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Influence of growth regulators on callus induction and plant regeneration from anthers of *Tagetes* spp.

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ABSTRACT

Marigold (*Tagetes* spp.) belongs to family Asteraceae, native to Mexico, is one of the most important flower crop grown commercially in different parts of India. The application of haploid techniques as anther culture can considerably accelerate marigold breeding programmes by providing homozygous doubled haploid (DH) lines for F_1 hybrid seed production. Therefore, the objective of the present investigation was to study the effect of growth regulators for *in vitro* regeneration in both African marigold and French marigold genotypes *viz*., Pusa Basanti Gainda and Pusa Arpita, respectively for haploid induction. Among the different treatments tested for callus induction, anthers cultured on MS medium supplemented with 1.0 mg/l BAP + 1.0 mg/l 2,4-D + 45 g/l sucrose significantly induced highest (38.74%) callus in the shortest period of time (16 days). Among two genotypes tested, embryogenic callus induction (27.70%) was significantly higher in Pusa Arpita over Pusa Basanti Gainda (23.37%). Among the different regeneration treatments, the highest adventitious shoot bud induction (13.32%), maximum (1.09) number of buds/callus in shortest duration (19.47 days) was observed in anther derived callus cultured on MS medium supplemented with 2.0 mg/l BAP + 0.5 mg/l NAA + 30 g/l sucrose. Among two genotypes evaluated for shoot bud induction, maximum (3.81%) regeneration was recorded in Pusa Arpita over Pusa Basanti Gainda (2.90%). In French genotype, the whole regeneration process was completed in 30 days whereas in African genotype it took nearly 40 days. This rapid regeneration system from anthers is highly useful in inducing haploids and di-haploids in African and French marigold lines, respectively.

Key words: African marigold, Anther culture, Callus induction, French marigold, Haploids, Regeneration

Marigold (*Tagetes* spp.) is a popular ornamental, industrial and medicinal crop belongs to Asteraceae family. It is native of Mexico and got naturalised in India about 350 years ago. Both African and French marigolds are gaining popularity and commercial importance in India on account of its easy cultivation, short duration, vast adaptability, wide spectrum of shape, size and good keeping quality. It is cultivated in an area of 56.04 thousand ha with 501.87 thousand MT production and occupied maximum area among the loose flowers (Anonymous 2015). It has been reported that leaves and flowers of this plant contain various bioactive compounds that exhibit anti-bacterial, anti-microbial, insecticidal, nematicidal, mosquitocidal, larvicidal, fungicidal, hepatoprotective, wound healing and analgesic activities (Priyanka *et al*. 2013). Besides loose flower and ornamental pot plants, the F_1 hybrids are also being used as cut flowers in Central America.

In recent years, F_1 hybrids are also gaining popularity in India for loose flower production but the costly imported marigold seed is a major hindrance for large-scale cultivation of this crop in farmer's field. In spite of its economic importance and availability of considerable genetic diversity, the genetic potentialities of marigold are practically unexplored in India and most of the F_1 hybrid seeds are being imported from other countries. For any successful hybrid seed production programme, homozygous parental lines are indispensable. The application of haploid techniques as anther culture or microspore culture can considerably accelerate breeding programmes by providing homozygous

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DH lines after a single *in vitro* culture step and avoiding the time-consuming process of developing inbred lines by selfing over several generations and years (Nichterlein and Horn 2005). Haploid induction through anther and/or ovule culture is the only way to achieve homozygosity in self-incompatible species, dioecious species, male sterile lines and species that suffer from inbreeding depression due to self-pollination. In a similar way, by employing anther culture homozygosity can be achieved in a single generation and eliminating the need for several generations of self-pollination thereby this doubled haploid (DH) lines can directly be used as parents. Other than this, DH lines have several other advantages such as, direct release as a new cultivar in self-pollinated crops (Veilluex 1994), isolation of recessive gene mutants (Hermsen and Ramana, 1981), reverse breeding (Wijnker *et al.* 2007), genomics, gene expression and genetic mapping (Ferrie and Caswell 2011). Furthermore, the haploid induction technique can nowadays be efficiently combined with several other plant biotechnological techniques, enabling several novel breeding achievements, such as improved mutation breeding, backcrossing, hybrid breeding and genetic transformation (Murovec and Bohanec 2012). To the best of our knowledge, successful reports are not available on plant regeneration from anther culture of African marigold (*Tagetes erecta* L.) although plant regeneration has been obtained from anthers of French marigold (Qi *et al*. 2011) but failed to induce haploids. Previously, regeneration in marigold was also reported from leaf explants (Belarmino *et al*. 1992, Misra and Datta, 2001, Venegas, 2002, Ying-chun *et al.* 2005, Vanegas-Espinoza *et al*. 2012), cotyledons and hypocotyls (Bespalhok and Hattori, 1998, Mohamed *et al.* 1999; Ying-Chun *et al*. 2005, Gupta and Rahman, 2015), stem segments (Venegas *et al.* 2002) and disc florets (Kothari and Chandra 1984).

