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Establishment of *T. erecta* and *T. patula* Suspension Cultures

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Abstract: *Tagetes erecta* and *T. patula* suspension cultures were established from aseptically grown seedlings after 30 days from germinated seeds on basal MS-medium used as sources of different explants i.e. leaf, stem and root. The results revealed that darkness significantly increased the cell fresh weight (g/flask) of leaf explants of both *T. patula* and *T. erecta* as compared with the incubation under light condition. Regarding the root explants the production of cell culture was significantly higher in *T. patula* compared with *T. erecta* under light or dark conditions. The maximum growth rate (expressed as growth index) of the leaf or root tissues were obtained after 8 and 10 days under light or dark conditions; respectively. The growth rate of the leaf cell culture suspension (g/day fresh or dry weight) was directly correlated with the concentration of the tryptophan added to the cell culture with a highest value at 15 mM. Meanwhile, the growth rate (g/day fresh or dry weights) in both *Tagetes species* was higher under dark incubation conditions. Moreover, the growth rate of *T. patula* was higher than *T. erecta* under light or dark conditions.

Key words: Suspension, *Tagetes erecta*, *T. patula*, Tryptophan.

INTRODUCTION

Marigolds (*Tagetes* species) are typically grown for ornamental purposes as bedding plants, studies have found that they can be highly toxic to plant parasitic nematodes and are capable a wide range up to (14 genera) of nematode pests (Suatmadji, 1969). The marigold species most often used for control are *Tagetes patula, T. erecta* and *T. minuta*. The key mode by which marigolds suppress plant parasitic nematodes is through biochemical interaction known as allelopathy. Allelopathy is a phenomenon when a plant releases compounds that are toxic to other plants, microorganisms or other organisms such as nematodes. Numerous reports have been published on the suppression of root-knot nematodes by marigold (Siddiqi and Alam, 1988; Mc Sorely and Frederick, 1994; Ploeg, 1999 and Ekaterini *et al*., 2005) found that *in vitro* study that the water extracts from seeds exudates of *Tagetes erecta* cv. Crackerjack and *T. patula* var. polynema caused significantly higher mortality to *Heterodera schachtii* **,** *Meloidogyne hapla* and *Pratylenchus penetrans* than the control extracts from radish, tomato and corn seeds; respectively. Natarajan *et al*. (2006) tested the ability of cold aqueous extracts (20% w/v, 100 ml aliquots) of pre and post flowering whole plants, roots and these portions of *T. erecta* to control *M. incognita* in infested soil (10 kg) in pots planted with susceptible *Lycopersicon esculentum*. Plant height and leaf number were significantly greater in *T. erecta, L. esculentum* than plant grown in untreated infested soil. They found that whole *T. erecta* plant extracts were more efficacious than stem extracts although both were more effective than root extracts and extracts from 40 day old plants were more efficacious than those from 70 day old plants. Root gall indices of *L. esculentum* treated with *T. erecta* plant extracts were significantly lower than untreated checks. Similarly, fruit yield from plants treated with *T. erecta* extracts was significantly better. Osman *et al*. (2008b) studied the nematicidal effects of ethanolic extracts of different *T. erecta* calli-derived from seed, leaf, stem and root explants grown on MS-medium supplemented by the three combinations of the two growth regulators namely; Naphthalene acetic acid (NAA) and 6-benzylamine purine (BAP) and cultured for 8 weeks under dark or light conditions on the percentage mortality of *M. incognita* (J_2) under laboratory conditions. Generally, the percentage net mortality was positively correlated with the concentrations of all callus extracts. The aim of this work is as follow: a- Cell cultures induction from different explants of *Tagetes erecta* and *T. patula.* b- Effect of DL-tryptophan as an enhancer compound on bioactive metabolities product in *Tagetes spp*.

MATERIALS AND METHODS

Aseptically grown *T. patula and T. erecta* seedlings after 30 days from germinated seeds on basal MS-medium were used as sources of different explants i.e. leaf, stem and root. Three sections 3-4 mm in diameter of

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each plant part were excised and cultured in 150 ml of glass jars containing 25 ml of MS-medium supplemented with 7.0 mg/l of NAA and 10.0 mg/1 of BAP.

