Indian Journal of Experimental Biology
Vol. 57, January 2019, pp. 30-39

Influence of genotypes, growth regulators and basal media on direct differentiation of shoot buds from leaf segments of marigold (*Tagetes* spp.)

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Received 06 December 2017; *revised 02 February 2018*

Marigold is a commercially important flower crop popular for its multipurpose use as loose flower, ornamental pot plant, pharmaceutical and industrial crop for lutein pigment extraction. The genus *Tagetes* is considered as a recalcitrant in terms of *in vitro* morphogenesis and plant regeneration. In the present study, we propose an efficient protocol for direct differentiation of shoot buds from immature leaf segments of African (*Tagetes erecta* L.) and French marigold (*Tagetes patula* L.) genotypes *viz*., Pusa Basanti Gainda and Pusa Arpita, respectively. We developed this protocol as follows. Leaf segments were collected from *in vitro* proliferated shoots established in aseptic culture from shoot tips of doubled haploids and dihaploids. Rapid and direct shoot bud induction was observed on enriched MS (EMS) medium supplemented with coconut water, $AgNO_3$, casein hydrolysate, polyvinylpyrrolidone and glutamine. Among the different treatments, the highest (89.7%) direct shoot bud induction, bud forming capacity index (BFC) (2.36) and rapid (14 days) organogenesis was observed from leaf segments of French marigold cv. Pusa Arpita cultured on EMS media supplemented with 2.0 mg L^{-1} 6-benzylaminopurine (BAP) and 0.5 mg L^{-1} 1-naphthalene acetic acid (NAA). African marigold cv. Pusa Basanti Gainda exhibited maximum regeneration (66.6%), BFC index (2.11) and early (20 days) shoot bud induction on EMS media supplemented with 0.5 mg L^{-1} BAP and 0.25 mg L^{-1} NAA. In order to improve the regeneration capacity, different maturity groups of distal and proximal leaf ends were cultured on genotype specific medium. Among the different explant groups, immature leaf basal segments exhibited highest BFC index of 4.2 and 2.6 in Pusa Arpita and Pusa Basanti Gainda respectively. All the regenerated shoots were excised, rooted in 0.5 mg L^{-1} IBA and transplanted under polyhouse conditions, where they exhibit normal morphological growth similar to mother plant.

Keywords: $AgNO_3$, Casein hydrolysate, Coconut water, Glutamine, Polarity, Regeneration, Shoot bud differentiation

Marigold (*Tagetes* spp.) is an Asteraceous plant and is native to South and Central America, specifically from Mexico. The genus *Tagetes* comprises of 55 species and the most commonly cultivated species are *Tagetes erecta* L. (African marigold) and *T. patula* L. (French marigold)[1]. These are commonly grown for loose flower production and also in landscape gardening owing to their alluring appearance, luminous colours, varied sizes, shapes, forms, etc. Besides loose flower and ornamental pot plants, the F_1 hybrids are also being used as cut flowers in Central America. Marigold is a rich source of lutein which is a yellow oxycarotenoid having beneficial pharmaceutical properties due to its strong antioxidant quality and it plays a critical role in maintaining normal eye vision[2]. Marigold petals are widely using as poultry feed to enrich the carotenoids in egg yolk and broiler chicken. It has been reported that leaves and flowers of this plant contains various bioactive compounds that exhibit antibacterial, antimicrobial, insecticidal, nematicidal, mosquitocidal, larvicidal, fungicidal, hepatoprotective, wound healing and analgesic activities[3].

Micropropagation is one of the viable alternatives for large-scale multiplication of marigold. This method is free of seasonal bounds and having other advantages like product uniformity, disease free plants, easy exchange of germplasm and planting material. Furthermore, this technique provides basis for application of different genetic improvement tools *viz*. *in vitro* mutagenesis, *in vitro* selection, genetic transformation, etc[4]. Rapid *in vitro* regeneration and multiplication of true-to-type planting material is highly needed as F_1 hybrid marigold seed is highly costly. Moreover, plant regeneration from leaves and

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other organs *via* adventitious shoot formation paved the way for mutation breeding to induce variability. The genus *Tagetes* is considered as a recalcitrant in terms of *in vitro* morphogenesis and plant regeneration[5]. Ketel *et al.*[6,7] reported severe browning and stoppage of growth of tertiary leaf-derived calli of *T. patula* and, primary and secondary leaf-derived calli of *T. erecta*, respectively. In addition, the formation of only roots from stem-derived calli of *Tagetes* spp. have earlier been reported[8]. These studies have indicated the difficulties in callus formation and plant regeneration in marigold genotypes. There are few reports on organogenesis from leaf segments of African marigold[5,9-12]. These protocols were highly inconsistent, genotype specific, callus mediated, took long time for regeneration and the results were not reproducible. All these protocols are based on the two step process *i.e* primary exposure of explants to cytokinin rich media for callus induction followed by transfer to low cytokinin media for organogenesis. This two-step process is difficult, uneconomical, takes long time for culture establishment, may produce somaclonal variations and culture contamination. Almost all of these protocols were only confined to *T. erecta* and reports on organogenesis or direct shoot bud induction from leaf segments of *T. patula* are not available. To our knowledge, no reports are available on the rapid and direct regeneration of shoot buds without any intermediate callus in both African and French marigold genotypes.

