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BRIEF COMMINICATION

In vitro bud regeneration of *Carthamus tinctorius* and wild *Carthamus* species from leaf explants and axillary buds

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Abstract

The organogenic competence of leaf explants of eleven *Carthamus* species including *C. tinctorius* on Murashige and Skoog (MS) medium supplemented with different concentrations of thidiazuron (TDZ) + α-naphthaleneacetic acid (NAA) and 6-benzyladenine (BA) + NAA was investigated. Highly prolific adventitious shoot regeneration was observed in *C. tinctorius* and *C. arborescens* on both growth regulator combinations and the shoot regeneration frequency was higher on medium supplemented with TDZ + NAA. Nodal culture of nine *Carthamus* species on media supplemented with BA and kinetin (KIN) individually revealed the superiority of media supplemented with BA over that of KIN in facilitating a higher shoot proliferation index. Proliferating shoots from axillary buds and leaf explants were transferred to medium supplemented with 1.0 mg dm^{-3} KIN or 0.5 mg dm^{-3} BA for shoot elongation. Elongated shoots were rooted on half-strength MS medium supplemented with 1.0 mg dm^{-3} each of indole-butyric acid (IBA) and phloroglucinol. The plantlets thus obtained were hardened and transferred to soil.

Additional key words: direct organogenesis, growth regulators, *in vitro* culture, tissue culture, wild safflowers.

Safflower (*Carthamus tinctorius* L.) of the family *Asteraceae* is one of oilseed crops. Successful cultivation of safflower is constrained by the vulnerability to biotic and abiotic stresses. Wild safflowers serve as a useful and rich source for resistance genes (Ashri 1971, Kumar 1991). The genus *Carthamus* is composed of 16 species with basic chromosome numbers of 10, 11, 12, 22 and 32 and *C. tinctorius* has 12 pairs of chromosomes and interspecific hybridization through conventional techniques is difficult. There is a need to develop protocols of tissue culture for large scale multiplication and germplasm maintenance and conservation. The amenability of safflower tissues to *in vitro* manipulations is evident from simple media requirements for direct and indirect caulogenesis and/or embryogenesis for Indian as well as American cultivars (George and Rao 1982, Ying *et al*. 1992, Orlikowska and Dyer 1993, Mandal *et al.* 1995, 2001, Sujatha and Suganya 1996, Nikam and Shitole 1999, Radhika *et al.* 2006). Murashige and Skoog (MS) medium supplemented with 6-benzyladenine (BA) and α-naphtha-leneacetic acid NAA has been frequently used for safflower tissue culture. Combination of thidiazuron (TDZ) + NAA also favoured high frequency of shoot regeneration from leaf explants (Orlikowska and Dyer 1993). Studies carried out in our laboratory revealed positive influence of TDZ + NAA and BA + NAA on induction of caulogenic response from seedling explants of *C. tinctorius* (Sujatha and Suganya 1996, Radhika *et al*. 2006). The present investigation has been undertaken to assess the efficacy of TDZ + NAA and BA + NAA combinations in stimulating the organogenic competence of the leaf explants of *Carthamus* species and to optimize conditions for axillary bud proliferation on MS with BA or KIN for establishment of *Carthamus* species *in vitro.*

Carthamus species (*C. boissieri, C. dentatus, C. glaucus, C. oxyacantha, C. palaestinus, C. arborescens, C. lanatus, C. turkestanicus, C. creticus* and *C. montanus*) obtained from Dr. Andreas Boerner, IPK Genebank, Gatersleben, Germany and *C. tinctorius* (cv. HUS-305) from the Germplasm Management Unit of Safflower, Hyderabad, India were maintained in a greenhouse. Leaves of 11 *Carthamus* species including *C. tinctorius* collected at 2 physiological stages (rosette stage and stem elongation stage) were used in the study.

