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Influence of Meta-Topolin on Efficient Plant Regeneration via Micropropagation and Organogenesis of Safflower (*Carthamus tinctorius* L.) cv. NARI-H-15

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Abstract

The effect of meta-Topolin (mT) was assessed to develop a reliable protocol for efficient plant regeneration of safflower (*Carthamus tinctorius* L.) cv. NARI-H-15. For micropropagation, 7 - 9 days old shoot-tip explants cultured on MS basal medium supplemented with 3.0 mg/L meta-Topolin (mT) + 0.5 mg/L CPPU showed 97.7% adventitious shoot formation (42.4 shootlets) than node after 45 days of culture. For organogenesis, the seedling explants of immature leaf cultured on 1.5 mg/L CPPU or 1.5 mg/L NAA fortified medium produced high amount of callus than cotyledon and stem calli after 60 days of culture. However, MS basal medium fortified with 4.0 mg/L mT + 1.5 mg/L CPPU was found beneficial to stimulate 100% organogenic response (74.7 shootlets) from immature leaf calli than cotyledon and stem derived calli after 45 days of culture. The healthy plantlets obtained from micropropagation and organogenesis process cultured on 1/4 MS basal salts, 1.5% sucrose (w/v) and 0.8% agar (w/v) medium supplemented with NAA (1.5 mg/L) and mT (0.1 mg/L) produced maximum of 96% (12.8 rootlets) and 84% (7.3 rootlets) adventitious rooting, respectively than mT and CPPU tested medium. However, maximum of 67% and 42% survival rate was noticed when *in vitro* raised plants from micropropagation and organogenesis were hardened in pots containing soil mix and maintained under green house condition. This optimized regeneration protocol might be helpful in regeneration of new genotypes and cultivars of safflower to improve agronomic traits through *in vitro* selection process and *Agrobacterium*-mediated genetic transformation system.

Keywords

Carthamus Tinctorius L., Meta-Topolin, N-(2-chloro-4-pyridyl-N'-phenylurea),

Micropropagation, Organogenesis

1. Introduction

Safflower (*Carthamus tinctorius* L.) belongs to the family Asteraceae, is a very important oil plant native to India, but is widely distributed in most warm countries. Seeds are used for the extraction of edible oil, flowers utilized for colouring foods and leaves are used as salad vegetable. In India, Pakistan and neighboring countries, a seed rate of 5 - 12 Kg/ha is common, the average seed yield of commercially grown safflower has increased steadily to around 1500 Kg/ha, nearly twice under irrigation [1]. The leaves and shoots of safflower are used as pot herb and salad. The mass of young plants are commonly sold as a green vegetable in markets in India and some neighboring countries [2]. Safflower and Sunflower seed oil have a significant amount of essential fatty acids Omega-3 and Omega 6. Safflower has attracted very little attention as far as tissue culture and genetic transformation are concerned. Initial efforts in safflower were directed to develop suitable culture conditions for whole plant regeneration. It has been demonstrated that regeneration frequencies are very high, and regeneration is possible through organogenesis and embryogenesis pathways. In earlier, the plant regeneration from different explants of safflower cultivars was studied by some researchers and very limited literature is available [3]-[18]. The choice of cytokinin to be used in a tissue culture is determined by its cumulative efficiency in inducing an acceptable rate of shoot multiplication which depends on safflower cultivars. BAP or TDZ along with auxins are reported to cause hyperhydricity with minimum rate of shoot multiplication in many cultivars of safflower plants. Therefore it is very important to find an alternative cytokinin to maintain a reasonable shoot development rate and sufficient plant quality under *in vitro* condition.

Recently, the use of meta-Topolins (mT) indicates a new source of cytokinins that could be suited to promote high morphogenetic development. mT is a highly active aromatic cytokinin from poplar leaves (*Populus* × *canadensis* Moench) [19]. The mT group compound can be considered as an alternative to other commonly used cytokinins in regeneration of recalcitrant plant species. In earlier studies, mT group cytokinin and its derivatives have a comparable effect on *in vitro* regeneration of sugar beet [20], *Curcuma longa* [21], *Aloe polyphylla* [22], banana cv. Williams [23]. Researchers found that mT was nearly twice as effective as BA in the induction of shoot growth. The structural difference with mT and synthetic phenylurea derivatives of *N*-(2-chloro-4-pyridyl)-*N*'-phenylurea (CPPU) could have a profound impact on plant regeneration during micropropagation and organogenesis. Thus, there are still no data concerning *in vitro* regeneration using mT as cytokinin for different safflower genotypes. Therefore, the study aimed to develop a reliable and efficient *in vitro* regeneration system of safflower cv. NARI-H-15 through micropropagation and organogenesis for

commercial propagation suitable for improving agronomic traits.

2. Material and Methods

2.1. Plant Material

Safflower (*C. tinctorius* L.) seeds of cv. NARI-H-15 were obtained from the Nimbhkar Agricultural Research Institute (NARI), Maharashtra, India for the present study. It is a spiny variety selected based on their area of cultivation in agro-climatic conditions.

2.2. *In Vitro* Seed Germination and Culture Condition

Safflower seeds (cv. NARI-H-15) were soaked with 1.0% Teepol (commercial bleach solution, 0.6 % sodium hypochlorite, Rockitt Benckiser (India) Ltd., Kolkata, India) solution for 30 sec and kept under tap water for 20 min to remove detergent. Further processes were carried out under aseptic conditions by treating with 70% ethanol (v/v) for 1 min and rinsed twice with sterile distilled water for 2 min followed by 0.1% $HgCl_2$ (mercuric chloride) for 2 min. The surface sterilized seeds were implanted in MS medium [24] containing 3.0% sucrose (w/v) and 0.8% agar (w/v) (Hi-media laboratories limited, Mumbai, India). All the cultures were initially incubated in darkness for 24 hr and later transferred to 16/8-hr light/dark conditions at 25˚C under 15 µmol $m^{-2}·s^{-1}$ provided by cool white fluorescent tubes (Philips, India) for *in vitro* seed germination.

