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Development of *in-vitro* protocol for direct regeneration from thalamus *ex-plant* of *Tagetes patula* L. var. Pusa Deep

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Abstract
The effect of growth hormones such as BAP, IAA and KINETIN in different treatment combinations on direct differentiation of shoots from thalamus *ex-plants* of French marigold (*Tagetes patula* L. var. "Pusa Deep") have been studied. A total of nine treatments was used to screen thalamus *ex-plant*s to determine the capability for plant regeneration and subsequently to find out the optimum medium conditions for high potentiality of direct shoot formation. Thalamus *ex-plant*s behaved differently in all over nine treatments and all the treatments are highly significant over the (T_0) control-MS devoid of hormones. The best medium found for direct, shoot organogenesis from the thalamus of Pusa Deep was treatment (T_7) MS + BAP 1.0 mg/l + KIN 0.5 mg/l + IAA 1.0 mg/. Pre-treatment with Carbendazim (0.2%) + Mancozeb (0.2%) + 8-HQC (200 mg/l) for 1.5hr resulted in minimum fungal (28.33%), bacterial (25.33%) contamination. Surface sterilization with 0.1% $HgCl_2$ for 5 min reduced microbial contamination up to (44.33%) simultaneously increased the survival percentage upto (55.67%). This technique can be utilized in gene transfer technology, *in vitro* mutagenesis, production of lutein rich pharmaceutical compounds and secondary metabolites using tissue culture techniques.

Keywords: French marigold, direct differentiation, thalamus, growth hormones, shoot organogenesis

Introduction
Marigold (*Tagetes* sp.) is a herbaceous ornamental plant grown all over the world as a loose and cut flower which belongs to the family Asteraceae. *T. erecta* (African marigold) and *T. Patula* (French marigold) are commonly cultivated species grown for decorative purpose while *T. minuta* L. (wild marigold) is cultivated for essential oil purposes. In India, it is one of the most commonly grown flowers and extensively used for decoration, social, religious, landscaping and carotenoid extraction purpose. To fulfil the increasing demand of quality planting material of marigold, micro-propagation is one of the viable approaches for large scale multiplication. There are very few published reports on the standardization of micropropagation protocols in marigold. Various research workers have used different types of *ex-plant*s in the development of *in-vitro* protocols. (Unopened capitula) Kothari and Chandra 1984, 1986 [7, 8] and Ram and Mehta 1982 [15], (leaves) Misra and Datta 1999, 2001 [9, 4] (hypocotyl and leaves) Belarmino *et al.* 1992 [1] and Venegas *et al.* 2002 [16], (cotyledon and hypocotyl) Mohamed *et al.* 1998 [11], Bespalhok and Hattori, 1998 [2], (shoot apexes) Miranda-Ham *et al.* 2006, (hairy roots) Mukundan and Hjortso, 1991b [12], (internode) Croes *et al.* 1989 [3] and (single-node stem segments) Pratibha *et al.* 2000, [14] (Anther) Kumar *et al.* 2018 [9] were used for successful *in vitro* establishment of cultures of *Tagetes* species. However, the regeneration through thalamus ex-plant have not yet reported in *Tagetes* for mass multiplication. The present study reports a highly efficient direct adventitious shoot regeneration system from thalamus *ex-plant*s. This protocol will be helpful for genetic transformation, *in-vitro* pollination and fertilization, large scale production of disease free quality material, somaclonal variants, in-vitro mutation breeding, *in-vitro* induction of pigments and secondary metabolites and *in-vitro* selection for biotic and abiotic stresses.

Materials and Methods
In this present study, French marigold (*Tagetes patula* L.) var. "Pusa Deep" plants were raised in an open field condition of the research farm of the Division of Floriculture and Landscaping, ICAR-IARI, New Delhi. The thalamus, which is present at the base of the flower is used as a non-axillary *ex-plant* in this study. Here, half opened, healthy, disease and insect free flower buds were collected directly from the field grown plants and excised only the

thalamus part. These were then washed with (0.1%) aqueous solution of Teepol for 5 minutes, followed by washing under running tap water for 10-15 minutes. Pre-treatment of thalamus *ex-plant*s were done with standardised doses of Carbendazim (0.2%) + Mancozeb (0.2%) + 8-HQC (200 mg/l) for 1.5hr.