In several plant species it was proven that, sole use of MS[13] media with alterations in hormonal ratios cannot be helpful in *in vitro* developmental process[14]. For such recalcitrant species, enriching the medium with organic compounds, such as coconut water, poly amines, such as casein hydrolysate may be a good alternative for rapid and efficient shoot bud induction and growth[15-18]. Considering all such studies, here, we enriched the tissue culture media with various organic and inorganic growth promoters in order to improve the efficiency of *in vitro* regeneration from leaf explants of African and French marigold.

Materials and Methods

Direct regeneration from leaf explants of African and French marigold genotypes

African marigold variety Pusa Basanti Gainda (PBG) and French marigold variety Pusa Arpita (PA) were selected to standardize the rapid and direct leaf regeneration. Leaves/leaf segments of approximately 0.3 cm^2 were collected from *in vitro* raised plantlets derived from anther culture induced doubled haploids and dihaploids. The leaves were cultured on MS[13] media containing 3% (w/v) sucrose and solidified with 0.26% (w/v) gelrite. The pH was adjusted to 5.8 prior to autoclaving at 121°C for 20 min at 1.05 kg cm^{-2} (15 lbs $inch^{-2}$). Leaf explants were excised aseptically and were placed onto MS medium and enriched MS (EMS) medium supplemented with various concentrations of phytohormones *viz*., BAP (0.25-2.0 mg L^{-1}) and NAA (0.25-0.5 mg L^{-1}). The enriched MS medium was supplemented with 2.0 mg L^{-1} $AgNO_3$, 6% coconut water (v/v), 250 mg L^{-1} casein hydrolysate, 250 mg L^{-1} polyvinylpyrrolidone and 60 mg L^{-1} glutamine. MS media without any phytohormones served as control. Thermolabile compounds were filter sterilized using 0.2 µm filters and added to media under laminar air hood. The effect of plant growth regulator (PGR) concentration and type of basal media on direct organogenesis from leaf explant was investigated in both the French and African marigold genotypes. Therefore, for plant regeneration, 17 treatments were tested on each genotype. The cultures were incubated at 25±1ºC, and RH (70%) under 16 h photoperiod (3000 lux) provided by Phillips white fluorescent tubes. After ascertaining the suitable basal medium and growth regulator, an independent experiment was conducted to test the morphogenetic ability of three kinds of explants *viz*., immature, young and mature leaf tips and basal leaf segments. Regeneration capabilities of proximal and distal ends of the above three types of leaves were examined separately. Based on our earlier studies, the regenerated shoot buds were transferred to MS medium devoid of phyto-hormones for elongation of shoots. After 20 days the elongated shoots were subcultured to ½ MS media supplemented with 0.5 mg L^{-1} IBA for root induction. The rooted plantlets were carefully removed from the bottles and the roots were rinsed with doubled distilled water to remove any residual culture medium. The rooted plants were successfully hardened in disposable plastic glasses in a 1:1 (v/v) mixture of sterilized peat and soil saturated with ½ MS macro and micro salts and fully hardened plants were transferred to field.

Experimental design and statistical analysis

All the experiments were conducted in a completely randomized design (CRD) with six replications. A replicate consisted of Petridish with 15-20 explants. Experiments were repeated at least

thrice and the data was pooled before analysis. The data indicated in the table are means of replicate values. The data in table were transformed using angular transformation and were subjected to analysis of variance (ANOVA). Comparison among treatment means were carried out using least square difference (LSD) values and are reported under critical difference (CD) at the end of each table. The bud forming capacity (BFC) index was calculated after 3 weeks of culture initiation according to Martinez-Pulido *et al*.[19].

$$\text{BFC index} = \frac{(\text{Mean number of buds per explant}) \times (\%\ \text{explants forming buds})}{100}$$

Results and Discussion

Culture establishment from leaf explants

Standardization of basal media and growth regulators

The leaf explants of Pusa Basanti Gainda (PBG) and Pusa Arpita (PA) were cultured in two different basal media compositions *viz*., MS and Enriched MS (EMS) media along with two different plant growth regulator combinations, such as BAP and NAA (Fig. 1A). Both the African (PBG) and French (PA) genotypes gave rapid regeneration without any intermediate callus in EMS basal media as compared to MS media in which, recorded poor callus mediated regeneration in prolonged durations (Fig. 1 B-C). Earlier reports on marigold regeneration have used only MS as basal medium for shoot bud induction[5,9-12]. However, reports on several other crops revealed that, regeneration efficiency of explant was highly depended on the basal medium and its components[20].

African and French marigold varieties showed specific genotype requirement of cytokinin (BAP) and auxin (NAA) combination for highest shoot bud induction in both MS and EMS mediums. Skoog and Miller[20] reported that organogenesis in tissue culture is governed by the balance of auxin and cytokinin in the medium. Earlier, regeneration of shoot from leaf explants of African marigold was reported by many workers [10,11,21,22]. All these reports on this crop mentioned the use of different cytokinins *viz*., BA, kinetin and TDZ in combination with auxins such as IAA and NAA. But, differentiation of shoots in leaf segments of African marigold with a combined use of cytokinin and auxin was highly associated with callus and which was detrimental for producing true-to-type plant material. Further, it requires two to three times culture transfer and demands more time for regeneration from leaf segments. Higher concentrations

