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Influence of different cytokinins on direct shoot regeneration from the different explants of *Carthamus tinctorius* L. Var Annigeri-2 (A high oil-yielding variety)

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ABSTRACT

The shoot tip, hypocotyls and cotyledons from the In vitro generated seedlings served as explant source for direct organogenesis in the present study. The influence on In vitro growth response and regeneration potentials of the shoot tip, hypocotyls, and cotyledons of safflower were evaluated on MS medium supplemented with different cytokines viz., TDZ/BAP/Kn and NAA (0.5-3.0 mg/l and 0.25-1.0 mg/l) evaluated. Among the different explants, the maximum mean shoot induction was recorded from shoot tip while cotyledons recorded minimum shoots induction. A protocol for large scale production of plantlets from shoot tip explant was standardized on MS medium supplemented with 1.5 mg/l TDZ and 0.5 NAA mg/l and 2.0mg/l BAP and 0.25 mg/l NAA. The In vitro derived microshoots from different explants were induced root initials on MS half strength medium supplemented with a range of 0.1 to 0.5mg/l NAA. The rooting response was maximum at 0.5 mg/l NAA supplemented medium. Complete rooting was observed within 45 days of culture. The rooted plantlets were subjected to an acclimatization process.

Keywords: *Carthamus tinctorius*, safflower, thidiazuron, micropropagation.
***Abbreviations**: IAA = Indole-3-acetic acid; IBA = Indole-3-butyric acid; BAP = 6-Benzyl amino purine; 2, 4-D = 2, 4-dichlorophenoxy acetic acid; TDZ = Thidiazuron; NAA: naphthalene acetic acid; Kn: kinetin; SE: Standard Error; MS medium = Murasighe and Skoog medium*

INTRODUCTION

Tissue culture is an effective way to offers quick and efficient methods to exploit crop or medicinal plants meaningfully to meet the measuring demands of growing population, industries and it also helps to study many basic aspects of cell development and differentiation [1]**.** Advances in plant tissue culture and other innovations in biotechnology have provided opportunities to plant breeders for creating a wide range of useful genetic variability and increased the precision and efficiency of selecting desirable genotypes. In particular, *In vitro* methods are being used progressively more as an adjunct to traditional breeding method for genetic improvement of crops [2]**.**

Safflower, *Carthamus tinctorius*L., is a member of the family Asteraceae and is one of the humanity's oldest crops and it is known as 'kusum' in India. It is cultivated mainly for its edible oil and it has high oil content (35%) with high amount of linoleic acid, which attributes the therapeutic value safflower seed oil [3]. It has been grown as a minor crop with world seed production of around 6 lakh tons/yrand India is the major producer in the world [4]**.**

Safflower has 4000yrs history which has been used for the several purpose such as coloring the cotton, silk, food and also flavoring the food [5]**.** In ayurveda suggests that the leaves are laxative, appetizer and diuretic also useful in urorrhea and ophthalmopathy[6, 7]**.** Safflower has several ethnomedicinal claims and many investigators has been investigated for its potential activity such as anti-tumor, sedative [8]**,** antimicrobial, anthelmintic [9, 10]**,** anti-inflammatory and analgesic effects [11] antihyperglycemic effects [12], hepatoprotective, antioxidant properties [13] and wound healing activity [14]**.** In India there are more than thirty varieties of safflower being cultivating, of which, three common varieties in Karnataka are Annigeri-1, Annigeri-2 and Annigeri-300. The variety Annigeri-2 of *Carthamus tinctorius*L. was developed in AICRP (All India Co-ordinated Research Project, Safflower), Agriculture Research Station, Annigeri, Gadag district [15]**.** Literature review indicated that there are varietal differences in terms of callusing and regeneration potentialities of safflower and such a study have not been undertaken in the commonly cultivated variety of Karnataka, Annigeri-2. Therefore, in this present paper focused on the rapid production protocol for the micro-propagation of *C tinctorius L*., var Annigeri-2 andto understand the influence of different cytokinins on direct shoot regeneration from the different explants of *Carthamus tinctorius*L. Var Annigeri-2.

