA	4.00	2	3.0 ± 0.75
2.22 BAP + 5.37 NAA	4.25	0	0
2.22 BAP + 10.74 NAA	8.10	0	0
4.44 BAP + 2.69 NAA	3.92	1.96	9.0 ± 4.5
4.44 BAP + 5.37 NAA	11.11	0	0
4.44 BAP + 10.74 NAA	6.12	2.04	10.0 ± 5.0
8.88 BAP + 2.69 NAA	1.89	1.89	1.0 ± 0.5
8.88 BAP + 5.37 NAA	8.70	0	0
8.88 BAP + 10.74 NAA	1.82	0	0
2.27 TDZ	6.60	5.50	35.0 ± 5.0*

In the experiment number five, zygotic embryos were released from the freshly isolated endosperms by pressing the later. Such embryo-less endosperms were either cultured as such on MS + 4.44 μM BAP or these were pretreated with GA_3 (1.52 - 3.03 μM) for 6 or 24 h prior to culture on the same medium. Both treated and untreated endosperms failed to elicit any morphogenic response thus indicating obligatory requirement of embryo for endosperm prolifertion. The obligate requirement for embryo has also been observed for *in vitro* proliferation of mature endosperms of *Croton* (Bhojwani 1966). However, for further growth, continued association of embryo was not required. Similarly, Kumar *et al.* (1985) observed association of zygotic embryo to be obligatory for proliferation of endosperms of *Cocos nucifera*. Thomas *et al.* (2000) also affirmed the requirement of embryo during initial phase of endosperm culture of *Morus.* It is thought that during initial stage of culture, embryo releases some hormone(s) that is/are necessary for proliferation from endosperms. The 'embryo factor' released by embryo is thought to be gibberellic acid (GA_3), which brings about metabolic changes in endosperms (Paleg 1960, Ingle and Hageman 1965). In the present study, the obligate requirement of embryo was not restricted only to the initiation of callus but even for its survival. The attempt to replace the 'embryo factor' with GA_3 met with little success. Probably, the removal of embryo and subsequent treatment with GA_3 led to washing away of endosperm nuclei and hence accounting for zero response in such cultures.

Stomatal frequency, guard cell length and stomatal plastid number have often been used as morphological markers for identifying ploidy levels in many plant species, *e.g*. alfalfa (Bingham 1968) and cotton (Krishnaswamy and Andal 1978). In *Carthamus tinctorius,* owing to the difficulty in rooting of shoots of *in vitro* raised plants, average stomata size of endosperm-derived shoots and the somatic tissue-derived shoots (control) was examined. The stomata from leaves of endosperm-derived shoots were slightly longer (0.027 ± 0.003 mm) and wider (0.025 ± 0.003 mm) than those from leaves of control derived-shoots (0.026 ± 0.003 and 0.020 ± 0.003 mm, respectively). Morphologically, the stomata from endosperm derived shoots appeared almost spherical as is also evident from the data.

Although, morphometric analysis of the shoots originating from endosperms points towards the differences in ploidy vis-à-vis with shoots from somatic explants, triploid nature could not be ascertained owing to the failure in rooting of the regenerated shoots. Thus, the application of this technique in breeding, would be possible only if triploid plants are regenerated. However, the present report though preliminary in nature, unequivocally demonstrates the morphogenic potential of safflower endosperms.

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