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Optimizing Callus Induction and Plantlet Regeneration in Marigold Anther Culture: Effects of Pretreatments and Culture Conditions

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ABSTRACT

Marigold (Tagetes spp.) holds significance in various industries, including pharmaceuticals, nutraceuticals, and pigments, due to its flower pigment and essential oil. Conventional breeding methods for genetic enhancement are time-consuming due to its cross-pollination nature. To expedite improvement, doubled haploid lines can be produced through androgenesis. In spite of the potential benefits, challenges such as genotype-specific responses, anther development stage, pretreatment and culture conditions pose significant hurdles. Addressing these factors could pave the way for more efficient application of androgenesis in marigold crop improvement, offering quicker pathways to developing superior plant varieties. This study aimed to determine the optimal temperature stress pretreatment, sucrose concentration in culture media and duration of darkness during anther incubation to enhance embryogenic callus proliferation and subsequent regeneration. It was found that subjecting marigold flower buds with anthers at the uninucleate stage to a temperature stress of 4 °C for 72 hours followed by culture in MS media containing 4% sucrose, supplemented with NAA (0.2 mg L^{-1}), and BAP (1 mg L^{-1}) yielded the best results. Ploidy analysis of fifty regenerated plantlets revealed varying ploidy levels, including haploid. These findings hold promise for advancing research on haploid and doubled haploid development in marigold and related species.

Keywords: *Anther culture, Androgenesis, Cytology, Doubled haploid, Embryogenesis, Haploid, Stress treatments, Tagetes erecta*
Abbreviations: *MS medium- Murashige and Skoog medium, BAP - Benzylamino purine, NAA- 1- Naphthalene-acetic acid*

1. Introduction

Marigold (*Tagetes* spp.), an admired annual flower native to Mexico, holds multifaceted importance as loose flowers, garden adornments, and as a trap crop. The essential oil extracted from *Tagetes erecta* flowers serves diverse functions, including perfumery and medicinal purposes such as antihaemorrhagic, anti-inflammatory, and antiseptic properties (1). Well-known for its lutein content, this crop finds applications across nutraceutical, cosmetic, and pharmaceutical industries (2,3). Marigold petals are extensively utilized in poultry feed, nutritional supplements, food coloring, and ophthalmic applications (4). The solubility of lutein esters in vegetable oils enhances their desirability as food colorants compared to synthetic carotenoids. Notably, studies suggest its potential in anticancer therapies (5). Because of the diverse uses of this crop, hybrid marigold varieties are in high demand owing to their profuse, early flowering, compact double flower heads, extended flowering duration, shelf-life, more lutein content and resilience to various stresses. In order to meet this demand, floriculture scientist aims to develop improved F1 hybrid of this crop. Conventional breeding methods for marigold F1 hybrids development are time-consuming due to its cross-pollinated nature; an issue alleviated by accelerating doubled haploid line production through androgenesis (6). Additionally, androgenesis can be improved by applying pre- and post-*in vitro* culture stress to anther. It has been stated that various factors *viz*; genotype, microspore developmental stage, temperature treatments, carbohydrate levels in culture media, PGR, and photoperiod exposure significantly impacts embryogenesis and callus induction from anther culture (7,8). This study seeks to elucidate optimal stress pretreatment, sucrose concentrations in culture media, and dark incubation durations for enhancing embryogenic callus proliferation and subsequent regeneration in marigold anther cultures.

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2. Materials and Methods

Marigold cultivar 'Pusa Narangi Gainda' plants were grown in experimental area of Division of Floriculture and Medicinal Crops-ICAR- Indian Institute of Horticultural Research, Bengaluru, Karnataka, India.

2.1. Cold stress pretreatment

Flower buds of marigold were collected from the field. These flower buds were kept at 4, 7 and 25 °C for four different durations (1, 24, 72 and 120 hr). After this pretreatment, buds were surface sterilized under aseptic condition. Anthers having microspore at uninucleate stage were excised carefully from individual disk floret and inoculated on Murashige & Skoog media (MS media) supplemented with NAA and BAP (0.2 mg L^{-1} and 1 mg L^{-1} respectively). Around hundred anthers were inoculated on each Petri plate and six sets per replication were maintained. The cultures were incubated for 25 days in dark room and future the induced calluses were exposed to 16/8 hr

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light-dark photoperiod. Calluses were later sub-cultured on same MS media composition for shoot differentiation.