The leaves and stem segments (7.5 to 10 cm) were

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Abbreviations: BA - 6-benzyladenine; IBA - indole-butyric acid; KIN - kinetin; NAA - α-naphthaleneacetic acid; TDZ - thidiazuron.
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washed thoroughly under running tap water for 30 min and surface sterilized with 0.1 % (m/v) mercuric chloride for 6 - 8 min depending on the species. Following four rinses of 5 min each in sterile distilled water, the leaves were cut into 1.0 cm^2 pieces and placed with the abaxial side in contact with the medium surface. Nodal explants of nine *Carthamus* species were collected at the stage when the rosette stage was completed and stem elongation occurred. Nodal sections of 1.0 cm along with the axillary buds were cut and explanted on the medium surface.

The culture medium consisted of full strength MS basal salts (Murashige and Skoog 1962) with 3 % sucrose. The growth regulator (*Sigma Co*, St Louis, USA) combinations tested for leaf segments included two combinations of 6-benzyladenine (BA) (1.0 mg dm^{-3}) + α-naphthaleneacetic acid (NAA) (0.5 - 1.0 mg dm^{-3}) and four combinations of thidiazuron (TDZ) (0.2 - 1.0 mg dm^{-3}) + NAA (0.2 - 1.0 mg dm^{-3}). Nodal segments were initially cultured on MS basal salt medium supplemented with 0.5 mg dm^{-3} BA for a week. Following bud break, the responding nodal cultures were transferred to medium supplemented with BA (0.5 - 1.0 mg dm^{-3}) and kinetin (KIN; 0.5 - 2.0 mg dm^{-3}).

Proliferating shoots were multiplied on medium supplemented with 0.5 mg dm^{-3} BA. For shoot elongation, proliferated shoots were transferred to medium supplemented with either 0.5 mg dm^{-3} BA or 1.0 mg dm^{-3} KIN. For rooting, initially elongated shoots of *C. tinctorius* were tested on medium supplemented with 0.25 to 5.0 mg dm^{-3} of 2,4,5-trichlorophenoxy-propionic acid (2,4,5-Cl_3-POP). Subsequently, rooting ability of *Carthamus* species including *C. tinctorius* was tested on half-strength MS medium supplemented with either 1.0 mg dm^{-3} NAA or 1.0 mg dm^{-3} inole-butyric acid (IBA) in combination with 1.0 mg dm^{-3} phloro-glucinol. Shoots with well-formed roots were transferred to sterile *Vermiculite* and maintained under high humidity for a week. After another week in *Vermiculite*, the established plantlets were transferred to soil.

The medium was adjusted to pH 5.7 ± 1.0 with 0.1 M NaOH, solidified with 0.7 % agar (*HiMedia*, Mumbai, India) and autoclaved for 20 min at 121 °C and 1.06 kg cm^{-2}. The cultures were maintained in a growth room at 26 ± 2 °C under a 16-h photoperiod, with irradiance of 30 μmol m^2 s^{-1} provided by cool white fluorescent tubes. The leaf explants were cultured either in culture tubes (25 × 150 mm) or 9.0 cm Petri dishes containing 20 cm^3 medium.

Observations on the number of explants responding to callus induction and shoot regeneration were made at weekly intervals and tabulated four weeks after culture initiation. Data on the number of shoots per regenerating explant was not recorded as the shoots were innumerable on medium supplemented with TDZ + NAA and the propensity to regenerate continued even after transfer of the material to subculture medium. Each treatment for leaf culture consisted of 6 Petri plates with 15 - 20 explants each and repeated thrice. For axillary bud proliferation, each treatment consisted of 12 culture tubes with one nodal explant each and replicated thrice. Number of shoots per proliferating explant was recorded 3 weeks after culture initiation. Data was subjected to analysis of variance using the *MSTATC* program and the means were separated according to LSD at $\alpha = 0.05$.

The cultured leaf explants of nine *Carthamus* species responded within a week after culture initiation and formed either callus or differentiated into shoots while leaf segments of *C. glaucus* and *C. creticus* failed to respond and remained green or turned brown. There were no differences in the response of the leaf segments collected either at rosette or stem elongation stage. With the exception of *C. tinctorius* and *C. arborescens,* the other species formed callus, which varied in its morphology. Leaf tissues of *C. lanatus* and *C. montanus* produced green nodular callus; those of *C. turkestanicus* formed flaky dry callus while those of other species formed white watery callus. Distinct differences were not detected in callus morphology in various combinations of the two tested cytokinins but the frequency of responding explants was higher in TDZ supplemented media when compared to BA supplemented media. Prolonged culture on the same medium or transfer of the calli to media supplemented with six combinations of BA (0.2 - 1.0 mg dm^{-3}) with NAA (0.1 - 0.5 mg dm^{-3}) failed to facilitate shoot differentiation from these calli. The callus induced from leaf segments of *C. oxyacantha* formed green nodular structures on the subculture medium but caulogenesis was not observed.

