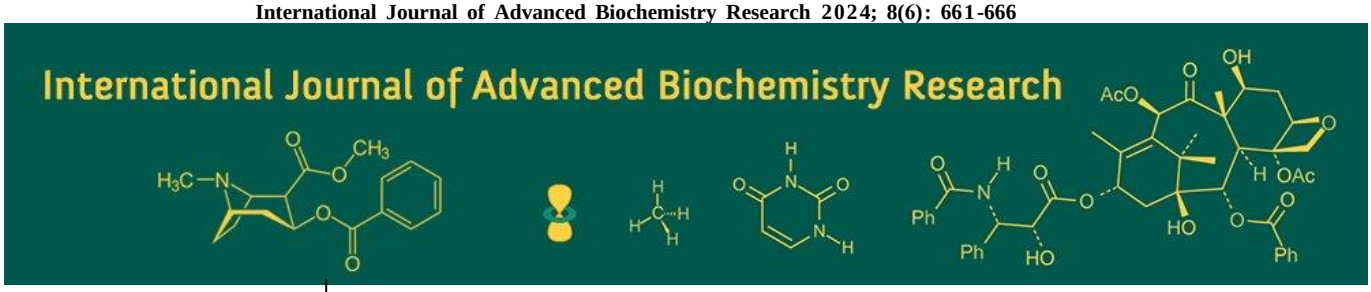

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Embryogenesis and haploid plant regeneration from anther culture of Marigold (*Tagetes erecta* L.)

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Abstract
An experiment was conducted to generate haploid marigold plantlet by examining the impact of plant growth regulators on callus induction and subsequent plant regeneration from anther cultures. The developmental stages of anthers were correlated with the morphological stages of flower bud development. Anthers containing microspores at the uninucleate stage were isolated from buds and cultured on Murashige and Skoog basal media supplemented with various combinations of growth hormones. The MS media supplemented with 4.44 µM BAP and 1.07 µM NAA was recorded to be most effective for; percent callus induction (69.16%), early callus induction (16.67 days) and induction of green colour calli with leaves. Shoot differentiation media, containing BAP (2.22–4.44 µM) and NAA (0.54–1.07 µM), yielded green regenerated plantlets at the highest frequency (58.91%) when using MS media supplemented with BAP (4.44 µM) and NAA (1.07 µM). MS media without PGR proved to be the most suitable rooting medium. Cytological analysis of root tip cells from fifty regenerated plants revealed three confirmed haploid individuals. This protocol serves as a valuable basis for further research aimed at developing homozygous *Tagetes erecta* and other *Tagetes species*.

Keywords: Anther culture, cytology, embryogenesis, haploid, plant growth regulator, *Tagetes erecta*

Introduction
Marigold (*Tagetes* spp.), a member of the Asteraceae family, includes commercially significant species such as *T. erecta, T. patula, T. minuta, T. lucida, and T. signeta*. This ornamental crop holds importance for loose flowers, garden plant, essential oil extraction, and lutein production. The essential oil derived from *Tagetes* spp. exhibits various beneficial properties including anti-inflammatory, antiseptic, antispasmodic, astringent, diaphoretic, anticancer, and antioxidant activities (Ghada *et al*., 2013; Abdoul-Latif *et al*., 2022; Bakshi and Ghosh, 2022, Tudora *et al*., 2024) [8, 1, 4, 24]. Marigold petals, abundant in lutein, serve diverse purposes ranging from nutritional supplements to food coloring, poultry feed additives, and ophthalmic applications (Alotaibi *et al*., 2021; Nam *et al*., 2021, Hussain *et al*., 2024) [2, 20, 9]. In India, marigold cultivation covers 73.15 thousand hectares, yielding 728.53 thousand tonnes of loose flowers and 26.57 thousand tonnes of cut flowers (NHB, 2021-2022) [21]. Productivity fluctuates with location and varieties, potentially attributed to its heterozygous nature. Developing homozygous lines for F1 hybrid production conventionally requires time-consuming processes. Hence, doubled haploid homozygous lines through anther culture offer a means to speedily obtain true breeding lines, bypassing multiple generations typically needed in conventional breeding methods (Kurimella *et al*., 2021, Al-Ashkar *et al*., 2023) [14, 3]. An essential factor for successful androgenesis is selecting flower buds at the appropriate microspore developmental stage, underscoring the necessity to discern various stages of microspore development in marigold flower buds. Additionally, the type and concentration of PGR in the induction medium significantly influence androgenesis, embryogenesis, and callus induction. Despite the importance, minimal research has been conducted on doubled haploid development in flower crops. Thus, this study aims to establish correlations between flower bud size, floret size, and microspore developmental stages, while also devising a protocol for embryogenesis from marigold anther cultures.