2.2. Heat stress pretreatment
Healthy marigold flower buds were kept at 25, 35 and 45 °C for 1, 3, 5 and 8 hr. These flower buds were surface sterilized and anthers were inoculated and callus was sub-cultured same way as in last experiment 2.1.

2.3. *in vitro* sucrose stress
Healthy marigold flower buds were kept at 4 °C for 72 hrs. These flower buds were surface sterilised in aseptic condition. Anthers having microspore at uninucleate stage from these buds were inoculated on MS media having deficient or excess of sucrose in it. Seven different sucrose concentration (0, 2.0, 4.0, 6.0, 8.0, 10.0, and 12.0%) in MS media supplemented with NAA and BAP (0.2 mg L^{-1} and 1 mg L^{-1} respectively) was used in this experiment. Around hundred anthers were inoculated on each Petri plate and six sets per replication were maintained. The cultures were incubated for 25 days in dark room for callus induction and future the induced calluses were exposed to 16/8 hr light-dark photoperiod.

2.4. *in vitro* light-dark cycle stress to cultured anther
Healthy marigold flower buds were kept at 4 °C for 72 hrs. These flower buds were surface sterilised in aseptic condition and then anthers having microspore at uninucleate stage were inoculated on MS media supplemented with NAA and BAP (0.2 mg L^{-1} and 1 mg L^{-1} respectively). These cultured plates were exposed to different dark duration (0, 1, 2, 3, 4, 5, and 6 weeks) at 25 °C. After dark period treatment, each plate was exposed to a 16/8-hour photoperiod.
Regenerated plantlets were hardened for few days in lab and then transferred to sterilized growing media in pot.

2.5 Determination of ploidy level by cytological analysis
Ploidy level of plantlets were analysed using chromosome counting of meristematic cells of root tip and method followed as described by Fukui and Nakayama in 1996 (9).

2.6 Data Analysis
Completely randomized designed was followed and data was analyzed using ICAR- Web Based Agricultural Statistics Software (WASP).

3. Result and discussion

3.1 influence of cold stress pretreatment
4 °C pretreatment stress to flower bud was found to be statistically superior for rate of callus induction (87.10 %), minimum days to callus initiation (17.00 days), calli size (31.02 mm) and shoot differentiation rate (76.83 %) from its *in vitro* anther culture (Tab. 1). Cold pretreatment for 72 hr was found to be ideal pretreatment as it significantly improved the callus induction rate (Tab. 1) and shoot differentiation rate (Tab. 2). However, cold pretreatment for 120 hrs decreased the response of anther for callus induction. Minimum days (17.27 days) to callus induction was reported when anthers were cold pretreated for 24 hrs. When interaction effect of temperature and duration was analysed, it was found that highest callus induction rate (96.60 %), miminum days to callus initiation (15.60 days), largest green colour embryogenic calli (34 mm) and maximum shoot differentiation rate (79.79 %) was reported from anthers which were pretreated at 4 °C for 72 hrs (Tab. 3). The influence of low-temperature pretreatment on anther culture is substantiated by its pivotal role in conserving microspore viability, thereby ensuring heightened androgenesis and callus induction rates. This phenomenon finds resonance in various studies across different plant species where researcher have reported similar outcomes in anther cultures of *Tagetes patula, Zantedeschia aethiopica, petunia, Tagetes erecta*, wheat, and bell pepper, respectively (10-15). This notion was further supported by suggesting that low-temperature pretreatment of flower buds or anthers can augment embryogenesis (16). Notably, another research demonstrated a significant enhancement in green plant regeneration in *Tagetes erecta* through a cold pretreatment of 4°C for 10 days before culture (17). Such pretreatment-induced stress on anthers plays a crucial role in impeding gametophytic development and initiating pollen embryogenesis in competent microspores. These findings was corroborated by observing improved embryogenic capacity with a 4-day cold pretreatment at 4°C in pepper hybrids (18).

Tab. 1. Effect of cold temperature pretreatment on callus induction and shoot differentiation from anther culture of marigold

Temprature (°C)	Callus induction rate (%)	Days to callus induction	Calli size (mm)	Shoot differentiation rate (%)
4	87.10[a]	17.00	31.02	76.83
7	84.95[b]	18.15	29.05	76.03
25	70.25[c]	18.75	25.97	66.40
SEm±	0.18	0.12	0.02	0.17
CD at 1 %	0.69	0.45	0.08	0.64

Tab. 2. Effect of cold temperature pretreatment duration on callus induction and shoot differentiation from anther culture of marigold.