MATERIALS AND METHODS

Plant material

Healthy seeds of *C tinctorius L*., var Annigeri-2 seeds were collected from All India Coordinated Research Project (Safflower), Agricultural Research Station, Annigeri, Gadag (D), Karnataka. Fresh seeds were used to raise the seedlings under sterile conditions. Seeds were pre-soaked for 2-3 hours and the seeds that settled down were selected for inoculation. Seeds were washed under running tap water and then treated with 0.1% (V/V) aqueous solution of Tween-20 for 10 min to remove dust particles and followed by bavistin(Biostadt India Ltd, Mumbai) to remove fungal contaminants. Finally, the seeds were washed thoroughly for several times with double distilled water. All the required sterilized materials were transferred to the laminar air flow cabinet. Seeds were then immersed in 70% ethanol for 1 min and surface disinfected with an aqueous solution of 0.1% HgCl2 for 10 min. The disinfectant was removed by rinsing the seeds with autoclaved distilled water for 5 to 6 times. Then the seeds were inoculated on pre-sterilized media with an appropriate concentration of phytohormones.

Media and explant preparation

In the present investigation, MS media [16] was used as basal nutrient medium for direct organogenesis. Following are the hormone supplements used either singly or in combination for the *In vitro* seed germination, direct organogenesis and for rooting of microshoots. For seed germination, Basal Medium (MS) and Basal Medium + 0.1mg/l 2, 4-D/0.5 mg/l Kn. The media for direct organogenesis was supplemented with cytokines viz., TDZ/BAP/Kn (0.5, 1.0, 1.5, 2.0, 2.5 or 3.0 mg/l) and auxins such as NAA/IAA/IBA (0.25, 0.5, or1.0 mg/l). The media were prepared by pippeting out appropriate volumes of macro, micronutrients, vitamins, amino acids, and iron source. The carbon source, sucrose was added to the solution at the concentration of 3% and made up to desired volume with sterilized double distilled water. The pH of the medium was adjusted between 5.6 -5.8 by adding 0.1N HCl or 0.1N NaOH prior to homogenization of the medium when necessary. The media were gelled with 0.8% of homogenized bacteriological grade Agar-agar. After homogenization, about 15 - 20 ml of molten medium was dispensed into each culture tube of 25 x 150 mm dimension and 50 ml of media was dispensed into culture bottles and conical flasks of 250 ml capacity. The tubes and the conical flasks were plugged with non-adsorbent cotton and the culture bottles were closed with screw caps. The culture media were steam sterilized in the autoclave at 15 lbs/in2 at a temperature of 1210 C for 15-20 min. For explant preparation, the *In vitro* germinated 10days old seedlings were used to prepare explants viz. shoot tip, hypocotyls, and cotyledon as a source for micropropagation studies. All the explants were excised and inoculated aseptically on to the media fortified with different concentrations and combinations of cytokines and auxins as mentioned above.

Culture conditions

Inoculated explants were maintained at 25 ± 20 C, 12 hrs of photoperiod with a light intensity of 2000 lux (Philips cool-white fluorescent tubes, India) and 65 to 70% relative humidity. Sub-culturing was done at the intervals of twenty days. Visual observations of the cultures were recorded on the basis of the percentage of cultures showing response in terms of proliferation and the number of regenerants per culture.

Rooting and acclimatization of *in-vitro* plantlets

For root induction *In vitro* grown, 45-50 days old microshoots were aseptically excised and transferred to full/half strength media (MS) supplemented with various concentrations of IBA, NAA and IAA (0.1, 0.3, 0.5 & 1.0 mg/l). Plantlets with well-grown roots were removed from the culture tubes/bottles and roots were washed in distilled water and the plants were transferred to pots containing sterilized mixture of vermicompost, sand and soil in 1:1:1 ratio. The regenerants were hardened for 2-3 weeks at the culture condition by covering with a thin perforated

transparent polythene bag to maintain humidity and were watered with 1/10th strength of MS basal salt solution. After 2-3 weeks of incubation, the polythene bags were removed and the plants were exposed to a natural environment at greenhouse.

Statistical analysis

The results were expressed as mean ± SE. Statistical analysis was performed with one-way analysis of variance (ANOVA) followed by Turkey's post-test. P<0.01 was considered as statistically significant.

RESULTS AND DISCUSSION

***In vitro* seed germination**

The seeds were inoculated on MS media supplemented with two different combinations of auxins and cytokinins. Even though the germination was observed in both the basal and hormone supplemented media maximum germination frequency (>95%) was noticed in Kn (0.5mg/l.) and 2, 4-D (0.1mg/l.). Therefore, for the entire subsequent explants source, *In vitro* germination was carried out utilizing this combination. The explants viz. shoot tip hypocotyls and cotyledon were taken from 10days old seedlings for further studies.

