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Optimising protocol for successful development of haploids in marigold (*Tagetes* spp.) through in vitro androgenesis

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Abstract

For the first time, we have developed a protocol for rapid and successful induction of haploids in *Tagetes* spp. using in vitro androgenesis techniques. Various factors affecting the in vitro androgenesis response in *Tagetes* spp. were optimised. At first, a correlation was established between floret size/bud size and stage of microspore development in different genotypes of *Tagetes erecta* and *Tagetes patula*. The floret size of 3.5–4.0 mm (French marigold) and 3.0–3.5 mm (African marigold) contained the highest percent of microspores at early-uninucleate to early-binucleate stage. The frequency of shoot formation from anthers ranged from 0 to 78.6% in *T. patula* cv. Pusa Arpita and 0 to 63.9% in *T. erecta* cv. Pusa Basanti Gainda. The French marigold cultivars exhibited higher androgenic response over the African marigold cultivars. The effects of basal media, plant growth regulator combinations and sucrose concentration for direct differentiation of shoot buds and haploid induction were studied. Marigold anthers cultured on newly formulated EMS_1 basal media (enriched with coconut water, $AgNO_3$, PVP etc.) exhibited a very high androgenic response over the other commercially available media. We have also optimised the cold temperature pre-treatments and culture condition for high frequency androgenesis in marigold. Ten days cold pre-treated anthers along with 20 days dark incubation of cultures proved highly beneficial for haploid induction. Among a total of 424 anther-regenerants, 56 plants were randomly selected for flow cytometry and cytological analysis. The ploidy analysis revealed 14.3% of anther regenerants as dihaploids, 66.1% as tetraploid (similar to donor mother plant) and 19.6% as polyploid. The determination of ploidy level by counting the number of chloroplast in stomatal guard cells of marigold was also established for rapid screening of haploids. The identified haploids were successfully diploidised and are being utilised in the hybrid breeding programme at our institute. The developed protocol will facilitate doubled haploids based breeding in *Tagetes* spp.

Key message

This is the first report of successful induction of haploids in *Tagetes* spp. using in vitro androgenesis techniques. We have studied various factors affecting the in vitro androgenesis response in *Tagetes* spp. A correlation was established between floret size/ bud size and stage of microspore development in different genotypes of *Tagetes erecta* and *Tagetes patula*. Basal media and cultural conditions were optimised for high frequency androgenesis in marigold. Haploids were successfully induced and their ploidy level was assessed through flowcytometry and cytological analysis.

Keywords African marigold · French marigold · Anther culture · Haploid · Flow cytometry · Cytology · Chloroplast

Abbreviations

2,4-D	2,4-Dichlorophenoxyacetic acid
BAP	Benzylamino purine

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EMS Enriched Murashige and Skoog medium
NAA 1-Naphthalene-acetic acid
TDZ Thidiazuron
PA Pusa Arpita
PBG Pusa Basanti Gainda

Introduction

Marigold (*Tagetes* spp.) is an Asteraceous plant and is native to South and Central America, specifically Mexico. The genus *Tagetes* comprises of 55 species (Godoy-Hernandez and Miranda-Ham 2007) and among them *Tagetes erecta* L. (African marigold; diploid; 2n = 2x = 24) and *T. patula* L. (French marigold; tetraploid; 2n = 4x = 48) are the most commonly cultivated species. Marigold is commonly grown for loose flower production and landscape gardening owing to their amazing flowers, attractive appearance, luminous colours, infinite-sizes, shapes, forms, fragrance etc. Its flowers are rich source of lutein which is a yellow oxy-carotenoid having beneficial pharmaceutical properties due to its strong antioxidant quality and plays a critical role in maintaining normal eye vision (Guerin et al. 2003). Accomplishing the beneficial effects of lutein, marigold petals are widely used as poultry feed to enrich the carotenoids in egg yolk and broiler chicken.

In recent years, F_1 hybrids of marigold are in great demand in India and other countries as they have many advantages like early flowering, profuse branching, uniform flowering, higher yield, biotic–abiotic tolerance, long post-harvest life, compact head and double flowers (Petalous male sterile forms). 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.

Homozygous parental lines are indispensable for commercial hybrid seed production. In Asteraceae family, more than 60% of species are estimated to be self-incompatible with sporophytic genetic determination (Theo Hendriks et al. 2014). The self-incompatibility is also reported in *Tagetes spp* (Eyster 1941). The in vitro induction of haploids and doubled haploids (DHs) through gametic embryogenesis is an effective approach for single-step development of complete homozygous lines from heterozygous parents. By employing anther culture technique, homozygosity can be attained in a single generation and thus eliminating the need for several cycles of selfing. The DH lines can directly be used as parents for development of hybrids. Other than this, DH lines has several other advantages like, direct release as a new cultivar in self-pollinated crops (Veilleux 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). The haploid induction technique can 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).

Many factors are responsible for successful production of DH plants viz., genotype, physiological condition of donor plant, developmental stage of the male and female gametophytes, pre-treatment, composition of the culture medium, physical factors like light and temperature during tissue culture (Zhang et al. 2009). Most of the Compositae species are regarded as recalcitrant in terms of microspore embryogenesis (Ferrie and Caswell 2011). Ketel et al. (1987) reported severe browning and stoppage of growth of tertiary leaf-derived calli of *Tagetes patula* and, primary and secondary leaf-derived calli of *T. erecta*, respectively. In addition, Croes et al. (1988) reported the formation of only roots from stem-derived calli of *Tagetes* spp. These studies pointed the difficulties in callus formation and plant regeneration in marigold cultivars. Compared to agronomic crops, very little work has been done in the ornamental species in terms of development and utilisation of DH protocols. The reports on development of DH lines and their utilization in breeding of genus *Tagetes* are lacking, although plant regeneration has been obtained from disc florets (Kothari and Chandra 1984), stem segments (Pablo et al. 2002), anthers (Qi et al. 2011), leaf explants (Vanegas-Espinoza et al. 2012) and hypocotyls (Gupta and Rahman 2015). Hence, there is an immediate need to standardize an efficient protocol for successful induction of haploids and DHs in the genus *Tagetes* by culturing male gametophytes. In this study we have tried to optimise various factors affecting in vitro androgenesis in marigold cultivars. For the first time we have developed a protocol for successful induction of haploids and DHs through anther culture in marigold. In this study we have optimised different factors such as cultivars, bud/floret size, temperature pre-treatments, culture conditions and basal media and growth regulators etc. for in vitro androgenesis through anther culture. The developed protocol will be instrumental for marigold breeding for development of F_1 hybrids and trait association studies.

Materials and methods

Plant material

Five African marigold (*T. erecta*) cultivars namely 'Pusa Basanti Gainda', Pusa Narangi Gainda', 'Pusa Bahar', 'IIHR-MYS-3' and 'Selection Af./W-1' (Online Resource 1; Supplementary Fig. 1a–e) and five French marigold (*Tagetes patula*) cultivars 'Pusa Arpita', 'Pusa Deep', 'Dainty Marietta', 'Valencia' and 'Selection Fr./W-20'(Online Resource 1; Supplementary Fig. 1f–j) were tested for androgenic response. The salient characters of the selected marigold cultivars are shown in the supplementary Table 1 (Online Resource 1). These selected germplasm represented the marigold cultivars from temperate, sub-tropical and tropical parts of India. The seeds were obtained from Division of Floriculture and Landscaping, ICAR-IARI, New Delhi and ICAR-IIHR, Bangalore. The seeds were germinated in plug tray containing a mixture of soil, coco peat and perlite (1:1:1) under shade net house with natural ventilation during the years 2014–2016. Thirty plants were raised for each variety. After 25 days after sowing, seedlings were transferred to naturally ventilated polyhouses. All the recommended cultural practices of ICAR-IARI, New Delhi were followed to raise the crop. The plants were weekly sprayed with liquid fertilizers (N:P:K = 19:19:19 and 13:0:45). The average maximum temperature ranged from 20 to 35 °C and the minimum temperature ranged from 5 to 18 °C during the active plant growth and flowering periods. The relative humidity of 65–75% was maintained by operating foggers. All precautionary measures were adopted to ensure for optimal growth conditions required for anther culture in marigold. Plant protection chemicals were not used either as spray or as soil drench, during the maintenance of donor plants to minimize the reduction of microspore viability. During morning hours, flower buds (capitulum along with 2–3 cm stalks) of different maturity were collected from healthy and strong growing plants. The excised flower buds were wrapped inside sterilized paper, packed in plastic bags and kept in ice box to avoid higher temperature exposure.

Association between flower bud/floret size and developmental stage of microspores

Young capitulum of all the ten cultivars were harvested at their early flower production period. The length of flower bud was measured, the buds were then fixed in Carnoy's solution-1 (3:1 of 100% ethanol:glacial acetic acid) at room temperature for 24 h and later on transferred to 70% ethanol and stored at 4 °C till analysis. To establish a correlation between floret size and stages microspore developmental, ten disc florets from each sized group of 1.5–1.9, 2.0–2.4, 2.5–2.9, 3.0–3.4, 3.5–3.9, 4.0–4.5 mm (African marigold) and 2.5–2.9, 3.0–3.4, 3.5–3.9, 4.0–4.4, 4.5–4.9, 5.0–5.5 mm (French marigold) were excised from each genotype. To determine the microspore stage, anthers were squashed in 1% aceto-carmine stain and the stage of microspores was checked under light microscope 'Nikon, ECLIPSE 50*i*model' at 400×. Microspore developmental stage of all the five anthers of a given floret length were examined. Based on the observations, suitable floret size group having anthers with the highest proportion of uni-nucleate microspores were determined in all the cultivars. We have also established an association between the capitulum size and floret size containing the highest proportion of microspores at early uni-nucleate to late uni-nucleate stages. For this, capitulum of different sizes 1.5, 2.0, 2.5, 3.0 cm (African marigold), 0.5, 1.0, 1.2, 1.5 cm (French marigold) were examined for the availability of suitable florets having early uni-nucleate to late uni-nucleate microspores in their anthers and the mean values calculated from ten buds.