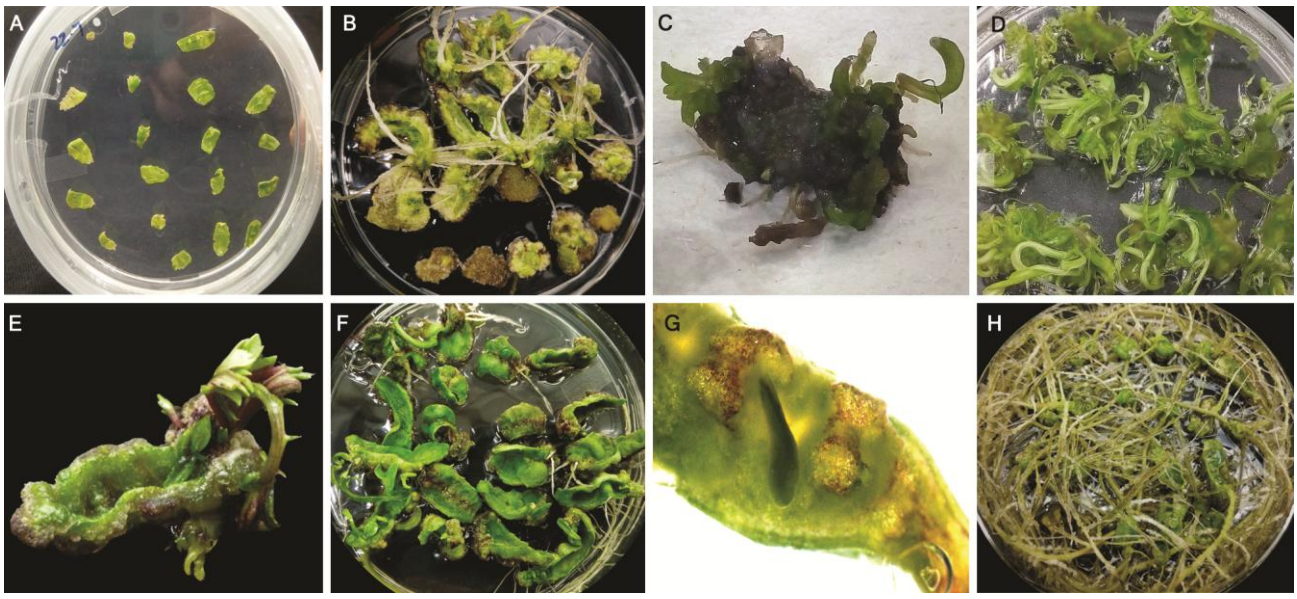

Fig.1—Effect of basal medium and growth regulators on *in vitro* shoot regeneration from leaf explants of African and French marigold genotypes. (A) Leaf segments on 1st day of inoculation; (B) Callusing and shoot bud induction in leaf segments of Pusa Arpita (PA) cultured on MS+2.0 mg L^{-1} BAP + 0.5 mg L^{-1} NAA; (C) Callus mediated regeneration in PA; (D) Direct differentiation of shoot buds from PA leaf segments on EMS+2.0 mg L^{-1} BAP + 0.5 mg L^{-1} NAA; (E) Regeneration of shoot buds from proximal end of PA leaf without any intermediate callus cultured on EMS medium; (F & G) Direct differentiation of shoot buds from Pusa Basanti Gainda (PBG) leaf segments on EMS+0.5 mgl^{-1} BAP + 0.25 mg L^{-1} NAA; and (H) Rhizogenesis from leaf segments of PBG cultured on MS + 0.25 mg L^{-1} BAP + 0.25 mg L^{-1} NAA.

of cytokinins (BA) became supraoptimal for explants to differentiate, as they showed hyperhydricity without any further increase in number of shoots or percent regeneration. Misra and Datta[10] and Gupta and Rahman[5] reported the use of GA_3 in combination with BA in the absence of auxin for direct shoot bud induction. They observed the direct differentiation of shoot buds from leaf segments after 4-5 weeks of culture initiation. However, they reported very poor response (14%) of leaf segments to the given GA_3 and BA combination so it was not useful for true-to-type plant material multiplication. However, till date reports are not available on the use of coconut water, $AgNO_3$ and other additional organics in marigold plant regeneration. It was found to be critical for adventitious shoot bud induction both in African and French genotypes. The genus *Tagetes* was considered as most recalcitrant for *in vitro* plant regeneration and development. To our knowledge however, there are no reports available on plant regeneration from leaf explants of French marigold, although plant regeneration has been reported from anthers[23].

In the present study, we incorporated coconut water, $AgNO_3$ and other additional organics in the culture initiation step for obtaining the maximum shoot bud induction with in less time from both African and French marigold genotypes. It is evident from Table 1 that, maximum shoot bud regeneration (55.8%) of leaf explants was observed on EMS medium supplemented with 0.5 mg L^{-1} BAP + 0.25 mg L^{-1} NAA, which was statistically at par with EMS medium supplemented with 2.0 mg L^{-1} BAP + 0.5 mg L^{-1} NAA (52.1%) and all other treatments were significantly differed with these two treatments. Maximum regeneration was recorded in EMS media as compared to MS media and was proved beneficial for both African and French genotypes. Among the two basal media, callus mediated regeneration was observed in both the genotypes cultured on MS medium supplemented with different growth regulator combinations which was undesirable for producing true-to-type plant material and genetic transformation studies. Moreover, explants cultured on EMS media have shown significantly earliest direct regeneration over MS media. Among the two genotypes, cultured on various growth regulator combinations, rapid shoot bud induction occurred in Pusa Arpita leaf explants cultured on EMS medium supplemented with 2.0 mg L^{-1} BAP + 0.5 mg L^{-1} NAA (14 days) (Fig. 1 D-E) and in Pusa Basanti Gainda leaf explants cultured on EMS medium supplemented with 0.5 mg L^{-1} BAP + 0.25 mg L^{-1} NAA (20 days) (Fig. 1F-G). Pusa Basanti

