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Protocol for *in vitro* regeneration and rapid mass multiplication of apetalous male sterile lines of marigold

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ABSTRACT

The apetalous form of male sterility, controlled by a single recessive gene (msms), is widely used for F1 hybrid seed production in marigold (Tagetes spp.). Maintenance of this form of male sterility through conventional methods is highly labour-intensive and costly. Hence, we have developed a viable in vitro regeneration protocol for rapid multiplication in three apetalous male sterile lines of marigold, MS-5, MS-7, and MS-8. The effect of Thidiazuron (TDZ) was assessed on in vitro establishment of apetalous male sterile lines using shoot-tip explants. The modified MSm medium supplemented with 1.5 mg/l TDZ and 0.2 mg/l NAA was suitable for culture establishment from shoot tip explants. We have also assessed the efficiency of two cytokinins, TDZ and Kinetin (KIN), for rapid multiplication of male sterile lines of marigold. The culture media, genotypes and their interactions significantly influenced the shoot proliferation of marigolds. Our findings have revealed the superiority of kinetin over TDZ in inducing quality shoots during in vitro proliferation phase. The highest proliferation of quality shoots was obtained on a modified MSm medium enriched with 0.5 mg/l kinetin and 0.1 mg/l NAA. Half-strength MS medium supplemented with 0.5 mg/l of IBA was found effective for the induction of quality roots in all the tested genotypes of marigold. Among the three male sterile lines of marigold, MS-5 exhibited better in vitro establishment, proliferation and rooting abilities over the MS-7 and MS-8 lines. Therefore, the developed protocol can be efficiently utilized for rapid in vitro mass multiplication of apetalous male sterile lines of marigold.

Keywords: *Tagetes erecta* L., Apetalous male sterility, Thidiazuron, *In vitro* proliferation, Shoot tip culture, Growth regulators.

INTRODUCTION

Marigold (*Tagetes spp*.) is a popular ornamental crop belonging to the *Asteraceae* family. The genus *Tagetes* is comprised of around 55 species (Godoy-Hernandez and Miranda-Ham, 6), among them only two species namely, *Tagetes erecta* L. (African marigold) and *Tagetes patula* (French marigold) are commercially cultivated. In recent years, F_1 hybrids are gaining tremendous popularity among marigold growers owing to their superior floral and vegetative characteristics. In India, the genetic potential of marigold remained relatively under-explored, and the majority of F_1 hybrid seeds are being imported from other countries (Kumar *et al*., 12). The discovery of male sterility in this crop have broadened the possibilities for improved hybrid seed production. Based on their floral morphology, marigold has three distinct male sterility systems; apetaloid (Flowers without petals), petaloid (Double flower types), and gyno-monoecious types (Tejaswini *et al.,* 16). The apetalous form of male sterility is commercially exploited for hybrid seed production in marigold due to its greater reliability and ease of pollination. The apetalous form of male sterility in marigold is genic male sterility and is reported to be controlled by a single recessive gene (*msms)* in the nucleus (He *et al.,* 9). The use of genetic male sterility in commercial hybrid seed production is often limited due to the resources required to maintain both sterile and fertile plants till they can be distinguished at flowering stage. Conventionally, the apetalous male sterile (*msms*) lines of marigold are maintained by crossing them with heterozygous (*Msms*) maintainers, which give both male sterile and fertile plants in a 1:1 ratio. As a result, only 50% of the male sterile plants can be exploited for hybrid seed development and the remaining 50% of the male fertile plants needed to be rogued out from the female line at the flowering stage.

The traditional method of maintaining male sterile lines is a time and space-consuming, and labor-intensive process that can significantly raise the cost of marigold hybrid seed production. As a result, alternative methods for multiplying male sterile lines by vegetative means must be investigated to avoid the expense of rouging fertile plants (Gupta *et al*.,

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8). *In vitro* plant tissue culture techniques could be of great assistance, particularly in the maintenance of male sterile parental lines (Bhatia *et al*., 2). Numerous factors such as species, genotype, culture medium, salts, organic matter, PGRs and environmental conditions play a significant role in vitro regeneration (Ahmad *et al*., 1). Thidiazuron (TDZ), a phenyl-urea-based plant growth regulator has emerged as an effective growth regulator in cell and tissue cultures in a wide array of plant species (Guo *et al*., 7; Chen and Wei, 4). However, this hormone has not been widely utilized in the micropropagation of marigold. Choosing the optimal concentration and type of plant growth regulator is a critical step for efficient regeneration in any crop (Chen and Wei, 4; Jones *et al*., 10). Hence, the present study was undertaken to optimize the explants and TDZ concentrations for rapid regeneration and mass proliferation of apetalous male sterile lines of marigold.

MATERIALS AND METHODS

The research work was undertaken at the Division of Floriculture and Landscaping, ICAR-Indian Agricultural Research Institute (IARI), New Delhi from 2020 to 2021. Three apetalous male sterile lines of African marigold namely, MS-5, MS-7, and MS-8 were chosen as mother plants to undertake the different experiments. The shoot tip explants were collected from healthy mother plants after the initiation of flowering when male-sterile male-fertile plants were easily identifiable. The well-prepared explants were surface sterilized in 0.2 percent mercuric chloride ($HgCl_2$) solution for 5 minutes and then subsequently, washed 4-5 times using autoclaved double-distilled water to remove any traces of toxic remnants of the surface sterilizing agent.

The salient characteristics of the three apetalous male sterile lines of African marigold are mentioned below.