Flower bud sterilization, anther excision and inoculation

The flower buds/capitulum of suitable size were collected from the healthy and disease-free plants and washed under running tap water for 10 min. The buds were treated in a solution containing 0.2% carbendazim (Biostadt, India), 200 mg l^{-1} 8-hydroxyquinoline citrate (8-HQC; Himedia, India) and 0.1% teepol for 15 min followed by thorough washings with distilled water. The buds were then sterilized with 0.1% mercuric chloride ($HgCl_2$; Himedia, India) for 4 min and finally rinsed in sterile double distilled water three times to remove the residue of $HgCl_2$.

Suitable lengths of florets, having anthers of uni-nucleate stage microspores were excised and 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 and immediately placed in Petri dishes (90 mm) containing 25 mL of solidified medium (30 anthers/each Petri dish).

In vitro androgenesis response of different African and French marigold cultivars

For studying the in vitro androgenic response, the anthers of African marigold cultivars were cultured on pre-standardized EMS medium from our laboratory (Modified MS based medium with organic supplements; Supplementary Table 2; Online Resource 1) supplemented with 0.5 mg l^{-1} BAP (Himedia, India), 0.25 mg l^{-1} NAA (Himedia, India), 45 g l^{-1} sucrose (Himedia, India). The anthers of French marigold cultivars were cultured on EMS medium supplemented with 2.0 mg l^{-1} BAP and 0.5 mg l^{-1} NAA and 45 g l^{-1} sucrose. The pH of media was adjusted to 5.8 and 2.5 g l^{-1} gelrite (Himedia, India) was added prior to their sterilization by autoclaving at 121 °C for 20 min (15 lbs/inch^2). All the thermolabile components were added to media after filter sterilization using 0.2 µm membrane filter (Merck, Millipore). The cultures were kept under darkness at 25 ± 1 °C till the emergence of shoots and then shifted to light. Based on the genotypic response study, the most responsive cultivar was utilized to optimize the various factors affecting the in vitro androgenesis in marigold. We have replicated all the in vitro experiments three times during the year 2014–2016 and each replicate had five petridish, containing 25–35 anthers each.

Optimization of basal media for direct shoot induction from anthers

Anthers of marigold genotype 'Pusa Arpita' were excised from 10 days cold pre-treated flower buds and cultured on different basal mediums. Five different commercially available basal medium, referred as MS medium (Murashige and Skoog 1962), N6 medium (Chu et al. 1975), B5 medium (Gamborg et al. 1968), Nitsch medium (Nitsch and Nitsch 1969) and NLN medium (Lichter 1982) were tested along with four newly modified basal media viz., Modified MS medium (MMS_1), Modified MS + 5% coconut water (EMS_1), Modified MS + 10% coconut water (EMS_2) and MS medium + 5% coconut water (EMS_3) were tested along with control i.e. devoid of any basal media. The composition of four modified basal media is shown in the file (Online Resource 1; Supplementary Table 2). All the basal media were supplemented with 2.0 mg l^{-1} BAP and 0.5 mg l^{-1} NAA with 45 g l^{-1} sucrose and solidified with 2.6 g l^{-1} gelrite. For NLN medium a sucrose concentration of 130 g l^{-1} was used. All the cultures were incubated in dark till the emergence of shoots/embryos and later shifted to 16/8 h light and dark cycles.

Optimization of sucrose concentration for direct embryo induction from anthers

In order to determine the optimum quantity of sucrose on androgenesis, the anthers of Pusa Arpita were cultured on solid EMS medium supplemented with 2.0 mg l^{-1} BAP + 0.5 mg l^{-1} NAA with different sucrose concentrations viz., 30, 45, 60, 75, 90, 105 and 120 g l^{-1}. All the cultures were incubated in dark till the emergence of shoot buds/embryos and later shifted to 16/8 h light and dark cycles. All the treatments had three replications with five petridish per replication.

Role of plant growth regulators for direct embryo/shoot bud induction from anthers

In order to standardize the growth regulators for direct regeneration of embryos from anthers, varieties *viz*., 'Pusa Basanti Gainda' from African type and 'Pusa Arpita' from French type were selected. Using EMS as the basic culture medium, twelve different induction media with three levels of BAP (0.5, 1.0 and 2.0 mg l^{-1}) in combination with three levels of NAA (0.25, 0.5 and 1.0 mg l^{-1}), two levels of TDZ (0.5 and 1.0 mg l^{-1}) were tested. All the induction media contained 45 g l^{-1} sucrose and 2.5 g l^{-1} gelrite.

Optimising cold pre-treatments for in vitro androgenesis

Pusa Arpita was chosen for the cold pre-treatment test. After sterilization, the flower buds were wrapped with moistened filter paper, sealed with polythene film of 100 gauge and then kept at 4 °C under dark conditions for 0, 2, 4, 6, 8, 10, 12, 14 and 16 days before culture. After cold pretreatment, anthers were excised and inoculated on solid EMS medium supplemented with 2.0 mg l^{-1} BAP, 0.5 mg l^{-1} NAA and 45 g l^{-1} sucrose.

Optimising the light conditions for in vitro androgenesis

Anthers of the Pusa Arpita were inoculated in solidified EMS medium supplemented with 45 g l^{-1} sucrose, 2.0 mg l^{-1} BAP, 0.5 mg l^{-1} NAA and the cultures were kept

in four different dark incubation durations viz., direct exposure of anthers to light, 2 days dark incubation followed by light, 7 days dark incubation followed by light and 20 days dark incubation followed by light exposure.

Shoot regeneration, rooting and hardening

Shoots/embryos were maintained and rooted on solid basal MS medium supplemented with 2.5 mg l^{-1} silver nitrate ($AgNO_3$; Himedia, India) and 30 g l^{-1} sucrose and 2.5 g l^{-1} gelrite. The culture were maintained at 25 ± 1 °C under fluorescent white light (47 µmol m^{-2} s^{-1}) at a photoperiod of 16:8 h light and dark cycle. The rooted plants were gradually acclimatized in glass jars (with polypropylene caps) containing sterile peat:perlite and vermiculite mixture (1:1:1) under controlled conditions.

Determination of the ploidy levels of anther regenerated plants of marigold

To determine the ploidy levels, 56 regenerants were randomly selected from total 424 anther derived plants in the cultivar Pusa Arpita based on the visible morphological differences. The ploidy level of each plant was analyzed by using chloroplast counting in stomatal guard cells (Indirect method), counting the chromosome number of root tip cells according to the method suggested by Zhang et al. (2011) and final confirmation was done by flow cytometry (FCM) as per the protocol described by Bhatia et al. (2016) (Direct methods). Nuclear DNA content was determined by using BDFACS Canto II (BD Bio-sciences, San Jose, CA, USA) flow cytometer with a 488 nm laser and fitted with a high throughput sampler (HTS). The ploidy analysis was done by using the tetraploid (2n = 4x = 48) Pusa Arpita donor plant as control against anther regenerants.

Statistical analysis

Completely randomized design (CRD) was followed and 't' test was arranged with different number of replications and Petri dishes/replications (P = 0.05). We have replicated the all the in vitro experiments three times during the year 2014–2016 with each replicate represented five petridish containing 25–35 anthers each. For studying the each individual factor, several times the experiments were conducted and determined the best factor. After 60 days of culture initiation and thereafter, direct adventitious shoot bud and embryo induction were counted and recorded. The frequency of shoot bud induction was calculated as the number of anthers produced adventitious shoot buds/embryos per 100 anthers cultured and the data were analysed using analysis of variance (ANOVA) with SAS 9.3 for windows statistical package (SAS Institute 1999). Significant differences between means were assessed by Duncan multiple range test by least significant difference (LSD) at P = 0.05 for CRD.

Results and discussion

Association between flower bud/floret size and developmental stage of microspores

The floret size, flower bud size and genotype determined the microspore developmental stage (Tables 1, 2). The cytological studies in marigold revealed six different developmental stages of the pollen grains viz., pre-tetrad, tetrad, early-uninucleate, late-uninucleate, early-binucleate and mature pollen (Fig. 1h–q). Differences were observed within and in-between the cultivars of African and French groups. In *T. patula* cultivars, the youngest florets located in the centre of the capitulum having < 3.5 mm length had maximum percent of pre-tetrad and tetrad stages of microspores in their anthers except in the genotype selection Fr. W-20 in which microspores are in pre-tetrad and tetrad stages up to 4.0 mm in floret length. The microspores at early-uninucleate to early-binucleate stage are considered to be the most responsive for induction of haploids. In case of French marigold cultivars, the florets of 3.5–4.0 mm sizes exhibited a predominance of this stage (Fig. 1c–o). Further, an indirect association between flower bud size and stage of microspore development was established by measuring the number of florets containing the ideal microspore developmental stage (Online Resource 1; Supplementary Table 3). The maximum number of florets having microspores at early-uninucleate to early binucleate stages were recorded with flower bud sizes of 1.2 cm. (Fig. 1a, b) except in selection Fr./W-20, in which flower buds of 1.5 cm length having maximum number of florets. It was also observed that the florets from unopened flower buds contained the microspores at early uninucleate to early binucleate stage.