Table 1—Effect of basal medium, growth regulators on direct shoot regeneration and callus growth from leaf explants in African and French marigold genotypes

Treatments	Regeneration (%)			Callusing	
	PA	PBG	Mean	PA	PBG
MS devoid of hormones (Control)	0.0 (0.00)*	0.0 (0.0)*	0.0 (0.0)*	-	-
MS + BAP (0.25 mg L^{-1}) + NAA (0.25 mg L^{-1})	2.0 (4.7)	0.0 (0.0)	1.0 (2.3)	+	+
MS + BAP (0.50 mg L^{-1}) + NAA (0.25 mg L^{-1})	13.3 (17.3)	10.4 (18.6)	11.9 (17.9)	+	+
MS + BAP (1.00 mg L^{-1}) + NAA (0.25 mg L^{-1})	13.3 (21.1)	4.4 (7.1)	8.9 (14.1)	++	++
MS + BAP (2.00 mg L^{-1}) + NAA (0.25 mg L^{-1})	5.6 (8.0)	1.9 (4.5)	3.7 (6.3)	+++	++
MS + BAP (0.25 mg L^{-1}) + NAA (0.50 mg L^{-1})	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	+	+
MS + BAP (0.50 mg L^{-1}) + NAA (0.50 mg L^{-1})	3.7 (6.5)	3.9 (6.7)	3.8 (6.6)	++	++
MS + BAP (1.00 mg L^{-1}) + NAA (0.50 mg L^{-1})	9.2 (14.5)	0.0 (0.0)	4.6 (7.2)	+++	++
MS + BAP (2.00 mg L^{-1}) + NAA (0.50 mg L^{-1})	47.2 (43.4)	0.0 (0.0)	23.6 (21.7)	+++	+++
EMS + BAP (0.25 mg L^{-1}) + NAA (0.25 mg L^{-1})	6.5 (14.6)	0.0(0.0)	3.3 (7.3)	-	-
EMS + BAP (0.50 mg L^{-1}) + NAA (0.25 mg L^{-1})	45.0 (42.1)	66.7 (54.8)	55.8 (48.4)	-	-
EMS + BAP (1.00 mg L^{-1}) + NAA (0.25 mg L^{-1})	46.4 (42.9)	24.4 (29.5)	35.4 (36.2)	-	-
EMS + BAP (2.00 mg L^{-1}) + NAA (0.25 mg L^{-1})	33.9 (35.6)	7.7(15.9)	20.8 (25.76)	-	-
EMS + BAP (0.25 mg L^{-1}) + NAA (0.50 mg L^{-1})	27.0 (31.3)	10.2 (18.2)	18.6 (24.7)	-	-
EMS + BAP (0.50 mg L^{-1}) + NAA (0.50 mg L^{-1})	52.0 (46.2)	25.6 (30.0)	38.8 (38.1)	-	-
EMS + BAP (1.00 mg L^{-1}) + NAA (0.50 mg L^{-1})	60.0 (50.9)	26.5 (30.6)	43.2 (40.8)	-	-
EMS + BAP (2.00 mg L^{-1}) + NAA (0.50 mg L^{-1})	89.7 (71.6)	14.5 (21.4)	52.1 (46.5)	-	-
Mean	26.8 (26.5)	11.5 (14.0)		-	-
	SEm±	CD (p=0.05)			
Treatment (T)	2.856	8.076			
Genotype (G)	0.979	2.770			
T × G	4.038	11.421			

[*Values in parenthesis represent arcsine transformed values]

Gainda and Pusa Arpita has taken 48 and 40 days, respectively for shoot bud induction from explants cultured on MS medium with the same set of growth regulator combinations (Table 2). These results are in accordance with the earlier reports on *in vitro* regeneration of marigold[10,11]. Among the two genotypes examined, leaf explants of Pusa Arpita exhibited highest regeneration (26.8%) and was statistically significant over Pusa Basanti Gainda (11.5%). Media and genotype interaction revealed that the maximum regeneration (89.7%) was observed in Pusa Arpita leaf explants cultured on EMS medium supplemented with 2.0 mg L^{-1} BAP + 0.5 mg L^{-1} NAA followed by Pusa Basanti Gainda (66.7%) cultured on EMS medium supplemented with 0.5 mg L^{-1} BAP + 0.25 mg L^{-1} NAA (Table 1). There was a significant difference between the two genotypes cultured on the same medium and Pusa Arpita responded well for most of the treatments compared to Pusa Basanti Gainda. Similar to these results, earlier studies also reported the necessary role played by the BAP for shoot formation from leaf explants[5,10-12]. The basal medium, combination of other growth regulators with BAP and the hormonal levels were highly contrasting with the present results. Kothari and Chandra[22] obtained the regeneration from African marigold leaf derived callus when cultured on MS medium supplemented with 7.0 mg L^{-1} BAP + 0.5 mg L^{-1} IAA. They reported superiority of BAP over kinetin for shoot bud regeneration. Misra and Datta[10] and Gupta and Rahman[5] obtained the direct shoot bud induction from African marigold leaf explants when cultured on MS medium supplemented with 14.43 mM GA_3 and 4.44 mM BA.