Materials and Methods

Selection of the flower bud for the proper microspore developmental stage

Marigold cultivar 'Pusa Narangi Gainda' plants were cultivated using standard practices. Buds ranging in size from 2 mm to 20 mm were harvested from the field early in the morning. The length of these buds was measured using a stainless-steel scale, and buds measuring 2 mm, 5 mm, 10 mm, 15 mm, and 20 mm were selected for further analysis. Disc florets from these selected buds were isolated, and their lengths were sequentially measured under a microscope. Subsequently, anthers were isolated from the respective florets. The anthers were placed on glass slides, stained with acetocarmine dye, and observed under a microscope (Olympus CX 41). The stage of microspore development and the size of microspores and pollen were recorded.

Protocol for induction media preparation and sterilization procedure in bud and anther culture

Murashige and Skoog (1962) [19] media, supplemented with seventeen different growth hormone combinations (I_1–I_{17}), were utilized as induction media to ascertain the optimal conditions for *in vitro* callus formation from cultured anthers (Table 2). All media formulations included 0.75% agar and 3% sucrose, with pH adjusted to range between 5.70 and 5.80. Following standard procedure, media were autoclaved at 121°C for 15 minutes and dispensed into 100 × 15 mm diameter Petri dishes in 25 ml aliquots.

Flower buds measuring 15 mm in length were collected from the field and subjected to surface sterilization under aseptic conditions. This involved treatment with 0.1% bavistin for 15 minutes, followed by rinsing with sterile distilled water. Subsequently, buds were disinfected by sequential immersion in 70% ethanol for 30 seconds and 1% NaOCl for one minute. The disinfected buds were then rinsed thrice with sterilized water and gently dried using aseptic filter papers.

From these prepared buds, disk florets of appropriate size (2-2.5mm) were excised, and all five anthers from each floret were immediately inoculated onto the induction media. A hundred anthers were placed on a single Petri plate. The experimental design employed a completely randomized design, with each type of induction medium replicated three times. The anther cultures were maintained at 25°C in darkness for 20 days, followed by incubation under fluorescent 20 W day light lamps for 16 hours light and 8 hours dark. Callus induction rates and calli size were assessed 25 days post anther culture. The initiation of callus formation was recorded when 50% of the anthers per plate exhibited callus growth.

Preparation of differentiation media and plant regeneration

Sixteen distinct types of differentiation media, designated as D_1–D_{16}, were evaluated alongside a control, labeled as D_{17} (Table 3). The preparation of differentiation media followed the same protocol as that for induction media. The calli were fragmented and ten pieces of calli measuring 5 × 5 mm each were sub- cultured to each Petri dish containing the differentiation media. These Petri dishes were then incubated under a 16-hour photoperiod at a temperature of 25°C for a duration of 30 days. A complete randomized design was employed, consisting of three replicates. Healthy differentiated shoots excised from the culture were subsequently transferred to MS media supplemented with varying concentrations of IBA (0, 0.49, 0.98, and 1.47 µM).

Determination of ploidy level

Cytological analysis

Fifty plantlets were randomly selected, and from each plantlet, four meristematic root tips were excised. The root tips were rinsed with distilled water and then immersed in a 0.002N solution of 8-Hydroxyquinoline (8HQ) at 4 °C for 2 hours. Subsequently, these treated root tips were transferred to Carnoy's solution for 24 hours for fixation. After fixation, these root tips were rinsed with distilled water and hydrolyzed in 1 N hydrochloric acid for 5 minutes at 65 °C, followed by another three rinses with distilled water. The root tips were then immersed in basic fuchsin stain in darkness for 2 hours. Prepared root tips were mounted on glass slides, stained with a 2% carmine for 2 minutes, and covered with a cover slip. The slides were briefly heated over a burner for approximately 5 seconds, and the root tips were gently pressed under the cover glass. Finally, the slides were examined and photographed under a 100x objective lens using a Olympus BX 51 microscope equipped with a Olympus BMXCLAMP camera.