Duration (hr)	Callus induction rate (%)	Days to callus induction	Calli size (mm)	Shoot differentiation rate (%)
1	80.40	17.40	28.98	74.87
24	84.73	17.27	29.41	74.96
72	86.53	17.73	30.65	75.48
120	71.40	19.46	25.69	67.04
SEm	0.21	0.13	0.02	0.19
CD at 1 %	0.79	0.52	0.09	0.74

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Tab. 3. Effect of cold temperature pretreatment and duration on callus induction and shoot differentiation from anther culture of marigold.

Temprature (°C) × duration (hr)		Callus induction rate (%)	Days to callus induction	Calli size (mm)	Shoot differentiation rate (%)
4 °C	1 hr	80.40	17.40	29.00	74.60
	24 hr	89.40	16.60	31.10	77.37
	72 hr	96.60	15.60	34.00	79.79
	120 hr	82.00	18.40	30.00	75.56
7 °C	1 hr	80.40	17.40	29.00	75.40
	24 hr	86.40	17.60	29.14	76.51
	72 hr	91.40	17.60	31.00	77.86
	120 hr	81.60	20.00	27.08	74.37
25 °C	1 hr	80.40	17.40	28.94	74.60
	24 hr	78.40	17.60	28.00	71.00
	72 hr	71.60	20.00	26.96	68.80
	120 hr	50.60	20.00	20.00	51.20
SEm		0.36	0.23	0.04	0.34
CD at 1%		1.38	0.90	0.16	1.29

Effect of heat stress pretreatment

Highest callus induction rate (76.80 %), minimum days to callus initiation (18.00 days), largest green colour calli (22.97 mm) and maximum shoot differentiation rate (58.90 %) was reported from anthers which were pretreated at 25 °C (Tab. 4). As the duration of heat pretreatment temperature increases, the callus induction rate, calli size and shoot differentiation rate decreases significantly. Maximum callus induction rate (80.40 %), minimum days to callus induction (17.13 days), largest size calli (28.98 mm) and maximum shoot differentiation rate (60.09 %) was reported when anthers were pretreated for 1 hr (Tab. 5). When interaction effect of heat pretreatment temperature with that of duration was analysed (Table 6), it was found that, maximum callus induction (80.40 %) was reported when anthers were pretreated at 25 °C, 35 °C and 45 °C for 1 hr. Minimum days to callus initiation (17 days) has been found when anthers were pretreated at 25 °C for 1 hr and this was found to be statistically at par with the treatment when anthers were heat pretreated at 35 °C and 45 °C for 1 hr (17.20 days). Maximum size of calli (29.00 mm) was reported in treatment where anther have been exposed to 25 °C, 35 °C for 1 hr and it was found to be statistically at par when anther were pretreated at 45 °C for 1hr (28.94 mm). Maximum shoot differentiation rate (60.26 %) was reported when anthers were pretreated at 25 °C and 35 °C for 1 hr. In the present study, the increase in heat pretreatment temperature and duration leads to less response of anther for adrogenesis and this may be due to reduction in microspore viability in high temperature (19). However, better response of *Dianthus chinensis* cultivar 'Carpet' anther culture was reported when flower bud was pretreated with 5-day heat shock (20). Similarly, 32 °C heat shock for 1 day to *Brassica napus* anther resulted better organogenesis and androgenesis (21).

Tab. 4. Effect of heat stress pretreatment on callus induction and shoot differentiation from anther culture of marigold.

Temprature (°C)	Callus induction rate (%)[1]	Days to callus induction	Calli size (mm)	Shoot differentiation rate (%)[2]
25	76.80	18.00	22.97	58.90
35	70.15	20.05	13.76	34.17
45	33.90	22.55	9.27	23.86
SEm	0.16	0.05	0.02	0.21
CD at 1 %	0.61	0.21	0.07	0.72

Tab. 5. Effect of heat stress pretreatment duration on callus induction and shoot differentiation from anther culture of marigold.

Duration (hr)	Callus induction rate (%)[1]	Days to callus induction	Calli size (mm)	Shoot differentiation rate (%)[2]
1	80.40	17.13	28.98	60.09
3	61.80	20.40	12.72	37.18
5	54.67	21.00	10.64	32.11
8	44.27	22.67	8.99	26.54
SEm	0.18	0.06	0.02	0.24
CD at 1 %	0.70	0.24	0.08	0.83

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Tab. 6. Effect of heat stress pretreatment and duration on callus induction and shoot differentiation from anther culture of marigold.