In the present study, root induction was also observed in all the treatments but differed for percent rhizogenesis. Significant differences were not observed among the two basal mediums for root induction. Among the two genotypes, Pusa Arpita recorded significantly highest rhizogenesis (52.4%) over Pusa Basanti Gainda (44.1%) (Table 2). Among the different treatments, highest rhizogenesis (91%) was recorded in MS + BAP (0.25 mg L^{-1}) + NAA (0.25 mg L^{-1}) (Fig. 1H). In general maximum root induction and poor shoot bud organogenesis was observed with equal or higher level of auxins with respect to cytokinins.

Perusal of data from Table 3 revealed that maximum number of shoots (2.78) per explant was recorded on EMS medium supplemented with 1.0 mg L^{-1} BAP + 0.5 mg L^{-1} NAA which was followed by EMS + 0.5 mg L^{-1} BAP + 0.25 mg L^{-1} NAA (2.52)

Table 2—Effect of basal medium, growth regulators on root organogenesis and number of days taken for shoot initiation in African and French marigold genotypes

Treatments	Rhizogenesis (%)			Days to shoot initiation		
	PA	PBG	Mean	PA	PBG	Mean
MS devoid of hormones (Control)	82.6 (65.7)*	42.6 (40.6)*	62.6 (53.2)*	0.0	0.0	0.0
MS + BAP (0.25 mg L^{-1}) + NAA (0.25 mg L^{-1})	100.0 (84.2)	82.0 (66.4)	91.0 (75.3)	45.0	0.0	45.0
MS + BAP (0.50 mg L^{-1}) + NAA (0.25 mg L^{-1})	85.9 (71.6)	48.8 (43.3)	67.3 (57.4)	42.0	48.3	45.2
MS + BAP (1.00 mg L^{-1}) + NAA (0.25 mg L^{-1})	51.7 (47.8)	30.1 (28.1)	40.9 (37.9)	43.7	52.3	48.0
MS + BAP (2.00 mg L^{-1}) + NAA (0.25 mg L^{-1})	42.3 (40.0)	0.0 (0.0)	21.2 (20.0)	43.7	52.0	47.8
MS + BAP (0.25 mg L^{-1}) + NAA (0.50 mg L^{-1})	26.0 (30.5)	45.8 (40.7)	35.9 (35.6)	0.0	0.0	0.0
MS + BAP (0.50 mg L^{-1}) + NAA (0.50 mg L^{-1})	67.0 (56.0)	46.1 (38.3)	56.6 (47.2)	47.0	46.0	46.5
MS + BAP (1.00 mg L^{-1}) + NAA (0.50 mg L^{-1})	86.1 (71.4)	2.4 (5.1)	44.3 (38.3)	43.3	0.0	43.3
MS + BAP (2.00 mg L^{-1}) + NAA (0.50 mg L^{-1})	25.2 (30.1)	6.3 (8.6)	15.7 (19.3)	40.0	0.0	40.0
EMS + BAP (0.25 mg L^{-1}) + NAA (0.25 mg L^{-1})	72.6 (58.8)	79.6 (66.1)	76.1 (62.5)	24.3	0.0	24.3
EMS + BAP (0.50 mg L^{-1}) + NAA (0.25 mg L^{-1})	24.2 (29.3)	26.3 (29.3)	25.2 (29.3)	17.0	20.0	18.5
EMS + BAP (1.00 mg L^{-1}) + NAA (0.25 mg L^{-1})	14.3 (22.1)	66.0 (56.2)	40.2 (39.2)	18.7	25.0	21.8
EMS + BAP (2.00 mg L^{-1}) + NAA (0.25 mg L^{-1})	5.3 (10.7)	31.1 (33.8)	18.2 (22.3)	20.0	24.0	22.0
EMS + BAP (0.25 m mg L^{-1}) + NAA (0.50 mg L^{-1})	29.2 (27.6)	79.9 (66.3)	54.6 (46.9)	21.0	26.3	23.7
EMS + BAP (0.50 mg L^{-1}) + NAA (0.50 mg L^{-1})	58.9 (51.2)	87.0 (71.1)	73.0 (61.2)	18.3	25.3	21.8
EMS + BAP (1.00 mg L^{-11}) + NAA (0.50 mg L^{-1})	49.9 (44.9)	21.7 (27.7)	35.8 (36.3)	18.3	25.7	22.0
EMS + BAP (2.00 mg L^{-1}) + NAA (0.50 mg L^{-1})	69.1 (56.8)	54.7 (47.7)	61.9 (52.3)	14.0	24.0	19.0
Mean	52.4 (47.0)	44.1 (39.4)	-	30.4	33.5	-
	SEm±	CD (p=0.05)		SEm±	CD (p=0.05)	
Treatment (T)	6.948	19.650		0.59	1.669	
Genotype (G)	2.383	6.740		0.202	0.572	
T × G	9.825	27.789		0.834	2.360	

[*Values in parenthesis represent arcsine transformed values]

Table 3—Effect of basal medium and growth regulators on number of shoot buds per leaf explant and bud forming capacity (BFC) index in African and French marigold genotypes