2.3. Microshoot Initiation and Proliferation

Approximately 0.5 to 1.5 cm long shoot-tip and node (7 - 9 days old) explants were trimmed by surgical blade and inoculated on MS medium supplemented with macro and micronutrients, 3.0% sucrose and 0.8% agar with various concentrations of an individual cytokinins, BAP or mT or TDZ or Kin or Zeatin or CPPU or 2-iP or Diuron or Monuron ranged from 1.0 - 9.0 mg/L alone or optimum level of BAP (7.0 mg/L) or mT (3.0 mg/L) or TDZ (5.0 mg/L) in combination with different concentration of CPPU (0.1 - 2.5 mg/L). All cultures were incubated at 25˚C ± 2˚C under continuous irridation with white fluorescent tube (15 µmol $m^{-2}·s^{-1}$) for 16 hr photoperiod for microshoot initiation and proliferation. Each experiment was repeated thrice. Every subculture was done after 2 weeks of interval. Data were collected on the frequency of plant regeneration, mean number of shoot formation and elongation of shoots in the same culture condition after 45 days of culture.

2.4. Indirect Organogenesis

2.4.1. Callogenesis

The primary explants of cotyledon, immature leaf and stem were excised from 7 - 9 days old *in vitro* seedlings. All explants were dissected into 0.5 - 1.0 cm length and wounded by using a sterile surgical blade. Cotyledon and immature leaf lobe explants were placed with the right side up (abaxial surface into the medium) or upside down (adaxial surface into the medium), and node segments

were placed horizontally into culture tubes (25 × 150 mm) each containing 15 ml MS basal salts, 3.0% (w/v) sucrose medium supplemented with with different ranges (0.5 - 2.5 mg/L) of individual auxins, NAA, IBA, IAA, 2,4-D, Dicamba, CPPU, pCPA and Picloram. The initiation of calli from wounded cotyledon, immature leaf and stem explants were subcultured at two weeks intervals. There were 35 explants/treatment and the experiment was repeated three times. Callusing efficiency was defined as the percentage of explants that produced callus per culture tube. The average fresh weight and dry weight of the callus was determined at each treatment after 60 days of culture.

2.4.2. Organogenesis

After calli initiation, approximately 200 mg of calli were isolated from both immature leaf and stem explants and subcultured on MS basal salts with 3.0% (w/v) sucrose medium supplemented with different concentrations of an individual cytokinins, BAP (3.0 - 9.0 mg/L) or mT (1 - 5 mg/L) or TDZ (3.0 - 9.0 mg/L) or CPPU (1 - 5 mg/L) or BAP (7.0 mg/L) + Kin (0.5 - 2.0 mg/L) or BAP (7.0 mg/L) + CPPU (0.5 - 2.0 mg/L) or BAP (7.0 mg/L) + NAA (0.5 - 2.0 mg/L) or BAP (7.0 mg/L) + IAA (0.5 - 2.0 mg/L) or BAP (7.0 mg/L) + IBA (0.5 - 2.0 mg/L) or mT (4.0 mg/L) + Kin (0.5 - 2.0 mg/L) or mT (4.0 mg/L) + CPPU (0.5 - 2.0 mg/L) or mT (4.0 mg/L) + NAA (0.5 - 2.0 mg/L) or mT (4.0 mg/L) + IAA (0.5 - 2.0 mg/L) or mT (4.0 mg/L) + IBA (0.5 - 2.0 mg/L) or TDZ (5.0 mg/L) + Kin (0.5 - 2.0 mg/L) or TDZ (5.0 mg/L) + CPPU (0.5 - 2.0 mg/L) or TDZ (5.0 mg/L) + NAA (0.5 - 2.0 mg/L) or TDZ (5.0 mg/L) + IAA (0.5 - 2.0 mg/L) or TDZ (5.0 mg/L) + IBA (0.5 - 2.0 mg/L) for organogenic calli formation and adventitious microshoot induction. There were 40 explants/treatment and the experiments were carried out three times. Data on percent of shoot organogenesis (percentage of explants producing shoot buds) and number of microshoot buds were recorded after 45 days of culture.

2.5. Rooting and Hardening of Plants

The individual plantlets were isolated from the clumps of shoots on shoot-tip, node explants culture (micropropagation) and also from cotyledon, immature leaf and stem derived calli (organogenesis) and trimmed to above 0.5 - 2.0 cm length and placed on quarter strength MS salts with 1.5 % sucrose medium supplemented with different levels of an individual auxins, NAA or IAA or IBA (0.5 - 2.5 mg/L) alone or NAA (2.0 mg/L) + BAP (0.1 - 0.5 mg/L) or NAA (1.5 mg/L) + CPPU (0.1 - 0.5 mg/L) or NAA (1.5 mg/L) + mT (0.1 - 0.5 mg/L) + NAA (1.5 mg/L) + $AgNO_3$ (0.1 - 0.5 mg/L) for the frequency of root induction. There were 35 plantlets/treatment and the experiments were carried out three times. Data were recorded on per cent of root induction after 30 days of culture. For first stage of hardening, plantlets with well developed roots were carefully removed from the culture tubes and washed under running tap water to remove agar gels from the roots and were transplanted onto 6.0 cm diameter plastic cups containing the mixture of sterile red soil, garden soil and sand in the ratio of 1:2:1. To maintain high humidity the plastic cups were covered with polythene bags. A

narrow cut (2.0 cm) was made in each bag after 24 h. Plantlets were irrigated with 5 ml of half-strength MS inorganic salts and subsequently with distilled water during the first two weeks. The polythene bags were then removed and plants were acclimatized in earthen pots containing soil mixture with vermiculite in the second stage of hardening. All acclimatized plants were maintained under 15 μmol $m^{-2} \cdot s^{-1}$ in 16-hr photoperiod provided by cool white fluorescent tubes at 25˚C ± 2˚C for another 2 weeks before the plants were transferred to the greenhouse.