In the present study, direct organogenesis was initiated using TDZ and NAA /IAA/ IBA, BAP and NAA /IAA/ IBA and Kn and NAA /IAA/ IBA hormones at various concentrations, utilizing different explants viz shoot tips, hypocotyl and cotyledon. The direct organogenesis tendency was however optimized in only two hormonal augmented media i.e. TDZ + NAA (Table 1) and BAP + NAA (Table 2) and the results for two parameters viz. regeneration percentage per explant and multiple shoots were evaluated.

Table 1. Direct shoot regeneration frequency from different seedling explants of *Carthamus tinctorius*, Annigeri-2 on MS medium supplemented with different concentration of TDZ & NAA

Plant growth regulators (mg/l)		Shoot tip		Hypocotyl		Cotyledon	
TDZ	NAA	Shoot Induction regeneration (%)	No. of Shoots/explant	Shoot induction regeneration (%)	No. of Shoots/ explant	Shoot induction regeneration (%)	No. of Shoots/ explant
0.5	0.25	45	4.30 ± 0.75	30.1	3.60 ± 0.75	17.7	1.30 ± 0.95
0.5	0.5	26.5	2.40 ± 0.19	24.3	1.70 ± 0.09	18.6	1.60 ± 0.16
0.5	1.0	---	---	---	---	---	---
1.0	0.25	77.7	6.40 ± 0.11	33.6	3.10 ± 1.09	18.1	1.06 ± 1.00
1.0	0.5	50.9	4.40 ± 1.86	51.1	3.30 ± 0.11	50.0	4.24 ± 0.11
1.0	1.0	47.7	3.30 ± 0.24	44.8	3.36 ± 0.26	37.32	2.90 ± 0.24
1.5	0.25	68.4	4.46 ± 0.68	70.22	4.60 ± 0.68	66.8	3.80 ± 0.39
1.5	0.5	100	12.70 ± 1.09**	88.9	6.30 ± 0.73	79.6	5.70 ± 0.36*
1.5	1.0	95.2	8.10 ± 0.82**	96.9	9.10 ± 0.39**	33.3	4.60 ± 0.68**
2.0	0.25	50.0	5.40 ± 0.75	70.5	6.80 ± 0.65*	31.6	4.10 ± 0.26*
2.0	0.5	66.7	6.80 ± 0.93**	58.4	4.30 ± 1.75	27.4	2.20 ± 0.30
2.0	1.0	60.6	5.80 ±0.68*	100	9.90 ± 1.07**	25.5	1.60 ± 0.28
2.5	0.25	44.6	5.00 ± 0.50	42.7	3. 25 ± 0.18	33.2	2.50 ± 0.23
2.5	0.5	33.3	2.70 ± 0.58	33.3	2.50 ± 0.55	65.4	5.30 ± 0.21
2.5	1.0	60.3	4.80 ± 0.63	38.9	2.60 ± 1.01	40.7	2.90 ± 0.81
3.0	0.25	27.1	2.10 ± 0.57	22.7	1.90 ± 0.76	43.3	3.05 ± 0.39
3.0	0.5	38.1	2.60 ± 0.69	29.4	2.06± 0.27	14.1	1.40 ± 0.58
3.0	1.0	43.7	3.73±0.45	32.33	2.06 ± 0.20	42.4	3.80 ± 0.71
F- Value Df			1570,92 17,162		1149 17,162		423 17,162

*Each value represents mean±SE of 10 observations. The *P value<0.05 was considered statistically significant*

Table 2. Direct shoot regeneration frequency from different seedling explants of *Carthamus tinctorius*, Annigeri-2 on MS medium supplemented with different concentration of BAP & NAA

