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Effect of cold pre-treatment on callus induction and shoots regeneration in ovary culture of marigold (*Tagetes* spp)

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Abstract
Marigold (*Tagetes* spp) is recognized to have potential usage in medicinal and pharmacological industries in addition to its use as an ornamental plant. The aim of the experiment was to investigate the effect of cold pre-treatment stress on the callus induction and its subsequent shoot differentiation in ovary culture of marigold cultivar Arka Agni. Ovaries from flower bud pretreated at 4 °c, 7 °c and 25 °c for 1, 24, 72 and 120 hrs were cultured on to callus induction and shoot differentiation media respectively. It was found that 7 ^{0}C temperature pre-treatment to be statistically superior for % callus induction (84.58 %), as well 4 ^{0}C temperature pre-treatment for minimum days to callus induction (20.30 days) and maximum shoot differentiation rate (10.52 %) followed by 7 ^{0}C pre-treatment. Whereas, in case of investigating on duration of exposure of flower buds to different cold pre-treatment, maximum callus induction (89.66 %) was reported from both 7 ^{0}C and 4 ^{0}C pretreated buds but duration of exposure varies 1hr and 24 hr respectively. However, flower buds pretreated at 4 for 24 hr achieved callus initiation in minimum days (20.00 days) and also resulted in highest shoot differentiation rate (14.15 %). Nevertheless, there is need for testing of the plantlets produced via gynogenesis for genetic variation as well as for the ploidy level.

Keywords: ovary culture, gynogenesis, cold pre-treatment, plant regeneration, *Tagetes erecta,* haploids

1. Introduction

Marigold (*Tagetes* spp) belongs to family Asteraceae and is recognized as a potential source of carotenoids. The essential oil from *Tagetes erecta* flowers is used in perfumery and its extract is used for coloration of 100 % cotton, silk and wool fabrics. In India, at present the area under marigold is 55.89 thousand hectare with a production of 511.31 thousand metric tons. Its productivity varies from 2 tons per acre in Uttar Pradesh to 8.2 tons per acre in Karnataka (National Horticulture Board, 2015). While a hybrid, Arka Agni, released by Indian Institute of Horticultural Research, Bangalore yields up to18 tons per acre. This variation in marigold productivity is due to its heterozygous nature that even hampers improvement of this valuable species. Producing homozygous lines by conventional methods is time consuming and difficult, because this species prefer cross pollination. However, production of homozygous lines by doubling the chromosome number of haploid lines produced through androgenesis and gynogenesis is a better alternative. The prospective of haploidy for crop improvement arose in 1964 with the success of androgenesis in Datura (Guha and Maheshwari, 1964, 1966) [1, 2]. Haploid embryos and plantlets can be successfully obtained by androgenesis (ovary/microspore culture) or gynogenesis (ovary/ovule culture), based on the type of cells targeted. One of the best-known methods for production of haploids in ornamental crops is ovary culture. As in androgenesis, gynogenic haploids may develop directly or indirectly via regeneration from the callus. It is used for production of haploids in species like wheat, maize, rice, onion, sugar beet, cucumber, carnation, squash, gerbera, sunflower, wheat, and barley etc (Yang and Zhou 1982; Sibi *et al*. 2001; Tang *et al*. 2006; reviewed by Chen *et al*., 2011) [3, 4, 5, 6]. Gynogenesis has not been investigated as thoroughly or with as many species as androgenesis; therefore, less information is available concerning the various factors that contribute to the successful production of haploids from the female than the male gametophyte. Thus, the major problems affecting the use of gynogenesis are the lack of established protocols for most species, poor yields, and production of diploid or mixoploid plants. Therefore, the objective of the present study was to investigate the effect of cold pre-treatment and its duration on callus induction and shoot differentiation in the ovary culture of marigold.

2. Materials and methods

2.1 Plant Material

Marigold cultivar 'Arka Agni' plants were grown using standard package and practice.

2.2 Effect of cold pre-treatment on callus induction and shoot differentiation rate

Healthy and young flower buds of length 20 mm were collected from the shade net house during morning hours between 9:00 am to 09:30 am were wrapped in tissue paper and aluminium foil, finally, sealed in the polythene bags separately. These wrapped flower buds were then stored in refrigerator at 4 °C, 7 °C and in growth chamber for 25 °C to different exposure time 1 hr, 24 hr, 72 hr and 120 hr and then ovaries were excised from buds for culturing.