Cultures of all treatments were divided into two groups. First group was maintained under light condition 16 h/day photoperiod at intensity of 3000 lux from cool light fluorescent lamps for 4 weeks, the second group was maintained under dark condition for 4 weeks. All cultures were incubated at 26 ± 1°C for each explant, five replicates were used.

1.Establishment of Tagetes Cell Suspension:

According to Torres (1989) suspension cultures were initiated by placing pieces of friable callus in an agitated liquid medium (Indirect cell suspension cultures).

Soft calli induced from leaf, stem and root cultures, which previously grown on MS-medium supplemented with 7.0 ppm of NAA+ 10.0 ppm of BAP, had been broken up in a hand-operated glass homogenize. Living, dead cells and debris were sieving through 90 µm nylon sieve in beaker glass, while the sieving operation the cells were washed 3 times with a new medium. After sedimentation the supplanted medium were removed; and the precipitant were transferred to a centrifuge tube, then centerifugated with 300 g (Haraeus-Christ centrifuge). One mg/ml of cells were transferred by using acuboy pipette to 15 ml cell culture induction medium in 75 ml Erlenmeyer flasks 200 ml. Flasks were incubated in shaker, speed 90 rpm/min. The incubation conditions were 26°C, 16/h light and 8/h dark, subcultures were repeated 3 times on the same culture medium. The fresh and dry weights (g/flask) were determined after 4 weeks.

2.Growth Index of Tagetes Patula Leaf and Root Cell Cultures:

Cell cultures were established from 2 months-old undifferentiated *T. patula* leaf and root calli cultures. These cultures were then grown in MS liquid medium supplemented with 7 ppm NAA and 10 ppm BAP (the best medium for calli production). As inoculums for starting the suspensions of ~ 0.50 gm fresh weight (FW) of the above calli were used. These suspensions were cultured in 75 ml medium in 200 ml conical flask and cultivated in growth chamber at 26± 1°C in an orbital shaker at 100 rpm under light (3000 lux of cooling florescent lamps) or dark condition. The suspensions were kept under the previous condition for two weeks. Final fresh weights were recorded. The growth index was determined as follow:

$$\frac{\text{Final FW- initial FW}}{\text{Initial FW}}$$

3.Effect of DL-tryptophan as an Enhancer Compound in Tagetes spp. Leaf Cell Cultures:

Enhancer compound of DL-tryptophan was added to ~ 0.5 g leaf suspension culture (MS liquid medium supplemented with 7 ppm of NAA and 10 ppm of BAP) of *T. erecta* and *T. patula* using sterile filter Whatman 0.22 μ m pores size catalog No. 0780-2504. DL-tryptophan was added at the concentrations of 5, 10, 15 and 20 mM. Cultures were incubated in growth chamber at 26 ± 1°C in an orbital shaker at 100 rpm under light or dark conditions for 10 days. Fresh weights (FW) and dry weights (DW) were recorded after 10 days. The growth rate (g/day) was determined as the following equation:

$$\frac{\text{Final weight - weight at 0 time}}{10}$$

RESULTS AND DISCUSSION

Data presented in Table (1) demonstrate the effect of light or dark incubation treatments on the production of cell cultures from the leaf, stem and root explants of *T. patula* and *T. erecta*. The results revealed that darkness significantly increased the cell fresh weight (g/flask) of leaf explants of both *T. patula* and *T. erecta* as compared with the incubation under light condition. This result in harmony with their obtained by Osman *et al*. (2008a). They found that the MS-medium supplemented with7.0 mg/l of NAA + 10.0 mg/l of BAP gave the highest growth rate and growth value under dark conditions. Further, the previous observation could be explained on the fact that these growth factors enhanced the protein synthesis, cell growth and cell division (Skoog & Schmitz, 1972 and Gamborg & Shyluk, 1981). Suspension fresh weights were 5.10 and 5.03 (g/flask) for cell cultures derived from leaf explants of *T. patula* and *T. erecta*; respectively. On the other hand, *T. patula* stem explants produced insignificantly higher amount of cell culture as compared with *T. erecta* under dark conditions. Regarding the root explants the production of cell culture was significantly higher in *T. patula* compared with *T. erecta* under light or dark conditions. Suspension fresh weights were 4.71 and 4.34 (g/flask) for cell cultures derived from root explants of *T. patula* compared with *T. erecta* under dark or light conditions; respectively.