Results and Discussion

Determining Ideal Flower Bud Stage for Microspore Development

The study revealed a close correlation between the developmental stages of microspores in marigold and the lengths of flower buds and florets (refer to Table 1 and Figure 1). The smallest flower bud, measuring 2.0 mm in length, contained disc florets of 0.28 mm in length, with green-colored anthers in an undifferentiated stage. As the bud length increased to 5.0 mm, the disc florets also grew to 0.35 mm, with anthers showing pollen mother cell developmental stage. Flower buds measuring 10.0 mm harbored florets ranging from 0.7 mm to 1.9 mm, with anthers containing mostly tetrad microspores of 27 µm. Buds of 15 mm length, contained three stages of pollen grains, with varying sizes of microspores found in different rows of disc florets. The experiment concluded that the middle three rows of disc florets, ranging from 2-2.5 mm in length in 15 mm long flower buds, containing microspores at the uninucleate stage, serve as the optimal explant for anther culture. Size of uninucleate microspore was 31.55 µm. Last row of disc floret (2.6 mm long floret) in this size bud contained most of the microspore at immature pollen grain stage. Size of this immature pollen grain was 50 µm. Floret of size 3-5 mm from flower bud of size ≥ 20 mm contained matured pollen grain of size 76.2 µm with well-defined spiny exine. For microspore culture, cell strainers of 45 µm can effectively isolate this stage of pollen mother cell from the microspore suspension. A significant co-relation between flower bud size and stage of microspore developmental was also observed in melon, kenaf, and eggplant, as reported by Nguyen *et al*. (2022) [22], Mahmood Ibrahim *et al*. (2014) [18], and Calabuig-Serna *et al*. (2021) [5], respectively. Similarly, Belwal *et al*. (2024) also illustrated the coorelation between onion flower bud size and the stages of microspore development. They observed a positive correlation between flower bud diameter, length, anther diameter, length, and microspore development stage.

Effect of different concentration of plant growth regulators on callus induction from anther culture of marigold

The MS media supplemented with BAP and NAA (4.44 µM and 1.07 µM respectively) resulted in the highest callus induction rate of 69.16%, with green-colored calli measuring 30.00 mm size (Table 2). This treatment exhibited significant superiority over all other treatments in both callus induction rate and calli size. Moreover, it showed similar effectiveness to other combinations of BAP and NAA (I_{13}, I_{15}, I_{16}) in terms of days required for callus induction (16.67 days). The combinations of Ki and 2,4-D (I_1-I_4) produced light yellow, friable, and very small calli (2 - 2.3 mm), while combinations of BAP and 2,4-D (I_5-I_8) resulted in light yellow-brownish yellow compact calli. Increasing the concentration of NAA from 0.54 to 1.07 µM in combination with Ki (I_{10}, I_{12}) led to the production of white fibrous roots from small yellow calli. The combination of BAP and NAA has been widely recognized as an effective PGR treatment for inducing callus from anther cultures in various plant species, including purple coneflower, marigold, chrysanthemum, lupinus, primula, saintpaulia, pumpkin, pepper, and rice (Zhao *et al*., 2006; Li *et al*., 2007; Gao *et al*., 2011; Kozak *et al*., 2012; Jia *et al*., 2014; Uno *et al*., 2016; Kurtar *et al*., 2016; İlhan and Kurtar, 2022; Chandravani and Lantos *et al*., 2023) [29, 17, 7, 13, 11, 25, 15, 10, 16].

Effect of different concentration of plant growth regulators on shoot differentiation from anther culture of marigold

After 20 days of culture on sixteen types of differentiation media alongside a control (D_1-D_{17}), calli exhibited various responses (Table 3). Notably, the highest shoot induction rate (58.91%) and the highest average number of shoots per callus (6.17) were observed on the shoot induction medium containing BAP and NAA (4.44 µM and 1.07 µM respectively). This treatment significantly outperformed all others. Conversely, calli cultured on medium D_9-D_{12} showed no shoot formation. Calli on media D_1-D_4 turned brownish and perished in 8 -10 days of transfer, while those on D_5-D_8 media, also turned brown and died after 15-20 days after transfer. In PGR-free MS medium, roots were healthy, long and widespread. As the concentration of IBA increased, roots became shorter and thicker. Similar outcomes have been reported in baby primrose, anthurium, and rice (Jia *et al*., 2014, Winarto *et al*., 2011 and Tripathy, 2022 respectively) [11, 26, 23].

