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*RESEARCH PAPER*

# Optimising protocol for direct differentiation of shoot buds from leaf *ex-plant* of *Tagetes erecta* L. var. Pusa Narangi Gainda

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**Abstract :** Marigold is an important loose flower crop has gained popularity for its pharmaceutical, industrial, medicinal and therapeutic importance. In this present study, we developed a protocol for direct regeneration using *in-vitro* raised immature leaves of African marigold (*Tagetes erecta* L.var. "Pusa Narangi Gainda"). A total of eight treatment combinations of growth hormones such as BAP, IAA and $GA_3$ with MS as a basal media was studied. The best medium found for direct, shoot organogenesis from leaf *ex-plant* was treatment ($T_3$) - MS + BAP 0.5 mg/l + IAA 0.25 mg/l. Pre-treatment with Carbendazim (0.2 %) + Mancozeb (0.2 %) + 8-HQC (200 mg/l) for 2hr followed by $HgCl_2$ for 4 min. resulted in minimum fungal (24.33%), bacterial (8.33%) contamination simultaneously it increased the survival percentage upto (67.33%). This study is helpful for rapid clonal propagation, production of lutein-rich pharmaceutical compounds and secondary metabolites using tissue culture techniques.

**Key Words :** African marigold, Shoot organogenesis, Leaf *ex-plant*, Growth hormones, Clonal propagation

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## INTRODUCTION

Marigold (*Tagetes* sp.) is a herbaceous asteraceous ornamental plant grown all over the world. It is a native of South and Central America especially Mexico. The genus *Tagetes* comprises of 55 species, of which the commonly cultivated species includes *T. erecta* (African marigold), *T. patula* (French marigold) are grown for decorative purpose and *T. minuta* L. (wild marigold) is cultivated mainly for essential oil purposes. The species African marigold (*Tagetes erecta* L.) is a hardy plant which bears semi or fully double large-sized globular heads of yellow, orange and white colour luminous, beautiful flowers. It can be grown in three seasons *viz.*, summer, winter and rainy. In India, marigold ranks first among loose flowers and accounts for more than half of the nation's loose flower production. It is commercially used for carotenoid extraction purpose which is widely used in pharmaceuticals. Besides, marigold is also known to possess anti-fungal properties (Kishore and Dwivedi, 1991 and Zygadlo *et al*., 1994), medicinal properties,

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repellent properties against flies, ants and mosquitoes, acts as a source of an emulsifying gum (Medina *et al*., 1993) as well as a very good source of xanthophylls for egg yolk pigmentation (Narahari *et al*., 1981). The therapeutic activities like anti-mutagenicity, anti-inflammatory, anti-tumourogenic, antiviral and immune-stimulating effects have also been reported in marigold by (Hamburger *et al*., 2003).

Micro-propagation is one of the viable approach for large scale multiplication of disease-free planting material, season bound production, *in-vitro* pollination and fertilization, *in-vitro* mutation breeding, induction of pigments and secondary metabolites, *in-vitro* selection for biotic and abiotic stresses etc. There are very few published reports on direct organogenesis from African marigold. These protocols are highly inconsistent, genotype-specific and callus mediated. Callus mediated regeneration consists of two-step process, *i.e.*, production of callus from *ex-plant* and again differentiate the callus to organogenesis using suitable auxin/cytokinin ratio, which is a long time process, difficult and not suitable for rapid propagation. Keeping these views under consideration, we proposed to study the direct organogenesis of shoot buds from leaves without intermediating a callus stage and influence of different growth regulators on it.