Therefore, the present study was undertaken for developing an efficient *in vitro* anther regeneration protocol in both African and French marigold species in order to observe *in vitro* response to various growth regulators and the possibility of producing haploid plants.

MATERIALS AND METHODS

The present experimentation was carried out at the Central Tissue Culture Laboratory, ICAR-National Research Centre on Plant Biotechnology, Pusa, New Delhi during 2014-2017. African marigold cv. Pusa Basanti Gainda (PBG) and French marigold cv. Pusa Arpita (PA) were used for the study. The flower buds were collected from the healthy and disease-free plants from 8.00 am to 9.00 am, kept in the ice box and immediately brought to the laboratory. Excess flower stalks were removed from the capitulum and washed under running tap water for 10 minutes. The flower buds were treated with 0.2% Bavistin® (Carbendazim) + 0.2% Ridomil® (Metalaxyl + Mancozeb) + 8-hydroxy quinoline citrate (200 mg/l) along with 1 - 2 drops of teepol (0.1%) solution and placed on a Max Q 4000 model (Barnstead/Lab-line) horizontal shaker (90 rpm) for 15 minutes. The buds were shifted to laminar air-flow chamber and thoroughly washed with autoclaved double distilled water to remove the residues of detergent and fungicides. The buds were then transferred to autoclaved sterile jam bottle and treated with $HgCl_2$ (0.1%) for 4 minutes by continuous manual stirring. Surface-sterilized explants were then given 3-4 washings with sterile double distilled water to remove the traces of sterilizing agent immediately after treatment. The sterilized buds were further used for anther excision.

Buds of suitable length were selected. After selection, the bracts and ray florets were removed carefully without any damage to inner disc florets. Based on the initial experiment results, 3.0-3.5 mm length of florets in Pusa Basanti Gainda and 3.5-4.0 mm length of florets in Pusa Arpita were excised as these florets having anthers in uninucleate to early binucleate stage microspores (Fig 1a and b). The isolated florets were immediately transferred to sterilized distilled water to prevent drying. The florets were dissected under magnifying operates by using sterilized needles and anthers were excised carefully without any physical damage and placed on culture medium. In order to standardize the growth regulators for anther culture, anthers of both the cultivars were inoculated in 90 mm petri-dish with 25 ml of Murashige and Skoog (1962) basal medium (MS) supplemented with 4.5% sucrose, 0.25% (w/v) gelrite and different combinations of BAP (0-2.0 mg/l) and 2, 4-D (0-1.5 mg/l) for callus induction. The percent responding anthers and callus induction was recorded 20 days after anther inoculation. After 30 days of anther inoculation, well developed, embryogenic callus was transferred to various concentrations of BAP (0.5 – 2.0 mg/l) and NAA (0.25-1.0 mg/l) for plant regeneration. The percent caulogenesis, rhizogenesis, days to shoot bud induction and number of micro-shoots per calli was recorded 25 days after transfer. Medium without any hormone served as control. The cultures were maintained at 24 ± 2°C under fluorescent white light (47 mol/m^2/s) at a photoperiod of 16/8 hr/ light and dark cycles. All cultures were examined periodically and observations on any morphological changes were recorded.

