BIOLOGIA PLANTARUM 50 (2): 174-179, 2006

Thidiazuron stimulates adventitious shoot regeneration in different safflower explants

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Abstract

Adventitious shoot regeneration from root, hypocotyl, cotyledon and primary leaf explants of safflower (*Carthamus tinctorius* L.) was studied. Shoot regeneration was promoted by benzyladenine (BA) + naphthaleneacetic acid (NAA), BA + indole-3-butyric acid (IBA), kinetin + NAA and thidiazuron (TDZ) + NAA incorporated in Murashige and Skoog (MS) basal medium. High frequency of shoot regeneration and high number of shoots per regenerating explant were obtained on a wide range of TDZ + NAA combinations. Proliferated shoots were elongated in MS + 0.5 mg dm^{-3} kinetin and well-developed shoots were rooted in half strength MS + 0.5 mg dm^{-3} NAA. Rooted shoots were successfully acclimatized and established in soil.

Additional key words: auxins, *Carthamus tinctorius* L., cytokinins, *in vitro* culture.

Introduction

Safflower (*Carthamus tinctorius* L.) is an important oilseed crop. Genetic improvement of this crop for agronomical attributes is constrained by the modest levels of variability available in the cultivar germplasm. Development of *in vitro* shoot regeneration techniques is essential for introgression of desirable traits from alien sources. The amenability of safflower tissues to *in vitro* manipulations is evident from simple media requirements and the use of Murashige and Skoog (1962) salts as the basal medium in conjunction with BA and NAA for direct as well as 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, Sujatha and Suganya 1996, Nikam and Shitole 1999, Mandal and Gupta 2003). In most of the earlier investigations on safflower tissue culture, the frequency of regeneration varied from 26 to 100 % with 8 to ~ 200 shoots per regenerating explant but shoot morphogenesis was confined to selected tissues and genotypes (George and Rao 1982, Ying *et al*. 1992, Orlikowska and Dyer 1993). Keeping in view the limitations in the regeneration experiments, the present investigation was undertaken to assess the efficacy of various growth regulator combinations in stimulating the organogenic competence of the seedling tissues of safflower.

Materials and methods

Explants: Seeds of the commercially cultivated Indian safflower (*Carthamus tinctorius* L.) genotypes A-1, Manjira and HUS-305 were washed thoroughly under running tap water and surface sterilized with 0.1 % (m/v) mercuric chloride for 8 min. Following four rinses in sterile distilled water, the seeds were transferred aseptically to half strength MS basal salt media (Murashige and Skoog 1962) with 1.5 % sucrose and gelled with 0.7 % agar (*Himedia*, Mumbai, India). After appearance of the first pair of true leaves, four types of explants – roots, hypocotyls, cotyledons and primary leaves were tested for callus induction and shoot

Received 28 May 2004, *accepted* 4 March 2005.
Abbreviations: BA - N^6-benzyladenine; 2,4-D - 2,4-dichlorophenoxyacetic acid; IBA - indole-3-butyric acid; MS - Murashige and Skoog (1962) medium; NAA - α-naphthaleneacetic acid; TDZ - 1-phenyl-3-(1,2,3-thiadiazol-5-yl) urea (thidiazuron).
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regeneration. The roots and hypocotyls were divided into 0.75 to 1.0 cm segments and placed horizontally on the medium. The cotyledonary and primary leaves were cut into 1.0 cm pieces and placed with the abaxial side in contact with the medium surface.