Identification of ploidy level in regenerated plantlets

Among the fifty plants examined, three exhibited a chromosome count of 12, indicating they were haploid, while one plant had 36 chromosomes, indicating triploidy. The remaining plants had 24 chromosomes, indicating diploidy (Fig. 3). Similar findings have been reported in various studies. For instance, anther cultures of *Brassica oleracea* (Zhao *et al*., 2022, Dhiman *et al*., 2024) [30, 6], *Citrus aurantium* (Jin *et al*., 2022) [12], and apple (Zhang *et al*., 2022) [28] yielded plantlets of different ploidy levels. It's conceivable that diploid plantlet may originate from residual filament tissues and anther walls. Haploid plantlet are unquestionably the product of embryogenesis from microspores in the cultured anthers. The variations in haploid and doubled haploid frequencies observed in this study and others are likely influenced by various factors, including flower bud pretreatments, anther pretreatments, media composition, environmental conditions, and dark period duration.

Table 1: Co-relation of flower bud size, floret size and microspore developmental stages

Bud size	Flore size	Anther colour	microspore developmental stages
2 mm	0.286 mm	Green	Undifferentiated anther
5 mm	0.357 mm	Green	Pollen mother cell
10 mm	0.7 – 1.9 mm	Green	Tetrad spore of size 27 µm
15 mm	1.849 mm	Green	Tetrad spore of size 28 µm
	2-2.5 mm	Light Green to greenish yellow	Mid uninucleate – late uninucleate microspore of size 31.55µm
	2.6 mm	Greenish yellow	Immature pollen grain
20 mm	3-5 mm	Yellow	Mature pollen grain with size 76.2 µm

Table 2: Effect of various induction media on callus induction from cultured anthers of marigold cv. 'Pusa Narangi Gainda'

PGR (µM)					Callus Induction Rate (%)a	Days to Callus Induction	Callus size (mm)	Callus growth pattern
Induction medium	Ki	BAP	2,4-D	NAA				
I_1	4.65	-	0.45	-	37.86m	19.33	2.00	Light yellow friable calli
I_2	4.65	-	0.91	-	38.45lm	19.00	2.00	Light yellow friable calli
I3	2.32	-	0.45	-	39.03lm	19.00	2.33	Light yellow soft calli
I_4	2.32	-	0.91	-	39.23l	18.67	2.00	Light yellow soft calli
I_5		4.44	0.45	-	45.38gh	18.00	4.67	Light yellow compact calli
I_6		4.44	0.91	-	45.95fg	18.00	4.83	Light yellow compact calli
I_7		2.22	0.45	-	46.91ef	17.67	4.67	Brownish yellow compact calli
I_8		2.22	0.91	-	47.48e	17.67	4.50	Brownish yellow compact calli
I_9	4.65	-	-	0.54	44.04hi	20.33	2.00	Yellow and soft calli
I_{10}	4.65	-	-	1.07	42.71ij	21.00	2.33	Yellow calli with whiteroot
I_{11}	2.32	-	-	0.54	41.74jk	21.00	2.00	Yellow and soft calli
I_{12}	2.32	-	-	1.07	40.98k	21.33	2.00	Yellow calli with whiteroot
I_{13}	-	4.44	-	0.54	65.69b	17.00	28.00	Green calli
I_{14}	-	4.44	-	1.07	69.16a	16.67	30.00	Green calli with leaves
I_{15}	-	2.22	-	0.54	53.13d	17.00	24.50	Yellowish green calli
I_{16}	-	2.22	-	1.07	55.56c	17.00	23.25	Yellowish green calli
I_{17}	-	-	-	-	0.29n	0.00	0.00	-

100 anthers were inoculated in one petri dish; 3 replicates of each medium were performed
A Callus Induction Rate (%) = (No. of callus formed / No. of anthers inoculated) ×100.
Data are provided as mean ± S.E., and those followed by different letters are significantly different at 1% level of significance

Table 3: Effect of various differentiation media on shoot regeneration from calli derived from marigold 'Pusa Narangi Gainda' anthers

