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Effect of Phytohormones on Multiple Shoot Bud Induction in cv. NARI-6 of Safflower (*Carthamus tinctorius* L.)

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Abstract

In the present study, *in vitro* multiple shoot induction was achieved from cotyledonary node and stem nodal explants of cv. NARI-6 of safflower (*Carthamus tinctorius* L.). Among various growth regulators tested, MS salts and B5 vitamins supplemented with BA (6-Benzylaminopurine) 17.76 μM and KN (Kinetin) 6.96 μM phytohormonal combination was found to be the most effective in initiating numerous shoot buds after 30 days of culture than BA (4.44-44.39 μM) or KN (2.32-46.40 μM) alone in the medium. In addition, 0.8% (w/v) agar (Hi-media) and 3.0% sucrose (w/v) was the optimum level for the formation of adventitious shoots. Further results showed the maximum shoot elongation occurred on MS medium with BA (8.88 μM) and GA_3 (11.56 μM) combinations. Efficient rooting occurred on quarter strength MS medium with NAA 10.74 μM. The regenerated plantlets were acclimatized and successfully transferred to the field.

Key words: Safflower, *Carthamus tinctorius* L., cv. NARI-6, Medicinal, plant, Multiple Shoot, Phytohormones

Introduction

Safflower, *Carthamus tinctorius* L., is an annual medicinal and aromatic oilseed crop grown for many centuries in China and the Mediterranean region. The Indian subcontinent has been assumed to be one of the centres of origin of this plant species (Vavilov, 1951). The Safflower contains nearly 75% linoleic acid, which is considerably higher than corn, soybean, cottonseed, peanut or olive oil. The oil seed of the plant is used primarily for edible oil products such as salad oils and soft margarines. The oil can also be used as a diesel fuel substitute. But like most vegetable oils, is currently too expensive for this use. Researchers disagree on whether oils high in polyunsaturated acids, like linoleicacids, help decrease cholesterol and the related heart and blood circulatory problems (Robinson and Otto, 1967). The plant has therapeutic value due to its high degree of polyunsaturation in the form of linoleic acid that is known to check antherosclerosis (Knowles, 1982). The oil also has an elevated level of α- tocopherol, an antioxidant that improves the utilization of vitamin A in human body (Furuya et al., 1987).

The dried flowers of *Carthamus tinctorius* L.(Safflower) have been used in traditional Asian medicine for thousands of years. The active compounds are red and yellow pigments, which have been experimentally shown to enrich blood, to decrease fatigue and to promote menstruation (Akihisa et al., 1994). Plant improvement program has been expanded by safflower breeders in order to develop desirable genotypes. However, the new biotechnological approaches via *in vitro* propagation techniques are the additional tools for genetic improvement of economically important plants (Mandal et al., 2000). The impressive progress has been made in development of transgenic plants from various oil seed crops. The improvements of crop plants are regeneration of plants preferably from direct and indirect organogenesis. George and Rao (1982) reported in safflower the induction of shoot from cotyledons on BA supplemented MS medium, but regeneration frequency was low and complete plants were rarely obtained. However, direct regeneration of shoot and inflorescence has been achieved from cotyledon on MS medium containing BA or Kinetin along with NAA in some cultivars

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of *C. tinctorius* L. (Tejovathi and Anwar, 1984; Tejovathi et al.1987). Tissue culture offers increased rates of vegetative propagation as well as the maintenances of pathogen free plants (Saito and Masaru Nakano, 2002).

In the current status of tissue culture studies, safflower does not present a promising out look in favour of plant regeneration either via organogenesis or embryogenesis. However, because of an increasing use of petals of *C. tinctorius* for pharmaceuticals and food pigments, the yield of petals from naturally grown Safflower plant is not enough to meet present needs (Yu and Xu, 1997). In the present paper, we have standardized an efficient protocol for the induction of multiple shoots in cv. NARI-6 of *C. tinctorius* L.

Materials and Methods

Plant Materials

Seeds of Safflower (*C.tinctorius* L.) cv. NARI-6 was obtained from Seed Unit, Nimbkar Agricultural Research Institute, Maharashtra, India. Seeds were surface sterilized with 70% ethanol (v/v) for 1 min followed by three minutes treatment with 0.1% (w/v) mercuric chloride and finally three washes for 5 minutes each in sterile distilled water. Seeds were then germinated and grown on MS semisolid medium without plant growth regulators in the dark at 25℃