Plant growth regulators (mg/l)		Shoot tip		Hypocotyl		Cotyledon	
BAP	NAA	Shootinduction regeneration (%)	No. of Shoots/explant	Shoot induction regeneration (%)	No. of Shoots/explant	Shoot induction regeneration (%)	No. of Shoots/explant
0.5	0.25	43	2.90 ± 0.75	30.7	2.10 ± 0.75	18.7	1.10 ± 0.05
0.5	0.5	26.5	1.80 ± 0.19	33.3	2.40 ± 0.09	11.6	1.00 ± 0.19
0.5	1.0	---	---	---	---	---	---
1.0	0.25	57.7	3.45±0.15	22.7	1.40 ± 0.09	27.7	2.80 ± 1.00
1.0	0.5	65.9	4.40±0.18	61.4	3.90 ± 0.11	48.7	3.30 ± 0.01
1.0	1.0	42.27	2.30±0.23	46.3	2.70 ± 0.86	66.7	4.20 ± 0.24
1.5	0.25	38.4	1.90±0.14	27.3	1.80 ± 0.68	66.8	4.40 ± 0.93
1.5	0.5	70	5.60±0.14*	67.9	4.30 ± 0.73*	78.6	6.60 ± 0.73**
1.5	1.0	66.2	4.10±0.17	68.6	4.70 ± 0.93*	53.3	3.60 ± 1.68
2.0	0.25	95.6	7.60±0.54**	84.5	6.80 ± 0.65**	41.6	3.80 ± 0.26*
2.0	0.5	100	8.20±0.88**	98.4	7.90 ±0.75**	27.4	1.70 ± 1.03
2.0	1.0	60.6	4.40±0.52	53.6	3.70 ±0.37	25.5	1.60 ± 0.52
2.5	0.25	44.6	3.10±0.32	45.1	3. 55 ± 0.18	33.2	2.50 ± 0.23
2.5	0.5	33.3	2.70±0.59	48	2.30 ± 0.55	55.4	3.80 ± 0.21
2.5	1.0	53.3	4.20±1.11	27.3	1.75 ± 1.01	15.7	1.40 ± 0.81
3.0	0.25	17.1	1.60±0.47	19.9	1.30 ± 0.76	22.3	2.05 ± 0.39
3.0	0.5	28.1	1.90±0.18	36.9	2.80± 0.92	42.5	3.40 ± 0.58
3.0	1.0	13.0	1.20±0.13	17.5	1.60 ± 1.00	12.4	1.08 ± 0.71
F- Value Df			773 17,162		644 17,162		410 17,162

*Each value represents mean±SE of 10 observations. The *P value<0.05 was considered statistically significant.*

Among the two combinations investigated TDZ and NAA has yielded better regeneration results when compared to BAP and NAA indicating its efficacy in direct organogenesis. In the TDZ + NAA plant growth regulator combinations, the shoot tip has recorded highest (100%) shoot regeneration at 1.5 mg/l and 0.5mg/l followed by 1.5mg/l and 1.0mg/l. Maximum multiple shoots (12.70±1.09 and 8.10±0.82) was also recorded at the same concentrations. In regard to hypocotyls, the highest regeneration potential (100%) and multiple shoot (9.90±1.07) was noted at 2.0 mg/l and 1.0 mg/l followed by 96.9 and 9.10± 0.39 at 1.5mg/l and 1.0mg/l. Whereas, cotyledon has shown maximum regeneration tendency for both the parameters (79.6% and 5.70 ± 0.36) at 1.5 mg/l and 0.5 mg/l. It is evident from the results that among the three explants, shoot tip has proved to be an ideal material for direct organogenesis by virtue of its higher regeneration tendency and also in the formation of multiple shoots followed by hypocotyl and cotyledon (Figure 1). It is interesting to note that the higher concentrations of TDZ (>3.5-5 mg/l) studied failed to elicit a favourable response for shoot induction and it results in the formation of vitrified and watery shoots from all the explants.

Similarly, in the BAP + NAA supplemented media the shoot tip has shown maximum shoot regeneration potential (100%) and the number of shoots per explant (8.20±0.88) at 2.0 g/l and 0.5mg/l followed by 95.6% and 7.60±0.54 respectively at 2.0 mg/l and 0.25 mg/l. Furthermore, hypocotyl and cotyledon have exhibited maximum shoot regeneration and multiple shoots (98.4 and 78.6% ; 7.90 ±0.75 and 6.60 ± 0.73) respectively at 2.0 mg/l and 0.5mg/l and 1.5mg/l and 0.5mg/l. Similar to TDZ and NAA combinations, the shoot tip has expressed better regeneration abilities for both the parameters evaluated (Figure 1).

Many investigators reported the use of TDZ+NAA combination for induction of adventitious shoots in safflower but constitutes the successful attempt of achieving shoot regeneration from all seedling explants of the Indian safflower cultivar, Annigere-2 using these growth regulators. The interaction of explant vs. growth regulator was found significant in the earlier investigations on safflower tissue culture. Despite the high frequency of shoot regeneration from leaves, cotyledon and hypocotyl, root sections produced only callus on medium supplemented with TDZ+NAA [17, 18]. Radhika et al.(2006) [19], reported high-frequency shoot regeneration in CV HUS-305 on MS medium supplemented with TDZ and NAA. They have also reported differential performance of regeneration of different explants in different varieties viz. manjira, A-1 and HUS-305 on all media with the exception of media supplemented with TDZ+NAA.