Treatments	Number of buds per explant			Bud forming capacity index		
	PA	PBG	Mean	PA	PBG	Mean
MS devoid of hormones (Control)	0.00	0.00	0.00	0.00	0.00	0.00
MS + BAP (0.25 mg L^{-1}) + NAA (0.25 mg L^{-1})	0.33	0.00	0.17	0.02	0.00	0.01
MS + BAP (0.50 mg L^{-1}) + NAA (0.25 mg L^{-1})	1.30	1.00	1.15	0.29	0.10	0.20
MS + BAP (1.00 mg L^{-1}) + NAA (0.25 mg L^{-1})	1.42	0.33	0.88	0.20	0.04	0.12
MS + BAP (2.00 mg L^{-1}) + NAA (0.25 mg L^{-1})	1.44	0.00	0.72	0.24	0.00	0.12
MS + BAP (0.25 mg L^{-1}) + NAA (0.50 mg L^{-1}	0.00	0.00	0.00	0.00	0.00	0.00
MS + BAP (0.50 mg L^{-1}) + NAA (0.50 mg L^{-1})	0.67	0.33	0.50	0.00	0.04	0.02
MS + BAP (1.00 mg L^{-1}) + NAA (0.50 mg L^{-1})	1.00	0.00	0.50	0.13	0.00	0.07
MS + BAP (2.00 mg L^{-1}) + NAA (0.50 mg L^{-1})	3.60	0.00	1.80	1.69	0.00	0.84
EMS + BAP (0.25 mg L^{-1}) + NAA (0.25 mg L^{-1})	1.00	0.00	0.50	0.07	0.00	0.03
EMS + BAP (0.50 mg L^{-1}) + NAA (0.25 mg L^{-1})	1.88	3.16	2.52	0.87	2.11	1.49
EMS + BAP (1.00 mg L^{-1}) + NAA (0.25 mg L^{-1})	1.61	1.92	1.76	0.75	0.45	0.60
EMS + BAP (2.00 mg L^{-1}) + NAA (0.25 mg L^{-1})	2.23	1.00	1.61	0.75	0.08	0.41
EMS + BAP (0.25 mg L^{-1}) + NAA (0.50 mg L^{-1})	1.60	1.00	1.30	0.44	0.10	0.27
EMS + BAP (0.50 mg L^{-1}) + NAA (0.50 mg L^{-1})	2.45	2.28	2.37	1.28	0.70	0.99
EMS + BAP (1.00 mg L^{-1}) + NAA (0.50 mg L^{-1})	1.89	3.67	2.78	1.15	0.96	1.06
EMS + BAP (2.00 mg L^{-1}) + NAA (0.50 mg L^{-1})	2.63	1.63	2.13	2.36	0.23	1.29
Mean	1.47	0.96		0.60	0.28	
	SEm±	CD (p=0.05)		SEm±	CD (p=0.05)	
Treatment (T)	0.295	0.833		0.105	0.298	
Genotype (G)	0.101	0.286		0.036	0.102	
T × G	0.417	1.179		0.149	0.421	

and EMS + 0.5 mg L^{-1} BAP + 0.5 mg L^{-1} NAA (2.37). The minimum number of shoots (0.17) per explant was recorded on MS medium supplemented with 0.25 mg L^{-1} BAP + 0.25 mg L^{-1} NAA. Furthermore, maximum number of shoots per explant was recorded in EMS medium as compared to MS medium. It was also evident from the data that Pusa Arpita leaf explants cultured on EMS medium produced maximum number of shoots per explant (1.47) as compared to Pusa Basanti Gainda (0.96). Medium and genotype interaction revealed that the maximum number of shoots (3.67) per explant was obtained on EMS medium supplemented with 1.0 mg L^{-1} BAP + 0.5 mg L^{-1} NAA in Pusa Basanti Gainda which was followed by MS + 2.0 mg L^{-1} BAP + 0.5 mg L^{-1} NAA (3.60) in Pusa Arpita.

For true determination of the efficiency of the suitable basal media and growth regulator combination the Bud Forming Capacity (BFC) index was calculated[14]. Perusal of data from Table 3 reveals that highest BFC index (1.49) was reported on EMS medium supplemented with 0.5 mg L^{-1} BAP + 0.25 mg L^{-1} NAA which was followed by EMS + 2.0 mg L^{-1} BAP + 0.5 mg L^{-1} NAA (1.29) and was found to be statistically at par with each other. Among the treatments lowest BFC index (0.01) was reported on MS medium supplemented with 0.25 mg L^{-1} BAP + 0.25 mg L^{-1} NAA. Among the two genotypes, Pusa Arpita leaf explants recorded maximum BFC index (0.60), which was statistically significant over Pusa Basanti Gainda (0.28). Lowest bud forming capacity of explants was observed in MS medium as compared to EMS in both Pusa Arpita and Pusa Basanti Gainda genotypes. Medium and genotype interaction was found to be significant and maximum bud forming capacity index (2.36) was obtained in Pusa Arpita explants cultured on EMS medium supplemented with 2.0 mg L^{-1} BAP + 0.5 mg L^{-1} NAA, which was statistically at par with Pusa Basanti Gainda explants cultured on EMS medium supplemented with 0.5 mg L^{-1} BAP + 0.25 mg L^{-1} NAA (2.11). The percent regeneration of explants represents the response capacity of the tissue to the suitable medium, and the number of shoots per explant represents the capacity of the explants to produce shoots[14]. The combination of these two components while calculating BFC index gave the better idea about the optimal requirements for the plant regeneration. Vanegas-Espinoza *et al*.[12] reported the highest BFC index (1.39) of African marigold leaf explants cultured on MS medium supplemented with 17.1 µM IAA and 13.3 µM BA. Vanegas-Espinoza *et al*.[12] reported the maximum BFC index (2.07) when the leaf segments were cultured on MS medium supplemented with 3.0 mg L^{-1}