2.6. Data Analysis

The direct shoot induction from meristem explants via micropropagation and shoot organogenesis from callus culture was determined. Regeneration frequency was evaluated based on plantlet and rootlet production per explants. Each experiment was conducted in three replicates in completely randomized block design. Data presented are means ± standard error for independent experiments. Data were analyzed using analysis of variance (ANOVA) and differences of means were evaluated by Duncan's multiple range test (DMRT) of SPSS statistical package version 12. This software was used for expressing the statistical significance obtained by comparing plant regeneration through micropropagation and organogenesis.

3. Results

3.1. Micropropagation Response

In order to have successful *in vitro* regeneration, factors such as explant type and culture conditions are very important. In this study, microshoot buds were initiated from 7 - 9 days old shoot-tip and node explants and grew into tiny visible shoots in cytokinin supplemented media (**Figure 1(a)**) In this case, MS basal salts, 3.0% sucrose (w/v), 0.8% agar (w/v) medium supplemented with BAP (7.0 mg/L) influenced 77.4% and 72.9% shoot regeneration with an average of 16.5 and 12 number of shootlet formation from shoot-tip and node explants, respectively after 45 days of culture. mT at 3.5 mg/L induced 88.6% and 80% shoot regeneration with an average of 21 and 19 shootlets per shoot-tip and node explants, respectively. TDZ at 5.0 mg/L influenced 80.6% and 73.7% shoot regeneration with an average of 18 and 17.2 number of microshoots per shoot-tip and node explants culture, respectively. CPPU at 3.0 mg/L induced 58% and 54.9% shoot regeneration with an average of 12 and 10 number of shootlets per shoot-tip and node explants, respectively. Diuron and Monuron supplemented medium showed moderate response to induce adventitious shoot formation in both shoot-tip and node explants than Kin, Zeatin and 2-iP tested medium. About 85.7% and 78.3% shoot induction frequency with an average of 33.5 and 31 number of shootlets was noticed from shoot-tip and node explants cultured on MS medium fortified with BAP (7.0 mg/L) + CPPU (1.5 mg/L). TDZ (5.0 mg/L) + CPPU (0.5 mg/L) induced 90.9% and 84.3% shoot regeneration frequency with an average of 39 and 35 number of shootlets in shoot-tip and node explants cul-

ture, respectively. However, about 97.7% shoot induction frequency with an average of 42.4 number of shootlets was noticed in shoot-tip than node explants cultured on MS medium fortified with mT (3.0 mg/L) + CPPU (0.5 mg/L) after 45 days of culture. Statistical analysis revealed that a significant correlation between growth regulators and microshoot induction response at $P < 0.05$ level (Figure 1(b); Table 1).

3.2. Callus Induction Response

Cotyledon and immature leaf and stem (one explant per tube) segments placed in contact with the solidified MS basal salts with 3.0% sucrose medium containing various types of hormones resulted different colour and texture of calli. They were allowed to grow up to 60 days. In this case, about 82%, 89.7%, 75.4% light green friable of conspicuous callus mass with 795, 810, 769 mg fresh weight and 220, 265, 210 mg dry weight was obtained per cotyledon, immature leaf and stem explants cultured on medium containing 2.0 mg/L IBA, respectively. IAA at 2.0 mg/L induced 80.9%, 85.7% and 71.7% light yellow green nodular callusing with an average of 720, 750 and 681 mg fresh mass and 200, 242 and 185 mg dry weight per cotyledon, immature leaf and stem explants after 60 days of culture. Dicamba (1.0 mg/L) influenced 85.7% and 94.6% yellow friable calli with the production of an average of 873 and 900 mg fresh mass and 263 and 293 mg dry weight per cotyledon and immature leaf explants, respectively, whereas the stem explants produced 77.1% yellow friable calli with an average of 830 mg fresh weight and 222 mg dry weight in the culture. 2,4-D/pCPA/Picloram showed slow response to induce white friable, yellow brown and yellow friable calli for three explants culture. Maximum of 100% light green nodular callusing efficiency with 975 mg fresh weight and 314 dry weight of callus was observed per immature leaf explants on MS basal medium supplemented with 1.5 mg/L NAA

Table 1. Effect of phytohormones on multiple shoot induction from shoot-tip explants of safflower cv. NARI-H-15.

MS medium composition (mg/L)									Shoot-tip			Node		
BAP	mT	TDZ	Kin	Zeatin	CPPU	2-iP	Diuron	Monuron	Mean no. of explants	% of response	Mean no. of shoots	Mean no. of explants	% of response	Mean no. of shoots
7.0	-	-	-	-	-	-	-	-	27.1cd	77.4cd	16.5e	25.5cd	72.9cd	14d
-	3.5	5.0	-	-	-	-	-	-	31b	88.6b	21d	28b	80b	19c
-	-	-	1.5	-	-	-	-	-	28.2c	80.6c	18de	25.8c	73.7c	17.2cd
-	-	-	-	-	-	-	-	-	11fg	31.4g	5.4hi	10.5fg	30fg	4.4g
-	-	-	-	5.0	-	-	-	-	14f	40f	6.0h	12f	34.3f	5.6fg
-	-	-	-	-	3.0	-	-	-	20.3d	58d	12f	19.2d	54.9d	10de
-	-	-	-	-	-	5.0	-	-	17ef	48.6ef	9.3gh	16.9ef	48.3ef	8.1f
-	-	-	-	-	-	-	3.0	-	19.5de	55.7de	11fg	18de	51.4de	9.5e
-	-	-	-	-	-	-	-	3.5	19e	54.3e	9.7g	17e	48.6e	8.5ef
7.0	-	-	-	-	1.5	-	-	-	30bc	85.7bc	33.5c	27.4bc	78.3bc	31b
	3.0	-	-	-	0.5	-	-	-	34.2a	97.7a	42.4a	33a	94.3a	38a
	-	5.0	-	-	0.5	-	-	-	31.8ab	90.9ab	39ab	29.5ab	84.3ab	35ab

Thirty five explants were taken for each experiment. Values are mean of three repeated experiments. Mean within a column followed by the different letters are significantly different according to one way ANOVA and Duncan's multiple range test ($P < 0.05$).

than cotyledon and stem explants after 60 days of culture. However, CPPU at 1.5 mg/L supplemented medium noticed 100% light green nodular calli in cotyledon (914 fresh weight/290 dry weight) and immature leaf explants (979 fresh weight and 318 dry weight), respectively than stem explants (**Figure 1(c)**; **Figure 2(a)-(c)**).