However, significantly higher amounts of cell cultures were only observed in the root explants of *T. patula* as compared with *T. erecta* under light and dark conditions expressed as suspension dry weight (g/flask) Table (2). Suspension dry weights were 0.29 and 0.36 (g/flask) for cell cultures derived from root explants of *T. patula* compared to *T. erecta* under dark or light conditions; respectively. Generally it was observed that, darkness showed equal or better results of cell culture induction as compared with light conditions in the most treatments.

Table 1: Fresh weight (g/flask) of leaf, stem and root cell cultures of *Tagetes erecta* and *Tagetes patula* cultured on MS-medium supplemented with 7 ppm of NAA and 10 ppm of BAP and incubated under light or dark conditions at 26±1°C for 4 weeks.

Treatments	Cell cultures		
	Leaf	Stem	Root
T. erecta (light)	4.116 ± 0.825	3.489 ± 0.752	3.366 ± 0.529
T. erecta (dark)	5.032 ± 0.801*	3.968 ± 0.501	3.450 ± 0.290
T. patula (light)	4.330 ± 0.623	3.536 ± 0.397	4.348 ± 0.449 @
T. patula (dark)	5.106 ± 0.423 *	4.279 ± 0.699	4.713 ± 0.354 @

Mean ± Standard Division (SD).
Each mean is the average of five replicates.
* Significant difference from light treatment at $p \leq 0.05$ according to T test.
@ Significant difference from *T. erecta* incubated under light or dark conditions at $p \leq 0.05$ according to T-test.

Table 2: Dry weight (g/flask) of leaf, stem and root cell cultures of *Tagetes erecta* and *Tagetes patula* cultured on MS-medium supplemented with 7 ppm of NAA and 10 ppm of BAP and incubated under light or dark conditions at 26±1°C for 4 weeks.

Treatments	Cell cultures		
	Leaf	Stem	Root
T. erecta (light)	0.2844 ± 0.0678	0.2383 ± 0.0554	0.2168 ± 0.0493
T. erecta (dark)	0.3343 ± 0.0890	0.2720 ± 0.0441	0.2286 ± 0.0189
T. patula (light)	0.3374 ± 0.0783	0.2752 ± 0.0487	0.3644 ± 0.0445 @
T. patula (dark)	0.3238 ± 0.0660	0.2693 ± 0.0460	0.2944 ± 0.0575 @

Mean ± Standard Division (SD).
Each mean is the average of five replicates.
@ Significant difference from *T. erecta* incubated under light or dark conditions at $p \leq 0.05$ according to T-test.

Growth Index of Tagetes Patula Leaf and Root Cell Cultures:

Data shown in Table (3) indicate that the fresh weights (g/flask) of derived suspension culture of leaf and root explants of *T. patula* under light or dark conditions for 2, 4, 6, 8, 10, 12 and 24 hours, and2, 4, 6, 8, 10, 12, and 14 days, increased as time increased. The highest values of leaf suspension fresh weight 2.92 (g/ flask) after 8 days and 3.57 (g/flask) after 10 days were recorded with light or dark incubation condition; respectively. Similar findings 1.33 (g/flask) after 8 days and 1.47 (g/flask) after 10 days were recorded using derived cell culture from root calli under light or dark incubation; respectively.

The maximum growth rate (expressed as growth index) of the leaf or root tissues were obtained after 8 and 10 days under light or dark conditions; respectively (Table 4). These values were found (4.615 and 5.73) for the leaf explant and (1.51 and 2.12) for the root explant under light and dark conditions; respectively.

Table 3: Fresh weight (g/flask) of leaf and root cell culture of *T. patula* cultured under light or dark conditions for two weeks at different time intervals.