In *T. erecta* cultivars, Pusa Basanti Gainda Pusa Narangi Gainda and IIHR-Mys-3, florets of 3.0–3.5 mm length contained the highest percent of microspores at early-uninucleate to early-binucleate stage. The other two cultivars, IIHR-Mys-3 (3.0–3.5 mm), Pusa Bahar and Sel/Af.W-1 (3.5-4.0 mm) exhibited significant variation (Table 2). The bud length of 2.5 cm contained the highest number of florets with early-uninucleate to early binucleate microspores (Online

Table 1 Ratio of microspore developmental stages in different French marigold cultivars with respect to different floret sizes

Floret size (mm)	Microspore developmental stages (%)					
	Pre tetrad stages	Tetrad stage	Early-uninucleate stage	Late-uninucleate stage	Early-binucleate stage	Binucleate stage
Dainty Marietta						
2.5–3.0	83.3 ± 3.1	16.7 ± 3.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
3.0–3.5	0.0 ± 0.0	66.66 ± 7.2	33.3 ± 7.2	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
3.5–4.0	0.0 ± 0.0	0.0 ± 0.0	45.8 ± 2.4	45.8 ± 2.4	8.3 ± 6.9	0.0 ± 0.0
4.0–4.5	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	29.2 ± 2.6	70.8 ± 2.6
4.5–5.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	12.5 ± 0.0	87.5 ± 0.0
5.0–5.5	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	100.00 ± 0.0
Pusa Arpita						
2.5–3.0	54.2 ± 2.4	45.8 ± 2.4	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
3.0–3.5	0.0 ± 0.0	20.8 ± 3.1	58.3 ± 2.4	20.8 ± 5.7	0.0 ± 0.0	0.0 ± 0.0
3.5–4.0	0.0 ± 0.0	0.0 ± 0.0	12.5 ± 8.9	58.3 ± 5.0	29.2 ± 2.6	0.0 ± 0.0
4.0–4.5	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	45.8 ± 2.4	54.2 ± 2.4
4.5–5.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	4.2 ± 6.9	95.8 ± 6.9
5.0–5.5	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	100.0 ± 0.0
Pusa Deep						
2.5–3.0	29.2 ± 2.6	70.8 ± 2.6	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
3.0–3.5	0.0 ± 0.0	25.0 ± 0.0	58.3 ± 2.4	16.7 ± 3.1	0.0 ± 0.0	0.0 ± 0.0
3.5–4.0	0.0 ± 0.0	0.0 ± 0.0	33.3 ± 2.6	50.0 ± 4.2	16.7 ± 3.1	0.0 ± 0.0
4.0–4.5	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	29.2 ± 2.6	70.8 ± 2.6
4.5–5.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	100.0 ± 0.0
5.0–5.5	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	100.0 ± 0.0
Selection Fr./W-20						
2.5–3.0	95.8 ± 6.9	4.2 ± 6.9	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
3.0–3.5	70.8 ± 2.6	29.2 ± 2.6	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
3.5–4.0	0.0 ± 0.0	70.8 ± 2.6	29.2 ± 2.6	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
4.0–4.5	0.0 ± 0.0	8.3 ± 6.9	75.0 ± 4.9	16.7 ± 3.1	0.0 ± 0.0	0.0 ± 0.0
4.5–5.0	0.0 ± 0.0	0.0 ± 0.0	33.3 ± 2.6	54.2 ± 2.4	12.5 ± 8.9	0.0 ± 0.0
5.0–5.5	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	41.7 ± 2.4	58.3 ± 2.4
Valencia						
2.5–3.0	58.3 ± 2.4	37.5 ± 4.3	4.2 ± 6.9	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
3.0–3.5	0.0 ± 0.0	66.7 ± 2.6	25.0 ± 0.0	8.3 ± 6.9	0.0 ± 0.0	0.0 ± 0.0
3.5–4.0	0.0 ± 0.0	0.0 ± 0.0	37.5 ± 4.3	37.5 ± 4.3	20.8 ± 3.1	4.2 ± 6.9
4.0–4.5	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	8.3 ± 6.9	25.0 ± 4.9	66.7 ± 2.6
4.5–5.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	4.2 ± 6.9	95.8 ± 6.9
5.0–5.5	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	100.0 ± 0.0

Resource 1; Supplementary Table 3). The half opened capitulum/flower buds contained the florets with microspores at ideal stage. Unopened and/or fully opened buds were not having any suitable stage of florets for anther culture.

At any given time, florets of varying maturity may exist in a single flower bud (Bal and Touraev 2009). The stage of microspore development at the time of anther excision and culture is the most critical factor for successful induction of pollen embryos or calli. In a vast majority of species successful induction of haploids have been achieved, when anthers were excised and cultured at uninucleate to early binucleate stage of microspores development (Touraev et al.

Table 2 Ratio of microspore developmental stages in different African marigold cultivars with respect to different floret sizes

Floret size (mm)	Microspore developmental stages (%)					
	Pre tetrad stages	Tetrad stage	Early-uninucleate stage	Late-uninucleate stage	Early-binucleate stage	Binucleate stage
Pusa Basanti Gainda						
1.5–2.0	70.8 ± 2.6	33.3 ± 2.6	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
2.0–2.5	0.0 ± 0.0	79.2 ± 3.1	20.8 ± 3.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
2.5–3.0	0.0 ± 0.0	16.7 ± 3.1	66.7 ± 2.6	16.7 ± 3.1	0.0 ± 0.0	0.0 ± 0.0
3.0–3.5	0.0 ± 0.0	0.0 ± 0.0	25.0 ± 0.0	45.8 ± 2.4	29.2 ± 2.6	0.0 ± 0.0
3.5–4.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	8.3 ± 6.9	91.6 ± 6.9
4.0–4.5	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	100.0 ± 0.0
Pusa Narangi Gainda						
1.5–2.0	62.5 ± 2.4	37.5 ± 2.4	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
2.0–2.5	0.0 ± 0.0	58.3 ± 2.4	29.2 ± 2.6	12.5 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
2.5–3.0	0.0 ± 0.0	8.3 ± 3.1	58.3 ± 2.4	33.3 ± 2.6	0.0 ± 0.0	0.0 ± 0.0
3.0–3.5	0.0 ± 0.0	0.0 ± 0.0	29.2 ± 2.6	41.7 ± 2.4	29.2 ± 2.6	0.0 ± 0.0
3.5–4.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	25.0 ± 4.9	75.0 ± 4.9
4.0–4.5	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	100.0 ± 0.0
Pusa Bahar						
1.5–2.0	83.3 ± 6.9	16.7 ± 6.9	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
2.0–2.5	0.0 ± 0.0	70.8 ± 5.7	25.0 ± 4.9	4.2 ± 6.9	0.0 ± 0.0	0.0 ± 0.0
2.5–3.0	0.0 ± 0.0	29.2 ± 2.6	58.3 ± 2.4	12.5 ± 8.9	0.0 ± 0.0	0.0 ± 0.0
3.0–3.5	0.0 ± 0.0	8.3 ± 6.9	29.2 ± 2.6	62.5 ± 4.3	0.0 ± 0.0	0.0 ± 0.0
3.5–4.0	0.0 ± 0.0	0.0 ± 0.0	16.7 ± 3.1	45.8 ± 2.4	37.5 ± 4.3	0.0 ± 0.0
4.0–4.5	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	16.7 ± 3.1	83.3 ± 3.1
Selection Af/W-1						
1.5–2.0	100.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
2.0–2.5	83.3 ± 3.1	16.7 ± 3.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
2.5–3.0	0.0 ± 0.0	91.7 ± 6.9	8.3 ± 6.9	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
3.0–3.5	0.0 ± 0.0	45.8 ± 2.4	45.8 ± 2.4	8.3 ± 6.9	0.0 ± 0.0	0.0 ± 0.0
3.5–4.0	0.0 ± 0.0	0.0 ± 0.0	54.2 ± 2.4	37.5 ± 0.0	8.3 ± 6.9	0.0 ± 0.0
4.0–4.5	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	37.5 ± 4.3	62.5 ± 4.3
IIHR-Mys-3						
1.5–2.0	83.3 ± 3.1	16.66 ± 3.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
2.0–2.5	0.0 ± 0.0	70.83 ± 2.6	20.8 ± 3.1	12.5 ± 8.9	0.0 ± 0.0	0.0 ± 0.0
2.5–3.0	0.0 ± 0.0	0.0 ± 0.0	45.8 ± 2.4	41.7 ± 2.4	12.5 ± 8.9	0.0 ± 0.0
3.0–3.5	0.0 ± 0.0	0.0 ± 0.0	8.3 ± 6.9	29.2 ± 2.6	41.7 ± 2.4	20.8 ± 5.7
3.5–4.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	8.3 ± 6.9	16.7 ± 3.1	75.0 ± 4.9
4.0–4.5	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	4.2 ± 6.9	95.8 ± 6.9

2001; Bhatia et al. 2016). The induction of higher ploidy level embryos were reported when late binucleate and advanced stages of pollen grain were cultured. After the deposition of starch and other storage reserves, they usually lose their embryogenic potentiality and follow the gametophytic pathway (Raghavan 1990). Similar observations have been recorded earlier in African marigold (Li 2007; Xue-Jiao et al. 2010) and in chamomile, sanvitalia and solidago (Bal and Touraev 2009). However, Qi et al. (2011) revealed that florets of 1.0–1.5 mm length contained uninucleate stage of