## MATERIAL AND METHODS

The present study was carried out at the Central Tissue Culture Laboratory, Division of Floriculture and Landscaping, ICAR-Indian Agricultural Research Institute, New Delhi. Harvested seeds of African marigold variety Pusa Narangi Gainda which were grown at the research farm of Floriculture were collected and washed with detergent teepol (0.1%) solution for 5 minutes, followed by washing under running tap water for 10-15 minutes to remove all the residues of the detergent. These seeds are then pre-treated with a standardised dose of Carbendazim (0.2 %) + Mancozeb (0.2 %) and 8-HQC (200 mg/l) for 2h with the help of a shaker which continuously shakenthese treated seeds at 500 rpm. Observations such as per cent fungal infection of *ex-plants*, per cent bacterial infection of *ex-plants*, per cent contamination of *ex-plant*s and per cent *ex-plant* survival were recorded during this experiment. After the completion of pre-treatment seeds were taken to the laminar air flow and were surface sterilized with a standardised dose of 0.1% $HgCl_2$ for 4 minutes, followed by immediate 3-4 times of washings with double distilled autoclaved water. Then the seeds were inoculated on culture medium and maintained at 25±1ºC temperature, with a photoperiod of 16:8 hours of light and dark cycles under fluorescent white light (47µmol/$m^2$/S). 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. After 7 days young, healthy leaves were excised from the *in-vitro* germinated seeds and were used as *ex-plant*s. The leaf lamina was transversely cut into squares (5mm × 5 mm) pieces and cultured on media for high regeneration. Culture media are supplemented with MS basal media and different plant growth regulators *viz.*, Thidiazuron (TDZ), Gibberellic acid ($GA_3$), µ-Naphthalene acetic acid (NAA), indole-3 acetic acid (IAA) and 6-benzylaminopurine (BAP) in various combinations. The regenerated shoot buds are sub-cultured inthe fresh medium within 1 week and maintained in the aseptic lab conditions for other studies. Observations such as days taken for callus induction, Number of *ex-plants* producing callus, days taken for shoot bud regeneration, number of *ex-plants* showing shoot bud regeneration, number of visible shoot buds per *ex-plant*, bud forming capacity (BFC) (Vanegas *et al.*, 2002) were recorded during this experiment.

**Experimental design and statistical analysis:**

The experiment was 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. The bud forming capacity (BFC) index was calculated after three weeks of culture, according to Martinez-pulido *et al*. (1992).

**BFC = (Mean number of buds per explant) × (% explants forming buds)/100**

## RESULTS AND DISCUSSION

The results obtained from the present investigation as well as relevant discussion have been summarized under following heads :

**Effect of pre-treatment on leaf *ex-plant*s of African marigold :**

In the present investigation, different experiments

were carried out to standardize the culture media for efficient regeneration from non-axillary leaf *ex-plants*. The effect of pre-treatments on seeds of African marigold var. Pusa Narangi Gainda was presented in the Table 1. The data elucidated that, pre-treatment of seeds with different fungicides and bacteriocides reduced the microbial contamination significantly and enhanced the survival percentage as compared to ($T_0$)- Control (Distilled water) for 2hrs. Among different pre-treatments, ($T_4$)- Carbendazim (0.2 %) + Mancozeb (0.2 %) + 8-HQC (200 mg/l) for 2hrs exhibited minimum fungal (24.33%), bacterial (8.33%) and total contamination (32.67 %) as compared to control (64.67 %, 12.33 % and 77.00 %, respectively). A maximum survival of *ex-plants* (67.33%) was also observed in ($T_4$) - Carbendazim (0.2 %) + Mancozeb (0.2 %) + 8-HQC (200 mg/l) for 2hrs as compared to control (23.00%). Among the two fungicides tested, Mancozeb played very effective in reducing contamination (40.33%) as compared to Carbendazim (57.33%). Moreover, it is observed that the absence of 8-HQC during pre-treatment resulted in higher bacterial and fungal contamination (64.67%). Plant material anchorages different types of endogenous and exogenous microbial (Fungal and bacterial) contaminants in the tissue culture techniques. Surface contaminants are comparatively easy to control as both fungicidal and bacterial compounds could be used efficiently as pre-treatments to eliminate such contaminants. Similar observations were also recorded during *in vitro* propagation of rose (Prasad, 1995) and chrysanthemum. The use of fungicides/ bactericides as pre-treatment before sterilization with 0.1% $HgCl_2$ to minimize contamination has been suggested by earlier workers (Krishnamurthy *et al*., 2001 and Bhatia, 2008).

**Effect of surface sterilization on leaf *ex-plant*s of African marigold:**

The effect of mercuric chloride on surface

**Table 1: Effect of pre-treatments on contamination (%) and survival (%) of the seeds of African marigold var. Pusa Narangi Gainda**

| Treatments | Bacterial (%) | Fungal (%) | Contamination (%) | Survival (%) |
|---|---|---|---|---|
| $T_0$ | 12.33 (20.48) ± 1.26 | 64.67 (53.55) ± 2.10 | 77.00 (61.53) ± 3.04 | 23.00 (28.43) ± 3.04 |
| $T_1$ | 12.00 (20.25) ±0.51 | 52.67 (46.51) ± 1.01 | 64.67 (53.51) ± 0.72 | 35.33 (36.45) ± 0.72 |
| $T_2$ | 8.33 (16.68) ±1.28 | 49.00 (44.41) ±2.17 | 57.33 (49.20) ± 1.51 | 42.67 (40.75) ± 1.51 |
| $T_3$ | 8.67 (17.04) ±1.20 | 31.67 (34.18) ± 1.93 | 40.33 (39.39) ± 1.52 | 59.67 (50.56) ± 1.52 |
| $T_4$ | 8.33 (16.76) ± 0.34 | 24.33 (29.53) ± 0.58 | 32.67 (34.83) ± 0.73 | 67.33 (55.12) ± 0.73 |
| S.E. ± | 1.00 | 1.80 | 1.80 | 1.73 |
| C.D. (P≤ 0.05) | 3.21 | 5.96 | 5.51 | 5.51 |