Table 1. Frequency of shoot regeneration [%] from leaf segments of *C. tinctorius* and *C. arborescens* on MS medium supplemented with TDZ or BA in conjunction with NAA [mg dm^{-3}]. Percentage values were angular transformed prior to analysis. Means followed by same letters are not significantly different according to LSD at $\alpha = 0.05$. Data was scored 4 weeks after culture.

NAA	TDZ	BA	*C. tinctorius*	*C. arborescens*
0.2	1.0	-	45.1^{e}	91.7^{b}
0.2	0.5	-	55.6^{e}	100.0^{a}
0.2	0.2	-	71.1^{d}	94.4^{b}
1.0	0.2	-	14.3^{fg}	100.0^{a}
1.0	-	1.0	23.3^{f}	76.7^{c}
0.5	-	1.0	13.3^{g}	85.0^{c}

Two species failed to respond while leaf tissues of 7 species failed to differentiate shoots either through direct or indirect organogenesis. In this study, leaf tissues were chosen because of their availability in abundance and in all seasons. Earlier studies on safflower tissue culture proved the amenability of various tissues including roots for morphogenic differentiation (Mandal *et al*. 1995, Sujatha and Suganya 1996). Hence, for the wild safflower species in which leaves expressed

recalcitrance, other tissues can be tested for their caulogenic ability.

Leaf segments of *C. tinctorius* produced, callus, callus + shoots or shoots from the cut ends (Fig. 1*A*). In *C. arborescens,* countless number of shoots originated directly without an intervening callus not only from cut ends but also from all over the explant (Fig. 1*B*). The shoot regeneration frequency in *C. arborescens* was high, reproducible and significantly higher than that of *C. tinctorius* (Table 1). The frequency of shoot regeneration in both the species was higher on TDZ + NAA supplemented media as compared to BA + NAA supplemented media. On TDZ + NAA supplemented media, the frequency of shoot regeneration from *C. tinctorius* varied from 14.3 to 71.1 % and on BA + NAA it was 13.3 to 23.3 %. Leaf segments of *C. arborescens* regenerated shoots with a high frequency of 91.7 to 100 % on TDZ + NAA supplemented media and 76.7 to 85 % on BA + NAA fortified media. Hyperhydricity is a common problem in such prolific adventitious shoot regenerating systems but such problem was not evident in *C. arborescens*. Proliferating cultures of *C. arborescens* formed well-differentiated shoots on transfer to medium supplemented with 0.5 mg dm^{-3} BA (Fig. 1*C*).