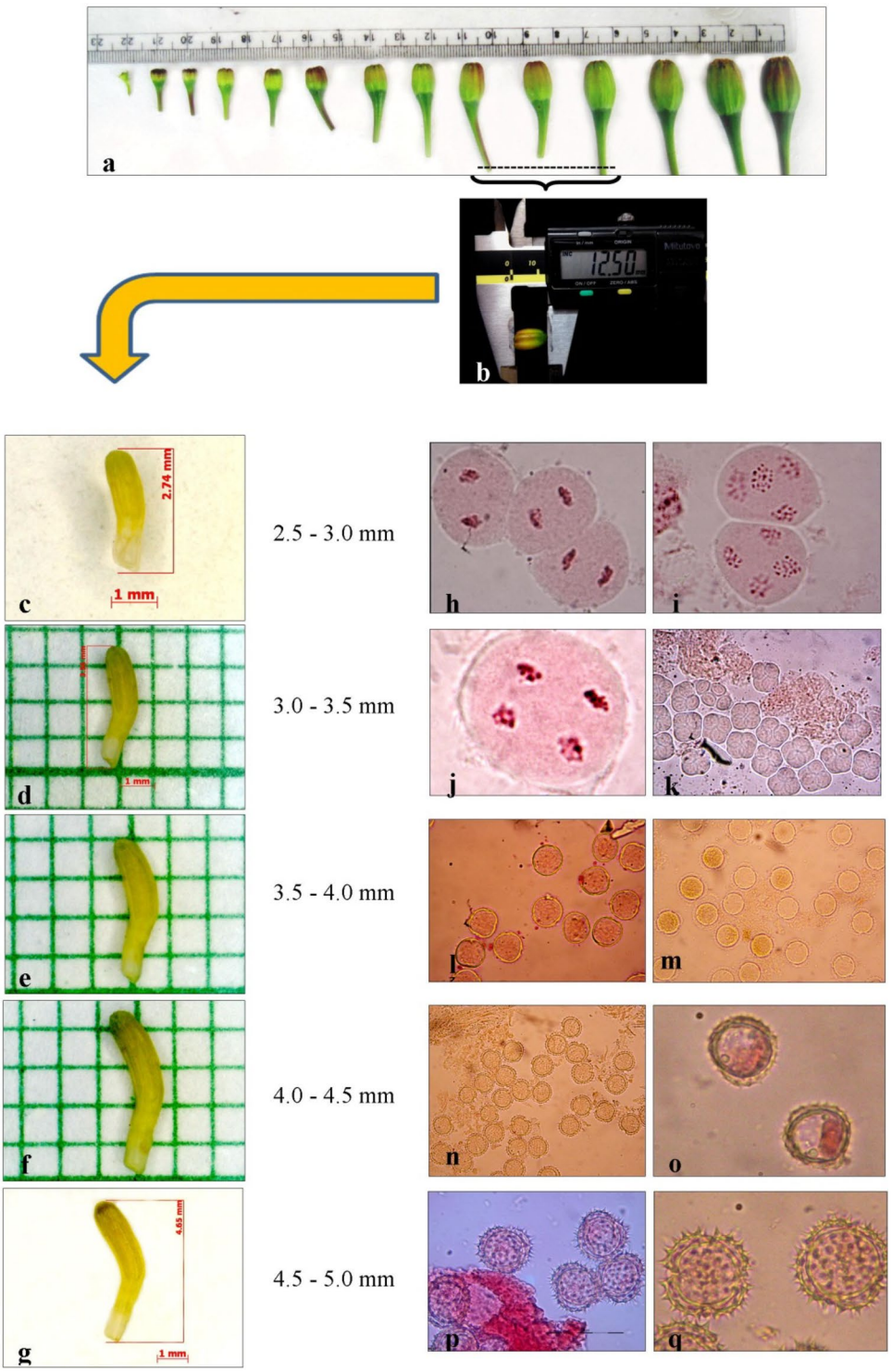

Fig. 1 Correlation of flower bud size, floret size and microspore development stage **a** *Tagetes patula* cv. Pusa Arpita flower bud developmental stages **b** measurement of flower bud length **c–g** different sizes of individual disc florets **h–j** pre-tetrad stages of microspores **k** tetrad stage **l** ealy-uninuncleate stage **m** late-uninucleate stage, **n, o** ealry-binucleate stage, **p, q** mature microspores (pollen grain)

microspores in French marigold. These differences in floret length might be due to the variation in genotype, season, soil nutritional status and external weather conditions.

Effect of genotype on androgenesis

Significant differences for in vitro androgenesis response was observed among different cultivars (Table 3). The

Table 3 In vitro androgenic response in different African and French marigold cultivars

Treatment	No. of anthers inoculated[1]	Caulogenesis[1] (%)	Days to shoot emergence[1]	Number of shoots per anther[1]
African marigold				
Pusa Basanti Gainda (PBG)	183	59.6 ± 1.3 (50.5)ab	23.2 ± 0.3c	2.3 ± 0.1b
Pusa Narangi Gainda (PNG)	183	35.7 ± 2.5 (36.6)dc	25.5 ± 0.2b	1.9 ± 0.1b
Pusa Bahar (PB)	173	0 ± 0 (0.0)e	0.0 ± 0.0 g	0.0 ± 0.0d
Selection Af./W-1	173	3.8 ± 13.1 (9.2)e	29.2 ± 9.7a	1.0 ± 0.0c
IIHR Mys-3	151	0 ± 0 (0.0)e	0.0 ± 0.0 g	0.00 ± 0.0d
French marigold				
Dainty Marietta	180	71.1 ± 7.6 (58.7)ab	17.1 ± 0.4f	4.5 ± 0.3a
Pusa Arpita	172	76.2 ± 6.9 (61.9)a	16.9 ± 0.4f	4.6 ± 0.2a
Pusa Deep	168	54.6 ± 3.6 (47.7)bc	18.0 ± 0.3ef	2.4 ± 0.3b
Selection Fr /W-20	170	40.5 ± 4.7 (39.4)dc	19.1 ± 0.7de	1.2 ± 0.2c
Valencia	168	31.7 ± 6.1 (33.8)d	19.8 ± 0.8d	1.1 ± 0.1c

Figures given in parentheses are angular transformed values

[1]Data provided as mean ± SE, and those followed by different letters are significantly different at 95% level by Duncan's test

direct shoot bud induction was significantly higher in French marigold cultivars as compared to the African marigold lines. Among the French cultivars, Pusa Arpita was the most responsive line which induced the highest shoot buds (76.2%) with the maximum number of shoots per anther (4.6). In African marigold cultivars, the maximum shoot bud induction (59.6%) with in the shortest period of 23.2 days was observed in Pusa Basanti Gainda. Three African marigold cultivars namely Sel. Af. W-1, Pusa Bahar and IIHR-Mys-3 failed to induce direct shoots from the cultured anthers. The in vitro androgenesis response is highly genotype and different lines or varieties within a species exhibit diverse responses in anther culture (Germana 2011). This study also revealed the higher androgenic response of French marigold cultivars over the African marigold cultivars. These results corroborates the earlier findings of Qi et al. (2011) in French marigold, Zhong et al. (1995) in sunflower, Gao et al. (2011) and Khandakar et al. (2014) in chrysanthemum. The genotypic response is controlled by an interaction between cytoplasmic and nuclear genes and modified by the environmental conditions.

Effect of basal medium on androgenesis

Among the different basal media used, significantly highest response (65.9%), direct differentiation of shoot buds (71.6%) with maximum number of shoots per anther (3.8) was recorded in EMS_1 media (modified MS basal + 5% coconut water) (Table 4). Direct differentiation of shoot buds from anthers were only recorded in EMS_1 media, whereas anthers cultured on all other commercially available basal media produced different types of callus and drying of anthers were recorded in N6, Nitsch and NLN-13 media. Different combinations of modified MS basal media, MS media with 5% and 10% coconut water were tested to reveal the favourable effects of the organic additives and combination effects. The results revealed that, marigold anthers cultured in modified MS basal media with 5% coconut water (EMS_1) had significantly highest androgenic capacity over other media tested. Additional supplementation of antioxidants, amino acids and poly-amines also played a key role in enhancing the direct embryogenic potential of marigold anthers in the present study. The composition of the basal medium serves as one of the crucial factor in eliciting successful haploid induction in anther culture. The requirement varies from genotype to genotype Khandakar et al. (2014). It was also reported that in addition to macro, micro nutrients, vitamins and organics in basal medium, supplementation of other substances, such as anti-oxidants, glutamine, proline, casein, biotin, inositol, coconut water, silver nitrate and polyvinylpyrrolidone significantly improved the haploid induction in many crops (Achar 2002). The enhanced embryogenic potential of recalcitrant marigold anthers were mainly attributed to the additional supplements like coconut water, silver nitrate, polyvinylpyrrolidone (PVP), amino acids, anti-oxidants and poly amines like putrescine. Earlier researchers (Jackson et al. 2004; Gopikrishna et al. 2008) have revealed the benefits of coconut water and found that it was rich in amino acids (cystine, lysine, histidine, methionine and other essential one), vitamins, minerals (potassium, calcium and magnesium) and sugars.

Table 4 Effect of different basal media on anther response, caulogenesis, days to shoot emergence and number of shoots per anther

Treatment	No. of anthers inoculated	Caulogenesis[1] (%)	Days to shoot emergence[1]	Number of shoots per anther[1]	Type of callus produced
Control (without any basal medium) (M0)	171	0.00 ± 0.0e (0.00)	0.00 ± 0.0e	0.00 ± 0.0f	No callus
MS medium (M1)	167	16.6 ± 10.4d (19.8)	40.2 ± 1.2a	2.20 ± 0.1c	Pale green colour callus
N6 medium (M2)	166	0.00 ± 0.0e (0.0)	0.00 ± 0.0e	0.00 ± 0.0f	Produced white non embryogenic callus
B5 medium (M3)	170	8.8 ± 7.2de (14.2)	37.5 ± 0.6b	1.16 ± 0.2e	Early phenolic exudation and turning of green callus to brown
Nitsch medium (M4)	175	0.00 ± 0.0e (0.0)	0.00 ± 0.0e	0.00 ± 0.0f	Yellow coloured callus
NLN-13 medium (M5)	167	0.00 ± 0.0e (0.0)	0.00 ± 0.0e	0.00 ± 0.0f	Early death of anthers
Modified MS medium (MMS_1)	175	22.7 ± 4.2dc (28.0)	39.0 ± 0.8ab	1.70 ± 0.2d	Yellowish green callus
Modified MS + 5% coconut water (EMS_1)	179	71.6 ± 3.5a (58.0)	17.2 ± 0.4d	3.81 ± 0.3a	Direct regeneration
Modified MS + 10% coconut water(EMS_2)	163	47.3 ± 3.0b (43.4)	17.6 ± 0.9d	3.76 ± 0.2a	Observed somatic embryo like structures on green callus
MS + 5% coconut water(EMS_3)	166	32.8 ± 4.8bc (34.6)	25.2 ± 1.2c	2.66 ± 0.2b	Greenish callus