Temprature (°C) × duration (hr)		Callus induction rate (%)[1]	Days to callus induction	Callus size (mm)	Shoot differentiation rate (%)[2]
25 °C	**1 hr**	80.40	17.00	29.00	60.26
	3 hr	79.60	18.00	22.00	59.74
	5 hr	74.60	18.00	20.94	58.05
	8 hr	72.40	19.00	19.94	57.54
35 °C	**1 hr**	80.40	17.20	29.00	60.26
	3 hr	76.40	20.00	12.00	31.19
	5 hr	72.60	21.00	8.00	24.04
	8 hr	51.40	22.00	6.04	21.72
45 °C	**1 hr**	80.40	17.20	28.94	59.73
	3 hr	29.40	23.20	4.16	20.62
	5 hr	17.00	24.00	2.98	14.23
	8 hr	8.80	25.80	1.00	0.34
SEm		0.32	0.11	0.04	0.42
CD at 1 %		1.21	0.42	0.14	1.44

Effect of sucrose concentration

Among all the sucrose concentration treatment, maximum callus induction rate (69.74 %) was reported in media having 4% sucrose (Tab. 7). Media with 0% sucrose and 12% sucrose recorded no callus initiation. The findings of this study align closely with those of Yingchun et al. (2011), who observed the highest rate of callus induction at a sucrose concentration of 3%. Moreover, they noted a gradual reduction in the rate of callus induction with increasing sucrose concentrations. A parallel discovery was documented in periwinkle anther culture (22).

Effect of dark treatment

In the present experiment, maximum value for callus induction (97.33%) rate was observed in treatments where anther were incubated in dark for 4, 5 and 6 weeks (97.00%) (Tab. 8). Additionally, it was noted that calli exhibited faster growth in darkness compared to continuous exposure to light. Exposure of anther culture plates to continuous light (designated as '0 week' of dark treatment) resulted in a minimal callus induction rate of 27.67% and the formation of small calli, measuring 8 mm. A similar outcome was documented in marigold (13).

Tab. 7. The effect of different concentration of sucrose on callus induction, days to callus induction, size and type of calli from anther culture of marigold

Sucrose concentration	Callus induction rate (%)[1]	Days to Callus Induction	Callus size	Callus growth pattern
0%	0.29[f]	0.00[e]	0.00[e]	Anther dried and became black
2%	66.69[b]	19.67[d]	30.33[b]	yellowish green callus
4%	69.74[a]	20.33[cd]	32.00[a]	White compact callus
6%	31.73[c]	21.00[bc]	5.67[c]	white calli
8%	17.12[d]	21.33[b]	1.00[d]	White calli with no shoot differentiation
10%	6.54[e]	22.67[a]	1.00[d]	White calli with no shoot differentiation
12%	0.29[f]	0.00[e]	0.00[e]	Anther remain greenish yellow for 2 week and no callus initiation

Tab. 8. The effect of different dark duration on callus induction rate (%), days to callus induction, size of calli and shoot differentiation rate from anthers culture of marigold

Dark period (week)	Callus induction rate (%)[1]	Days to callus induction	Callus size	Shoot differentiation rate (%)[2]
0	27.67[d]	23.67 [a]	8.00 [f]	25.22 [e]
1	75.00[c]	21.67 [b]	11.33 [e]	36.90 [d]
2	79.67[b]	20.33 [c]	20.33 [d]	73.22 [b]
3	97.00[a]	17.33 [d]	30.00 [c]	82.82 [a]
4	97.33[a]	17.33 [d]	34.07 [b]	70.55 [c]
5	97.33[a]	17.33 [d]	34.67 [b]	10.27 [f]
6	97.33[a]	17.33 [d]	37.33 [a]	8.21 [f]

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Determination of ploidy level by cytological analysis

Cytological analysis revealed 6% of anther regenerants as haploid, 6% as triploid, 2% as mixoploid and the rest as diploid (Fig. 2). Different ploidy level in anther regenerated population has also been reported in *Lilium formosanum* (23), gentian (24), bamboo (25), marigold (13) and potato (26). The recovery of haploid plantlets in the current experiment undoubtedly stemmed from embryogenesis originating from the microspores of the cultured anther. However, the phenomenon of generating plantlets with varying ploidies observed in this research is multifaceted. Hence, enhancing the efficiency of haploid regeneration through anther culture in this crop necessitates further investigation.