Multiple Shoot Bud Induction

Cotyledonary node (5-6 days old) (1-2 cm long) and stem nodal explants (1-2 cm long) were excised from a healthy plant, inoculated into culture tubes (150×25mm) each containing 15ml Murashige and skoog's nutrient salts (1962) and B5 vitamins (Gamborg et al. 1968) media supplemented with individual phytohormones or a combinations of cytokinins in the concentration ranges BAP (4.44-44.39 μM), KN (2.32-46.40 μM) alone and BA (8.88,17.76,26.64,35.52 and 44.39 μM) and KN (2.32,4.64, 6.96,9.28,11.60 and 13.92 μM) combinations (Table 1.) Sucrose (3.0, 3.5, 4.0, 4.5 and 5.0 %) was used as a Carbon source and 0.50, 0.60 and 0.8 % Agar-Agar Type I (Hi-Media) was tested as a gelling agent. The pH of the medium was adjusted to 5.7 before autoclaving at 15lb for 15 min (121℃). The explants inoculated in culture tubes were maintained at 23 ± 2℃ and 16h photoperiod. Subculture was done once in two weeks of inoculation. After 30 days of culture, numerous shoots were initiated from the cotyledonary node and stem node explants. The percentage of shoot proliferation was determined.

Shoot elongation, Rooting and Acclimatization

The efficiency of *in vitro* propagated primary shoots (5-20 mm) bearing at least two leaves with. 1 or 2 shoots were excised from the shoot clump, were transferred to MS medium with B_5 vitamins, supplemented with BA (2.22, 4.44, 6.66, 8.88 and 11.10 μM) and GA_3 (2.89, 5.78, 8.67, 11.56 and 14.45 μM) combinations for shoot elongation (Table 2.). Frequency of rooting from elongated shoots was observed with the phytohormones NAA, IAA and IBA (2.69, 5.37, 8.06, 10.74, 13.43 and 16.11 μM) alone in ¼ strength MS media after 45 days of culture (Table 3.). Regenerated plantlets after rooting were carefully taken out from the medium and then transferred to the plastic cups containing sterile soil with vermiculite (1:1) for hardening. Humidity was maintained by covering the plastic cups with polythene bags. Finally disease free young plantlets were transferred to the fields.

Statistical analysis

The experiments were consisted of 20 explants and the experiments were repeated thrice. The cultures were observed periodically, percentage of response was recorded on the basis of observation. Mean value with standard error was used in all experiments and analysis of the variance and mean separations were carried out using Duncan's Multiple Range Test (DMRT), and the significance was determined at 5% level (Gomez and Gomez 1984).

Results and Discussion

In the present study, the cotyledonary node explants of safflower (*Carthamus tinctorius* L.) cv. NARI-6 exhibited better regeneration potential than stem nodal explants. When the MS and B_5 vitamins supplemented media with various concentrations of BA (4.44-44.39 μM) or KN (2.32-46.40 μM) alone and BAP (4.44, 8.88, 17.76, 26.64, 35.52 and 44.39 μM) and KN (2.32,4.64,6.96,9.28 and11.60 μM) combinations tested, multiple shoot induction from both cotyledonary node and stem node explants of *C. tinctorius* was significant on MS and B5 vitamins supplemented media with BA (17.76 μM) and KN (6.96 μM) combinations (Table 1.). Cotyledonary leaves exhibited no response in this cultivar. Earlier, induction and proliferation of shoot buds has been reported in cotyledons in the presence of BA with different cultivars tested. No shoot bud formation was noticed in the presence of KN or 2-ip or in the absence of cytokinin (Mandal et al., 2000). George and Rao (1982) also reported the necessity of BA for shoot induction from cotyledons of

Table 1. Effect of cytokinins on multiple shoot induction of safflower (*Carthamus tinctorius* L.) on MS medium with B_5 vitamins