All the experiments were conducted in a completely randomized design (CRD) with five replications. A replicate consisted of petridish with 30-35 explants. Experiments were repeated at least twice and the data were pooled before analysis. The data indicated in the table are means

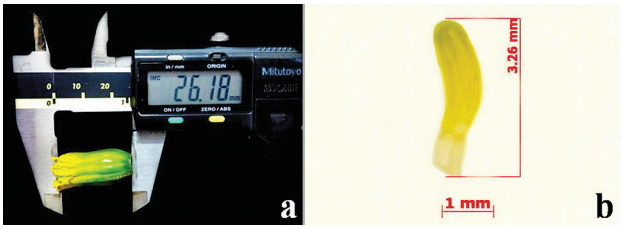

Fig 1 African marigold cv. Pusa Basanti Gainda explants for anther culture, a) Flower bud having maximum number of suitable disc florets; b) Disc floret of 3.0 -3.5 mm length having anthers of early uni-nucleate to early binucleate stage microspores.

of replicate values. The data in the 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.

RESULTS AND DISCUSSION

Effect of BAP and 2, 4-D on callus induction

In the present study, the structures of the anthers were found to be similar in both the species. The anthers were inflated after one week on the induction medium. It seems that the key step in the culture procedure for obtaining haploid plants is the induction of high quality callus from the anthers, and the key strategy is the application of right kinds of plant growth regulators in the callus induction medium. Perusal of data from Table 1 revealed that all the treatments significantly differed from MS medium without growth regulators (control) for per cent anthers swelling. Among the treatments, maximum (18.67%) swelling of anthers was observed when they were cultured on MS medium supplemented with 2.0 mg/l BAP + 1.5 mg /l 2,4-D which was statistically at par with MS + 1.0 mg/l BAP + 1.0 mg/l 2,4-D (18.51%) and MS + 2.0 mg/l BAP + 1.0 mg/l 2,4-D (17.45%). Among the two genotypes, maximum swelling (14.93%) of anthers was recorded in cultivar Pusa Arpita, which was significantly superior to cultivar Pusa Basanti Gainda (5.75%). The interaction between treatment and genotype revealed that the maximum (27.85%) percent swelling of anthers of Pusa Arpita was observed when they were cultured on MS medium supplemented with 2.0 mg/l BAP + 1.5 mg/l 2,4-D which was statistically at par with MS + 1.0 mg/l BAP + 1.0 mg/l 2,4-D (25.51%) and MS + 2.0 mg/l BAP + 1.0 mg/l 2,4-D (23.38%). Both the genotypes did not show any response to control medium.

It is evident from Table 1 that media devoid of growth regulators (control) showed maximum non-responsive anthers over all other treatments. Among the different treatments, least (42.75%) number of non responding anthers were reported on MS medium supplemented with 1.0 mg/l BAP + 1.0 mg/l 2,4-D, which was significantly different with MS + 2.0 mg/l BAP + 1.5 mg/l 2,4-D (47.32%) and MS + 2.0 mg/l BAP + 1.0 mg/l 2,4-D (47.57%). Among the two genotypes, PBG showed maximum (70.88%) non responding anthers, which was significantly different from PA (57.37%). Both the genotypes failed to respond on MS medium devoid of hormones (control). Treatment × genotype interactions revealed that significantly lowest non responding anthers were observed in PA cultured on MS medium supplemented with 1.0 mg/l BAP + 1.0 mg/l 2,4-D% (28.54%) which significantly differed from MS + 2.0 mg/l BAP + 1.5 mg/l 2,4-D (38.16%) in the same genotype.

Table 1 Effect of BAP and 2, 4-D on *in vitro* response of anthers in African marigold cv. Pusa Basanti Gainda (PBG) and French marigold cv. Pusa Arpita (PA)