PGRs (µM)					Number of calli cultureda	Shoot Differentiation Rate (%)b	Average number of shoot per callus
Differentiation Media	Ki	BAP	2,4-D	NAA			
D_1	4.65	-	0.45	-	60	0.37e	0.00^e
D_2	4.65	-	0.91	-	60	0.37e	0.00^e
D_3	2.32	-	0.45	-	60	0.37e	0.00^e
D_4	2.32	-	0.91	-	60	0.37e	0.00^e
D_5		4.44	0.45	-	60	0.37e	0.00^e
D_6		4.44	0.91	-	60	0.37e	0.00^e
D_7		2.22	0.45	-	60	0.37e	0.00^e
D_8		2.22	0.91	-	60	0.37e	0.00^e
D_9	4.65	-	-	0.54	60	0.37e	0.00^e
D_{10}	4.65	-	-	1.07	60	0.37e	0.00^e
D_{11}	2.32	-	-	0.54	60	0.37e	0.00^e
D_{12}	2.32	-	-	1.07	60	0.37e	0.00^e
D_{13}	-	4.44	-	0.54	60	32.51b	3.83^c
D_{14}	-	4.44	-	1.07	60	58.91a	6.17^a
D_{15}	-	2.22	-	0.54	60	30.00d	3.00^d
D_{16}	-	2.22	-	1.07	60	31.09c	4.00^b
D_{17}	-	-	-	-	60	0.37e	0.00^e

1. each treatment consist of 3 replicate, each with 6 petriplates, at a density of 10 callus per plates.
2. Shoot Differentiation Rate from green callus (%) = (No. of callus that generating shoots in differentiation medium / No. of callus inoculated) ×100
3. Data are provided as mean ± S.E., and those followed by different letters are significantly different at 1% level of significance

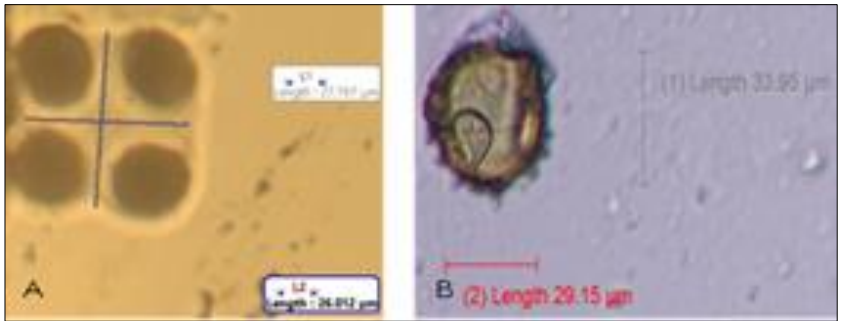

Fig 1: Stages of microspore development in marigold cv. 'Pusa Narangi Gainda'.A) Tetrad; B) Uninucleate

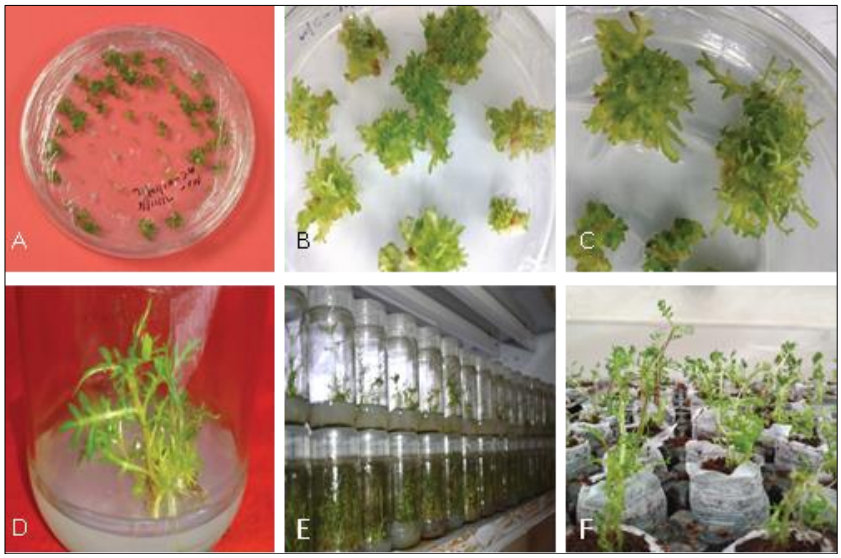

Fig 2: Callus induction and plantlet differentiation from anther culture of marigold *cv.* 'Pusa Narangi Gainda' (A) Embryogenic calluses; (B, C) differentiation of embryogenic calluses; (D) plantlet in differentiation media; E) Anther cultured grown plant in rooting media; F) hardening of anther cultured grown plants in cocopeat media.