Media composition and culture conditions: The culture medium consisted of full strength Murashige and Skoog (MS) basal medium with 3 % sucrose. The growth regulator (*Sigma*, St. Louis, USA) combinations tested included 20 combinations of benzyladenine (BA) (0.1 - 2.0 mg dm^{-3}) + naphthaleneacetic acid (NAA) (0 - 2.0 mg dm^{-3}); 12 combinations of BA (0.1 - 2.0 mg dm^{-3}) + indole-3-butyric acid (IBA) (0.1 - 1.0 mg dm^{-3}); 9 combinations of kinetin (0.1 - 2.0 mg dm^{-3}) + NAA (0 - 2.0 mg dm^{-3}); 12 combinations of 2,4-dichlorophenoxy acetic acid (2,4-D) (0.1 - 1.0 mg dm^{-3}) + kinetin (0.1 - 2.0 mg dm^{-3}) and 20 combinations of thidiazuron (TDZ) (0.5 - 5.0 mg dm^{-3}) + NAA (0 - 2.0 mg dm^{-3}). As sufficient number of primary leaf segments was not obtained from seedling explants, the shoot apices were maintained on medium supplemented with 0.5 mg dm^{-3} kinetin to serve as a continuous source of leaf tissue.

The medium was adjusted to pH 5.7 ± 1.0 with 0.1 M NaOH, solidified with 0.7 % agar 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} (cool white fluorescent lamps).

The 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 formation and shoot induction were recorded three 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. Data was subjected to statistical analysis (FRBD) in experiments assessing TDZ+NAA induced shoot regeneration in different seedling explants and genotypes. The experiments were completely randomized and each treatment consisted of 16 culture tubes with 4 explants each and replicated thrice.

Results

All explants showed symptoms of swelling 3 d after culture initiation followed by callus formation at their cut ends, which were in contact with the medium. Depending on the growth regulator concentration and combination, and the explant, direct as well as callus mediated shoot differentiation was observed on the primary medium itself. The callus induced on medium with BA+NAA was loose, friable and nodular with green spots. BA at 2.0 mg dm^{-3} resulted in shoot formation (1 - 3 shoots) from primary leaf of A-1 after the induction of low amount of green nodular type of callus. The callus formed on medium with TDZ+NAA was white, loose and friable with green dots on lower concentrations of TDZ, while it was green to dark green and occasionally hyperhydric on medium with higher concentration of TDZ (> 2.0 mg dm^{-3}). Kinetin in combination with low concentrations of 2,4-D produced loose, friable, translucent white callus and in higher concentrations (> 0.5 mg dm^{-3}) resulted in pale yellow callus with round nodular structures. Medium supplemented with BA+IBA favoured production of watery callus with white flaky patches.

Differential response of the four explants of two safflower genotypes A-1 and Manjira, was observed for shoot differentiation. Of the leaf tissues, response of primary leaf to shoot regeneration (5.5 to 50 %) was superior to that of the cotyledonary leaf (8.3 %). Of the two genotypes, Manjira responded well as evidenced by the induction of shoots on a wider range of BA and NAA combinations besides a comparatively higher frequency of shoot regeneration (5.6 to 50 %) as compared to A-1

Table 1. Frequency of shoot induction [%] from different seedling explants of safflower cv. HUS 305 on MS medium supplemented with TDZ and NAA. Data were scored 3 weeks after culture. Percentage values were angular transformed prior to analysis.

TDZ/NAA [mg dm^{-3}]	Root	Hypocotyl	Cotyledon	Primary leaf
0.5/0.0	50.0	25.0	33.3	27.7
0.5/0.1	26.5	67.1	86.3	41.6
0.5/0.5	98.5	72.3	25.0	58.1
0.5/1.0	97.0	67.1	0	53.4
0.5/2.0	0	67.1	14.8	55.7
1.0/0.0	27.7	33.3	8.1	41.6
1.0/0.1	77.8	52.6	33.3	33.8
1.0/0.5	58.4	84.1	50.0	19.3
1.0/1.0	67.1	44.3	27.7	38.7
1.0/2.0	33.3	75.0	0	58.7
2.0/0.0	33.3	58.7	50.0	31.6
2.0/0.1	50.0	100.0	67.1	27.4
2.0/0.5	66.7	89.6	43.3	37.6
2.0/1.0	60.6	89.5	12.0	15.5
2.0/2.0	44.3	58.7	0	33.2
5.0/0.0	38.7	8.1	0	33.2
5.0/0.1	50.0	67.1	16.3	75.0
5.0/0.5	27.7	58.7	0	66.7
5.0/1.0	38.7	75.0	11.1	22.1
5.0/2.0	0	100.0	0	72.3
	explant (E)	medium (M)	E × M	
SE_m	0.924	2.067	4.134	
$CD_{0.05}$	2.582	5.774	11.55	

(5.5 to 25 %). Overall the explants and genotypes, primary leaf of Manjira recorded maximum shoot regeneration (50%) on medium supplemented with 0.5 mg dm^{-3} BA without NAA.