Future scope of study

There are numerous factors influencing the outcome of in vitro androgenesis. In addition to those studied in the present investigation, other variables such as donor plant growth conditions, genotype, media type, and composition also play crucial roles in directing the gametophytic pathway towards the sporophytic pathway. Therefore, future studies focusing on these factors in *Tagetes* spp. could prove highly beneficial. Developing a protocol for microspore culture in this crop holds considerable promise for future advancements.

Author contribution statement

Dr. Thaneshwari and Dr. Aswath C has contributed for research work in lab. Dr. Aswath, Dr. Thaneshwari, Dr. Tejaswini Prakash, Dr. Ab Waheed Wani and Glomeraz Abdi has contributed for writing the manuscript.

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Conflict of Interest

The authors declare that they have no conflict of interest.

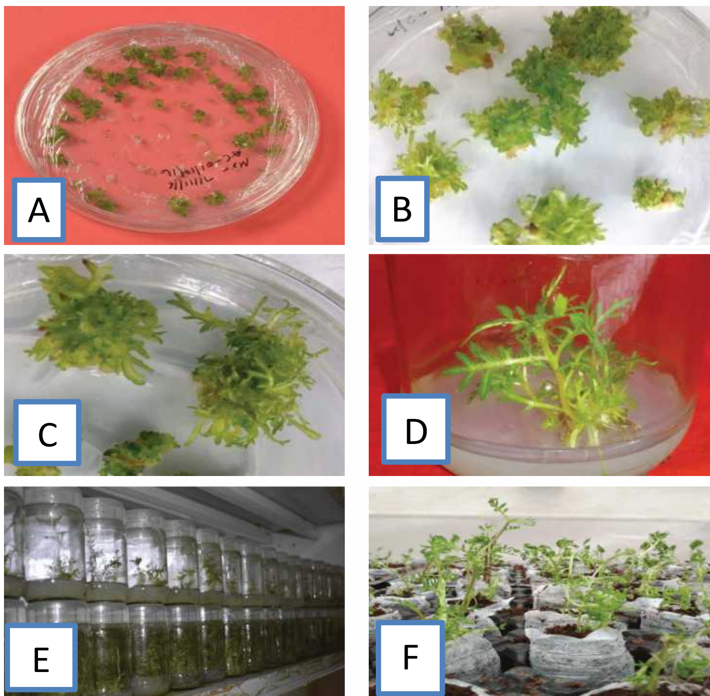

Fig. 1 Callus induction, embryogenesis and plantlet differentiation from anther of marigold pretreated at 4 °C for 72 hr and cultured on MS media supplemented with 4.44 µM BAP and 1.07 µM NAA.

(A) Embryogenic calluses induced from anthers of marigold; (B, C) subculture and 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.

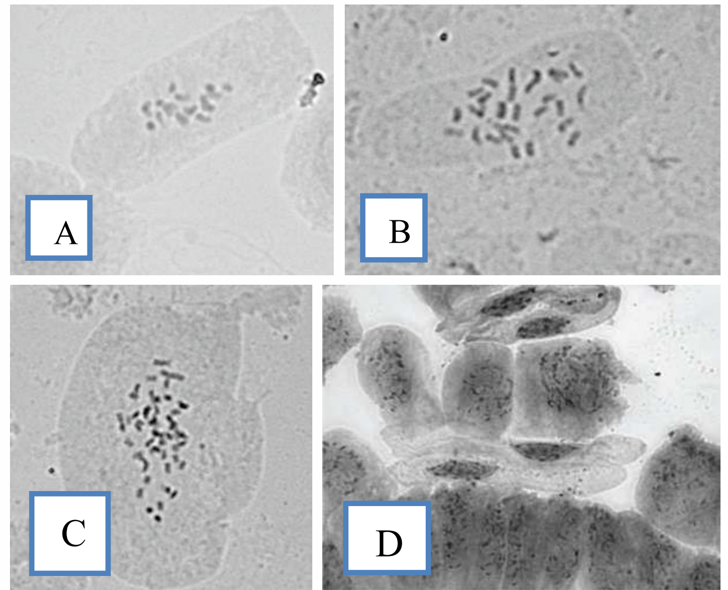

Fig. 2 Images of marigold metaphase stage chromosomes (from root tips of regenerated plants); A) An image of 12 chromosomes (haploid); B) An image of 24 chromosomes (diploid); C) An image of 36 chromosomes (Triploid); D) An image of 12 and 36 chromosomes in different cells of same root tip (mixoploid).

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