3.3. Organogenesis Response

All type of calli were turned organogenic after 7 - 14 days of culture on plant regeneration medium. The callus tissues cultured on MS basal salts, 3.0% sucrose and 0.8% agar medium supplemented with plant growth regulators and their interactions were significant for organogenesis. Among the four cytokinin hormones tested, about 61%, 64.3% and 57.5% shoot organogenesis with an average of 48.2, 49.5 and 47 number of microshoot buds were observed from cotyledon, immature leaf and stem derived calli, respectively on 7.0 mg/L BAP tested medium after 45 days of culture. mT at 4.0 mg/L induced maximum of 72.5%, 82% and 71% shoot organogenesis with an average of 58, 62.7 and 55 number of microshoots per cotyledon, immature leaf and stem calli. The explants derived calli cultured on CPPU tested medium showed low response in shoot organogenesis. TDZ at 5.0 mg/L showed 67.5%, 71%, 64% microshoot induction response with an average of 54, 56.5 and 52 number of shoots per cotyledon, immature leaf and stem calli. Further, cotyledon, immature leaf and stem calli cultured on optimum level of TDZ (5.0 mg/L) and CPPU (1.5 mg/L) influenced maximum of 95.5%, 98% and 85.8% shoot induction frequency with an average of 49.7, 53.4 and 45 numbers of microshoots, respectively than that of BAP + Kin or BAP + CPPU or TDZ + Kin tested medium. Optimum level of BAP (7.0 mg/L) and CPPU (1.5 mg/L) tested medium noticed 80%, 84.8 and 78% shoot organogenesis in cotyledon, immature leaf and stem calli. BAP in combinations with Kin or NAA or IAA tested medium showed moderate response in shoot organogenesis of three explants derived calli than BAP + IBA treatment. TDZ (5.0 mg/L) in combinations with CPPU (1.5 mg/L) tested medium induced 86.8%, 89% and 83.3% shoot organogenesis with an average of 63.2, 68.5 and 59 number of microshoots per cotyledon, immature leaf and stem calli than TDZ + NAA or TDZ + Kin treatment. TDZ in combinations with IAA/IBA showed slow response. However, 100% adventitious shoot organogenesis with an average of 74.7 number of microshoots were noticed to be highest from immature leaf derived calli on mT (4.0 mg/L) + CPPU (1.5 mg/L) fortified medium than cotyledon and stem calli. mT (4.0 mg/L) + NAA (1.5 mg/L) or mT (4.0 mg/L) + Kin (1.5 mg/L) showed moderate response in shoot organogenesis than mT + IAA or mT + IBA treatment in all culture condition (**Figure 1(d)**, **Figure 1(e)**; **Tables 2-4**). TDZ and Picloram tested medium did not show useful results in shoot organogenesis through callus culture (data not shown).

3.4. Rooting and Acclimatization Response

Root initiation was observed from cutting edge of stem portion of elongated

Table 2. Effect of phytohormones on multiple shoot induction from cotyledon derived callus explants of safflower cv. NARI-H-15.

MS medium composition (mg/L)	Mean no. of callus explants	% of organogenesis	Mean no. of shoots/callus	Mean shoot length (cm)
7.0 mg/L BAP	24.4hi	61hi	48.2ij	0.6ab
4.0 mg/L mT	29ef	72.5ef	58fg	0.4b
5.0 mg/L TDZ	27h	67.5h	54h	0.5ab
1.5 mg/L CPPU	19.5k	48.8k	22.7j	0.8a
7.0 mg/L BAP + 1.5 mg/L Kin	27.7fg	69.3fg	57.4g	0.5b
7.0 mg/L BAP + 1.5 mg/L CPPU	32d	80d	59e	0.4bc
7.0 mg/L BAP + 1.0 mg/L NAA	30.8de	77de	58.5f	0.5b
7.0 mg/L BAP + 1.5 mg/L IAA	27.4g	68.5g	54.2gh	0.5b
7.0 mg/L BAP + 1.0 mg/L IBA	24ij	60ij	49.7i	0.5b
4.0 mg/L mT + 1.5 mg/L Kin	34.4bc	86bc	61bc	0.4bc
4.0 mg/L mT + 1.5 mg/L CPPU	39.7a	99.3a	68.2a	0.3c
4.0 mg/L mT + 1.0 mg/L NAA	35.2ab	88ab	66.6ab	0.4bc
4.0 mg/L mT + 1.5 mg/L IAA	29.5e	73.8e	60.4c	0.4bc
4.0 mg/L mT + 1.0 mg/L IBA	27.2gh	68gh	59.9d	0.5b
5.0 mg/L TDZ + 1.5 mg/L Kin	32.5cd	81.3cd	58.7ef	0.5b
5.0 mg/L TDZ + 1.5 mg/L CPPU	34.7b	86.8b	63.2b	0.4bc
5.0 mg/L TDZ + 1.0 mg/L NAA	33.3c	83.3c	60cd	0.4bc
5.0 mg/L TDZ + 1.5 mg/L IAA	27.9f	69.8f	59.3de	0.4bc
5.0 mg/L TDZ + 1.0 mg/L IBA	24.1i	60.3i	52.8hi	0.5b

Forty explants were taken for each experiment. Values are mean of three repeated experiments. Mean within a column followed by the different letters are significantly different according to one way ANOVA and Duncan's multiple range test ($P < 0.05$).

Table 3. Effect of phytohormones on multiple shoot induction from immature leaf derived callus explants of safflower cv. NARI-H-15.