Cytokinins (μM)	Cotyledonary node			Stem node		
	% of response	Mean No. of Shoots/Explant ± S.E	Mean Shoot length (mm) ± S.E	% of response	Mean No. of Shoots/Explant ± S.E	Mean Shoot length (mm) ± S.E
BAP						
4.44	20	2.0 ± 0.024^{h}	9.7 ± 0.162^{ij}	20	1.9 ± 0.052^{i}	8.9 ± 0.079^{j}
8.88	30	5.6 ± 0.042^{g}	12.1 ± 0.196^{g}	30	4.2 ± 0.091^{g}	11.6 ± 0.136^{gh}
13.32	50	6.0 ± 0.216^{fg}	14.2 ± 0.154^{f}	40	4.9 ± 0.139^{f}	13.2 ± 0.099^{f}
17.76	70	15.4 ± 0.130^{cd}	17.4 ± 0.229^{cd}	65	10.0 ± 0.164^{de}	15.4 ± 0.205^{e}
22.20	70	19.4 ± 0.201^{ab}	17.5 ± 0.234^{c}	65	15.4 ± 0.109^{ab}	15.7 ± 0.114^{c}
26.64	80	19.9 ± 0.299^{a}	22.0 ± 0.099^{a}	75	16.0 ± 0.114^{a}	20.8 ± 0.303^{a}
31.08	70	16.2 ± 0.107^{c}	21.4 ± 0.239^{ab}	60	12.4 ± 0.105^{c}	20.1 ± 0.198^{ab}
35.52	65	10.8 ± 0.089^{e}	15.6 ± 0.146^{e}	60	10.7 ± 0.069^{d}	15.5 ± 0.052^{cd}
39.95	60	6.3 ± 0.011^{f}	12.0 ± 0.120^{gh}	60	4.8 ± 0.184^{fg}	12.0 ± 0.146^{g}
44.39	60	6.0 ± 0.085^{fg}	9.8 ± 0.105^{i}	40	4.1 ± 0.146^{gh}	8.8 ± 0.164^{ij}
KIN						
2.32	0	0.0 ± 0.000^{e}	0.0 ± 0.000^{de}	0	0.0 ± 0.000^{de}	0.0 ± 0.000^{f}
4.64	0	0.0 ± 0.000^{e}	0.0 ± 0.000^{de}	0	0.0 ± 0.000^{de}	0.0 ± 0.000^{f}
9.28	30	2.7 ± 0.076^{d}	12.0 ± 0.114^{bc}	25	2.4 ± 0.209^{e}	11.7 ± 0.146^{c}
18.56	50	11.5 ± 0.139^{ab}	20.4 ± 0.120^{a}	45	9.6 ± 0.120^{a}	16.9 ± 0.105^{a}
27.84	60	11.8 ± 0.164^{a}	14.6 ± 0.120^{b}	60	6.9 ± 0.171^{b}	15.4 ± 0.189^{ab}
37.12	50	4.5 ± 0.099^{c}	10.0 ± 0.094^{c}	40	2.0 ± 0.146^{cd}	8.1 ± 0.069^{d}
46.40	40	1.0 ± 0.069^{de}	4.3 ± 0.069^{d}	40	1.0 ± 0.146^{d}	3.5 ± 0.120^{e}
BAP + KIN						
4.44 + 2.32	60	12.0 ± 0.114^{e}	20.9 ± 0.136^{bc}	50	7.8 ± 0.069^{de}	20.0 ± 0.079^{cd}
8.88 + 4.64	75	19.5 ± 0.136^{c}	22.7 ± 0.091^{b}	60	11.2 ± 0.114^{c}	20.3 ± 0.145^{c}
17.76 + 6.96	90	23.9 ± 0.160^{a}	25.6 ± 0.171^{a}	85	15.6 ± 0.114^{a}	22.1 ± 0.158^{a}
26.64 + 9.28	90	23.3 ± 0.167^{ab}	25.1 ± 0.045^{ab}	85	15.5 ± 0.069^{ab}	22.0 ± 0.139^{ab}
35.52 + 1.60	80	23.0 ± 0.146^{b}	22.8 ± 0.092^{b}	80	8.1 ± 0.094^{d}	17.8 ± 0.225^{e}
44.39 + 13.92	70	17.1 ± 0.120^{d}	18.2 ± 0.052^{d}	70	7.5 ± 0.464^{e}	15.9 ± 0.094^{f}

Table 2. Effect of phytohormones on shoot elongation of safflower (*Carthamus tinctorius* L.) on MS medium with B_5 vitamins.

Phytohormones (μM)	Cotyledonary node		Stem node	
	% of Response	Mean Shoot Length (mm) ± S.E	% of Response	Mean Shoot Length (mm) ± S.E
BAP + GA_3				
2.22 + 2.89	60	19.2 ± 0.068^{e}	50	15.1 ± 0.099^{de}
4.44 + 5.78	60	22.9 ± 0.103^{cd}	60	31.8 ± 0.130^{bc}
6.66 + 8.67	80	48.1 ± 0.081^{a}	75	45.0 ± 0.026^{a}
8.88 + 11.56	60	40.0 ± 0.133^{b}	50	37.0 ± 0.188^{b}
11.10 + 14.45	40	35.5 ± 0.089^{c}	40	20.5 ± 0.144^{d}

C. tinctorius L. In their study, KN, 2-ip and Zeatin were also found to be ineffective in inducing shoot buds from cotyledon and hypocotyl explants. Combination of BA with NAA was reported to induce shoot buds (Tejovathi and Anwar, 1987; Orlikowska and Dyer, 1993; Zhanming and Biwen, 1993). The mechanism by which BA initiates shoot organogenesis is not clear but it has been suggested that BA interrupted chromosomal DNA replication and repro-

Table 3. Effect of NAA on rooting of elongated shoots of safflower (*Carthamus tinctorius* L.)