C. arborescens with 12 pairs of chromosomes belongs

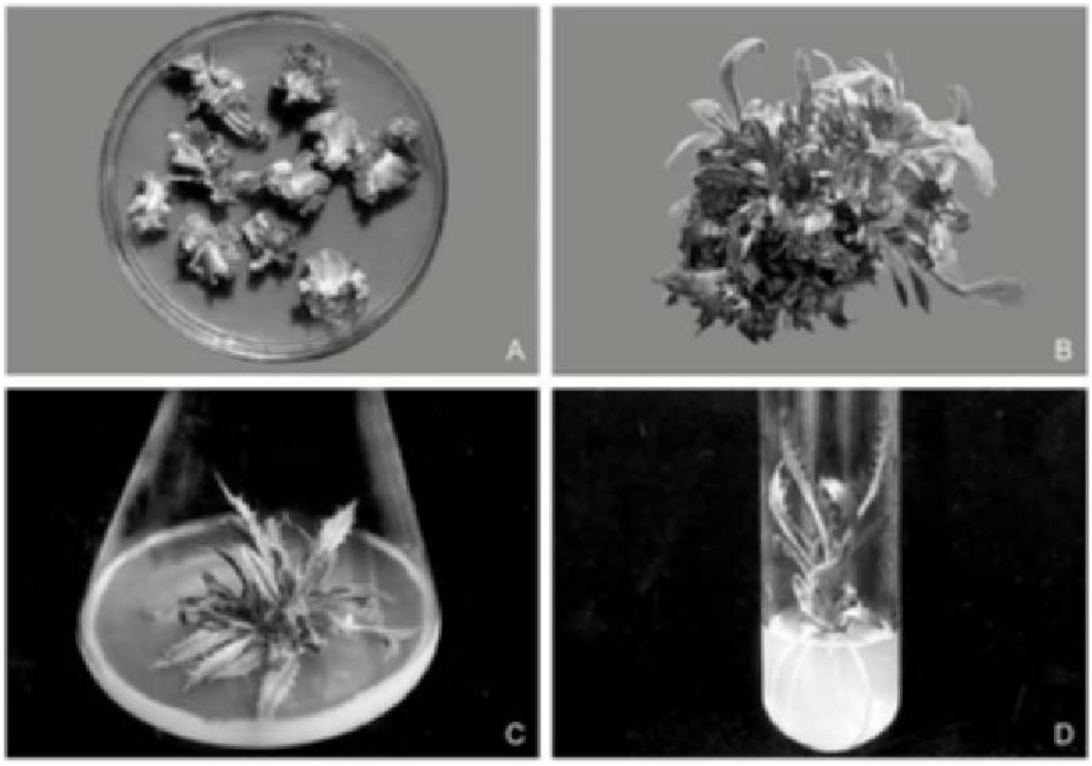

Fig. 1. Adventitious shoot regeneration from leaf segments of *Carthamus* species. *A* - Leaf explants of *C. tinctorius* showing the formation of callus, callus + shoots and shoots from cut ends on medium supplemented with 0.5 mg dm^{-3} TDZ + 0.2 mg dm^{-3} NAA. *B* - Prolific shoot regeneration from entire leaf explant of *C. arborescens* on medium supplemented with 0.5 mg dm^{-3} TDZ + 0.2 mg dm^{-3} NAA. *C* - Multiplication and elongation of shoots of *C. arborescens* on medium supplemented with 0.5 mg dm^{-3} BA. *D* - Rooting of shoots of *C. arborescens* on medium supplemented with 1.0 mg dm^{-3} IBA and 1.0 mg dm^{-3} phloroglucinol.

to the same chromosomal subsection as that of *C. tinctorius*. This species failed to hybridize with other *Carthamus* species having 12 pairs of chromosomes including *C. tinctorius* (Ashri and Knowles 1960). *C. arborescens* is found to be highly resistant to *Alternaria carthami* (results unpublished). Based on its perennial nature and the genetic distance from *C. tinctorius*, *C. arborescens* has been reclassified in the genus *Phonus* (Lopez-Gonzalez 1989, Vilatersana *et al.* 2000). The high frequency of shoot regeneration from leaf explants indicates that it could be a good model system for protoplast isolation and culture for interspecific/intergeneric hybridization in the genus *Carthamus*. The superiority of TDZ was reported in *C. tinctorius* (Radhika *et al.* 2006) and *Psoralea corylifolia* (Faisal and Anis 2006).

Axillary buds responded to the cytokinins incorporated in the medium and the number of shoots per responding explant varied from 2 to 11 depending on the genotype and the cytokinin type and concentration (Table 2). In *C. oxyacantha*, *C. lanatus* and *C. montanus,* response was good and the shoot buds proliferated to

form multiple shoots, which elongated simultaneously. Buds of *C. boissieri* and *C. turkestanicus* proliferated without base callus but required a transfer to a low cytokinin medium for shoot elongation. Axillary nodes of *C. glaucus* showed extreme vitrification and formed either callus or vitrified watery shoots. For reducing vitrification and promoting proliferation, the vitrified shoots were transferred from medium supplemented with 0.5 mg dm^{-3} BA to 0.5 or 1.0 mg dm^{-3} KIN. In *C. palaestinus*, the entire explant formed callus through which shoots originated. In few cases vitrification was high and transfer to low concentration of cytokinin or low potent cytokinin (BA to KIN) did not improve the condition.