Medium supplemented with kinetin and NAA either singly or in combination failed to promote caulogenesis from root explants of both genotypes and primary leaf explants of Manjira. As concerns other explants, only one or two of the nine tested combinations facilitated shoot induction of particular explant type. The frequency of shoot regeneration was lower (8.3 to 16.7 %) as compared to that on medium fortified with BA+NAA (5.5 to 50 %).

Medium supplemented with kinetin+2,4-D favoured only callusing from all seedling explants, while shoot differentiation failed to occur on media supplemented with these growth regulators at the concentrations tested.

Shoot regeneration was not observed in cotyledonary leaf explants of the genotype A-1 on media supplemented with BA and IBA. In A-1, maximum shoot regeneration (33.3 %) was obtained with hypocotyl explants on medium supplemented with 1.0 mg dm^{-3} BA and 0.5 mg dm^{-3} IBA. In Manjira, roots recorded higher shoot regeneration frequencies (16.7 %) on medium with BA+IBA in a 2:1 ratio (1.0 mg dm^{-3} BA + 0.5 mg dm^{-3} IBA; 2.0 mg dm^{-3} BA + 1.0 mg dm^{-3} IBA). Media supplemented with 0.5, 1.0 and 2.0 mg dm^{-3} BA in combination with 0.5 and 1.0 mg dm^{-3} IBA facilitated shoot regeneration from primary leaf explants of A-1.

Initially, the response of the four seedling explants of the genotype HUS-305 was assessed using 20 combinations of TDZ+NAA (Experiment I). Since vitrification was observed on medium with high concentration of TDZ (5.0 mg dm^{-3}), nine combinations of these growth regulators were selected for testing the variability to caulogenesis in seedling explants of three selected safflower genotypes (Experiment II).