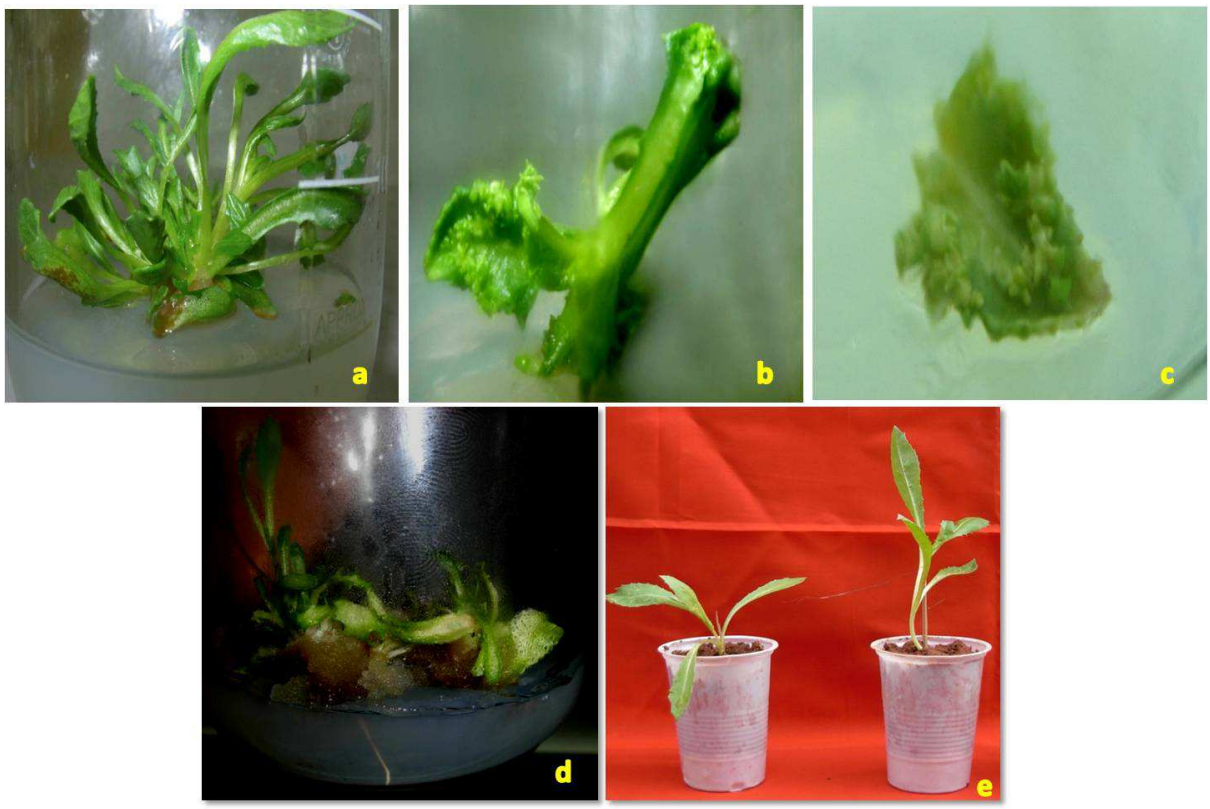

Figure.1 Direct organogenesis from different explants. a) Shoot induction from shoot tip explant on a medium, b) Shoot induction from hypocotyls explant on a medium, c) Shoot induction from cotyledon explant on a medium, d) Regenerated shoot showing rhizogenesis and e) Hardened plantlet regenerated from different explants

It is evident from the comparison of the results that, among the three explants, shoot tip proved to be a suitable material for direct organogenesis mode of multiplication for safflower Annigeri-2 variety. Further, it has shown the higher mean values for both shoot regeneration frequency and multiple shoots in both the hormonal combinations which might be due to the fact that tissues from same organs are more disposed to rapid cell division than others [20]**.** Similar observation of differential performance of various explants in terms maximum shoot regeneration and multiple shoots per explant in safflower was made by George & Rao (1982) [21] , Tejovathi& Anwar (1987) [22], Prasad et al. (1991) [23], Orlikowska& Dyer (1993) [17], Sujatha&Suganya (1996) [24], Nikam&Shitole (1999) [3], Sujatha& Dinesh (2007) [25] and Dilek et al. (2008) [18] utilizing different varieties of safflowers. The importance of choosing the ideal explant for the successful establishment of *In vitro* culture has been emphasized by Gamborg et al. (1974) and Torres &Carlisi (1986) [26, 27]. They have showed that the expressions of totipotency also vary with the physiological state of the explant derived from the same plant.