Table 2. Number of shoots per axillary bud in *Carthamus* species cultivated on MS medium supplemented with BA or KIN for 3 weeks.

Species	BA [mg dm^{-3}]		KIN [mg dm^{-3}]		
	0.5	1.0	0.5	1.0	2.0
C. tinctorius	3.3±0.5	3.7±0.5	2.3±0.5	3.0±0.0	4.0±0.8
C. boissieri	8.0±0.8	10.3±0.5	3.0±0.8	4.7±1.2	6.3±0.5
C. glaucus	2.7±0.9	3.7±0.5	0	0	1.7±0.5
C. oxyacantha	6.3±0.5	6.0±0.8	3.7±1.2	3.0±0.0	4.7±1.2
C. palaestinus	7.3±1.2	8.0±2.2	6.7±0.9	3.3±0.5	3.3±0.9
C. lanatus	4.0±0.8	5.0±0.8	4.7±1.2	3.7±0.5	3.7±0.5
C. turkestanicus	4.3±0.5	4.7±0.5	3.0±0.0	4.7±1.7	3.3±0.5
C. creticus	5.7±1.7	4.7±0.9	3.7±0.5	4.0±0.0	3.7±0.5
C. montanus	3.0±0.8	4.7±0.9	2.3±0.5	2.7±0.5	3.0±0.8

Across the genotypes, medium supplemented with BA was significantly superior to that with KIN and maximum shoot proliferation was obtained on medium with 1.0 mg dm^{-3} BA. Averaged over genotypes, the species *C. boissieri* and *C. palaestinus* recorded the highest shoot multiplication index. However, in case of *C. palaestinus,* shoots originated from the base callus.

Elongated shoots of *C. tinctorius* and the species, *C. lanatus* and *C. oxyacantha* produced flowers *in vitro.* The flowers opened normally but the number of florets was few (4 - 7). Pollen was fertile but the capitula failed to set seeds in culture.

One interesting feature in safflower tissue cultures is the *in vitro* induction of capitulum (Tejovathi and Anwar 1984, Nikam and Shitole 1999). Singh (1991) attempted to induce floral buds in *C. oxyacantha* - the wild safflower to overcome the problem of asynchrony in flowering. In the present investigation, shoots of *C. oxyacantha* and *C. lanatus* formed flowers *in vitro.* These two species have been identified as sources of resistance to rust, *Ramularia* leaf spot, and powdery mildew (Kumar 1991). *C. tinctorius* crosses readily with *C. oxyacantha.* Alloploids of *C. tinctorius* with *C. lanatus* have been achieved artificially (Ashri and Knowles 1960). The major problem limiting interspecific hybridization between *C. tinctorius* and *C. lanatus* is the long duration. Maintenance of cultures *in vitro* will facilitate hybridization between the two species by overcoming the problem of temporal asympatry.

Shoots of *C. tinctorius* and *C. arborescens* developed roots on half-strength MS medium supplemented with either 1.0 mg dm^{-3} NAA (36.0 and 62.5 %, respectively) or 1.0 mg dm^{-3} IBA in combination with 1.0 mg dm^{-3} phloroglucinol (58.0 and 85.7 %, respectively) (Fig. 1*D*). In both the cases, frequency of rooting was higher in shoots cultured on medium supplemented with IBA + phloroglucinol. Hence, elongated shoots of all the *Carthamus* species were rooted on medium fortified with 1.0 - 2.0 mg dm^{-3} IBA in combination with 1.0 mg dm^{-3} phloroglucinol and the rooting frequency varied between 25 to 83.3 % depending on the species.

Rooting of *in vitro* regenerated shoots of safflower has been consistently problematic, thus reducing the overall efficiency of whole plant regeneration (George and Rao 1982, Nikam and Shitole 1999). Less than 10 % of shoots regenerated from cotyledons rooted on hormone-free MS medium with 6 to 8 % sucrose (George and Rao 1982) or 9 % sucrose (Tejovathi and Anwar 1987). Most of the other reports define the use of growth regulator free MS medium or medium supplemented with NAA for rhizogenesis but the frequency of rooting was not up to the desired extent (Singh 1991, Sujatha and Suganya 1996).

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