Treatment	Treatment details (mg/l)		Plumpy anthers (%)		Mean	Non responding anthers (%)		Mean
	BAP	2,4-D	PBG	PA		PBG	PA	
T_0	0.0	0.0	0.00 (0.00)*	0.00 (0.00)	0.00 (0.00)	100.00 (88.15)	99.10 (85.61)	99.55 (86.88)
T_1	1.0	0.5	1.61 (5.95)	8.42 (16.67)	5.01 (11.31)	82.09 (64.95)	66.35 (54.55)	74.22 (59.75)
T_2	1.0	1.0	11.51 (19.77)	25.51 (30.26)	18.51 (25.02)	56.96 (48.99)	28.54 (32.15)	42.75 (40.57)
T_3	1.0	1.5	4.35 (11.86)	17.60 (24.72)	10.98 (18.29)	68.25 (55.70)	44.71 (41.94)	56.48 (48.82)
T_4	1.0	2.0	3.41 (10.49)	10.33 (18.66)	6.87 (14.58)	74.05 (59.48)	62.83 (52.42)	68.44 (55.95)
T_5	2.0	0.5	4.10 (11.60)	6.31 (14.47)	5.20 (13.03)	77.59 (61.75)	75.71 (60.46)	76.65 (61.10)
T_6	2.0	1.0	11.51 (19.76)	23.38 (28.24)	17.45 (24.30)	51.59 (45.89)	43.56 (41.23)	47.57 (43.56)
T_7	2.0	1.5	9.49 (17.88)	27.85 (31.83)	18.67 (24.85)	56.49 (48.76)	38.16 (38.12)	47.32 (43.44)
		Mean	5.75 (12.17)	14.93 (20.69)		70.88 (59.21)	57.37 (50.81)	
			SEm±	*CD (P=0.05)*		*SEm*±	*CD (P=0.05)*	
	Treatment (T)		0.994	2.877		1.345	3.891	
	Genotype (G)		0.497	1.438		0.672	1.946	
	T × G		1.406	4.069		1.902	5.503	

*Figures given in parentheses are angular transformed values.

Based on the microscopic observations, callus originated from somatic tissues like anther walls, filaments and vascular tissues were not considered for calculating percent callus induction (Fig 2c). Among the different treatments, highest embryogenic compact callus emerging from inside the anthers (Fig 2d) (38.74%) was obtained on MS medium supplemented with 1.0 mg/l BAP + 1.0 mg/l 2,4-D, which was statistically significant over all other treatments (Fig 2b). Poor (0.45%) callus induction was observed on MS medium without growth regulators (control) (Fig 2a). Significant differences were observed between the two genotypes for callus induction. Maximum (27.70%) callus induction was observed in PA as compared to PBG (23.37%). PA anthers produced maximum (45.95%) callus with MS + 1.0 mg/l BAP + 1.0 mg/l 2,4-D, whereas, in PBG maximum (36.90%) callus induction was observed with MS + 2.0 mg/l BAP + 1.0 mg/l 2,4-D. Treatment × genotype interactions revealed that the maximum callus (45.95%) induction was observed when PA anthers were cultured on MS medium supplemented with 1.0 mg/l BAP + 1.0 mg/l 2,4-D, which was statistically significant to MS + 1.0 mg/l BAP + 1.5 mg/l 2,4-D (37.69%) (Table 2). These results are in close agreement with Gao *et al*. (2011), who found the maximum callus induction (18.88%) from chrysanthemum anthers cultured on MS medium supplemented with 2.0 mg/l BAP, 1.0 mg/l 2, 4 -D and 90 g/l sucrose. Similarly, Khandakar *et al*. (2014) reported a good amount of callus when the chrysanthemum anthers cultured on MS medium supplemented with 1.0 mg/l 2, 4-D + 2.0 mg/l BA + 250 mg/l casein hydrolysate and 45 g/l sucrose. Similar results were also reported by Jia *et al*. (2014) in baby primrose and Mosquera *et al*. (1999) in carnation.

The number of days taken for callus induction considerably reduced with the increase in the concentration of BAP and 2, 4 - D. Among the different treatments, minimum days (16.00) to callus induction was recorded on MS medium supplemented with 1.0 mg/l BAP + 1.0 mg/l 2, 4-D, which was statistically at par with MS + 1.0 mg/l BAP + 1.5 mg/l 2, 4-D (16.67). All the treatments exhibited early induction of callus over control (Table 2). The two genotypes significantly differed with days to callus induction. Significantly less number of days (17.50) required for callus induction in PBG over PA (18.35 days). The interaction between treatment and genotype varied