MS medium composition (mg/L)	Mean no. of callus explants	% of organogenesis	Mean no. of shoots/callus	Mean shoot length (cm)
7.0 mg/L BAP	25.7i	64.3ij	49.5i	0.5ab
4.0 mg/L mT	32.8de	82de	62.7d	0.4b
5.0 mg/L TDZ	28.4g	71gh	56.5gh	0.4b
1.5 mg/L CPPU	23.7ij	59.3j	25ij	0.6a
7.0 mg/L BAP + 1.5 mg/L Kin	31.4ef	78.5ef	58fg	0.4b

Continued

7.0 mg/L BAP + 1.5 mg/L CPPU	33.9cd	84.8cd	61.3de	0.4b
7.0 mg/L BAP + 1.0 mg/L NAA	31.6e	79e	59.8f	0.5ab
7.0 mg/L BAP + 1.5 mg/L IAA	28gh	70h	56h	0.5ab
7.0 mg/L BAP + 1.0 mg/L IBA	26.2hi	65.5i	51.5hi	0.5ab
4.0 mg/L mT + 1.5 mg/L Kin	35.3bc	88.3bc	69.9b	0.4b
4.0 mg/L mT + 1.5 mg/L CPPU	40a	100a	74.7a	0.3bc
4.0 mg/L mT + 1.0 mg/L NAA	38.4ab	96ab	71.2ab	0.4b
4.0 mg/L mT + 1.5 mg/L IAA	30.6ef	76.5f	60.8e	0.5ab
4.0 mg/L mT + 1.0 mg/L IBA	29.4f	73.5fg	58fg	0.5ab
5.0 mg/L TDZ + 1.5 mg/L Kin	33d	82.5d	64.2cd	0.4b
5.0 mg/L TDZ + 1.5 mg/L CPPU	35.6b	89b	68.5bc	0.5ab
5.0 mg/L TDZ + 1.0 mg/L NAA	35.2c	88c	65.9c	0.5ab
5.0 mg/L TDZ + 1.5 mg/L IAA	29fg	72.5g	60.4ef	0.4b
5.0 mg/L TDZ + 1.0 mg/L IBA	26.5h	66.3hi	57.3g	0.4b

Forty explants were taken for each experiment. Values are mean of three repeated experiments. Mean within a column followed by the different letters are significantly different according to one way ANOVA and Duncan's multiple range test ($P < 0.05$).

Table 4. Effect of phytohormones on multiple shoot induction from stem derived callus explants of safflower cv. NARI-H-15.

MS medium composition (mg/L)	Mean no. of callus explants	% of organogenesis	Mean no. of shoots/callus	Mean shoot length (cm)
7.0 mg/L BAP	23i	57.5ij	47.1hi	0.7a
4.0 mg/L mT	28.4e	71ef	55e	0.5ab
5.0 mg/L TDZ	25.6g	64gh	52.4f	0.5ab
1.5 mg/L CPPU	17.2j	43j	21i	0.5ab
7.0 mg/L BAP + 1.5 mg/L Kin	26.2f	65.5fg	56de	0.5ab
7.0 mg/L BAP + 1.5 mg/L CPPU	31.2c	78cd	57cd	0.4b
7.0 mg/L BAP + 1.0 mg/L NAA	29.8d	74.5de	56.8d	0.4b
7.0 mg/L BAP + 1.5 mg/L IAA	26fg	65g	52fg	0.5ab
7.0 mg/L BAP + 1.0 mg/L IBA	23.3hi	58.3i	48h	0.5ab
4.0 mg/L mT + 1.5 mg/L Kin	32bc	80c	58.5bc	0.5ab
4.0 mg/L mT + 1.5 mg/L CPPU	37.1a	92.8a	61.1a	0.4b
4.0 mg/L mT + 1.0 mg/L NAA	34ab	85b	59.3ab	0.5ab
4.0 mg/L mT + 1.5 mg/L IAA	28.5de	71.3e	55e	0.5ab
4.0 mg/L mT + 1.0 mg/L IBA	24.9gh	62.3h	48.4gh	0.5ab
5.0 mg/L TDZ + 1.5 mg/L Kin	30.4cd	76d	57.8c	0.5ab

Continued

5.0 mg/L TDZ + 1.5 mg/L CPPU	33.3b	83.3bc	59b	0.5ab
5.0 mg/L TDZ + 1.0 mg/L NAA	32bc	80c	57cd	0.5ab
5.0 mg/L TDZ + 1.5 mg/L IAA	26.8ef	67f	54.2ef	0.4b
5.0 mg/L TDZ + 1.0 mg/L IBA	23.8h	59.5hi	50g	0.4b

Forty explants were taken for each experiment. Values are mean of three repeated experiments. Mean within a column followed by the different letters are significantly different according to one way ANOVA and Duncan's multiple range test ($P < 0.05$).

shoots (>0.5) on rooting medium within 7 - 14 days of culture. In this case, individual excised microshoots subcultured on quarter strength MS basal salts, 1.0% sucrose and 0.8% agar medium supplemented with optimum level of NAA (2.0 mg/L) induced 92.8% (10.5 rootlets/plantlet) and 64% (5.0 rootlets/plantlet) rooting through micropropagation and organogenesis process, respectively after 30 days of culture. Other auxins such as IAA/IBA showed low rooting response for both *in vitro* culture methods. High level of NAA and low level of BAP showed 94.8% (11.5 rootlets/plantlet) and 68% (5.7 rootlets/plantlet) rooting response through micropropagation and organogenesis methods, respectively. About 95.6% (12 rootlets/plantlet) and 73.6% (6.4 rootlets/plantlet) root induction frequency was recorded in plantlets regenerated from micropropagation and organogenesis at NAA (1.5 mg/L) and CPPU (0.3 mg/L) treated medium, respectively. However, maximum of 96% rooting with an average of 12.8 number of rootlets were noticed per plantlet cultured on quarter strength MS basal medium supplemented with NAA (1.5 mg/L) and mT (0.1 mg/L) via micropropagation while about 84% rooting frequency with an average of 7.3 number of microroots were recorded through organogenesis process after 30 days of culture (**Figure 1(f)**; **Table 5**). In rooting media, the basal callus formation was higher in IBA or IAA alone tested medium than NAA via organogenesis process while IBA or IAA alone induced low amount of basal callus formation around the stem base of plantlets via micropropagation. The regenerated plants with vigorous healthy roots via micropropagation and organogenesis were acclimatized and showed 67% and 42% survival in potting mix after 14 - 21 days of transfer (**Figure 1(g)**). The *in vitro* raised plants did not show any morphological abnormalities compared to donor plant during the maturation period after 75 days of transfer to field under green house condition (**Figure 1(h)**).