After that, they were continuously shaked with the help of a shaker at 500 rpm. Observations such as per cent fungal infection of explants, per cent bacterial infection of explants, per cent contamination of *ex-plant*s and per cent explant survival were recorded during this experiment.

Then, the pre-treated thalamus were taken into laminar airflow and washed with sterilized double distilled water for 3-4 times to remove all the traces of Pre-treated fungicides and detergents. Then after, they were subjected to standardized dose of mercuric chloride ($HgCl_2$ 0.1% for 5 min) as a surface sterilization treatment. Observations such as Per cent fungal infection of *ex-plant*s, Per cent bacterial infection of *ex-plant*s, Per cent contamination of *ex-plant*s and Per cent *ex-plant* survival were recorded during this experiment.

*Ex-plant*s were then washed with sterilized double distilled water for 3-4 times to remove the traces of mercuric chloride ($HgCl_2$) followed by drying with autoclaved tissue paper for 5 minutes. After complete drying, individual thalamus is cut equally into two pieces and cultured on prepared autoclaved MS media. The MS culture media was prepared using double distilled water at 121 ºC for 1hr (15-20 lbs/inch² pressure) and pH of the medium was adjusted to 5.75-5.8. Agar (8.0 g/L) was added to the medium to solidify. All the cultures were maintained at 25±1 °C temperature and RH (70% with a photoperiod of 16:8 hours of light and dark cycles under fluorescent white light (47µmol/m^2/S). The calli derived from different explants were transferred to fresh medium at 1 week interval to avoid browning of the cultures.

Experimental design and statistical analysis

The experiments were laid out in completely randomized design (CRD) with three replications. The complete data was analysed using OPSTAT software. All the percentage data were subjected to Angular transformation before calculating ANOVA.

Results

Effect of pre-treatment on thalamus *ex-plant*s of French marigold

Thalamus *ex-plant*s are subjected to different pre-treatment methods with a several combinations of bacteriocides and fungicides. Data related to effect of pre-treatments on contamination (%) and survival (%) of ray florets and thalamus *ex-plant*s were presented in the Table 1. Here, except (T_0) - Control (distilled water) for 1hr all other treatments reduced the microbial contamination significantly and enhanced the *ex-plant* survival percentage. Among the different pre-treatments, (T_8) - Carbendazim (0.2%) + Mancozeb (0.2%) + 8-HQC (200 mg/l) for 1.5hr showed minimum fungal (28.33%), bacterial (25.33%) contamination with maximum survivability (46.33%) as compared to control (31.00%, 69.00% and 0.00%). Mancozeb played here more effectively in reducing contamination 79.67% in (T_3) Mancozeb (0.2%) + 8 HQC (200mg/l) for 1hr and 69.67% in (T_7) Mancozeb (0.2%) + 8 HQC (200mg/l) for 1.5hrs as compared to Carbendazim 85.67% in (T2) Carbendazim (0.2%) + 8 HQC (200mg/l) for 1hr and (79.67%) in (T6) Bavistin (0.2%) + 8 HQC (200mg/l) for 1.5hr.

Effect of surface sterilization on thalamus *ex-plant*s of French marigold

The effect of mercuric chloride as surface sterilization of thalamus *ex-plant* of French marigold is performed after the best pre-treatment with Carbendazim (0.2%) + Mancozeb (0.2%) + 8-HQC (200 mg/l) for 1 hour. These were surface sterilized with 0.1 percent $HgCl_2$ for different durations before inoculation. The presented data in the Table 2 elucidates that, surface sterilization of thalamus with 0.1% $HgCl_2$ for 5 min reduces microbial contamination up to (44.33%) and simultaneously increases the survival percentage (55.67%). When *ex-plant*s were exposed to extremity (8 min) to 0.1% $HgCl_2$ it reduced the contamination but the survivability chance was very less. Moreover, it caused browning of the *ex-plant*s followed by subsequent drying within a week. Here, all the treatments are significantly different over control T_0 (Distilled water).