Figures given in parentheses are angular transformed values

[1]Data provided as mean ± SE, and those followed by different letters are significantly different at 95% level by Duncan's test

Effects of sucrose concentration on direct embryo induction from anthers

The direct shoot bud induction rate from culture anthers of 'Pusa Arpita' varied with the concentration of sucrose in culture media (Table 5). Among the different sucrose concentrations tested, the best androgenic response with the maximum number of shoots per anther (3.8) was recorded with 45 g l^{-1} sucrose. Increasing sucrose concentration beyond 45 g l^{-1} up to 105 g l^{-1} had detrimental effects on shoot bud embryogenesis. The anthers cultured on media with higher sucrose level showed delayed shoot bud differentiation with significantly reduced embryogenic potential. These observations were in close conformity with Qi et al. (2011), where they found maximum callus induction and regeneration was obtained with 30 g l^{-1} sucrose and anthers failed to regenerate at 8, 9 and 13% sucrose concentration. Gao et al. (2011) obtained the highest callus induction at 9% sucrose concentration in chrysanthemum, whereas, Khandakar et al. (2014) obtained highest callus induction and regeneration with 4.5% sucrose concentration in chrysanthemum anther culture. The higher sucrose concentration can lead to beneficial morphogenic potential by suppressing the proliferation of somatic tissues Agarwal et al. (2006).

Table 5 Effect of sucrose concentration on anther response, caulogenesis, days to shoot emergence and number of shoots per anther

Treatment (g l^{-1})	No. of anthers inoculated	Caulogenesis[1] (%)	Days to shoot emergence[1]	Number of shoots per anther[1]
30	173	64.2 ± 6.2 (53.3)ab	18.4 ± 0.6e	3.8 ± 0.5a
45	170	70.4 ± 14.7 (57.5)a	18.3 ± 0.7e	3.8 ± 0.2a
60	171	52.6 ± 15.7 (46.5)ab	21.3 ± 1.8d	2.1 ± 0.2b
75	165	49.6 ± 18.8 (44.9)ab	28.41 ± 2.0c	1.2 ± 0.3c
90	176	34.4 ± 15.0 (36.0)bc	41.5 ± 1.8b	1.0 ± 0.0c
105	168	16.7 ± 15.0 (15.0)cd	48.0 ± 0.2a	0.3 ± 0.6d
120	171	0.0 ± 0.0 (0.0)d	0.0 ± 0.0f	0.0 ± 0.0d

Figures given in parentheses are angular transformed values

[1]Data provided as mean ± SE, and those followed by different letters are significantly different at 95% level by Duncan's test

Role of plant growth regulators on direct differentiation of shoot buds

The response of anthers culture on twelve different growth regulator combinations varied among both African and French marigold cultivars (Table 6). The anthers inoculated in EMS_1 medium containing no plant growth regulator has shown least response in PA (6.5%) and no response in PBG, the anthers gradually became necrotic and dried (Fig. 2d). The anthers of PA and PBG cultured on EMS_1 media supplemented with TDZ failed to induce shoot buds. Bulging of the cultured anthers became evident within a week, later on gradual turning of anther wall to brown colour from yellowish green colour, surface cracked from the middle and direct formation of embryo and/or adventitious shoot bud induction was recorded in suitable culture media (Fig. 2e, f). Among the different growth regulator combinations, the highest embryogenesis/direct differentiation of shoot buds (78.6%) were recorded in PA anthers cultured in media No. 7 (2.0 mg l^{-1} BAP and 0.5 mg l^{-1} NAA) within a short period (17.1 days) (Fig. 2a–c). The micro-shoots emerged from anthers of Pusa Arpita has shown green and purple colour pigmentation and it was clearly indicating the microspore origin (Fig. 2j–l). Whereas, in *T. erecta* cv. Pusa Basanti Gainda, the cultured anthers had shown highest direct differentiation of shoot buds (63.9%) in media No. 2 (0.5 mg l^{-1} BAP and 0.25 mg l^{-1} NAA) after 22.6 days of culture initiation. The maximum number of shoot bud induction (4.6) per anther was recorded in PA anthers cultured in media No.7 (Fig. 2g–i).

The role of growth regulators on androgenesis is widely discussed in many crop species. The presence of growth regulators mostly auxins and cytokinin is essential for microspore derived embryo production, particularly in recalcitrant plant species. The auxin type and concentration either singly or in combination with cytokinin determines the pathway of microspore development (Ball et al. 1993). Earlier, Qi et al. (2011) reported two step process of callus induction and regeneration in French marigold anthers cultured on MS basal medium supplemented with 0.5 mg l^{-1} BA + 0.5 mg l^{-1} NAA + 45 g l^{-1} sucrose, but the ploidy of all the regenerated shoots was similar to donor parent and indicating somatic tissue origin. Poor callus induction and regeneration was also recorded in the two step process of our earlier studies (Ravindra et al. 2018). Both the cultivars showed specific requirement for a growth regulator combination. These results are first of its kind in recalcitrant Asteraceous ornamental crops.

Table 6 Effect of different plant growth regulator combinations on anther response, caulogenesis, days to shoot emergence and number of shoots per anther

S. No.	Treatment (mg l^{-1})			No. of anthers inoculated[1]		Caulogenesis[1] (%)			Days to shoot emergence[1]			Number of shoots per anther[1]		
	BAP	NAA	TDZ	PA	PBG	PA	PBG	Mean	PA	PBG	Mean	PA	PBG	Mean
1	0	0	0	76	183	0 (0.0)d	0 (0.00)d	0 (0.00)e	0j	0j	0f	0f	0f	0f
2	0.5	0.25	0	81	192	16.4 (23.6) bcd	63.9 (53.1)a	40.2 (38.3)b	24.2de	22.6ef	23.4 cd	1de	2.3bc	1.7bc
3	1	0.25	0	73	177	11.7 (19.7)cd	5.8 (11.2)d	8.7 (15.5)cde	22.5efg	27.6bc	25.0b	1.4d	1de	1.2cde
4	2	0.25	0	74	175	27.2 (31.4)bc	3.6 (8.9)d	15.4 (20.2)cd	18.8hi	28.8ab	23.8 bc	1.7dc	de	1.4bcd
5	0.5	0.5	0	81	195	3.4 (8.7)d	16.9 (24.2)bcd	10.2 (16.4)cde	24.5de	25.6dc	25.0b	1.1de	1.0de	1.1de
6	1	0.5	0	77	179	30.7 (32.9)b	7.1 (15.3)d	18.9 (24.1)c	20.5fgh	29.0ab	24.8bc	1.1de	1.2d	1.2cde
7	2	0.5	0	81	182	78.6 (62.7)a	26.9 (31.1)bc	52.8 (46.9)a	17.1i	24.2de	20.7e	4.6a	1.4d	3.0a
8	0.5	1	0	76	179	7.4 (15.5)d	1.7 (4.3)d	4.6 (9.9)de	24.2de	30.5a	27.3a	1de	0.3ef	0.7e
9	1	1	0	72	179	60.7 (51.2)a	17.6 (24.5)bcd	39.2 (37.9)b	18.3hi	25.9dc	22.1de	2.6b	1.0de	1.8b
10	2	1	0	75	180	15.3 (22.8)	63.7 (52.9)a	39.5 (37.9)b	20.2hg	22.8ef	21.5e	1.4d	2.2bc	1.8b
11	0	0	0.5	71	174	0 (0.00)d	0 (0.00)d	0 (0.00)e	0j	0j	0f	0f	0f	0f
12	0	0	1.00	71	179	0 (0.00)d	0 (0.00)d	0 (0.00)e	0j	0j	0f	0f	0f	0f
	Mean					20.94a	17.27b		15.8b	19.7a		1.3a	0.96b	

Figures given in parentheses are angular transformed values

[1]Data provided as mean ± SE, and those followed by different letters are significantly different at 95% level by Duncan's test