4. Discussion

4.1. Shoot Induction and Multiplication via Micropropagation

The development of plant regeneration systems for safflower has progressed in spite of the high economic value edible crop. The *in vitro* regeneration response was influenced by the genotypic and seedling age of the explants in incorporated basal salts as well as different plant growth regulators supplemented media at varied concentrations. The use of mT in tissue culture for enhanced plant regeneration has increased in recent period. The positive effect of exogenous mT on plant regeneration has been reported in other plant species [25] [26]. The

Table 5. Effect of phytohormones on root induction in safflower (*Carthamus tinctorius* L.) cv. NARI-H-15.

¼ MS medium composition (mg/L)	Micropropagation				Organogenesis			
	Mean no. of plantlets	% of rooting	Mean no. of roots/plantlets	Basal callus formation	Mean no. of plantlets	% of rooting	Mean no. of roots/plantlets	Basal callus formation
NAA 2.0	23.2c	92.8c	10.5c	+ Low basal callus	16cd	64d	5.0c	+ Low basal callus
IAA 1.5	18d	72d	2.5d	+ Low basal callus	13e	52e	1.7d	++ More basal callus
IBA 1.5	9.4e	37.6e	1.1e	+ Low basal callus	8.1f	32.4f	0.8e	++ More basal callus
NAA + BAP 2.0 + 0.1	23.7b	94.8b	11.5b	-	17bc	68c	5.7b	-
NAA + CPPU 1.5 + 0.3	23.9ab	95.6ab	12.0ab	-	18.4b	73.6b	6.4ab	-
NAA + mT 1.5 + 0.1	24a	96a	12.8a	-	21a	84a	7.3a	-
NAA + $AgNO_3$ 1.5 + 0.5	23.6bc	94.4bc	11.1bc	-	16.7c	66.8cd	5.2bc	-

+ Low basal callus, ++ More basal callus, - No basal callus; Twenty five explants were taken for each experiment. Values are mean of three repeated experiments. Mean within a column followed by the different letters are significantly different according to one way ANOVA and Duncan's multiple range test ($P < 0.05$).

information regarding utilization of mT on plant regeneration is less focused till now. Hence, in the present study, an attempt was made to investigate the synergetic effect of BAP or mT or TDZ and CPPU of various levels on plant regeneration from 7 - 9 day old shoot-tip and node explants of safflower cv. NARI-H-15 via micropropagation. The effect of BAP or TDZ has no major impact on rate of plant regeneration than mT in the media. In earlier, Amoo *et al.* [27] reported that the treatment of mT has shown a promise role to influence high frequency plant regeneration when compared to TDZ in micropropagation of *Merwilla plumbea.* However, in our study, mT had highly significant effects ($P < 0.05$) in addition with CPPU on plant regeneration from shoot-tip explants. Similarly, the aromatic cytokinin mT promotes *in vitro* propagation, shoot quality and micrografting was produced in *Corylus colurna* [28]. These finding are in contrast with Basalma *et al.* [14] reports that have proved that the highest percentage of shoot regeneration per explants of safflower cv. Dinçer achieved on a MS medium containing TDZ and IBA. Our present finding is also not supported with other reports demonstrating that the urea-type cytokinins, TDZ and CPPU was more effective for the induction and the morphogenesis of adventitious multiple shoots from cotyledonary node explants of *M. crystallinum* plants than BAP [29].

4.2. Callus Induction and Proliferation

In addition, callus induction from cotyledon, immature leaf and stem explants of safflower cv. NARI-H-15 was investigated on MS medium supplemented with various levels of an individual hormones, NAA, IBA, IAA, 2,4-D, Dicamba, CPPU, pCPA and picloram at 0.5 to 2.5 mg/L. There were significant differences in the callus induction from the variations of the explants and various types of