Effect of growth regulators on direct shoot organogenesis from thalamus *ex-plant* of French marigold

Thalamus *ex-plant*s were cultured on the basal medium using MS (Murashige and Skoog, 1962) [13] with different concentrations of BAP, IAA and KIN and they responded differently. The results of direct shoot regeneration from thalamus *ex-plant*s were described in Table 3. From the day 4th the entire thalamus region cultured were started swelling and transformed into a mass with differentiation of cells. Here, treatment (T_7) MS + BAP 1.0 mg/l + KIN 0.5 mg/l + IAA 1.0 mg/l showed the maximum number of explants producing regeneration followed by (T_8) MS + BAP 2.0 mg/l + KIN 0.5 mg/l + IAA 1.0 mg/l. While, (T_5) medium supplemented with BAP 3.0 mg/l + IAA 0.5 mg/l (23 days) and treatment (T_6) MS + BAP 0.5 mg/l + KIN 0.5mg/l + IAA 1 mg/l (21.33) showed minimum days for callus induction but no regeneration. Here, all the treatments were significantly different over (T0) control-MS devoid of hormones. Although, treatment (T_7) MS + BAP 1.0 mg/l + KIN 0.5 mg/l + IAA 1.0 mg/l showed maximum (16.67%) percent regeneration followed by (T_8) MS + BAP 2.0 mg/l + KIN 0.5 mg/l + IAA 1.0 mg/l (12.28%). The no. of days taken for direct regeneration were (53.17days) and (51.00 days) for T_8 and T_7 respectively. Bud forming capacity was found to be highest in (T_7) MS + BAP 1.0 mg/l + KIN 0.5 mg/l + IAA 1.0 mg/l (0.85) with (5.27) no. of visible buds followed by (T_8) MS + 2.0 mg/l BAP + KIN 0.5 mg/l + IAA 1.0 mg/l (0.48) with (3.67) no. of visible buds. Thalamus *ex-plant*s did not show roots in any of the treatments supplemented with different concentrations of growth regulators.

Discussion

Regeneration protocol using thalamus *ex-plant* is developed using different growth regulators with MS medium (Murashige and Skoog, 1962) [13] as a basal medium. Here, Time of collection of flower played a very important role in successive regeneration. Flowers were more likely attacked by pathogens, when they fully opens. Keeping with this view, *ex-plant*s were collected from half opened bud stage flowers to reduce the contamination in cultures. (T_8) - Carbendazim (0.2%) + Mancozeb (0.2%) + 8-HQC (200 mg/l) for 1.5hr showed minimum fungal (28.33%), bacterial (25.33%) contamination with maximum survivability (46.33%) and found the best pre-treatment for thalamus. When *ex-plant* is subjected to a longer period of pre-treatments all the *ex-plant*s were dried due to toxicity. Similarly, 0.1% $HgCl_2$ for 5 min was found best as a surface sterilization for thalamus. It

reduced microbial contamination up to (44.33%) and the maximum survival percentage was found (55.67%). When *ex-plant*s were exposed to extremity of time (8 min) to 0.1% $HgCl_2$ it reduced the contamination but the survivability chance was very very less. Although, it caused browning of the *ex-plant*s followed by subsequent drying within a week. For regeneration, treatment (T_7) MS + BAP 1.0 mg/l + KIN 0.5 mg/l + IAA 1.0 mg/l found to be the best medium with maximum number of *ex-plant*s producing regeneration followed by (T_8) MS + BAP 2.0 mg/l + KIN 0.5 mg/l + IAA 1.0 mg/l. Although, the percent of regeneration was very less from thalamus *ex-plant* but through this protocol, thalamus also can be taken as an *ex-plant* for whole plant regeneration of *Tagetes* spp. and its further studies. In the whole experiment, it was observed that, Auxins and cytokinins are compatible to each other and played a very important role in obtaining regeneration successfully from the thalamus.

Table 1: Effect of pre-treatments on contamination (%) and survival (%) of thalamus explant of French marigold var. Pusa Deep