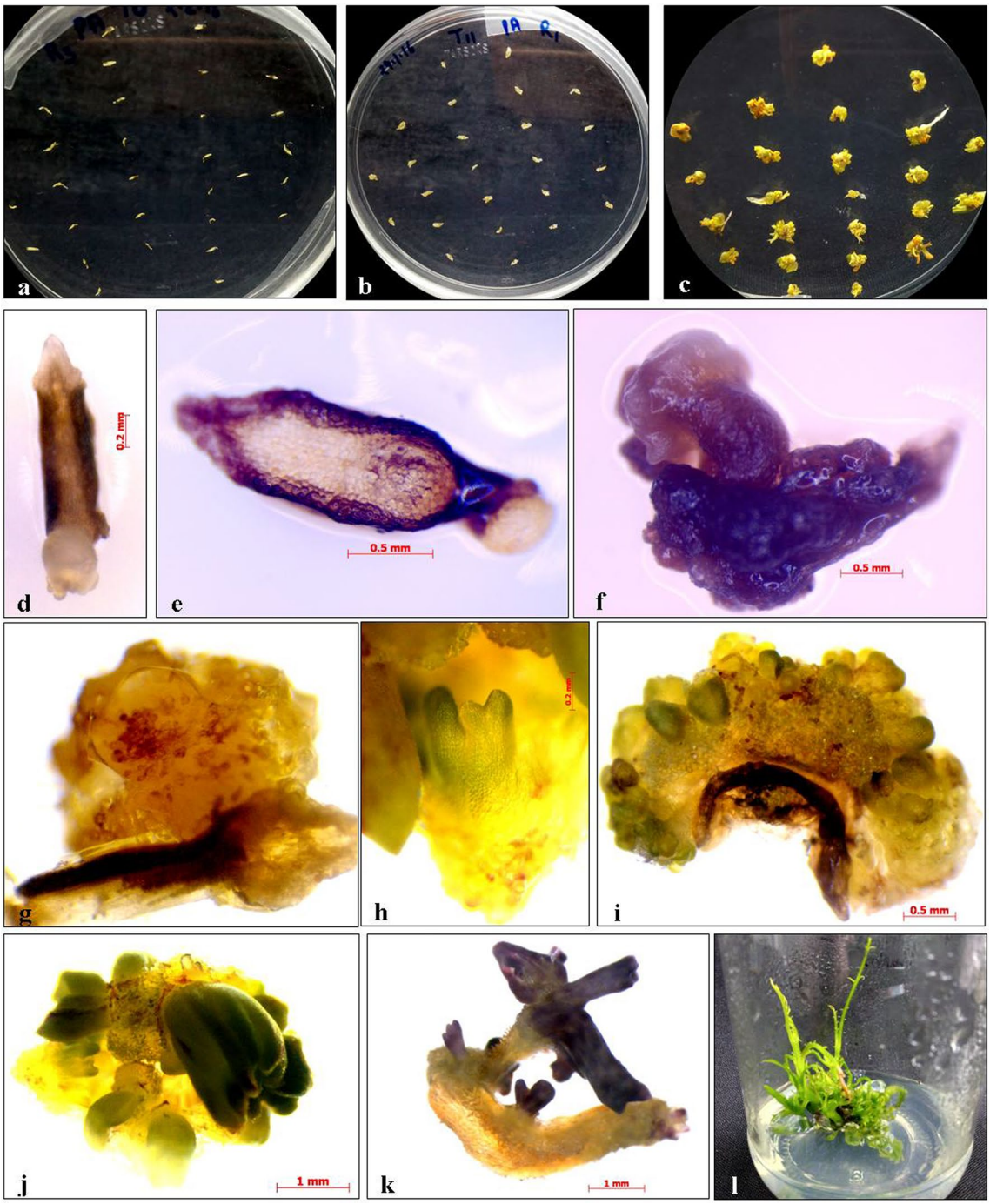

Fig. 2 Direct differentiation of shoot bud/embryo from anthers of *Tagetes patula* cv. Pusa Arpita. **a** the culture initiation day 1, **b** 7 days and **c** 25 days after anther inoculation in EMS media supplemented with 2 mg/l BAP + 0.5 mg/l NAA + 45 g/l sucrose and incubated in 20 days dark. Microscopic observations of anther development **d** non responsive anther **e** bulging of anther, vertical crack and emerging embryo/embryogenic callus from the basal end, **f** embryo derived from anther, **g** embryogenic callus emerging from anther, **h** formation of cotyledonary embryos, **i** globular embryos emerging from embryogenic callus and degradation of anther wall, **j**, **k** germinating embryo and formation of shoots with green and purple pigmentation, **l** formation of well developed shoots from anther

Table 7 Effects of low temperature pre-treatment at 4 °C on anther response, caulogenesis, days to shoot emergence and number of shoots per anther

Treatment (days)	No. of anthers inoculated	Caulogenesis[1] (%)	Days to shoot emergence[1]	Number of shoots per anther[1]
0	173	25.8 ± 3.7 (30.3)d	18.7 ± 1.2a	2.4 ± 0.2bcd
2	173	37.5 ± 8.2 (37.2)dc	19.3 ± 0.3a	1.3 ± 0.3d
4	168	29.7 ± 0.8 (33.0)d	18.4 ± 0.6a	1.5 ± 0.3dc
6	167	49.4 ± 3.1 (44.7)bc	18.5 ± 1.3a	2.2 ± 0.2bcd
8	176	56.8 ± 1.1 (48.9)ab	19.3 ± 0.7a	2.4 ± 0.4bcd
10	175	68.4 ± 2.1 (55.8)a	19.8 ± 0.6a	3.7 ± 0.2a
12	174	55.8 ± 1.0 (48.3)abc	19.2 ± 0.4a	2.9 ± 0.2ab
14	173	59.4 ± 4.8 (50.5)ab	19.6 ± 0.9a	2.6 ± 0.8abc
16	183	61.0 ± 3.2 (51.4)ab	19.5 ± 0.3a	2.3 ± 0.6bcd

Figures given in parentheses are angular transformed values

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Effects of cold pre-treatments for in vitro androgenesis in marigold

The cold pre-treatment of excised flower buds at 4 °C had significant influence on direct differentiation of shoot buds from anthers (Table 7). Among the different pre-treatments flower buds exposed to 10 days cold treatment recorded the highest direct differentiation of shoot buds (68.4%) over all other treatments. However, the results were non-significant for days to shoot bud induction. Among the exogenous factors, the stress inducing physical or chemical pre-culture treatment are very crucial for DH production from the male gametophyte since it directs the microspore to change its initial pollen developmental program to the embryo development program (Jacquard et al. 2009). In the present investigation, cold pre-treatment was given to the excised flower buds at 4 °C before culturing the anthers. Pre-treatment significantly enhanced the regeneration capacity of anthers. The maximum regeneration capacity was recorded in 10 days pre-treated anthers. Similar to these observations, Qi et al. (2011) also reported the improvement in embryogenic capacity of anthers with cold pre-treatment at 4 °C for 4 days. Recently, Bhatia et al. (2017) have demonstrated the role of cold pre-treatments in different cauliflower cultivars. In many crops cold pre-treatment at 4 °C for 2–3 weeks has been recommended for successful haploid induction and its effect was also genotype dependent (Osolnik et al. 1993).

It has been suggested that cold pre-treatment may cause re-orientation of spindle axis, which results in the production of two similar nuclei at the first pollen mitosis (Nitsch and Norreel 1973) or by increasing the accumulation of inducible microspores (Wenzel et al. 1977) or by delaying the anther senescence, thus ensuring the survival of induced microspores for longer duration. According to Vasil and Nitsch (1975) cold pre-treatment results in a general reduction in the metabolic activity within the anther, thus making it possible for the accumulation of a large percentage of microspores at the required developmental stage.

Optimization of light conditions for in vitro androgenesis

Light conditions played a significant role in eliciting in vitro androgenic response in marigold genotype 'Pusa Arpita' (Table 8). Among the different treatments, significantly highest direct differentiation (76.2%) of shoot buds with

Table 8 Effect of duration of light exposure on anther response, caulogenesis, days to shoot emergence and number of shoots per anther

Treatment (g l^{-1})	No. of anthers inoculated	Caulogenesis[1] (%)	Days to shoot emergence[1]	Number of shoots per anther[1]
Direct exposure to light	174	16.1 ± 1.5 (23.6)b	26.6 ± 0.8a	1.1 ± 0.1b
2 days dark followed by light	167	18.7 ± 2.9 (25.3)b	26.3 ± 0.9a	1.2 ± 0.1b
7 days dark followed by light	166	19.8 ± 1.4 (26.9)b	25.9 ± 0.4a	1.4 ± 0.1b
20 days dark followed by light	171	76.2 ± 0.9 (60.5)a	18.2 ± 0.3b	3.1 ± 0.2a

Figures given in parentheses are angular transformed values

[1]Data provided as mean ± SE, and those followed by different letters are significantly different at 95% level by Duncan's test