These cold pretreated buds were placed under running tap water for 15 minutes to remove dirt and dust adhering to them. Under aseptic conditions, these buds were surface sterilised with 0.1% carbendazim for 15 minutes, followed by rinsing with sterile distilled water. These buds were then disinfected by dipping in 70% ethanol for 30 seconds and in 1% NaOCl for 5 minutes, followed by rinsing with sterilized distill water for 3 times and finally, blotted dry on aseptic filter papers. Ovaries were precisely excised from the florets, cultured immediately on to MS media (Murashige & Skoog, 1962) [7] supplemented with 4.44 µM BAP and 4.52 µM 2,4 D for callus induction and maintained at 25 ± 1 ^{0}C in darkness until callus formed. Further, calli were transferred to MS regeneration medium supplemented with 4.44 µM BAP and 1.07 µM NAA and maintained at 25 ± 1° C under 16 h/8 h light/dark period. Finally, the regenerated shoots were transferred to hormone free MS medium for rooting. The experiment had twelve treatment combinations (4 °C, 7 °C and 25 °C for 1, 24, 72, 120 hr) tested using factorial complete random design, with 5 replicates for each type of treatment and seventy ovaries in each plate were placed.

2.3 Data Collection and Analysis

The ovary cultured plates were examined and data was recorded on percentage of callus induction, days to callus initiation and size of callus. However, days to callus initiation was recorded when 50% of the ovaries callused per plate and the size of the callus was recorded 25 days after ovary culture. The callus induction and regeneration frequencies were calculated as callus induction rate (%) = number of ovaries producing calli/number of ovaries plated x 100; shoot differential rate (%) = number of calli differentiated in to shoot/ number of calli transferred x 100. Finally, analysis of variance (ANOVA) was conducted using Web Based Agricultural Statistics Software (WASP).

3. Result

3.1 Effect of cold pre-treatment on callus induction and shoot differentiation rate

Cold pre-treatment at 7 ^{0}C to marigold flower buds was found to be significantly superior for callus induction rate (84.58 %), while buds pretreated at 4 ^{0}C took minimum days for callus induction (20.30 days). However, largest calli size was reported in flower buds exposed to 25 ^{0}C and as the temperature decreased from 25 ^{0}C to 4 ^{0}C, size of calli decreased significantly. In addition, pre-treatment of buds at 4 ^{0}C was found to be statistically superior for % shoot differentiation rate (10.52 %) but with the increase in the temperature of pre-treatment from 4 ^{0}C - 25 ^{0}C, shoot differentiation frequency decreased drastically (Table 1).

3.2 Effect of cold pre-treatment duration on callus induction and shoot differentiation rate

Cold pre-treatment for 1 hr. was found to be ideal for callus induction rate as it significantly increased the induction rate of callus, reaching the maximum of 89.66 %. Nonetheless, with the increase in duration from 1 hr to 120 hrs, callus induction rate decreased significantly. Further, minimum days to callus initiation was reported from ovaries cold pretreated for 24 hrs (20.33 days), while maximum calli size (7.98 mm) was reported when ovaries were cold pretreated for 1 hr that was found to be statistically superior to all other treatments. Besides, shoot formation rate increased as ovaries were cold pretreated for 1 to 24 hrs, while prolonged cold pre-treatment (120 hrs) dramatically decreased the potential of ovaries to produce shoots, but increased the frequency of callusing, however ovaries turned necrotic. Therefore, buds exposed to 24 hrs cold pre-treatment showed highest shoot differentiation rate (10.60 %) that was statistically superior to all other treatments, while flower buds cold pretreated for 120 hrs reported minimum shoot differentiation of 2.98 % (Table 2 & Figure 1).

Table 1: Effect of cold pre-treatment temperature to flower buds on callus induction in unfertilized ovary culture of marigold cv. 'Arka Agni'

Temperature (°C)	callus induction Rate (%)[a]	Days to callus induction	Size of callus (mm)	Shoot differentiation Rate (%)[b]
4	81.09	20.30	3.50	10.52
8	84.58	20.70	6.65	7.15
16	76.67	21.65	6.75	3.76
SEm ±	0.16	0.05	0.03	0.03
CD at 5 %	0.46	0.15	0.08	0.09

Table 2: Effect of cold pre-treatment duration to flower buds on callus induction in unfertilized ovary culture of marigold cv. 'Arka Agni'

Duration (hr)	callus induction Rate (%)[a]	Days to callus induction	Size of callus (mm)	Shoot differentiation Rate (%)[b]
1	89.66	21.00	7.98	8.38
24	84.66	20.33	6.65	10.60
72	76.58	20.80	4.06	6.61
120	72.22	21.40	3.83	2.98
SEm ±	0.18	0.06	0.03	0.04
CD at 5 %	0.54	0.17	0.09	0.11

[a]**Callus Induction Rate (%)** = (No. of callus formed / No. of ovaries inoculated) × 100.
[b]**Shoot Differentiation Rate (%)** = (No. of callus generating shoots in differentiation medium / No. of callus inoculated) × 10