Treatment	Bacterial (%)	Fungal (%)	Contamination (%)	Survival (%)
T_0	69.00 (56.14)±0.35	31.00 (33.82)±0.35	100.00 90±0	0.00 0±0
T_1	39.33 (38.81)±1.18	28.33 (32.10)±1.65	67.67 (55.39)±2.328	32.33 (34.57)±2.32
T_2	46.00 (42.68)±1.84	39.67 (38.98)±1.99	85.67 (67.73)±0.55	14.33 (22.22)±0.55
T_3	28.33 (32.13)±0.92	51.33 (45.74)±1.66	79.67 (63.34)±2.67	20.33 (26.61)±2.67
T_4	34.67 (35.96)±2.99	22.67 (28.39)±0.99	57.33 (49.22)±2.05	42.67 (40.74)±2.05
T_5	29.33 (32.74)±1.50	39.33 (38.81)±1.36	68.67 (56.07)±2.98	31.33 (33.88)±2.98
T_6	45.33 (42.30)±0.69	34.33 (35.74)±3.05	79.67 (63.40)±3.03	20.33 (26.56)±3.03
T_7	32.67 (34.73)±2.89	37.00 (37.44)±1.02	69.67 (56.60)±1.83	30.33 (33.35)±1.83
T_8	25.33 (30.16)±1.46	28.33 (32.01)±2.76	53.67 (47.08)±1.63	46.33 (42.87)±1.63
±SE(M)	1.76	1.84	2.14	2.14
C.D ($P \leq 0.05$)	5.28	5.51	6.40	6.40

*Values in parenthesis are angular values

Treatment details:

T_0 = Control (Distilled water) for 1hr
T_1 = Carbendazim (0.2%) + Mancozeb (0.2%) for 1hr
T_2 = Carbendazim (0.2%) + 8 HQC (200mg/l) for 1hr
T_3 = Mancozeb (0.2%) + 8 HQC (200mg/l) for 1hr
T_4 = Carbendazim (0.2%) + Mancozeb (0.2%) + 8 HQC (200mg/l) for 1hr
T_5 = Carbendazim (0.2%) + Mancozeb (0.2%) for 1.5hr
T_6 = Carbendazim (0.2%) + 8 HQC (200mg/l) for 1.5 hr
T_7 = Mancozeb (0.2%) + 8 HQC (200mg/l) for 1.5 hr
T_8 = Carbendazim (0.2%) + Mancozeb (0.2%) + 8 HQC (200mg/l) for 1.5hr

Table 2: Effect of mercuric chloride on contamination (%) and survival (%) of the thalamus explant of French marigold var. Pusa Deep

Treatment	Bacterial (%)	Fungal (%)	Contamination (%)	Dried (%)	Survival (%)
T_0	42.67 (40.74)±1.84	17.33 (24.53)±1.35	60.00 (50.81)±2.89	0.00 0±0	40.00 (39.15)±2.89
T_1	37.00 (37.44)±1.02	19.67 (26.25)±1.47	56.67 (48.81)±0.19	0.00 0±0	43.33 (41.15)±0.19
T_2	26.00 (30.60)±1.51	22.00 (27.86)±2.08	48.00 (43.83)±3.04	0.00 0±00	52.00 (46.13)±3.04
T_3	25.00 (29.00)±0.34	19.33 (26.06)±0.29	44.33 (41.72)±0.44	0.00 (0±0)	55.67 (48.23) ±0.66
T_4	40.67 (39.59)±1.18	23.33 (28.77)±2.15	64.00 (53.11)±0.68	36.00 (36.85)±0.68	0.00 0±0
±SE(M)	0.43	0.30	0.51	0.20	0.47
C.D (P≤0.05)	1.38	0.97	1.64	0.64	1.52

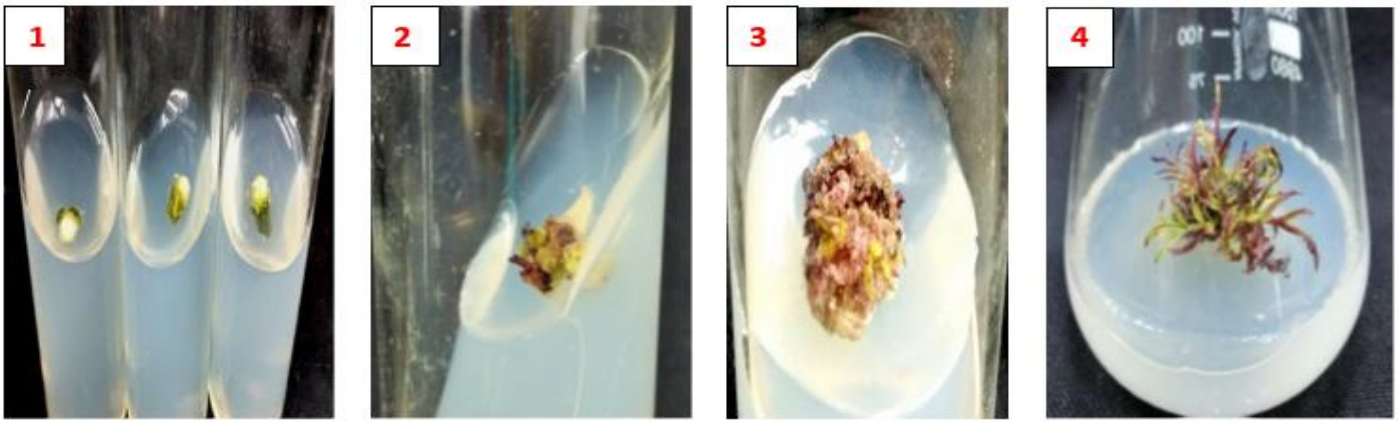
*Values in parenthesis are angular values