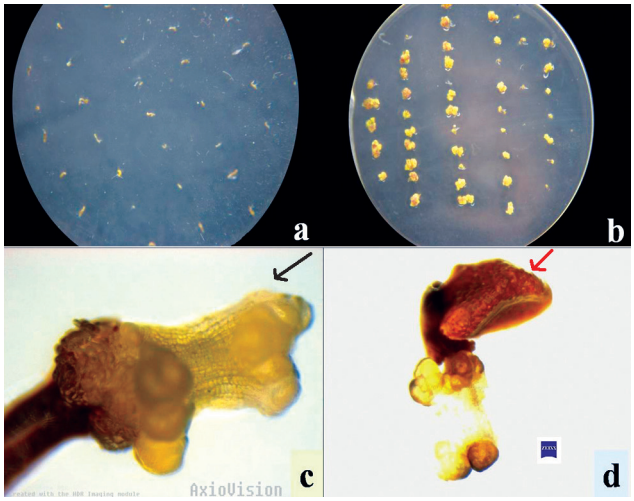

Fig 2 Callus induction in African marigold cv. Pusa Basanti Gainda anthers 20 days after inoculation a) Anthers cultured on MS medium devoid of growth regulators (Control); b) Anthers cultured on MS medium supplemented with BAP (1.0 mg/l) + 2,4-D (1.0 mg/l) + sucrose (45 g/l); c) Callus induction from somatic tissues of anther (indicated with arrow); d) Compact embryogenic callus emerging from inside the anthers (indicated with arrow).

Table 2 Effect of BAP and 2, 4-D on callus induction and days to callus initiation from anthers in African marigold cv. Pusa Basanti Gainda (PBG) and French marigold cv. Pusa Arpita (PA)

Treatment	Treatment details (mg/l)		Callusing (%)		Mean	Days to callus induction		Mean
	BAP	2,4-D	PBG	PA		PBG	PA	
T_0	0.0	0.0	0.00 (0.00)*	0.90 (3.15)	0.45 (1.57)	0.00	29.00	29.00
T_1	1.0	0.5	16.31 (23.74)	25.24 (30.12)	20.77 (26.93)	21.17	19.17	20.17
T_2	1.0	1.0	31.52 (34.12)	45.95 (42.66)	38.74 (38.39)	18.83	13.17	16.00
T_3	1.0	1.5	27.39 (31.54)	37.69 (37.84)	32.54 (34.69)	19.83	13.50	16.67
T_4	1.0	2.0	22.54 (28.16)	26.83 (31.13)	24.69 (29.64)	21.00	17.00	19.00
T_5	2.0	0.5	18.31 (25.31)	17.98 (25.04)	18.14 (25.17)	22.17	20.50	21.33
T_6	2.0	1.0	36.90 (37.38)	33.06 (35.04)	34.98 (36.21)	18.33	18.33	18.33
T_7	2.0	1.5	34.02 (35.51)	33.99 (35.63)	34.01 (35.57)	18.67	16.17	17.42
		Mean	23.37 (26.97)	27.70 (30.08)		17.50	18.35	
			SEm±	*CD (P=0.05)*		*SEm*±	*CD (P=0.05)*	
	Treatment (T)		1.231	3.563		0.403	1.166	
	Genotype (G)		0.616	1.781		0.201	0.583	
	T × G		1.741	5.039		0.57	1.649	

*Figures given in parentheses are angular transformed values.

significantly for days required for callus induction. Minimum days (13.17) required for callus induction was observed in PA anthers cultured on MS medium supplemented with 1.0 mg/l BAP + 1.0 mg/l 2,4-D which was statistically at par with MS + 1.0 mg/l BAP + 1.5 mg/l 2,4-D (13.50 days) in the same genotype (Table 2).

The effect of growth regulators on androgenesis was widely discussed in most of the crop plants. The presence of growth regulators mostly auxins and cytokinins are highly essential for microspore derived embryo production, particularly in recalcitrant plant species. The auxin type and concentration are mostly determining the pathway of microspore development singly or with the combination of cytokinin (Ball *et al*. 1993). Several authors reported the callus formation with 2,4-D and direct embryogenesis with NAA or IAA (Armstrong *et al*. 1987, Liang *et al*. 1987). The synthetic auxin 2,4-D has been reported as an effective plant growth regulator for the induction of androgenesis in many plant species, including wheat (Ball *et al*. 1993) and legumes (Croser *et al*. 2006).

The present investigation revealed that all the different concentrations of 2,4-D, when combined with BAP, allowed successful callus induction from marigold anthers. However, the overall embryogenic callus induction from inside the anthers was low, with the highest callus induction rate achieved at the equal dose of 2,4-D with BAP (Fig 2b). Thus, it is possible that the further optimization of the medium could induce higher rates of callus formation or direct adventitious shoot bud induction from marigold anthers. Aside from growth regulator concentrations, donor plant genotype may have also influenced callus induction, as this has been reported as a controlling factor on androgenesis in many species (Doi *et al*. 2010).