Shoot regeneration was prolific and a countless

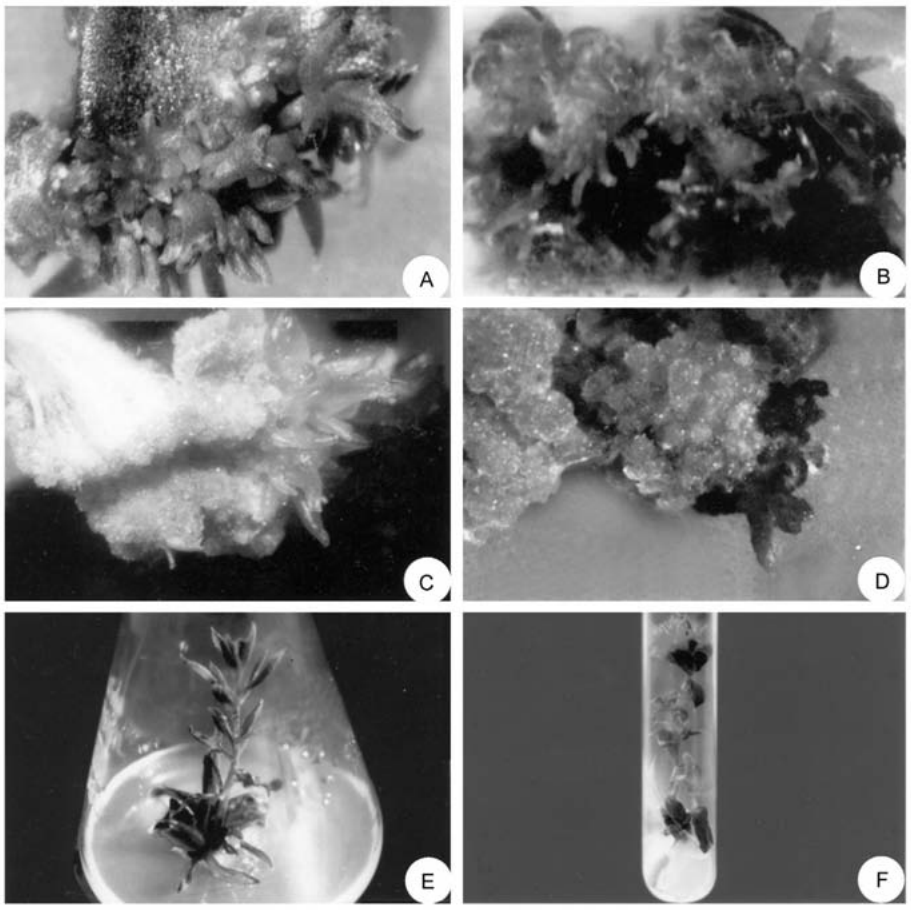

Fig. 1. Shoot regeneration from seedling explants of safflower on MS medium supplemented with TDZ and NAA: *A* - prolific shoot regeneration without callus intervention from the cut margin of the primary leaf; *B* - leafy shoots without any callus formation from all over the hypocotyl segment; *C* - simultaneous induction of callus and shoots from cotyledonary leaf; *D* - shoot regeneration through callus induced from root tissue; *E* - differentiation of leafy shoots induced on medium with TDZ+NAA into well-developed shoots on medium supplemented with 0.5 mg dm^{-3} kinetin; *F* - shoots bearing capitula fail to develop roots.

Table 2. Shoot regeneration frequency [%] from different seedling explants (R - root, H - hypocotyl, CL - cotyledon, PL - primary leaf) of 3 safflower genotypes on MS medium supplemented with TDZ and NAA. Data were scored 3 weeks after culture. Percentage values were angular transformed prior to analysis.

TDZ/NAA [mg dm^{-3}]	Manjira				A-1				HUS 305			
	R	H	CL	PL	R	H	CL	PL	R	H	CL	PL
0.5/0.0	50.0	0	0	33.2	27.7	0	0	0	50.0	25.0	33.3	27.7
0.5/0.5	58.7	53.3	11.1	21.8	57.5	84.1	8.3	16.3	98.5	72.3	25.0	58.1
0.5/1.0	41.6	11.1	0	11.1	67.1	23.7	33.2	8.1	97.0	67.1	0	53.4
1.0/0.0	27.4	11.1	0	11.1	44.3	58.7	64.5	16.3	27.7	33.3	8.1	41.6
1.0/0.5	41.6	33.3	33.3	5.6	55.7	27.7	11.1	25.0	58.4	84.1	50.0	10.3
1.0/1.0	37.5	33.3	44.9	33.3	8.3	38.7	8.1	50.0	67.1	44.3	27.7	38.7
2.0/0.0	22.1	30.5	14.3	28.3	41.6	38.7	37.5	0	33.3	58.7	50.0	31.6
2.0/0.5	39.8	50.0	18.6	36.1	37.5	72.3	41.6	8.1	66.7	89.6	43.3	37.6
2.0/1.0	8.1	11.1	33.3	24.7	29.8	27.7	13.8	0	60.6	89.6	12.0	15.5
	genotype	explant	treatment	G × E	G × T	E × T	G × E × T					
SE_m	0.534	0.616	0.924	1.067	1.601	1.848	3.202					
$CD_{0.05}$	1.488	1.718	2.576	5.273	4.463	5.135	8.925					

number of leafy shoots were induced. Regardless of the media, explant and genotype, three types of shoot regeneration was observed; *1*) direct shoot regeneration without callus intervention (Fig. 1*A*,*B*); *2*) callus formation and shoot differentiation occurring simultaneously (Fig. 1*C*); and *3*) formation of callus and subsequent regeneration of shoots through the induced callus (Fig. 1*D*).