Time	Leaf		Root	
	Light	Dark	Light	Dark
0 time	0.52 j ±0.01	0.53 l ±0.02	0.53 hi ±0.02	0.47 gh ±0.04
2 h	0.48 j ±0.03	0.50 l ±0.02	0.45 ij ±0.01	0.43 gh ±0.04
4 h	0.43 j ±0.02	0.45 l ±0.02	0.34 k ±0.05	0.37 h ± 0.03
6 h	0.48 j ±0.03	0.53 l ±0.03	0.39 jk ±0.04	0.48 gh ±0.03
8 h	0.62 i ±0.06	0.69 k ±0.07	0.42 jk ±0.04	0.55 g ±0.05
10 h	0.73 h ±0.07	0.84 j ±0.09	0.57 gh ±0.07	0.66 f ±0.06
12 h	0.79 h ±0.04	1.00 i ± 0.04	0.65 g ±0.04	0.74 ef ±0.02
24 h	0.97 g ±0.02	1.17 h ±0.05	0.78 f ±0.03	0.81 e ±0.02
2 D	1.26 f ±0.11	1.39 g ±0.04	0.92 e ±0.04	0.93 d ±0.05
4 D	1.53 e ± 0.10	1.84 f ±0.06	1.03 d ±0.07	1.11 c ±0.13
6 D	2.77 b ±0.03	2.26 e ±0.11	1.17 bc ±0.08	1.26 b ±0.09
8 D	2.92 a ±0.08	3.36 c ±0.11	1.33 a ±0.09	1.36 b ±0.06
10 D	2.73 b ±0.08	3.57 a ±0.06	1.23 b ±0.08	1.47 a ±0.06
12 D	2.41 c ±0.06	3.46 b ±0.04	1.11 cd ±0.05	1.25 b ±0.09
14 D	2.21 d ±0.04	3.16 d ±0.05	0.87 ef ±0.07	1.14 c ±0.12

Mean ± Standard Division (SD).
Each mean is the average of five replicates.
Incubation conditions: 26±1°C, MS medium supplemented with 7 ppm of NAA and 10 ppm of BAP.

Table 4: Growth index (Gi) of cell derived from leaf and root calli cultures of *T. patula* under light or dark conditions for two weeks at different time intervals.

Time	Leaf		Root	
	Light	Dark	Light	Dark
2 h	-0.077	-0.056	-0.151	- 0.085
4 h	-0.173	-0.151	-0.358	- 0.212
6 h	-0.077	0.0	-0.264	0.021
8 h	0.192	0.302	-0.208	0.170
10 h	0.404	0.585	0.075	0.404
12 h	0.520	0.887	0.226	0.574
24 h	0.865	1.208	0.472	0.723
2 D	1.423	1.623	0.736	0.979
4 D	1.942	2.472	0.943	1.362
6 D	4.327	3.264	1.208	1.681
8 D	4.615	5.340	1.509	1.894
10 D	4.250	5.736	1.321	2.128
12 D	3.635	5.528	1.094	1.660
14 D	3.250	4.962	0.642	1.426

Growth index(Gi) = $\frac{\text{Final FW- initial FW.}}{\text{Initial FW}}$

Incubation conditions: 26±1°C, MS medium supplemented with 7 ppm of NAA and 10 ppm of BAP.

In the present studies, tryptophan was used to inhance thiophene production in leaf cell cultures of *Tagetes patula*. Tryptophan was added to the culture media at the concentrations of 5, 10, 15 and 20 mM. This concept depends on the metabolic conversion of tryptophan to natural IAA in cell culture. The important of IAA as natural auxin in cell division and cell enlargement was reported by Havsteen (2002). The addition of tryptophan to the culture medium enhanced the production of flavonoids. Furthermore, Murch *et al.* (2000) reported that using tryptophan as a precursor enhanced the production of melatonin and serotonin biosynthesis in *in vitro* regenerated cultures of *Hypericum perferatum* plants.

Data in Table (5, 6) showed that the effect of different concentrations of DL- tryptophan at 0, 5, 10, 15 and 20 mM on growth rate (g/day) of leaf cell cultures of *T. erecta* and *T. patula*. The incubation had been carried out under light (16/8 h) or under darkness incubation for 10 days at 26±1°C.

The highest values of growth rate (g/day fresh weight) of *T. patula* leaf cell culture was found to be 0.096 and 0.091under dark or light conditions; respectively using 15 mM tryptophan concentration. As for *T. erecta* the highest growth rate (g/day) of fresh weight values were found to be 0.077 and 0.070 at dark or light conditions; respectively using 15 mM tryptophan. The highest values of growth rate (g/day) of dry weight was obtained in leaf cell culture of *T. patula* it was 0.009 using tryptophan at concentration of 15 mM under dark condition. While, the highest growth rate value for *T. erecta* leaf cell culture suspension was 0.004 at 15 mM under light conditions.