*Values in parenthesis are angular values

**Treatment details:** $T_0$ = Control (Distilled water) for 2h followed by $HgCl_2$ for 4min.
$T_1$ = Carbendazim (0.2 %) + Mancozeb(0.2 %) for 2h followed by $HgCl_2$ for 4min.
$T_2$ = Carbendazim (0.2 %) + 8-HQC (200 mg/l) for 2h followed by $HgCl_2$ for 4min.
$T_3$ = Mancozeb (0.2 %) + 8-HQC (200 mg/l) for 2h followed by $HgCl_2$ for 4min.
$T_4$ = Carbendazim (0.2 %) + Mancozeb (0.2 %) + 8-HQC (200 mg/l) for 2h followed by $HgCl_2$ for 4 min.

**Table 2: Effect of mercuric chloride on contamination (%) and survival (%) of the seeds of African marigold var. Pusa Narangi Gainda**

| Treatments | Bacterial (%) | Fungal (%) | Contamination (%) | Survival (%) |
|---|---|---|---|---|
| $T_0$ | 14.33 (22.24) ±0.27 | 61.33 (51.74) ±4.87 | 75.67 (61.12) ±5.56 | 24.33 (28.84) ±0.89 |
| $T_1$ | 11.33 (19.65) ±0.61 | 18.67 (25.54) ±1.28 | 30.00 (33.19) ±0.72 | 70.00 (56.77) ±0.72 |
| $T_2$ | 7.33 (15.67) ±0.75 | 3.33 (10.49) ±0.52 | 10.67 (19.05) ±0.31 | 89.33 (70.91) ±0.31 |
| $T_3$ | 8.00 (16.40) ±0.61 | 13.33 (21.32) ±1.53 | 21.33 (27.46) ±1.28 | 40.33 (39.40) ±1.52 |
| $T_4$ | 8.67 (17.11) ±0.34 | 9.67 (18.07) ±0.86 | 18.33 (25.32) ±0.89 | 0.00 (0.00) ±0.00 |
| S.E. ± | 0.55 | 2.40 | 2.60 | 2.60 |
| C.D. (P≤0.05) | 1.75 | 7.66 | 8.32 | 8.31 |

*Values in parenthesis are angular values

**Treatment details:** $T_0$ = Control (Distilled water) for 2h by $HgCl_2$ (0.1%) for 4min.
$T_1$ = Carbendazim (0.2 %) + Mancozeb (0.2 %) + 8-HQC (200 mg/l) for 2h followed by $HgCl_2$ for 2 min.
$T_2$ = Carbendazim (0.2 %) + Mancozeb (0.2 %) + 8-HQC (200 mg/l) for 2h followed by $HgCl_2$ for 4min.
$T_3$ = Carbendazim (0.2 %) + Mancozeb (0.2 %) + 8-HQC (200 mg/l) for 2h followed by $HgCl_2$ for 6 min.
$T_4$ = Carbendazim (0.2 %) + Mancozeb (0.2 %) + 8-HQC (200 mg/l) for 2h followed by $HgCl_2$ for 8 min.