Effect of BAP and NAA on regeneration from callus

In the present experiment, compact, green, embryogenic callus emerging from inside the anthers only considered for regeneration and callus originated from other somatic tissues eliminated based on the microscopic observations. The data presented in Table 3 revealed that treatments for percent regeneration significantly varied with each other. Best regeneration or caulogenesis (13.32%) was observed in MS medium supplemented with 2.0 mg/l BAP + 0.5 mg/l NAA which was statistically significant with all other treatments. It was also observed that higher concentration of auxin over cytokinin and in equal concentrations prevented the regeneration from callus. Regeneration was not observed in T_4, T_7 and T_8 treatments. Significant variation was not observed among the two genotypes, viz. PBG and PA. Among the genotypes, maximum regeneration (3.81%) was recorded in PA followed by PBG (2.90%). Treatment × genotype interaction revealed that highest regeneration (18.87%) was obtained from the PA anther callus cultured on MS medium supplemented with 2.0 mg/l BAP + 0.5 mg/l NAA (Fig 3a) which was statistically significant over PBG anther callus cultured on MS + 2.0 mg/l BAP + 1.0 mg/l NAA (9.20%) (Fig 3b) and MS + 0.5 mg/l BAP + 0.25 mg/l NAA (9.14%). These results are in close agreement with Gao *et al*. (2011), who found the maximum (67.1%) regeneration from chrysanthemum anther derived callus with 2.0 mg/l BAP + 0.2 mg/l NAA + 30 g/l sucrose. Similarly, Khandakar *et al*. (2014) reported haploid induction when the chrysanthemum anther derived calli were allowed to differentiate on MS medium supplemented with 2.0 mg/l BA + 0.1 mg/l NAA and 30 g/l sucrose. However, Qi *et al*. (2011) reported high callus induction and regeneration of French marigold anthers on MS basal medium supplemented with 0.5 mg/l BA + 0.34 or 0.5 mg/l NAA + 45 g/l sucrose.

Table 3 Effect of BAP and NAA on shoot organogenesis and root organogenesis from anther derived callus in African marigold cv. Pusa Basanti Gainda (PBG) and French marigold cv. Pusa Arpita (PA)

Treatment	Treatment details (mg/l)		Caulogenesis (%)		Mean	Rhizogenesis (%)		Mean
	BAP	NAA	PBG	PA		PBG	PA	
T_1	0.5	0.25	9.14 (17.28)*	0.00 (0.00)	4.57 (8.64)	24.95 (29.84)	0.00 (0.00)	12.47 (14.92)
T_2	1.0	0.25	0.00 (0.00)	2.08 (4.82)	1.04 (2.41)	11.59 (19.7)	0.00 (0.00)	5.79 (9.85)
T_3	2.0	0.25	0.00 (0.00)	8.93 (17.27)	4.46 (8.64)	2.22 (4.99)	0.00 (0.00)	1.11 (2.49)
T_4	0.5	0.50	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	52.10 (46.19)	38.97 (38.52)	45.54 (42.35)
T_5	1.0	0.50	0.00 (0.00)	4.44 (9.97)	2.22 (4.99)	45.08 (42.16)	15.00 (22.12)	30.04 (32.13)
T_6	2.0	0.50	7.78 (16.01)	18.87 (25.70)	13.32 (20.86)	3.70 (9.09)	8.70 (16.77)	6.20 (12.93)
T_7	0.5	1.00	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	75.87 (61.00)	70.32 (57.14)	73.09 (59.07)
T_8	1.0	1.00	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	67.89 (55.49)	63.80 (53.01)	65.85 (54.26)
T_9	2.0	1.00	9.20 (17.45)	0.00 (0.00)	4.60 (8.73)	23.86 (29.18)	20.35 (26.55)	22.10 (27.87)
		Mean	2.90 (5.64)	3.81 (6.42)		34.14 (33.07)	24.13 (23.79)	
			SEm±	*CD (P=0.05)*		*SEm*±	*CD (P=0.05)*	
	Treatment (T)		1.336	3.847		2.040	5.876	
	Genotype (G)		0.630	NS		0.962	2.770	
	T × G		1.889	5.440		2.885	8.309	

*Figures given in parentheses are angular transformed values.