Experiment I: Shoot regeneration frequencies on medium with TDZ+NAA were high (8.1 to 100 %) and shoot buds were induced on a wide range of the concentrations used and from all the explants as compared to the other growth regulator combinations tested in this study (Table 1). Maximum mean shoot induction (67.7 %) was recorded from hypocotyl, while cotyledonary leaf recorded minimum shoot induction (18.8 %) and the differences were significant. Media with all tested concentrations of TDZ+NAA facilitated shoot regeneration from primary leaf tissues and hypocotyls. Higher concentration of TDZ (5.0 mg dm^{-3}) failed to elicit a favourable response for shoot induction from cotyledonary leaves and the regeneration frequency was either low or nil. Medium fortified with 5.0 mg dm^{-3} TDZ singly or in combination resulted in the formation of vitrified and watery shoots from all the explants.

Experiment II: The frequency of shoot induction from the seedling explants of three safflower genotypes, Manjira, A-1 and HUS-305 on MS media supplemented with nine combinations of TDZ and NAA ranged from 5.6 to 98.5 % (Table 2). The individual as well as the interaction effects of genotype, explant and media were found to be highly significant.

Over all genotypes, shoot regeneration was maximum from root (47 %) followed by hypocotyl (41.7 %) while cotyledons were found to be less responsive (19.4 %). Medium supplemented with 0.5 mg dm^{-3} NAA with 0.5 or 2.0 mg dm^{-3} TDZ facilitated maximum frequency (45 - 47 %) of shoot induction. Among the three genotypes, mean shoot induction was maximum in HUS 305 (47.2 %) and was significantly superior to Manjira and A-1 (22.9 to 26.5 %, respectively). The mean shoot regeneration frequencies from different explants of HUS 305 root (65.4 %), hypocotyl (64.2 %), cotyledonary leaf (24.8 %) and primary leaf (35.3 %) were also higher as compared to those from the other two genotypes. Root segments of all tested genotypes, primary leaf tissues of HUS 305 and Manjira and hypocotyls of HUS 305 differentiated shoots on all media combinations tried although at varied frequencies.

For shoot eleongation and rooting, explants with callus and callus + shoots were transferred to medium supplemented with 1.0 mg dm^{-3} each of BA and NAA. Explants with shoots alone were transferred to medium supplemented with 0.5 mg dm^{-3} kinetin where 2-6 well-developed shoots were observed within 2 - 3 weeks (Fig. 1*E*). Elongated shoots (4 - 5 cm) were transferred to half-strength MS salt medium with 3.0 % sucrose and 0.5 mg dm^{-3} NAA for rooting (Sujatha and Suganya 1996). After 5 - 6 subcultures in two week intervals, few of the elongated shoots and proliferating shoot cultures developed flower buds. The frequency of capitulum induction varied with genotype and was 12.0, 8.0 and 33.3 % in A-1, Manjira and HUS 305, respectively. Interestingly, only the shoots devoid of flower buds developed roots. Shoots with capitula (Fig. 1*F*) were transferred to sterilized vermiculite saturated with a solution of 0.5 mg dm^{-3} NAA and maintained under high humidity for a week by covering the pots with polythene bags. The plantlets were maintained in the growth room for another week prior to transfer to pots. The survival frequency of rooted shoots was 32.4 % while that of the rootless micro shoots was 18.1 %.

Discussion

The frequency of shoot regeneration from seedling explants of safflower reported in this study is very high as compared to those reported earlier. Moreover in most cases, where callus formation was either low or negligible, countless number of shoots originated from the responding region. George and Rao (1982) reported a maximum of 8 - 10 buds per cotyledon in 65 - 70 % of the cultures. Ying *et al.* (1992) obtained a maximum of 26 % bud induction from leaf-derived calli. Nikam and Shitole (1999) reported a maximum of 7.5 shoots from entire cotyledon explants. The best shoot regeneration frequencies through direct organogenesis reported so far are those of Sujatha and Suganya (1996) where 48.9 % shoot regeneration with 32.1 shoots per explant was achieved. With regard to embryogenesis, a maximum embryogenic response of 54 % with a mean of 14.7 ± 4.1 somatic embryos per responding explant was reported (Mandal *et al*. 1995). Thus, the TDZ+NAA combinations tested in the present investigation proved superior to those reported earlier as the frequency of shoot induction was high (up to 98.5 %) and the number of regenerating shoots in the responding explants was enormously high (countless). Thus, TDZ which is known for induction of adventitious shoots and proliferation of axillary shoots in woody species (Huettemann and Preece 1993) holds promise for an herbaceous annual like safflower.