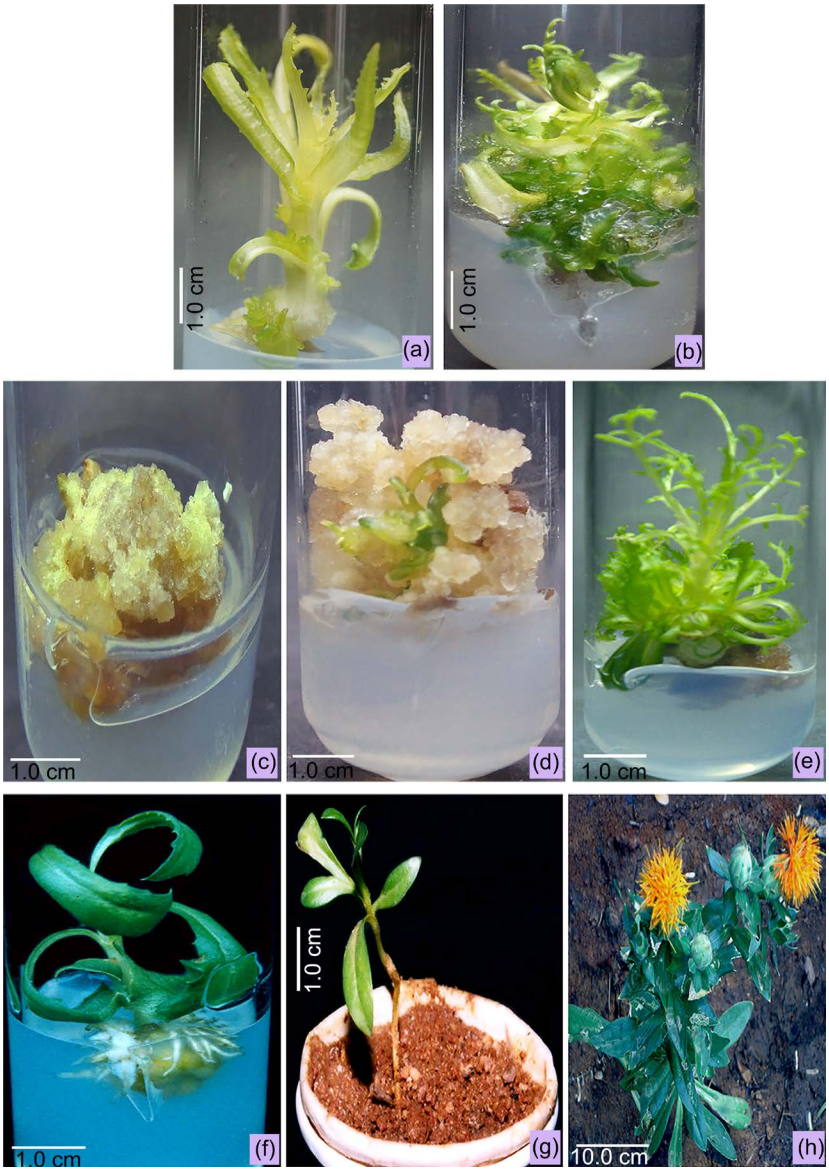
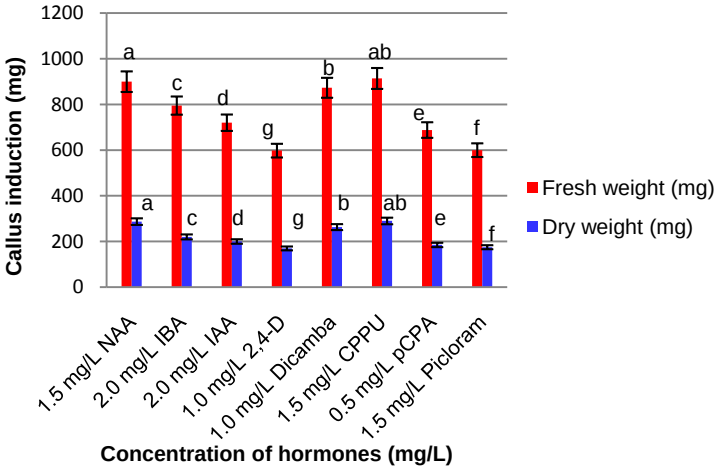
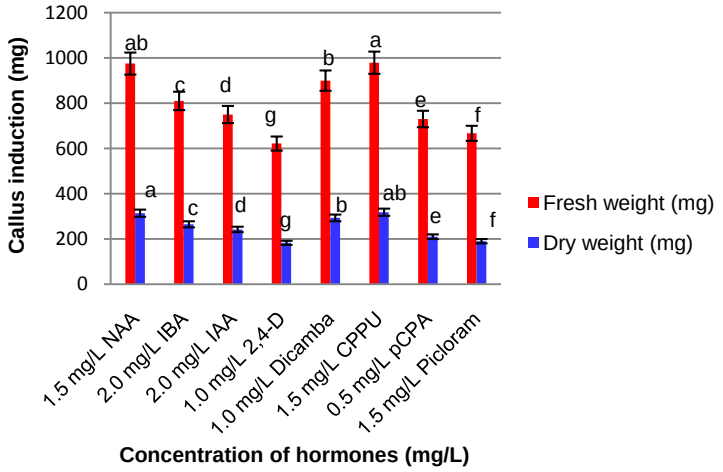


Figure 1. Effect of mT and CPPU on plant regeneration from shoot-tip meristem and immature leaf calli of safflower cv. NARI-H-15. (a, b) Initiation and proliferation of microshoots from a cultured shoot-tip explants on MS basal medium supplemented with mT (3.0 mg/L) + CPPU (0.5 mg/L) after 45 days in culture. (c) Induction of light green nodular calli from immature leaf explants on 1.5 mg/L CPPU fortified medium after 60 days in culture. (d, e) Initiation and proliferation of microshoots from organogenic calli on mT (4.0 mg/L) + CPPU (1.5 mg/L) fortified medium after 45 days in culture. (f) In vitro shoots rooted on 1/2 MS supplemented with NAA (1.5 mg/L) and mT (0.1 mg/L) after 30 days of culture. (g) An acclimatized plant in paper cup containing red soil, garden soil and sand (1:2:1) mixture after 14 - 20 days of transfer. (f) Field survived mature plants under green house condition after 75 days of transfer to field under green house condition.

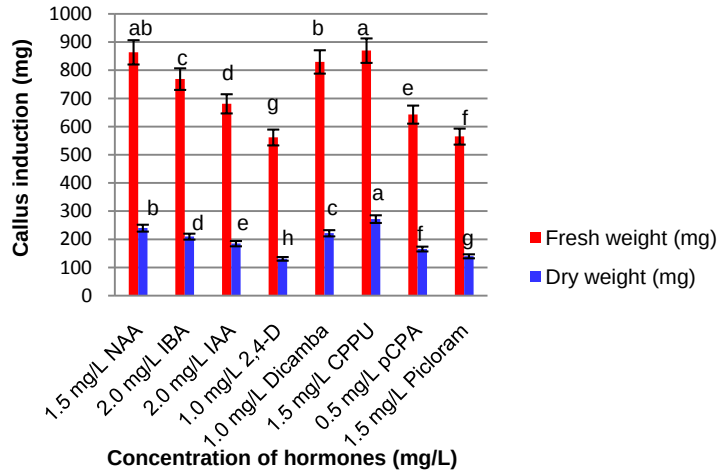
growth regulators tested media after 14 - 20 days of culture initiation (**Figure 1(a)**). Most of explants cultured on CPPU or NAA formed callus in all concentrations than other auxins tested. Earlier, Chavhan *et al.* [17] reported that the level of NAA and BAP was found best for callus induction from cotyledonary



(a)



(b)



(c)

Figure 2. (a). Effect of phytohormones on callus induction from cotyledon explants of safflower cv. NARI-H-15. (b) Effect of phytohormones on callus induction from immature leaf explants of safflower (*C. tinctorius* L.) cv. NARI-H-15. (c) Effect of phytohormones on callus induction from stem explants of safflower cv. NARI-H-15. Mean within a column followed by the different letters are significantly different according to one way ANOVA and Duncan's multiple range test ($P < 0.05$).

leaf disc explants of safflower cv Shardha and PBN 12 in comparison with other levels of NAA. Ghasempour *et al.* [18] study showed that the best result of significant callus induction was obtained from leaf of safflower cv. Lesaf on BAP tested medium after 25 - 30 days whereas hypocotyls produced moderate amount of callus on 2,4-D, NAA and BAP tested medium. But our earlier study showed that cotyledon explants of safflower cv. NARI-6 on MSG basal salts, myo-inositol, T. HCl medium supplemented with picloram produced more amount of callus [15]. However, in the present study we observed that CPPU was found superior to reveal significant result in callusing from cotyledon and immature leaf explants than NAA. Dicamba or IBA tested medium showed moderate response in callusing in all three explants than IAA, pCPA, 2,4-D and Picloram treated medium.