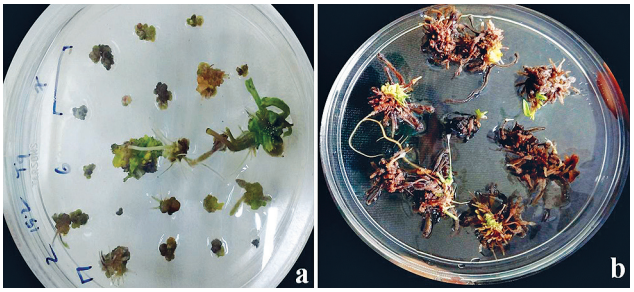

Fig 3 Regeneration of adventitious shoot buds from callus a) Regeneration from anther derived callus of Pusa Basanti Gainda on MS + BAP (2.0 mg/l) + NAA (1.0 mg/l); b) Regeneration from anther derived callus of Pusa Arpita on MS + BAP (2.0 mg/l) + NAA (0.5 mg/l).

These anther regenerated plantlets were subjected for ploidy analysis and they found that all the regenerated shoots have similar to donor parent ploidy and determined as somatic tissue origin.

Considerable percent of rhizogenesis was observed in all the treatment combinations. Among the different treatments tested maximum (73.09%) rhizogenesis was recorded on MS medium supplemented with 0.5 mg/l BAP + 1.0 mg/l NAA which was statistically significant over MS + 1.0 mg/l BAP + 1.0 mg/l NAA (65.85%). Further, it was observed that root induction was maximum from the cultured callus where the auxin concentration was higher than cytokinins. Among the two genotypes, significantly maximum (34.14%) root induction was observed from PBG as compared to PA (24.13%) (Table 3).

Perusal of data from Table 4 revealed that the early (19.47 days) shoot emergence was observed on MS medium supplemented with 2.0 mg/l BAP + 0.5 mg/l NAA, which was statistically at par with MS + 1.0 mg/l BAP + 0.5 mg/l NAA (19.50 days) and MS + 2.0 mg/l BAP + 0.25 mg/l NAA (19.83 days). Maximum days (24.33) to shoot emergence was recorded on MS medium supplemented with 0.5 mg/l BAP + 0.25 mg /l NAA. Further, it was observed that higher cytokinin concentration induced shoot buds earlier than lower levels. Significant difference was not observed among the two genotypes for days to shoot emergence. Early (19.53 days) shoot bud induction was recorded in PA followed by PBG (22.36 days). Treatment × genotype interaction revealed that the earliest (16.77 days) regeneration was observed from PA anther derived callus cultured on MS medium supplemented with 2.0 mg/l BAP + 0.5 mg/l NAA which was statistically at par with MS + 1.0 mg/l BAP + 0.50 mg/l NAA (19.50 days) in the same genotype.

Among the different treatments, maximum (1.09) shoots per callus were recorded on MS medium supplemented with 2.0 mg/l BAP + 0.5 mg/l NAA which were significantly higher than MS + 2.0 mg/l BAP + 1.0 mg/l NAA (0.71). Significant difference was not observed among the two genotypes for number of shoots emerged from callus. Maximum (0.38) number of shoots was recorded from PBG followed by PA (0.35). It is evident from Table 4 that, the interaction between treatment and genotype revealed that callus derived from PBG anthers showed maximum (1.42) shoots when cultured on MS + 2.0 mg/l BAP + 1.0 mg/l NAA which was statistically at par with MS + 2.0 mg/l BAP + 0.5 mg/l NAA in the genotype PA (1.17). Among the different treatments, highest culture establishment index (0.145) was recorded on MS medium supplemented with 2.0 mg/l BAP and 0.5 mg/l NAA. Among the two genotypes, Pusa Arpita recorded maximum establishment index (0.221) in the same treatment (T_6) where as callus derived from Pusa Basanti Gainda showed maximum regeneration (0.131) in T_9, i.e. MS + 2.0 mg/l BAP + 1.0 mg/l NAA (Fig 4).