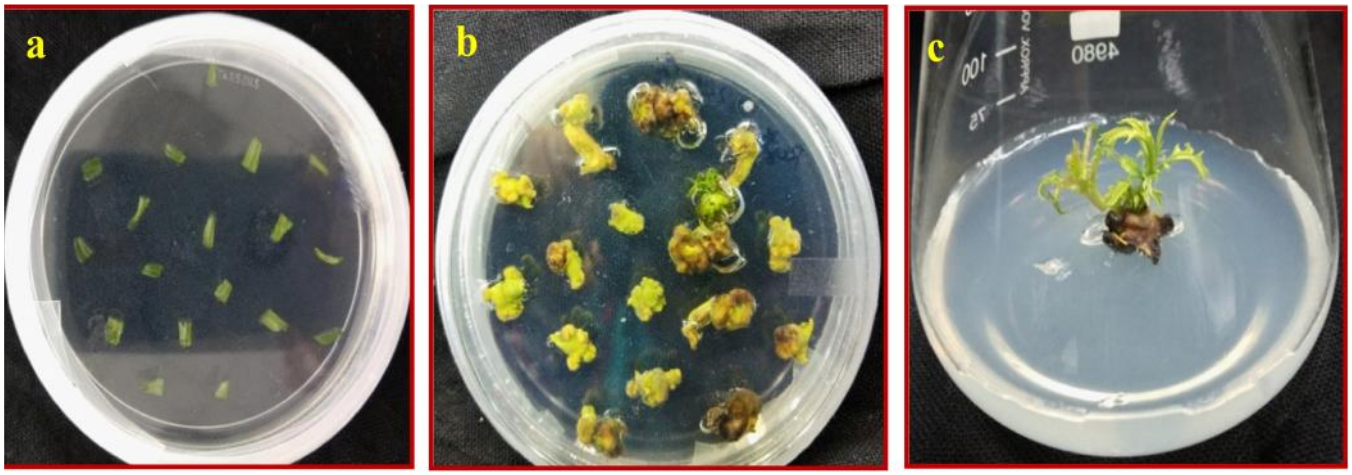
sterilization of seeds is shown in Table 2. Here, all the treatments were significantly different over control $T_0$ (Distilled water). After the best pre-treatment of seeds with ($T_4$) - Carbendazim (0.2 %) + Mancozeb (0.2 %) + 8-HQC (200 mg/l) for 2hrs, these were surface sterilized with 0.1% $HgCl_2$ for different durations before inoculation. The presented data concluded that, surface sterilization of seeds with 0.1% $HgCl_2$ for 4min reduced maximum fungal (3.33%), bacterial (7.33%) and total microbial contamination (10.67%) and simultaneously increased the survival percentage (89.33%). When the seeds were exposed for a prolonged period (6 and 8 min) to 0.1% $HgCl_2$ it significantly reduced the microbial contamination (21.33% and 18.33%) but the survival of seeds affected adversely. Surface sterilizing agents, their levels and duration of exposure are already known to affect the *in-vitro* cultures. Surface sterilization procedure for marigold has been outlined by different workers in the past, but it also varies with species, genotypes and type of explant. Seeds showed the least contamination. The reason may be due to seeds have a hard coat that is why showed minimum infestation by pathogens. Venegas *et al*. (2002) surface-sterilized seeds of different varieties of marigold with absolute ethanol for 1 min, 70 per cent ethanol for 5 min, 2 per cent sodium hypochlorite for 15 min and 1 per cent sodium hypochlorite for 15 min. Similar observation in gerbera carried out by Thakur *et al.* (2004) *i.e.*, the shoot tips in 5 per cent teepol for 15 min. and then under running water for 30 minutes, followed by surface sterilized with 0.1% $HgCl_2$ for 5 minutes and washing under running tap water.

**Effect of growth regulators on direct differentiation of leaf *ex-plants* of African marigold:**

Direct differentiation of shoot buds from Pusa Narangi Gainda using leaf *ex-plants* is shown in the Table 3. Maximum number of explants producing callus like structure (13.67) was observed in treatment ($T_3$) medium supplemented with BAP 0.5 mg/l + IAA 0.25 mg/l followed by treatment ($T_2$)- BAP 3.0 mg/l + IAA 3.0 mg/l (13.67) which was significantly at par with treatment ($T_3$). Maximum per cent regeneration (1.83%) was found in treatment ($T_3$) - MS + BAP 0.5 mg/l + IAA 0.25 mg/l followed by treatment ($T_2$) - MS + BAP 3.0

**Table 3: Effect of growth regulators on direct shoot organogenesis from leaf explant African marigold var. Pusa Narangi Gainda**