In tissue culture, the effect of cytokinins is most noticeable to stimulate cell division and control morphogenesis. cytokinins in shoot culture media overcome apical dominance and release lateral buds from dormancy. Cytokinins are very effective in promoting direct or indirect shoot initiation. They are used for adventitious shoot formation purpose in combination with auxins. Most oftenly balance between auxin and cytokinin normally gives the most effective organogenesis [28]. The effect of cytokinins on tissue or organ cultures can vary according to the particular compound used. The type of culture, a variety of plant from which it was derived and whether the explant is derived from juvenile or mature tissues. In Corylusavellana, 5 mg/l BA gave the best rate of shoot multiplication from juvenile explants, but 10 mg/l zeatin was required for nodal sections of plants in the adult phase [29]**.** A requirement for a particular cytokinin is sometimes noted for the induction of embryogenesis [30]**.** The promotion of direct or indirect adventitious shoot formation; for example, cultures of Browallia viscose required iP for the initiation of adventitious shoots whereas, kinetin, BA and zeatin were ineffective [31]**.**

A low concentration of auxin is often beneficial in conjunction with high levels of cytokinin when shoot multiplication is required, although in some cases cytokinin alone is sufficient. It is important to choose an auxin at a concentration that will promote growth without inducing callus formation. Many investigators viz. Lane (1979), Bhojwani (1980), Paterson &Rost (1981) and Garland &Stolz(1981)[32-35] found cytokinins are very effective in promoting direct or indirect shoot initiation. A wide range of cytokinins like Kinetin, BAP and TDZ have been employed in shoot proliferation [34, 36]**.** The reason for effectiveness of the BAP and TDZ may lie in the ability to

increase in the polyribosome content in the cells due to which the production of D-type cyclin (CycD3) which stimulate the cell cycle progression [37-40]**.**

The thidiazuron (TDZ) (N-phenyl-N′-1, 2, 3-thidiazol-5-ylurea), is a thiadiazole- substituted phenylurea and reported possessing high cytokinin activity [41]. In some plants, it is proved to be more effective than adenine-based compounds (e.g. BAP) by 100 folds as in tobacco callus [42]and produced more shoots in azalea shoot cultures. It is useful for inducing adventitious shoot regeneration e.g. in Rhododendron [43] and Malus[44]**.** TDZ can also be highly effective for inducing axillary shoot formation in shoot cultures e.g. in Rorippa[45];Linum [46]; Mulberry [47];*Hyoscyamusniger*[48**]** and *Mucunapruriens*[49]**.**Thidiazuron was also adopted for the micropropagation of woody plants in the family Oleaceae[50]**.**

The induction of rhizogenesis usually requires an adjustment in the levels of auxins and cytokinins. Rhizogenesis is usually achieved by treatment with auxin alone. In the present investigation, rooting of the micro shoots was transferred to half strength MS medium supplemented with a range of 0.1 to 0.5mg/l NAA, IAA and IBA. The rooting response was 100% at 0.5 mg/l NAA supplemented half strength MS medium. The roots emerged from the basal nodes which were grew crookedly down words and penetrated into the media. Rooted shoots were successfully acclimatized and established in the soil. The present results on rhizogenesis in an *In vitro* generated shoots are similar to the observations of Radika et al. (2006) [19] suggesting *In vitro* rooting behavior is identical in Annigeri-2 variety of safflower with other Indian cultivars. Further, Walia et al. (2005) [51] reported in their studies that among the three auxins viz. IBA, NAA and IAA, even though IBA exhibited highest rhizogenic response followed by NAA, the roots formed on NAA supplemented media were found to be thick, elongated and with lateral roots suggesting its benefit in rhizogenesis. Friedman et al. **(1985)**[52], suggested the auxin-induced root formation is thought to require or induce the promotion of polyamine synthesis.

Considering the crop, industrial and medicinal value of safflower, micropropagation studies in different cultivars is of paramount importance in many crop improvement strategies. The present study documents the report on the high-frequency shoot regeneration from seedling explants of safflower, C. tinctorious var., Annigeri-2 by direct organogenesis mode.

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