IAA and 1.5 mg L^{-1} BA. The present results showed the superiority of EMS medium over MS medium as compared to earlier results and also revealed the important role of basal medium along with the hormones for adventitious bud formation from leaf explants. The enhanced regeneration capacity of both African and French genotypes mainly attributed to the optimum levels of cytokinin: auxin along with the addition of 6% coconut water, amino acids, vitamins and silver nitrate. Several researchers reported the use of coconut water for regeneration in recalcitrant species, as coconut water is rich source for cystine, lysine, methionine, histidine and other essential amino acids along with high minerals and vitamins[24,25]. These results are in close conformity with the findings of Tefera and Wannakrairoj[26] in Karwan, Agampodi and Jayawardena[15] in *Dracaena purplecompacta* and Michael[16] in sweet potato. Incorporation of silver nitrate also enhanced the regeneration of leaf explants in marigold and similar observations were recorded by Geetha *et al*.[17] in *Solanum nigrum*. The enhanced plant regeneration by $AgNO_3$ mainly attributed to inhibiting the ethylene action on explant. Often the presence of ethylene in *in vitro* culture vessel, negatively affect the callus growth, shoot regeneration and somatic embryogenesis[27,28]. Misra and Datta[10] suggested the use of ethylene inhibitors such as $AgNO_3$ for obtaining higher proliferation by reducing ethylene levels and reducing *in vitro* recalcitrance of *Tagetes erecta*.

Selection of suitable maturity stage of leaf explant for enhanced in vitro organogenesis

In the initial experiments it was observed that most of the shoot bud induction occurred from the basal petiolar end, while the tip portion did not differentiate much. Further different maturity stages of leaf explants collected from *in vitro* shoots showed varied shoot morphogenic ability. To select the suitable explant for enhancing BFC index, a separate experiment was initiated with three leaf maturity groups (old basal leaves, middle leaves and top leaves) along with their proximal and distal ends. In this experiment, Pusa Arpita and Pusa Basanti Gainda explants were cultured on EMS medium supplemented with 2.0 mg L^{-1} BAP + 0.5 mg L^{-1} NAA and 0.5 mg L^{-1} BAP + 0.25 mg L^{-1} NAA respectively as per the standardization of basal media and growth regulators in the earlier experiment.

The data presented in Table 4 shows that maximum regeneration (86.7%) was observed in immature leaf bases over all other treatments. In both the species and irrespective of maturity groups, lowest percentage of regeneration was recorded from distal part of leaf as compared to proximal end. Maximum regeneration (93.3%) was recorded in immature leaf bases of Pusa Arpita which was statistically at par with mature leaf base explants (86.5%). Similar trend was also recorded in African marigold cv. Pusa Basanti Gainda in which immature leaf bases exhibited highest regeneration (80.1%) followed by mature leaf base (72.0%) and were statistically at par with each other. Perusal of data also revealed that the number of shoots emerged per leaf explant were significantly high (3.84) in immature leaf bases over all other treatments. In this experiment also maximum number of shoots per explant (3.76) was recorded in Pusa Arpita genotype over Pusa Basanti Gainda (2.04) as in our preliminary experiments. Genotype and type of explant interaction revealed that basal part of immature leaves of Pusa Arpita and Pusa Basanti Gainda was recorded maximum number of shoots per explant *viz*., 4.40 and 3.28, respectively.

Table 4—Effect of maturity of leaf explant and its polarity on *in vitro* plant regeneration capacity of African and French marigold genotypes

Treatments	Regeneration (%)		Number of buds per explant		Bud forming capacity index	
	PA	PBG	PA	PBG	PA	PBG
Immature leaf tip	82.0±2.23 (65.2)*	67.6±3.06 (55.6)*	3.7±0.29	1.4±0.13	3.1±0.26	0.9±0.07
Immature leaf base	93.3±4.90 (78.5)	80.1±2.27 (63.8)	4.4±0.29	3.3±0.31	4.2±0.30	2.6±0.20
Young leaf tip	73.0±1.16 (58.7)	29.2±3.48 (32.3)	3.4±0.46	1.2±0.13	2.6±0.37	1.1±0.05
Young leaf base	82.3±2.52 (65.6)	54.9±2.56 (47.8)	4.2±0.43	1.5±0.13	3.5±0.46	2.2±0.18
Mature leaf tip	64.6±1.71 (53.6)	60.1±2.41 (50.9)	2.7±0.10	1.8±0.08	1.8±0.10	0.4±0.10
Mature leaf base	86.5±5.52 (71.1)	72.0±2.53 (58.3)	3.8±0.41	3.1±0.17	3.3±0.39	0.8±0.08
Mean	80.3 (65.4)	60.7 (51.5)	3.8	2.04	3.07	1.33
SEm±	3.417	2.750	0.352	0.173	0.332	0.127
CD (p=0.05)	10.033	8.076	1.034	0.508	0.974	0.373

[*Values in parenthesis represent arcsine transformed values. Values represent the mean±standard error]

Bud forming Capacity (BFC) Index was calculated for a more realistic determination of explant selection. Among the different stages tested, immature leaf base part recorded maximum BFC (3.4) index over all other treatments. Among the two genotypes, explants of Pusa Arpita recorded highest BFC (3.07) index over Pusa Basanti Gainda (1.33). Genotype and type of explant interaction revealed that Pusa Arpita immature leaf proximal ends recorded maximum BFC (4.20) index over all other treatments. However, a significant increase in BFC (2.60) index was also recorded in Pusa Basanti Gainda when the immature leaf basal part used as explant for initiation of culture.