Table 3: Effect of cold pre-treatment temperature and duration to flower buds on callus induction in ovary culture of marigold cv. 'Arka Agni'

Temperature (°C)× duration (hr)		callus induction Rate (%)[a]	Days to callus induction	Size of callus (mm)	Shoot differentiation Rate (%)[b]
4°C	1 hr	89.66	21.00	8.00	8.52
	24 hr	89.66	20.00	3.00	14.15
	72 hr	76.32	20.00	1.50	11.29
	120 hr	68.71	20.00	1.50	8.13
8°C	1 hr	89.66	21.00	7.96	8.52
	24 hr	89.66	20.00	9.94	11.54
	72 hr	81.11	20.80	4.70	8.13
	120 hr	77.91	21.00	4.00	0.41
16°C	1 hr	89.66	21.00	8.00	8.13
	24 hr	74.68	21.00	7.00	6.11
	72 hr	72.32	21.60	6.00	0.41
	120 hr	70.04	23.00	6.00	0.41
SEm ±		0.32	0.11	0.06	0.06
CD at 5 %		0.93	0.31	0.17	0.18

[a]**Callus Induction Rate (%)** = (No. of callus formed / No. of ovaries inoculated) × 100.
[b]**Shoot Differentiation Rate (%)** = (No. of callus generating shoots in differentiation medium / No. of callus inoculated) × 100.

Fig 1: Callus induction and shoot regeneration **a)** Ovaries excised from cold pre-treated flower buds **b)** Callus initiation **c)** Shooted and rooted plantlet of marigold

4. Discussion

Successful recovery of haploids and doubled haploids in ovule/ovary culture depends on several factors, including the genotypes of donor plants, low/high temperature pre-treatment, female gametophyte developmental stages, growth regulators or other components of media, and culture conditions. Many experiments have shown that cold-pre-treatment of flower buds has a strong influence on *in vitro* gynogenesis (Lakshmi Sita 1996; Forster *et al*. 2007; Shalaby, 2007) [8, 9, 10]. In the present experiment, it was found that pre-treatment to flower buds at 7 °C was statistically superior for callus induction, whereas 4 °C pre-treatment for shoot differentiation rate. However, pre-treatment of flower buds at 4 °C for 24 hrs resulted in maximum callus induction rate with minimum days to callus induction, as well maximum shoot differentiation rate. Similar results were reported by [11] Pathirana *et al* in 2011 that pre-treatment at 4 °C for 48 hr to *G. triflora* ovaries enhanced its gynogenic response. They also observed most prolific root formation in *G. triflora* from the same treatment. Likewise, flower buds pretreated at 4 °C for 4-5 days increased the development of haploid embryos in cultured ovules of *Beta vulgaris* (Ferrant and Bouharmont 1994) [12]. Similarly, [13] Malik *et al* in 2011 reported that melon ovaries exposed to 4 °C for 4 days produced significantly high number of gynogenic ovules (63.3 %). On the contrary, [14] Bhat and Murthy in 2007 did not observe any beneficial effects of cold-pre-treatment (at 4 °C) in Niger on gynogenesis as compared to the control; rather it significantly decreased the embryogenic potential of unpollinated ovules. Likewise, [15] Rakha *et al*. in 2012 also reported that cold pre-treatment had negative effects on gynogenesis in six interspecific cucurbita hybrids. Also, prolonged cold pre-treatment apparently has detrimental effect on gynogenic responses, as reported by Malik *et al* in 2011 where they observed no gynogenic responses in melon ovaries pre-treated at 4 °C for 8 days.

5. Conclusion

In summary, the present investigation resulted into flower buds cold pretreated at 4 °C for 1 hr and24 hr), 7 °C (1 hr

and24 hrs) reported highest callus induction rate (89.66 %), while minimum days to callus induction (20.00 days) was reported from flower buds pretreated at 4 °C for 24 hrs, 72 hrs and 120 hrs respectively and 7 °C for 24 hrs. Furthermore, largest calli size (9.94 mm) resulted from buds pretreated at 7 °C for 24 hr. However, 4 °C pre-treatment for 24 hrs resulted in highest shoot differentiation rate (14.15 %) which was found to be statistically superior to all other treatment combinations followed by treatment combination of 7 °C for 24 hrs (11.54 %). To conclude flower buds pretreated at 4 °C for 24 hrs showed maximum callus induction rate, minimum days to callus induction as well as maximum shoot differentiation rate in *in vitro* ovary culture of marigold. Yet, there is need for testing of the plantlets obtained via gynogenesis for genetic variation as well as for the ploidy level, as ability for gynogenesis is highly genotypic dependent; therefore more studies involving different marigold cultivars are required.

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