This is not the first report of the use of TDZ+NAA combination for induction of adventitious shoots in safflower but constitutes the first successful attempt of achieving shoot regeneration from all seedling explants of the Indian safflower cultivars using these growth regulators. Orlikowska and Dyer (1993) reported high frequency of shoot regeneration associated with formation of large number of leafy shoots that were comparable to those reported in this study with the same growth regulator combination. However, the investigations of Orlikowska and Dyer (1993) failed to prove the amenability of safflower roots for caulogenesis unlike in the present study where root tissues had the highest regenerability in the genotypes tested. This probably could be due to the differences in the genetic determinants controlling caulogenesis in American *vs*. Indian cultivars or due to the varying concentrations of TDZ and NAA used in these two studies. Furthermore, in the studies of Orlikowska and Dyer (1993), the effect of polarity was evident and explants closest to the apical meristems developed leaf primordia. However, in this present investigation the distinctness of polarity was not observed and all the seedling tissues including roots developed adventitious shoots.

Earlier studies on safflower tissue culture pointed out the crucial role played by the genotype to *in vitro* culture conditions (George and Rao 1982, Prasad *et al*. 1991, Ying *et al*. 1992). In the present study also, medium supplemented with other growth regulator combinations (BA+IBA, BA+NAA, kinetin+2,4-D and kinetin+NAA) failed to induce organogenesis in the tested genotypes. However, medium supplemented with TDZ+NAA elicited a positive response and the seedling tissues of the tested genotypes responded to organogenesis although the frequencies varied with the genotype, explant and the concentration of these growth regulators in the medium. The studies unequivocally reveal that the external stimulus in the form of growth regulators triggering the morphogenic competence of the cultured tissues is as important as the genotype and use of TDZ+NAA for shoot regeneration has minimized the effects of genotype in safflower.

The interaction of explant *vs*. growth regulator was found significant in the earlier investigations on safflower tissue culture. In the studies of George and Rao (1982), hypocotyl explants did not respond to treatments with BA. Similarly, root derived callus of Manjira lacked morphogenic competence when cultured on medium with 1.0/1.0 or 0.5/0.1 mg dm^{-3} of BA+NAA (Sujatha and Suganya 1996). Despite a high frequency of shoot regeneration from leaves, cotyledons and hypocotyls, root sections produced only callus on medium supplemented with TDZ+NAA (Orlikowska and Dyer 1993). Shoot regeneration from root and leaf explants was not observed when cultured on medium supplemented with BA+NAA or BA+casein hydrolysate (Nikam and Shitole 1999). In the present study as well, differential response was exhibited by the various seedling explants on all media with the exception of media supplemented with TDZ+NAA. Growth and morphogenesis *in vitro* are regulated by the interaction and balance between the growth regulators provided in the medium and those produced endogenously by an explanted tissue. While most growth regulators exert a direct effect on cellular mechanisms, many synthetic regulants modify the level of endogenous growth substances (George 1993). Stimulation of regenerative capacity of a large number of cells along the entire seedling of safflower on medium supplemented with TDZ alone and in combination with NAA indicates a positive role of TDZ in the modification of the endogenous levels of growth regulators in this crop as well.

Incorporation of TDZ in conjunction with NAA resulted in a high frequency of shoot regeneration from the tested explants and genotypes of safflower. TDZ singly or in combination has been used to induce adventitious shoots and axillary bud proliferation in a number of plant species (Bhattacharya 2003/4, Hosseini-Nasr and Rashid 2003/4). The protocols described are currently being used in our laboratory for development of transgenic male sterility and fertility restoration system in safflower by deploying the appropriate candidate gene(s) as it is hitherto difficult to develop a viable cytoplasmic male sterility system through conventional methods.

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