Treatment details

T_0 = Control (No treatment of 0.1% $HgCl_2$) for 0 min
T_1 = 0.1% $HgCl_2$ for 1min
T_2 = 0.1% $HgCl_2$ for 3min
T_3 = 0.1% $HgCl_2$ for 5min
T_4 = 0.1% $HgCl_2$ for 7min

Table 3: Effect of growth regulators on direct shoot organogenesis from thalamus explant of French marigold var. Pusa Deep

Treatment	Number of explants producing callus	Days taken for callus formation	Number of explants producing regeneration	Per cent regeneration	Days taken for regeneration	Number of visible buds/ explant	Bud forming capacity (BFC)	Number of explants producing roots
T_0 (Control- MS medium devoid of hormones)	0.00±0.00	0.00±0.00	0.00±0.00	0.00 (0.00 ±0.00)	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
T_1(MS +BAP 3.0 mg/l +IAA 3.0 mg/l)	10.33±0.88	22.17±2.05	0.00±0.00	0.00 (0.00 ±0.00)	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
T_2 (MS +BAP 0.5 mg/l +IAA 0.5 mg/l)	6.00±0.58	22.50±1.32	0.00±0.00	0.00 (0.00 ±0.00)	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
T_3 (MS +BAP 1.0 mg/l +IAA 0.5mg/l)	8.67±0.33	22.50±1.04	0.00±0.00	0.00 (0.00 ±0.00)	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
T_4 (MS +BAP 2.0 mg/l +IAA 0.5 mg/l)	7.67±0.88	22.67±0.73	0.00±0.00	0.00 (0.00 ±0.00)	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
T_5 (MS +BAP 3.0 mg/l +IAA 0.5 mg/l)	5.33±0.33	23.00±1.53	0.00±0.00	0.00 (0.00 ±0.00)	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
T_6 (MS +BAP 0.5 mg/l +KIN 0.5 mg/l + IAA1.0 mg/l)	10.00±0.58	21.33±1.45	0.00±0.00	0.00 (0.00 ±0.00)	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
T_7 (MS +BAP 1.0 mg/l + KIN 0.5 mg/l + IAA 1.0 mg/l)	13.00±0.58	21.17±0.73	3.00±0.58	16.67 (23.89 ±2.50)	51.00±1.16	5.27±1.58	0.85±027	0.00±0.00
T_8(MS + BAP 2.0 mg/l +KIN 0.5 mg/l +IAA 1.0 mg/l)	13.33±1.20	20.00±1.26	2.33±0.33	12.28 (20.42 ±1.49)	53.17±0.44	3.67±0.93	0.48±0.19	0.00±0.00
±SE (m)	0.69	1.25	0.22	0.97	0.41	0.61	0.11	0.00
CD (P<0.05)	2.05	3.75	0.67	2,91	1.23	1.83	0.33	0.00



1. Inoculation of thalamus
2. Regeneration on 51th day
3. Regeneration on 63th day
4. Final regeneration of shoot buds T7 (MS +BAP 1.0 mg/l + KIN 0.5 mg/l + IAA 1.0 mg/l)

Conclusion

For the first time direct shoot organogenesis from thalamus *ex-plant* is successfully standardised. This protocol will be helpful for future studies, i.e., large scale production of disease free quality materials, genetic transformation, *in-vitro* pollination and fertilization, somaclonal variants, mutagenic studies, *in-vitro* induction of pigments and secondary metabolites and *in-vitro* selection for biotic and abiotic stresses.

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