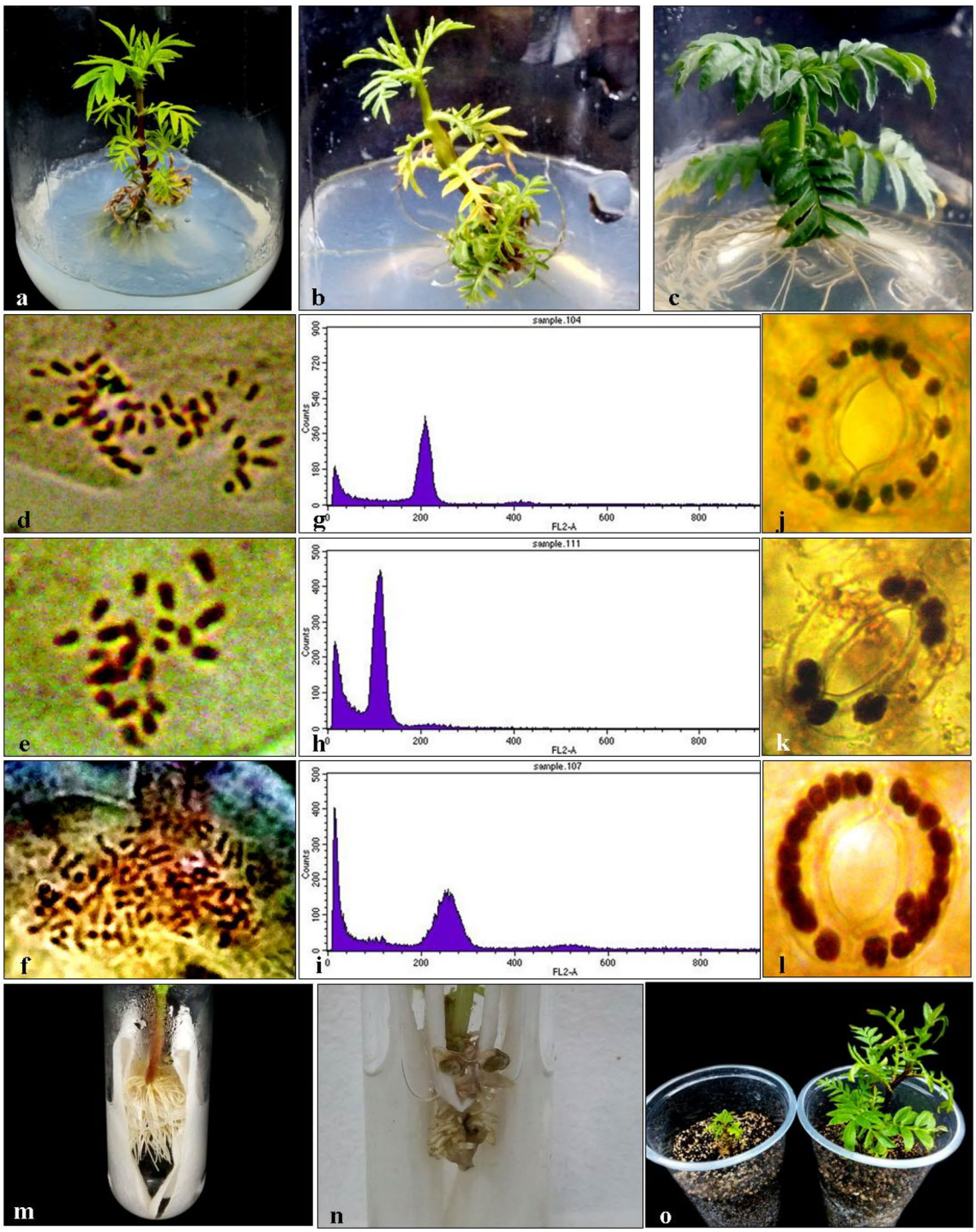

Fig. 3 Comparison of regenerated plantlets with different ploidy level in culture (**a**–**c**), determination of their ploidy levels by chromosome counts (**d**–**f**), flow cytometry histograms (**g**–**i**), chloroplast number in stomatal guard cells (**j**–**l**), morphological differences in rooting (**m, n**) and hardening (**o**). **a**, **d**, **g**, **j**, **m** Pusa Arpita donor tetraploid plant, 2n = 4x = 48; **b**, **e h**, **k**, **n** dihaploid plant, 2n = 2x = 24; **c, f, I, o** polyp loid plant; rooting of donor plant (**m**) and dihaploid (**n**); dihaploid (left) and donor plant (right) in hardening (**o**)

maximum number of shoots per anther (3.1) recorded with 20 days dark incubation. Anthers incubated in light, 2 and 7 days dark period produced green calli with reduced shoot bud induction and took more days for shoot regeneration. In general dark incubation seemed to be the best for anther culture in many species. Similarly, in the present investigation, it was observed that initial 20 days dark incubation followed by exposing to photoperiod light cycle gave maximum

Table 9 Cytological and flowcytometry analysis for determination of ploidy level in anther culture derived regenerants of *Tagetes patula* cv. Pusa Arpita

Technique	No. of plants screened for ploidy analysis	Ploidy level		
		Dihaploid (2x)	Tetraploid (4x)	Polyploids (4x–6x)
Chromosomal count in root tip cells	56	8 (14.3%)	37 (66.1%)	11 (19.6%)
Flowcytometry analysis	56	8 (14.3%)	37(66.1%)	11 (19.6%)

caulogenesis of anthers as compared to direct incubation in light. The detrimental effect of light on anther cultures of *Hordeum vulgare* has earlier reported by Xu (1990). These results are contradictory to earlier finding of Qi et al. (2011) where no significant differences were observe on the regeneration capacity of the French marigold anthers when incubated under light and dark conditions. The genotypic and environmental effects might be the reason for these varied results.

Ploidy determination of anther regenerated plants

Cytological and flow cytometry analysis

For ploidy analysis, 56 anther regenerated plants of Pusa Arpita were randomly selected from a population 424 plants. Among the 56 plants tested, 37 plants had a chromosome number of 48 (Fig. 3d) similar to their tetraploid donor mother plant (2n = 4x = 48), 8 plants had a chromosome number of 24 (Fig. 3e) and were dihaploid (2n = 2x = 24), 3 and 11 plants were found to be polyploids having more than 48 chromosomes (Fig. 3f). These cytological results were reconfirmed by conducting flow cytometry analysis by using Pusa Arpita as a tetraploid standard (Table 9) (Fig. 3g–i). The morphological characters of anther regenerated plants of different ploidy levels were observed visually. The Dihaploid plants were found to be dwarf, less vigorous and slow growing. They showed poor in vitro root induction and morphological abnormalities like angular growth of stems (Fig. 3b, n, o) compared with the tetraploid donor plant (Fig. 3a, m, o). However, the polyploid plants were highly vigorous and had strong plant growth (Fig. 3c).

The present results are in agreement with Winarto et al. (2010) in anthurium, Gao et al. (2011); Khandakar et al. (2014) in chrysanthemum, Hoveida et al. (2017) in borage. The complex reasons involved in the induction of different frequencies of haploids, DH s, mixoploids, endopolyploids and aneuploids. These frequencies could be influenced by factors like genotypic differences in source materials, microspore/female gametophyte developmental stage, anther or ovule pre-treatments, media composition, plant growth regulators, culture duration and culture environmental conditions (Kim et al. 2003). Flow cytometry is a direct ploidy assessment method and is more efficient, rapid and easy for estimation of DNA quantity in cell nuclei using specific dyes. It is the attractive option for ploidy analysis owing to its reliability, simplicity of sample preparation and ability to measure DNA content quickly in a large number of cells. Several researchers have utilised flow cytometry for screening of large-scale anther and ovule regenerants for ploidy determination in different crops (Bhatia et al. 2016; Hoveida et al. 2017; Wang et al. 2018).

Counting chloroplast number in stomatal guard cells

The ploidy levels of the anther regenerated plants was also confirmed via counting the chloroplast of the guard cells. To establish a correlation between the number of chloroplasts in the stomatal guard cells and the ploidy of the anther derived regenerated plants, the chloroplast number in the stomata of the all the 56 individuals were studied (Table 10). In the present investigation, the correlation between the chloroplast number and ploidy level was determined and it was found that the tetraploid donor

Table 10 Comparison of average number of chloroplasts per pair of stomatal guard cells of anther derived di-haploids, tetraploids and other ploidy levels in *Tagetes patula* cv. Pusa Arpita

Ploidy assessed through chromosome counts and flowcytometry analysis	No. of plants studied	Chloroplasts		
		$\bar{X}^a$	Variance	Standard deviation (SD)
Di-haploids (2x)	8	8.5	0.57	0.76
Tetraploids (4x)	37	16.3	1.38	1.18
Polyploids (4x–6x)	11	23.7	2.41	1.56

[a]Means are significantly different at the 5% level according to F-test with

parent had a mean of 16.3 chloroplasts in their guard cells, whereas the di-haploids had 8.5 and the polyploids had 23.7 (Fig. 3j–l). Assessing the ploidy level through cytological studies is time consuming and analysis through flow cytometry is more expensive. Hence, inexpensive chloroplast counting method one can use for rapid assessment of the ploidy of regenerants. Like in other crops, counting the chloroplast number in stomatal guard cells is a more convenient and a reliable indicator to determine plant ploidy level in marigold. These results corroborate the results obtained by Ganga et al. (2002) in banana, Winarto et al. (2010) in anthurium, Gao et al. (2011) in chrysanthemum and Zhou et al. (2017) in cassava.

Though, direct screening methods like cytological screening is more reliable it takes more time for analyzing the samples and cumbersome whereas the usage of flow cytometry analysis is limited due to its high cost of equipment and maintenance. Hence, a reliable, cheap, rapid and efficient indirect screening method needs to be standardized for initial screening of large-scale anther or ovule derived regenerants. Several screening methods like stomatal size, stomatal density, leaf size, microspore number per anther, microspore abnormalities etc. but in most of the studies counting chloroplast number in the stomatal guard cells was considered as most convenient and highly correlated with the ploidy level of the plants (Winarto et al. 2010).

Conclusion

1. This is the first report on successful induction of haploids through in vitro androgenesis in the genus *Tagetes*. We have established a correlation between bud size, floret size and microspore developmental stages for efficient haploid induction.
2. Anthers having uni-nucleate microspores produced most abundant embryos, shoots and haploids when they were cultured on newly developed enriched MS basal media (EMS_1) supplemented with 2.0 mg l^{-1} BAP and 0.5 mg l^{-1} NAA.
3. Important factors responsible for successful androgenesis were keenly studied. Our study revealed the role of cold pre-treatment and dark incubation on high frequency androgenesis in marigold.
4. It was established that for direct shoot induction, the anthers may be subjected to 10 days of cold pretreatment and thereafter cultured under dark incubation for 20 days.
5. The newly formulated basal media (EMS_1), combination of PGRs can be widely applied within the *Tagetes* species and cultivars. The optimized anther culture protocol will be highly useful for producing haploids and homozygous DH line in marigold.

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Author contributions KRK: He is responsible for conducting all the tissue culture related experiments and analysing the ploidy levels of anther induced regenerants. KPS: He has planned the experiments and provided the basic research facility for conducting these experiments. RB: She has planned the experiments and assisted in conducting the ploidy analysis of anther induced regenerants. DVSR: He has assisted in planning and data analysis of this study. SP: She has contributed in raising the marigold cultivars and maintenance of anther induced regenerants.

Compliance with ethical standards

Conflict of interest The consent of all the authors for submission of this manuscript has been taken. There is no conflict of interest among the authors of this manuscript.