| Treatments | 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 1.0 mg/l +$GA_3$ 5.0 mg/l) | 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 | 0.00059±0.03 | 0.00±0.00 |
| $T_2$( MS + BAP 3.0 mg/l +IAA3.0 mg/l) | 13.67±0.88 | 17.67±0.33 | 1.33±0.33 | 0.09 (1.68±0.29) | 18.33±0.88 | 0.67±0.33 | 0.00047±0.01 | 0.00±0.00 |
| $T_3$ ( MS + BAP 0.5 mg/l +IAA 0.25 mg/l) | 13.67±0.67 | 17.33±0.33 | 1.67±0.33 | 0.10 (1.83±0.20) | 20.00±0.58 | 3.33±0.33 | 0.0026±0.00 | 0.00±0.00 |
| $T_4$( MS +BAP 0.5 mg/l +IAA0.5 mg/l) | 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 | 0.000±0.005 | 0.00±0.00 |
| $T_5$( MS +BAP 1.0 mg/l +IAA0.5 mg/l) | 11.67±1.33 | 17.67±0.88 | 1.17±0.17 | 0.07 (1.54±0.13) | 20.67±0.88 | 2.67±0.33 | 0.00011±0.09 | 0.00±0.00 |
| $T_6$ ( MS + BAP 1.0 mg/l +IAA0.5mg/l) | 10.33±1.45 | 20.00±0.58 | 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 3.0 mg/l+IAA0.5 mg/l) | 7.67±0.67 | 22.67±0.33 | 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 |
| S.E. ± | 0.83 | 0.43 | 0.18 | 0.13 | 0.49 | 0.20 | 0.0037 | 0.00 |
| C.D. (P<0.05) | 2.52 | 1.29 | 0.54 | 0.40 | 1.47 | 0.62 | 0.018 | 0.00 |




a. Inoculation of leaf *ex-plant* b. Direct shoot organogenesis c. Sub-cultured regenerated shoot

**Fig. 1: Direct shoot organogenesis from leaf *ex-plant* of African marigold var. Pusa Narangi Gainda from $T_3$ (MS + BAP 0.5 mg/l +IAA 0.25 mg/l) culture media**

mg/l + IAA 3.0 mg/l (1.68%) and the minimum per cent regeneration (1.54%) was observed in treatment ($T_5$) - MS + BAP 1.0 mg/l + IAA 0.5 mg/l. There was no per cent regeneration found in control ($T_0$) -MS devoid of hormones, ($T_1$) - MS + BAP 1.0 mg/l + $GA_3$ 5.0 mg/l and ($T_4$) -MS + BAP 0.5 mg/l + IAA 0.5 mg/l. Treatments $T_6$ (MS + BAP 1.0 mg/l +IAA 0.5mg/l) and $T_7$ (MS + BAP 3.0 mg/l + IAA 0.5 mg/l) produced a little callus like structure but there was no regeneration. Here, all the treatments are found significantly different over control ($T_0$) -MS devoid of hormones except treatments $T_1$, $T_4$, $T_6$ and $T_7$. Maximum number of days for regeneration (20.67 days) was taken by treatment ($T_5$)- MS + BAP 1.0 mg/l + IAA 0.5mg/l followed by treatment ($T_3$)- MS + BAP 0.5 mg/l + 0.25 mg/l (20.00 days) which was significantly at par to treatment ($T_5$) followed by treatment ($T_2$)- MS + BAP 3.0 mg/l + IAA 3.0 mg/l (18.33 days) which has taken minimum number of days for regeneration. The treatment ($T_3$) -MS + BAP 0.5 mg/l + IAA 0.25 mg/l shown a maximum mean number of visible buds/explant (3.33) followed by treatment ($T_5$)- MS + BAP 1 mg/l + IAA 0.5 mg/l (2.67). Although, ($T_3$) MS + BAP 0.5 mg/l + IAA 0.25 mg/l showed maximum bud forming capacity (0.00047) followed by the treatment ($T_2$) MS+ 3 mg/l BAP + IAA 3 mg/l (0.0026). Here, no root formation was observed in any of the treatments. *In-vitro* growth and morphogenesis of plant tissue is largely governed by the composition of the culture medium. MS (Murashige and Skoog, 1962) medium is widely used to induce direct or indirect shoot organogenesis/regeneration from different *ex-plants* inoculated on to culture media. Here, high concentration of cytokinin and low concentration of auxin are resulted in shoot differentiation. Our results are similar to the findings of Ram and Mehta (1982) who worked on Barren capitula of African marigold, Kothari and Chandra (1985) in marigold using MS medium with BAP (1.0 mg/l) + NAA (2.0 mg/l) + $GA_3$ (0.5 mg/l), Kaul *et al.* (1990) in chrysanthemum, Misra and Datta (2001) in African marigold, Kantia and Kothari (2002) in *Dianthus chinensis* and Benitz-Garcia *et al.* (2014) in *T. erecta.*

## Conclusion:

Since regeneration capacity varies with genotype, the type of *ex-plant*, hence, in the present study efforts have been made to develop *in- vitro* protocol for efficient regeneration in African marigold. Here, treatment ($T_3$) medium supplemented with MS + BAP 0.5 mg/l + IAA 0.25 mg/l was found to be the best medium for shoot bud differentiation and direct regeneration from PNG (Pusa Narangi Gainda) using the leaf as a non-axillary *ex-plant*.

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