4.3. Shoot Organogenesis Response

Further, the callus morphology and adventitious shoot development was varied depend upon types, combination and concentrations of growth hormones in plant regeneration medium. In the present study, callus derived from cotyledons, immature leaf and stem of safflower cv. NARI-H-15 cultured on MS medium supplemented with various concentrations of individual hormones, BAP or mT or TDZ or CPPU alone or BAP or mT or TDZ in combinations with Kin or CPPU or NAA or IAA or IBA on shoot organogenesis was investigated. Among them BAP in combinations with CPPU showed satisfactory results in production of shoots from immature leaf calli than cotyledon and stem calli while BAP and NAA showed slow response in shoot organogenesis. In contrast, shoot regeneration was best from different explants derived calli of safflower cv Shardha, PBN 12 [17], Lesaf [18] and wild safflower (*C. persicus* Wild) [30] on MS supplemented with high level of BAP with low level of NAA. Earlier, we reported that embryogenic callus subcultured on MSG basal medium with TDZ and Picloram was developed more numbers of shoots from organogenic calli [15]. Basalma *et al.* [14] also reported the efficacy of various concentrations of TDZ and IBA on adventitious shoot regeneration from calli of cotyledonary leaves of safflower cv. Dinçer. Nikhil *et al.* [31] study also showed that best shoot induction response was obtained in different explants of safflower cv. AKS-207 in TDZ and NAA selective medium while leaf segments of *Carthamus arborescens* showed high frequency of shoot regeneration on TDZ and NAA treatment [13]. The contrast could be due to differences in cultivars, physiological state and age of explants. However, in the present observation, calli derived from immature leaf produced highest number of shoots than other explants in TDZ and CPPU tested medium. CPPU has been shown to possess a stronger cytokinin like activity to TDZ than combined action of other hormones for induction of high frequency of healthy adventitious shoot organogenesis from immature leaf derived calli. In other hand, the use of mT could be a new source of cytokinins with high morphogenetic activity. Proportion-dependent effects were recorded for larger numbers of shoots regeneration from different explants derived calli

on mT alone or in combinations with Kin or NAA or IAA or IBA. However, the shoot induction frequency was significantly ($P < 0.05$) increased and found to be greatest without formation of hyperhydricity in immature leaf calli than cotyledon and stem derived calli of safflower cv. NARI-H-15 on mT and CPPU treated medium. Similar results were reported with the mT treatments of potato [32] and *Aloe polyphylla* [22]. The use of mT and its derivatives has been recommended as a potential replacement for BAP [26], TDZ and other cytokinins in plant tissue culture industry. This is evident that callusing and shoot regeneration responses in safflower are purely genotype as well as species specific.

4.4. Rooting and Acclimatization Response

Moreover, the influence of culture conditions in rooting of safflower cv. NARI-H-15 plantlets was investigated further by transferring of plantlets from initial culture conditions to newly prepared medium with different plant growth regulators. The regenerated shoots were rooted after 30 days of culture in all treatment. There was no significant difference between MS media supplemented with NAA alone or NAA + BAP or NAA + $AgNO_3$ in terms of rooting per plantlets. The plantlets cultured on NAA + CPPU tested medium showed moderate response in terms of high frequency rooting of plantlets regenerated via micropropagation and organogenesis process (**Figure 1(a)**). Our earlier reports showed that the better rooting response was observed in plantlets of safflower cv. NARI-6 on NAA [11] or NAA and $AgNO_3$ tested media [15]. In contrast, the necessity of using IBA for roots induction of tissue culture plants of wild safflower was reported by Özdemir and Türker [30]. Chavhan *et al.* [17] study indicated that the rooting response of the regenerated cv. Sharda and PBN-12 plantlets was very poor on MS supplemented with 7.0% sucrose while inefficient rooting result was recorded on MS medium containing 7% - 8% sucrose and IAA [9]. However, it was evident that NAA in addition with mT exhibited significant ($P < 0.05$) results of better rooting without callus interception from regenerated plantlets of safflower cv. NARI-H-15 via micropropagation than organogenesis. In a similar fashion, low concentration of mT was resulted more roots in *Musca* [33] and turmeric [21] plants. The average root length within short time and reduce the expenditure coupled with tissue culture may have an optimistic influence on the survival of the plants. However, the acclimatized plants showed maximum survival rate in pots containing of soil mixture under green house condition. The transplanted *in vitro* plants in the field exhibited better survival and grown to mature plants.

5. Conclusion

The establishment of a successful efficient regeneration system suggests that meta-topolin in addition with CPPU may act as a new source of potential cytokinins and it is highly recommended for *in vitro* regeneration of safflower cv. NARI-H-15 for large scale production. It could be highly applicable to maintain the genetic stability of many cultivars of this genotype and would certainly help

in increasing safflower cultivation area in the world. In addition, the optimized regeneration protocol might be efficiently preferred to overcome the demand of raw material of safflower for plant based drug production, regeneration of new genotypes with improved productivity, resistance to biotic and abiotic factors through *in vitro* selection process and *Agrobacterium* mediated genetic transformation to modify genetic characteristics of agronomic traits in improvement of fatty acid contents in seed oil with enhancing the level of α tocopherol production in many cultivars of single genotypic safflower plant.

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