References

Achar PN (2002) A study of factors affecting embryo yields from anther culture of cabbage. Plant Cell Tissue Organ Cult 69:183–188

Agarwal PK, Agawam P, Custers JBM, Liu C, Bhojwani SS (2006) PCIB an anti auxin enhances microspore embryogenesis in microspore culture of *Brassica juncea*. Plant Cell Tissue Organ Cult 86:201–210

Bal U, Touraev A (2009) Microspore embryogenesis in selected medicinal and ornamental species of the Asteraceae. In: Touraev A (ed) Advances in Haploid Production in Higher Plants, Chap. 19. Springer, Dordrescht, pp 219–229

Ball ST, Zhou HP, Konzak CF (1993) Influence of 2,4-D, IAA and duration of callus induction in anther culture of spring wheat. Plant Sci 90:195–200

Bhatia R, Dey SS, Shritika S, Sharma K, Sharma VK, Parkash C, Kumar R (2016) Optimizing protocol for efficient microspore embryogenesis and doubled haploid development in different maturity groups of cauliflower (*B. oleracea* var. *botrytis* L.) in India. Euphytica 212:439–454

Bhatia R, Dey SS, Shritika S, Sharma K, Parkash C, Kumar R (2017) Efficient microspore embryogenesis in cauliflower (*Brassica oleracea* var. *botrytis* L.) for development of plants with different ploidy level and their use in breeding programme. Sci Hort 216:83–92

Chu CC, Wang CC, Sun CS, Hsu C, Yin KC, Chu CY, Bi FI (1975) Establishment of an efficient medium for anther culture of rice through comparative experiments on the nitrogen sources. Sci Sin 18:659–668

Croes AF, Bosveld M, Wullems GJ (1988) Control of thiophene accumulation in *Tagetes*. In: Breteler JH, Arnason T, Hansen L (eds) chemistry and biology of naturally occurring acetylenes and related compound (NOARC). Elsevier publishers, Amesterdam, pp 255–265

Eyster WH (1941) The induction of fertility in genetically self-sterile plants. Science 94:144–145

Ferrie AMR, Caswell KL (2011) Isolated microspore culture techniques and recent progress for haploid and doubled haploid plant production. Plant Cell Tissue Organ Cult 104:301–309

Gamborg OL, Miller RA, Rancillac M (1968) Nutrient requirements of suspension cultures of soybean root cells. Exp Cell Res 50:151–158

Ganga M, Chezhiyan N, Kumar N, Soorianathasundaram K (2002) Stomatal and chloroplast traits as ploidy assessment techniques for ploidy screening of in vitro induced tetraploids of banana. Phytomorphology 52(2–3):113–120

Gao Y, Chen B, Zhang J (2011) Anther culture of garden Chrysanthemum. Proceedings of XXVIIIth IHC-IS on micro and macro technologies for plant propagation. Acta Hort 923:103–110

Germana MA (2011) Anther culture for haploid and double haploid production. Plant Cell Tissue Organ Cult 104:283–300

Godoy-Hernandez G, Miranda-Ham ML (2007) Marigold biotechnology: Tissue culture and genetic transformation. Transgenic Plant J 1(1):169–174

Gopikrishna V, Baweja PS, Venkateshbabu N, Thomas T, Kandaswamy D (2008) Comparison of coconut water, propolis, HBSS, and milk on PDL cell survival. J Endod 34:587–589

Guerin M, Huntley ME, Olaizola M (2003) Haematococcusastaxanthin: applications for human health and nutrition. Trends Biotechnol 21:210–216

Gupta V, Rahman L (2015) An efficient plant regeneration and *Agrobacterium*-mediated genetic transformation of *Tagetes erecta*. Protoplasma 252:1061–1070

Hendriks T, Gonthier L, Blassiau C, Legrand S, Gagneul D, Huss B, Bergs H, Quillet M (2014) Unravelling self-incompatibility in the Asteraceae: cloning of the S Locus in Chicory (*Cichorium intybus* L). International Plant and Animal Genome Conference XXII

Hermsen JGT, Ramanna MS (1981) Haploidy and plant breeding. Philos Trans R Soc Lond B 292:499–507

Hoveida ZS, Abdollahi MR, Mirzaie-Asl A, Moosavi SS, Seguí-Simarro JM (2017) Production of doubled haploid plants from anther cultures of borage (*Borago officinalis* L.) by the application of chemical and physical stress. Plant Cell Tissue Organ Cult 130:369–378

Jackson JC, Gordon A, Wizzard G, McCook K, Rolle R (2004) Changes in chemical composition of coconut. J Sci Food Agric 84:1049–1052

Jacquard C, Nolin F, He´cart C, Grauda D, Rashal I, Dhondt-Cordelier S, Sangwan R, Devaux P, Mazeyrat-Gourbeyre F, Cle´ment C (2009) Microspore embryogenesis and programmed cell death in barley: effects of copper on albinism in recalcitrant cultivars. Plant Cell Rep 28:1329–1339

Ketel DH, Breteler H, De Groot B (1987) Distribution and accumulation of thiophenes in plants and calli of different *Tagetes* species. J Exp Bot 38:322–330

Khandakar RK, Jie YU, Sun-Kyung MIN, Mi-Kyoung WON, Choi HG, Ha-Seung PARK, Tae-Sung KIM (2014) Regeneration of haploid plantlet through anther culture of Chrysanthemum. Not Bot Horti Agrobot Cluj-Na 42(2):482

Kim KM, Baenziger PS, Rybczynski JJ, Arumuganathan K (2003) Characterization of ploidy levels of wheat microspore-derived plants using laser flow cytometry. In vitro Cell Dev Biol Plant 39:663–668

Kothari SL, Chandra N (1984) In vitro propagation of African marigold. HortScience 19:703–705

Li Fu (2007) Identification of the microspore development stages and study of anther vitro culture on *Tagetes erecta*. Thesis submitted to Qinghai University, China (Abstract)

Lichter R (1982) Induction of haploid plants from isolated pollen of *Brassica napus*. Z Pflanzenphysiol 105:427–434

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassay with tobacco tissue cultures. Physiol Plant 15:473–497

Murovec J, Bohanec B (2012) Haploids and doubled haploids in plant breeding. In: Abdurakhmonov I (ed) Plant breeding. In Tech, Zagreb

Nitsch JP, Nitsch C (1969) Haploid plants from pollen grains. Science 163:85–87

Nitsch C, Norreel B (1973) Effect d'un choc the rmiquesurIepouvoir embryogenedu pollen de *Datura* culture dansI' anthereouisole de l' anthere. Comptes Rendus Acad Sci 276:303–306

Osolnik B, Bohanec B, Jelaska S (1993) Stimulation of androgenesis in white cabbage (*Brassica oleracea* var. *capitata*) anthers by low temperature and anther dissection. Plant Cell Tissue Organ Cult 32:241–246

Pablo E, Vanegas PE, Cruz-Hernandez A, Valverde ME, Paredes-Lopez O (2002) Plant regeneration via organogenesis in marigold. Plant Cell Tissue Organ Cult 69:279–283

Qi Y, Ye Y, Manzhu B (2011) Establishment of plant regeneration system from anther culture of *Tagetes patula*. Afr J Biotechnol 10(75):17332–17338

Raghavan V (1990) From microspore to embryo: faces of the angiosperm pollen grain. In: Nijkamp HJJ, van der Plas LH, van Hartrigik J (eds) Progress in plant cellular and molecular biology. I.A.P.T.C. Kluwer, Dordrecht, pp 213–221

Ravindra Kumar K, Singh KP, Jain PK, Raju DVS, Kumar P, Bhatia R, Sapna Panwar (2018) Influence of growth regulators on callus induction and plant regeneration from anthers of *Tagetes* spp. Indian J Agr Sci 88(6):970–977

Touraev A, Pfosser M, Heberle-Bors E (2001) The microspore: a haploid multipurpose cell. Adv Bot Res 35:53–109

Vanegas-Espinoza PE, Ramos-Viveros V, Rios-Salome LB, Orbe-Rogel JC, Parredes-Lopez O, Cruz-Hernandez A, Villar-Martineza AAD (2012) Analysis of callus development and transient expression in *Tagetes erecta*. Acta Hort 937:963–968

Vasil IK, Nitsch C (1975) Experimental production of pollen haploids and their uses. Z Pfianzenphysiol 76:191–212

Veilleux RE (1994) Development of new cultivars via anther culture. Hortscience 29(11):1238–1241

Wang G, Qin H, Sun D, Fan S, Yang Y, Wang Z, Xu P, Zhao Y, Liu Y, Ai J (2018) Haploid plant regeneration from hardy kiwifruit (*Actinidia arguta* Planch.) anther culture. Plant Cell Tissue Organ Cult 134:15–28

Wenzel G, Hoffmann F, Thomas E (1977) Increased induction and chromosome doubling of androgenetic haploid rye. Theor Appl Genet 51:81–86

Wijnker E, Vogelaar A, Dirks R, van Dun K, de Snoo B, van den Berg M, Lelivelt C, de Jong H, Chunting L (2007) Reverse breeding: Reproduction of F_1 hybrids by RNAi-induced asynaptic meiosis. Chromosome Res 15(2):87–88

Winarto B, Mattjik NA, Teixeira da Silva JA, Purwito A, Marwoto B (2010) Ploidy screening of anthurium (*Anthurium andreanum* Linden ex André) regenerants derived from anther culture. Sci Hort 127:86–90

Xu ZH (1990) Barley (*Hordeum vulgare* L.) anther culture and the production of haploids. In: Bajaj YPS (ed) Haploids in crop improvement biotechnology in agriculture and forestry. Springer, Berlin, pp 125–175

Xue-jiao S, TAO CG, WANG P (2010) Interrelation of cytological development period of marigold microspore the morphology of flower organ. Hubei Agri Sci 9:039

Zhang XQ, Yang JS, Zheng ZX (2009) Study on induction of embroid through in vitro anther of *Cyclamen persicum*. Jiang Su Agri Sci 108:57–58

Zhang P, Zeng L, Su Y, Gong X, Wang X (2011) Karyotype studies on *Tagetes erecta* L. and *Tagetes patula* L. Afr J Biotechnol 10(72):16138–16144

Zhong D, Michaux-Ferriere N, Coumans M (1995) Assay for double haploid sunflower (*Helianthus annus*) plant production by

androgenesis: fact or artifact? Part 1. In vitro anther culture. Plant Cell Tissue Organ Cult 41:91–97
Zhou H, Zeng W, Yan H (2017) In vitro induction of tetraploids in cassava variety 'Xinxuan 048' using colchicine. Plant Cell Tissue Organ Cult 128:723–729