Leaf explant origin, maturity and its polarity also plays a significant role in *in vitro* organogenesis along with the concentration and combination of growth regulators and basal medium. In the present investigation, maximum bud forming capacity was obtained by culturing the immature leaf base of 'Pusa Arpita' (4.2) and 'Pusa Basanti Gainda' (2.6). The regeneration capacity substantially increased from the tip towards basal petiolar end of the marigold leaf. The organogenesis ability increases from the tip towards base of the leaf had also been observed in apple[29].

After standardizing the reliable and reproducible organogenesis in both African and French marigold genotypes, the regenerants along with leaf segments were transferred to MS media devoid of any growth regulators for further growth and elongation of shoots (Fig. 2A). Shoot buds transferred to the same regeneration media showed hyperhydricity and heavy callusing in the basal portion of shoots. After three weeks, healthy, elongated shoots in blank media were transferred to a low concentration of BA (0.5 mg L^{-1}), NAA (0.1 mg L^{-1}) was used along with 2.5 mg L^{-1} AgNO3 for shoot proliferation (Fig. 2 B-C). After achieving satisfactory proliferation in 3-5 cycles, micro-shoots were transferred to MS medium without any growth regulators for obtaining healthy and sturdy shoots. Thereafter, isolated shoots were excised and rooted using ½ MS medium supplemented with 0.5 mg L^{-1} IBA, where 100% rooting was achieved (Fig. 2D). Rooted shoots were hardened in low-cost polyethylene plastic glasses and recorded maximum (95-98%) field survival[30] (Fig. 2 E-F).

To the best of our knowledge, we have not come across any report on the use of coconut water, $AgNO_3$ and additional organic substances for enhancing the explant regeneration in the genus *Tagetes*. The results of the present study was demonstrated the crucial role of coconut water, $AgNO_3$ and other organic addenda for direct differentiation of shoot buds from both African and French marigold genotypes with the specific concentrations of auxin and cytokinin ratio. Culturing immature leaf basal segments of both genotypes in EMS medium greatly enhanced the BFC index. Coconut water is a rich source of amino acids, nucleic acids, organic acids, vitamins, sugars, sugar

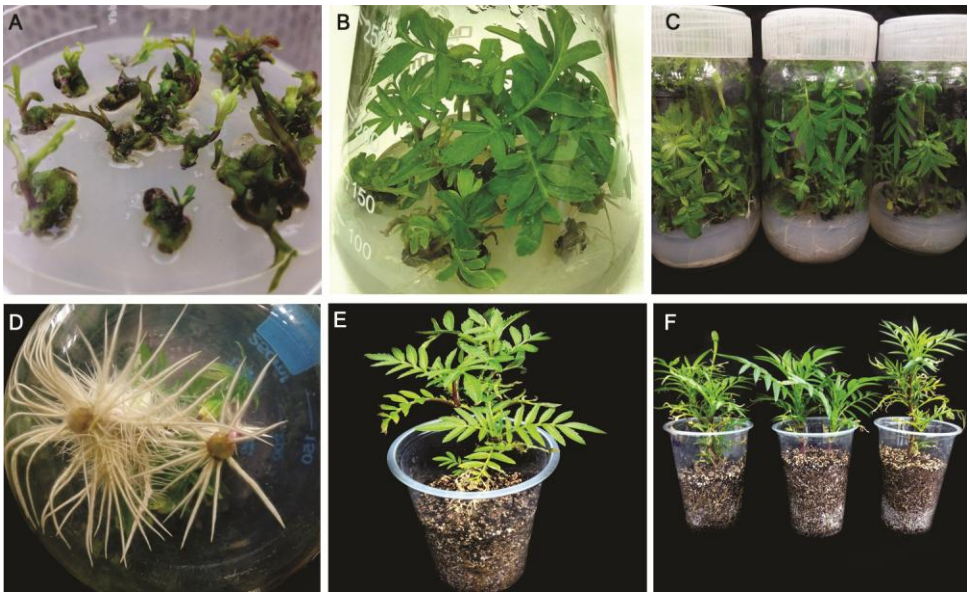

Fig. 2—(A) Proliferation of shoot buds accompanied with shoot elongation in *in vitro* cultures of African marigold cv. PBG in MS media devoid of growth regulators; (B & C) *In vitro* development of healthy elongated shoots of French (PA) and African (PBG) marigold; (D) Rooting of elongated marigold shoots on MS + 0.5 mg L^{-1} IBA; (E) Fully hardened PA plant; and (F) Fully hardened PBG plants

alcohols, plant hormones (cytokinins, auxins) and other unidentifiable compounds, none of which is solely responsible for growth promoting qualities. Unlike other complex nutrients, coconut water has proved harder to replace by fully defined media[18]. Many workers try to avoid using coconut water in their protocols due to the lack of reproducibility. However, incorporating coconut water gives a simple way to obtain satisfactory growth or shoot bud differentiation without the need to work out a suitably defined medium[31]. If satisfactory results are not obtained by employing the standard component of the media, in case of specific requirement of a particular species or tissues, other components like amino acids, anti-oxidants, and a variety of complex natural extracts, can be used for obtaining regeneration. When completely defined media did not give desired results, employing coconut milk, coconut water, malt/yeast extract, banana, tomato, carrot and orange juice have beneficial effects on *in vitro* plant cell and tissue cultures[32].

Conclusion

The present study on recalcitrant African and French marigold genotypes reiterates that, as coconut water, $AgNO_3$ and additional organic addenda act synergistically and helpful in achieving rapid shoot bud induction with highest Bud Forming Capacity index in the process of regeneration in African and French marigold genotypes.

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