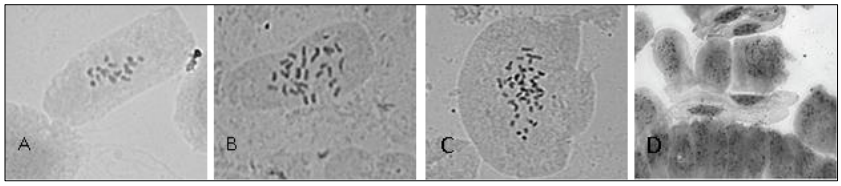

Fig 3: Images of marigold metaphase stage chromosomes. (from root tips of regenerated plants); A) An image of 12 chromosomes (haploid); B) An image of 24 chromosomes (diploid); C) An image of 36 chromosomes (Triploid); D) An image of 12 and 36 chromosomes in different cells of same root tip (mixoploid)

Conclusion

The current study documents the successful generation of haploid plantlets from marigold cultivar 'Pusa Narangi Gainda' through anther culture. It was observed that florets, ranging from 2 to 2.5 mm in length and located in the middle three rows of 15 mm long buds, contained anthers having microspores at the uninucleate stage. Optimal callus induction and shoot differentiation occurred when anthers from these florets was cultured (in the dark) and sub-cultured (under a 16-hour photoperiod) on MS media supplemented with BAP and NAA (4.44 µM and 1.07 µM). Shoots derived from anthers readily rooted on hormone free MS medium. Among 50 randomly selected plantlets regenerated from anther culture, cytological analysis revealed that three were haploid and one was triploid.

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References

1. Abdoul-Latif FM, Elmi A, Merito A, Nour M, Risler A, Ainane A, Ainane T. Essential oils of *Tagetes minuta* and *Lavandula coronopifolia* from Djibouti: Chemical composition, antibacterial activity and cytotoxic activity against various human cancer cell lines. Int J Plant Biol. 2022;13(3):315-29.
2. Alotaibi HN, Anderson AK, Sidhu JS. Influence of lutein content of marigold flowers on functional properties of baked pan bread. Ann Agric Sci. 2021;66(2):162-8.
3. Al-Ashkar I, Al-Doss A, Ullah N. Accelerating Crop Improvement through Speed Breeding. In: Climate-Resilient Agriculture, Vol 1: Crop Responses and Agroecological Perspectives. Cham: Springer International Publishing; 2023. p. 821-47.
4. Bakshi L, Ghosh R. Marigold Biopesticide as an Alternative to Conventional Chemical Pesticides. J Adv Sci Res. 2022;13(05):26-33.
5. Calabuig-Serna A, Porcel R, Corral-Martínez P, Seguí-Simarro JM. Anther and isolated microspore culture in eggplant (*Solanum melongena* L.). In: Doubled haploid technology. New York: Humana; 2021. p. 235-50.
6. Dhiman MR, Kumar R, Kumar S, Prakash C, Rana A. Development and characterization of elite doubled haploid lines of ornamental kale. Crop Breed Appl Biotechnol. 2024;24(2).
7. Gao Y, Chen B, Zhang J. Anther culture of garden Chrysanthemum. Acta Hortic. 2011;923:103-10.
8. Ghada IM. Biological effects, antioxidant and anticancer activities of marigold and basil essential oils. J Med Plants Res. 2013;7(10):561-72.
9. Hussain S, Gulfreen E, Abid S, Khalil S, Rizwan M, Batool N, et al. Investigating the Impact of Marigold Supplementation on Egg Yolk Color Intensity: A Study on Dietary Additives. J Health Rehabil Res. 2024;4(1):1744-51.
10. İlhan M, Kurtar ES. Double haploidization efficiency of selected pepper genotypes Via in Vitro Anther Culture. Selcuk J Agr Food Sci. 2022;36(2):253-9.
11. Jia Y, Zhanga QX, Pana HT, Wanga SQ, Liub QL, Sun LX. Callus induction and haploid plant regeneration from baby primrose (*Primula forbesii* Franch.) anther culture. Sci Hort. 2014;176:273-81.
12. Jin SB, Kim MJ, Choi C, Yun SH. Induction and genetic verification of Haploid-derived plants using anther culture of *Citrus aurantium* L. (Sour Orange). 2022.
13. Kozak K, Galek R, Waheed MT, Sienkiewicz ES. Anther culture of *Lupinus angustifolius*: callus formation and the development of multicellular and embryo-like structures. Plant Growth Regul. 2012;66:145-53.
14. Kurimella RK, Bhatia R, Singh KP, Panwar S. Production of Haploids and Doubled Haploids in Marigold (*Tagetes* spp.) Using Anther Culture. In: Doubled Haploid Technology. New York: Humana; 2021. p. 271-87.
15. Kurtar ES, Balkaya A, Kandemir D. Evaluation of haploidization efficiency in winter squash (*Cucurbita maxima* Duch.) and pumpkin (*Cucurbita moschata* Duch.) through anther culture. Plant Cell Tissue Organ Cult. 2016;127:497-511.
16. Lantos C, Jancsó M, Székely Á, Szalóki T, Venkatanagappa S, Pauk J. Development of in vitro anther culture for doubled haploid plant production in indica rice (*Oryza sativa* L.) genotypes. Plants. 2023;12(9):1774.
17. Li F, Tang D, Liang S. Effects of growth regulators on anther culture of marigold. J Qinghai Univ Nat Sci. 2007;1:12.
18. Mahmood Ibrahim A, Binti Kayat F, Ermiena Surya Mat Hussin Z, Susanto D, Ariffulah M. Determination of suitable microspore stage and callus induction from anthers of kenaf (*Hibiscus cannabinus* L.). Sci World J. 2014;2014:284342.
19. Murashige T, Skoog F. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant. 1962;15(3):473-97.
20. Nam S, Lee CY, Shim SM, Lee DU, Lee S. Functional Characterization of Marigold Powder as a Food Ingredient for Lutein-Fortified Fresh Noodles. Appl Sci. 2021;11(2):861.
21. National Horticulture Board (NHB). India production of Marigold (apeda.gov.in). 2021-22 (1st Adv. Estimate).
22. Nguyen ML, Huyen TNB, Trinh DM, Voronina AV. Association of bud and anther morphology with developmental stages of the male gametophyte of