The results in Tables (5, 6) and Fig. (1) indicate that the growth rate of the leaf cell culture suspension (g/day fresh or dry weight) was directly correlated with the concentration of the tryptophan added to the cell culture with a highest value at 15 mM. Meanwhile, the growth rate (g/day fresh or dry weights) in both *Tagetes species* was higher under dark incubation conditions. Moreover, the growth rate of *T. patula* was higher than *T. erecta* under light or dark conditions.

Table 5: Growth rate of leaf fresh cell cultures (g/day) of *T. erecta* and *T. patula* leaf cell cultures cultured on MS medium supplemented with 7 ppm of NAA and 10 ppm of BAP at 26±1°C and treated with different concentrations of DL- tryptophan.

Concentrations of DL-tryptophan	Growt h rate (g/day) fresh weight			
	T. erecta		*T. patula*	
	Light	Dark	Light	Dark
5 Mm	0.030 ± 0.005	0.041 ± 0.006	0.061 ± 0.009	0.072 ± 0.007
10 mM	0.042 ± 0.003	0.047 ± 0.009	0.082 ± 0.008	0.086 ± 0.005
15 mM	0.070 ± 0.007	0.077 ± 0.009	0.091 ± 0.007	0.096 ± 0.006
20 mM	0.061 ± 0.004	0.067 ± 0.004	0.078 ± 0.005	0.086 ± 0.005
LSD at α 0.05	0.0094	0.0133	0.0134	0.0107

Mean ± Standard Division (SD).

Each mean is the average of five replicates.

Growth rate (g/day) = $\frac{\text{Final weight- weight at 0 time}}{10}$

Table 6: Growth rate of leaf dry cell cultures (g/day) of *T. erecta* and *T. patula* leaf cell cultures cultured on MS medium supplemented with 7 ppm of NAA and 10 ppm of BAP at 26±1°C and treated with different concentrations of DL- tryptophan.

Concentrations of DL-tryptophan	Growth rate (g/day)dry weight			
	T. erecta		*T. patula*	
	Light	Dark	Light	Dark
5 mM	0.002 ± 0.001	0.003 ± 0.001	0.005 ± 0.001	0.004 ± 0.000
10 mM	0.003 ± 0.000	0.003 ± 0.001	0.008 ± 0.003	0.004 ± 0.000
15 mM	0.004 ± 0.000	0.003 ± 0.001	0.009 ± 0.001	0.009 ± 0.001
20 mM	0.004 ± 0.000	0.003 ± 0.001	0.008 ± 0.001	0.009 ± 0.001
LSD at α 0.05	0.0012	N.S	N.S	0.0019

Mean ± Standard Division (SD).
Each mean is the average of five replicates.

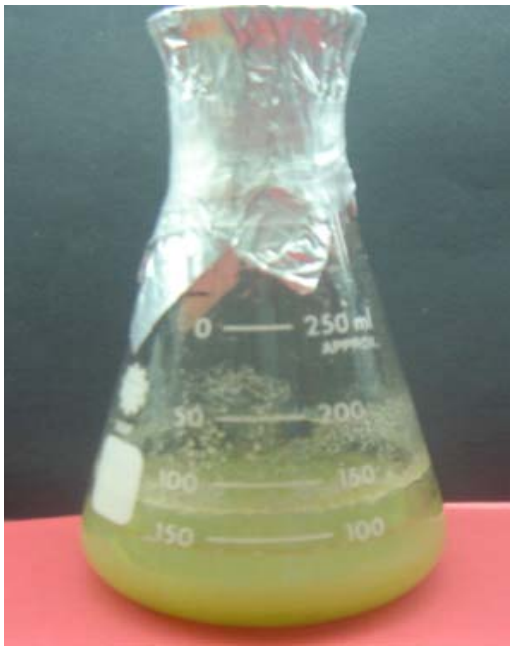

Fig. 1: Leaf suspension of *T. patula* cultured on MS medium supplemented with 7 ppm of NAA and 10 ppm of BAP and treated with 15 mM of DL-tryptophan as enhancer compound.

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