The concentration of BAP played an important role

Table 4 Effect of BAP and NAA on number of days required for shoot regeneration and number of shoots per anther derived callus in African marigold cv. Pusa Basanti Gainda (PBG) and French marigold cv. Pusa Arpita (PA)

Treatment	Treatment details (mg/l)		Days to shoot initiation		Mean	Number of micro-shoots per calli		Mean
	BAP	NAA	PBG	PA		PBG	PA	
T_1	0.5	0.25	24.33	0.00	24.33	1.00	0.00	0.50
T_2	1.0	0.25	0.00	22.00	22.00	0.00	0.33	0.17
T_3	2.0	0.25	0.00	19.83	19.83	0.00	1.00	0.50
T_4	0.5	0.50	0.00	0.00	0.00	0.00	0.00	0.00
T_5	1.0	0.50	0.00	19.50	19.50	0.00	0.67	0.34
T_6	2.0	0.50	22.17	16.77	19.47	1.00	1.17	1.09
T_7	0.5	1.00	0.00	0.00	0.00	0.00	0.00	0.00
T_8	1.0	1.00	0.00	0.00	0.00	0.00	0.00	0.00
T_9	2.0	1.00	20.58	0.00	20.58	1.42	0.00	0.71
		Mean	22.36	19.53		0.38	0.352	
			SEm±	*CD (P=0.05)*		*SEm*±	*CD (P=0.05)*	
	Treatment (T)		1.645	4.739		0.091	0.262	
	Genotype (G)		0.776	NS		0.043	NS	
	T × G		2.327	6.702		0.129	0.371	

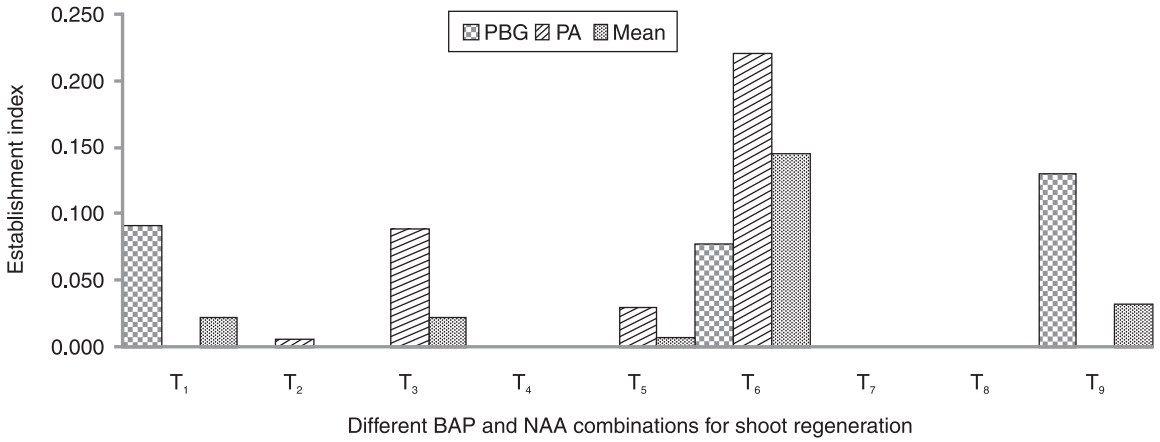

Fig 4 Effect of different BAP and NAA on establishment index of anther derived callus in African marigold cv. Pusa Basanti Gainda (PBG) and French marigold cv. Pusa Arpita (PA).

in regulating shoot regeneration from marigold calli in our study. Concentrations of BAP greater than 2.0 mg /l along with the low concentration of NAA were essential for shoot bud induction in marigold. However, for shoot bud elongation and healthy growth MS medium devoid of any growth regulators were ideal (Ravindra *et al*. 2017). Too high concentrations of auxins in the media prevent the shoot bud induction and promote root induction directly from the calli under such conditions. Anther culture of *Primula forbesii* (Jia *et al*. 2014), *Lupinus angustifolius* (Kozak *et al*. 2012) recorded similar results, with calli forming roots, then turning brown and dying a month later, in regeneration medium.

From the present studies, it is concluded that the current protocol can be effectively used for haploid induction and genetic manipulation for improvement of marigold species. Considering the findings, further investigation is required for enhancing the callus induction, plant regeneration and haploid production of marigold by experimenting with the type of basal medium, pre-treatment temperatures, concentration of carbohydrates and various other factors responsible for androgenesis in marigold.

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