NAA (μM)	% of Response	Mean No. of Roots/Explant	Mean Root Length/ Explant (mm) ± S.E
2.69	25	1.5 ± 0.069^{bc}	12.2 ± 0.114^{d}
5.37	40	2.3 ± 0.079^{b}	18.5 ± 0.160^{c}
8.06	40	2.7 ± 0.114^{ab}	25.9 ± 0.079^{b}
10.74	60	4.1 ± 0.094^{a}	34.8 ± 0.094^{a}
13.43	20	1.1 ± 0.114^{c}	23.4 ± 0.167^{bc}
16.11	0	0.0 ± 0.000^{cd}	0.0 ± 0.000^{de}

Total number of explants used for each concentration = 120
Experiments were repeated thrice.
Values are represented as mean ± S.E.
Mean with in a row followed by the same letters are not significant at p = 0.05 according to DMRT.

grammed the developmental fate of certain cells (Buising et al., 1994). In our results, we have investigated the MS and B5 vitamins fortified media with BA (26.64 μM) alone exhibited moderate response while KN (27.84 μM) supplemented media showed low response, but BAP (17.76 μM) and KN (6.96 μM) combinations have given high frequency of adventitious shoot bud formation after 30 days of culture (Fig 2.). Further more, cotyledonary node explants responded well than stem node explants. Studies of sunflower and other oil seed plants showed more number of multiple shoot buds in shoot tip or cotyledonary nodal explants in different culture media.

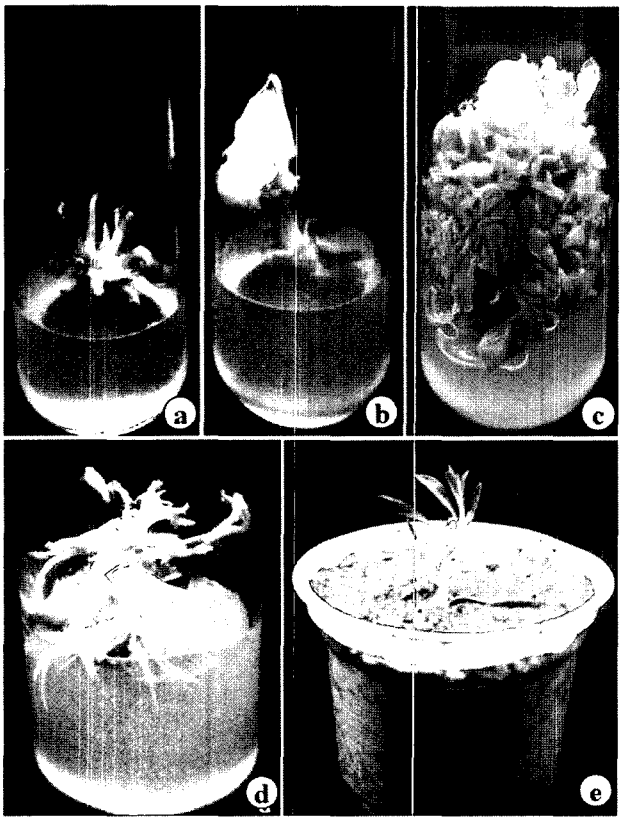

Figure 1. *Effect of phytohormones on multiple shoot induction of safflower (C. tinctorius* L.) cv. NARI-6. a. Stem node, b. Cotyledonary node, c. Multiple shoot buds, d. Rooted plant, e. Hardened plant

Our observation revealed the maximum number of shoots (23) were obtained in cotyledonary node than stem node (15) explants on MS salts, B5 vitamins and sucrose (3.0%) containing medium fortified with BA (17.76 μM) and KN (6.96 μM) in combinations (Table 1). High efficiency of rooting was achieved in NAA (10.74 μM) from the elongated shoots (6.66 μM BA, 8.64 μM GA_3 in combinations). Our results are in agreement with those of George and Rao (1982) and Mandal (1996) who reported the high concentration of sucrose level decrease the number of shoot formation. However, high concentration of BA in the medium increased the percentage of shoot normally and low BA level increased the percentage of shoot abnormality. Moreover, this study concluded that cv. NARI-6 of safflower was more responsible for the enhancement of multiple shoots in cotyledonary node and stem node explants in BA and KN supplemented medium. Cotyledonary leaf explants fully failed to develop shoot buds. MS salts with B_5 vitamin media composition were highly preferable for adventitious shoot buds formation in cotyledonary node with shoot tip and leaf node explants. In the absence of B5 vitamins, MS media alone promoting minimum number of shoot buds in cv. NARI-6. This is the first report demonstrating multiple shoot buds induction from cotyledonary node and stem node explants of *C. tinctorius* L. (Fig 1.). The more prevalent mode of regeneration is via direct organogenesis may be useful to wide range of clonal propagation to produce disease free genotype of safflower, which has good demand in the world market.

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