melon (*Cucumis melo* L.). Vavilov J Genet Breed. 2022;26(2):146.
23. Tripathy SK. High throughput anther culture response in an upland rice cross 'Khandagiri x Dular'. J Environ Biol. 2022;43(3):420-9.
24. Tudora C, Nenciu F, Muscalu A, Burnichi F, Gatea F, Boiu-Sicuia OA, et al. Pesticidal Potential of Essential Oil Obtained from a New Variety of Marigold (*Tagetes patula* L., fam. Asteraceae). Appl Sci. 2024;14(8):3159.
25. Uno Y, Koda-Katayama H, Kobayashi H. Application of anther culture for efficient haploid production in the genus Saintpaulia. Plant Cell Tissue Organ Cult. 2016;125:241-8.
26. Winarto B, Rachmawati F, Pramanik D, Jaime A, Silva TD. Morphological and cytological diversity of regenerants derived from half-anther cultures of anthurium. Plant Cell Tissue Organ Cult. 2011;105:363-74.
27. Yingchun Q, Yaomei Y, Manzhu B. Establishment of plant regeneration system from anther culture of Tagetes patula. Afr J Biotechnol. 2011;10:17332-8.
28. Zhang C, Deng S, Cao Q, Komori S, Xiao R. Plantlet regeneration from apple (*Malus*× *domestica* Borkh.) anther culture derived embryos. Plant Cell Tissue Organ Cult. 2022;1:20.
29. Zhao FC, Nilanthi D, Yang YS, Wu H. Anther culture and haploid plant regeneration in purple coneflower (*Echinacea purpurea* L.). Plant Cell Tissue Organ Cult. 2006;86:55-62.
30. Zhao Y, Zheng W, Li J, Qi X, Feng H, Zhang Y. Effects of genotype and sodium p-nitrophenolate on microspore embryogenesis and plant regeneration in broccoli (*Brassica oleracea* L. var. italica). Sci Hortic. 2022;293:110711.