S. No.	Genotypes/ Lines	Flower colour	Flower form	Season
1.	MS-5	Yellow	Apetalous	Rainy & Winter
2.	MS-7	Light Orange	Apetalous	Rainy & Winter
3.	MS-8	Dark Orange	Apetalous	Rainy & Winter

Modified MS^m medium comprising of MS (Murashige and Skoog, 13) basal medium enriched with 125 mg/l of Polyvinylpyrrolidone (PVP), 0.2 mg/l GA_3, 30 mg/l sucrose and 2.6 mg/l gelrite was used as basal media to conduct all the experiments. All the media were sterilized by steam sterilization in an autoclave at 121 ºC for 22 minutes at 15 lbs/inch^2 pressure. The thermolabile components of the media including Thidiazuron (TDZ) were sterilized through filter sterilization using 0.22 µm syringe filters. The autoclaved culture media was allowed to cool to 60ºC prior to the addition of TDZ. The effects of different doses of TDZ (0.0, 0.5, 1.0 & 1.5 mg/l) in combination with 1-Naphthaleneacetic Acid (NAA: 0.0, 0.2 & 0.5 mg/l) were assessed on *in vitro* establishment from different explants in all the three apetalous male sterile lines of marigold.

We have also assessed the efficiency of two cytokinins, TDZ and Kinetin (KIN), for rapid multiplication of male sterile lines of marigold. The regenerated micro-shoots were sub-cultured on Modified MS^m medium supplemented with different concentrations of TDZ (0.0, 0.25, 0.5 & 0.75 mg/l), KIN (0.0, 0.25, 0.5 & 0.75 mg/l) in combination with NAA (NAA: 0.0, 0.1 & 0.2 mg/l) for shoot proliferation. The well-developed shoots were transferred individually to the rooting medium consisting of ½ strength solidified basal MS medium supplemented with 45 gl^{-1} sucrose and different concentrations of IBA (0.0, 0.25, 0.5 & 1.0 mg/l) and NAA (0.0, 0.25, 0.5 & 1.0 mg/l) either alone or in combinations. All the cultures were maintained at 25±1 °C temperature with a photoperiod of 16:8 hours of light and dark cycles under fluorescent white light (47µmol/m²/s). The *in vitro* rooted plants were gradually hardened in plastic pots covered with a polythene cover and then shifted to field conditions.

The experiments were laid out in a completely randomized design (CRD). The data were subjected to standard analysis of variance (ANOVA) to test significance among different lines and media. Comparisons among treatment means were carried out using least square difference (LSD) values and are reported under a critical difference (CD) at the end of each table. Each treatment consisted of three replications with 10 explants/shoots were used in each replication during the establishment, proliferation and rooting stages.

RESULTS AND DISCUSSION

The modified MS^m media enriched with different levels of growth regulators significantly improved the culture establishment from shoot tip explants over the control in all the three apetalous male sterile lines (Table1 & 2). The maximum culture establishment with significantly early sprouting was obtained on medium fortified with 1.5 mg/l TDZ and 0.2 mg/l NAA (T_3). While, the maximum number of micro shoots were induced in T_2 – MS^m + 1.0 mg/l TDZ + 0.2 mg/l NAA (3.0 shoots/ explant). The shoot tip explants cultured on medium devoid of any hormones exhibited the minimum culture establishment, delayed emergence of shoots,

Table 1. Effects of growth regulators on culture establishment and days to shoot initiation in apetalous male sterile lines of marigold.

Concentration of growth regulators (mg/l)**			Culture Establishment from shoot tips (%)				Days taken for shoot initiation (days)			
	TDZ	NAA	MS-5	MS-7	MS-8	Mean	MS-5	MS-7	MS-8	Mean
T_0	-	-	55.55 (48.17)*	42.22 (40.50)*	44.45 (41.78)*	47.41 (43.48)*	11.31	12.30	12.48	12.03
T_1	0.5	0.2	60.00 (50.78)*	48.89 (44.33)*	64.44 (53.49)*	57.78 (49.53)*	7.48	9.16	8.66	8.43
T_2	1.0	0.2	64.45 (53.39)*	55.55 (48.17)*	66.67 (54.78)*	62.22 (52.11)*	5.64	7.58	6.38	6.53
T_3	1.5	0.2	77.78 (61.90)*	64.45 (53.39)*	73.33 (59.00)*	71.85 (58.09)*	4.20	5.50	5.80	5.16
T_4	0.5	0.5	62.22 (52.10)*	51.11 (45.63)*	62.22 (52.10)*	58.52 (49.94)*	8.04	8.71	8.73	8.49
T_5	1.0	0.5	68.89 (56.10)*	57.78 (49.49)*	68.89 (56.10)*	65.19 (53.90)*	6.72	6.39	7.06	6.72
T_6	1.5	0.5	75.55 (60.39)*	66.67 (54.78)*	71.11 (57.49)*	71.11 (57.55)*	5.21	6.78	5.68	5.89
Mean	-	-	66.35 (54.69)*	55.24 (48.04)*	64.45 (53.53)*		6.94	8.06	7.83	
			C D (p=0.05)		SEm±		C D (p=0.05)		SEm±	
Genotype			3.71		1.30		0.46		0.16	
Treatment			5.67		1.98		0.70		0.24	
Genotype × Treatment			N/S		3.43		N/S		0.42	

(* Arc sin transformed data)
**MS^m: MS+ PVP (125 mg/l) + GA_3 (0.2 mg/l)

Table 2. Effects of growth regulators on number of shoots induced and mean length of shoots induced from shoot tip explants in apetalous male sterile lines of marigold.

Concentration of growth regulators (mg/l)*			Number of shoots induced per shoot tip explant				Mean length of shoots (cm)			
	TDZ	NAA	MS-5	MS-7	MS-8	Mean	MS-5	MS-7	MS-8	Mean
T_0	-	-	1.44	1.33	1.22	1.33	1.00	0.80	0.70	0.83
T_1	0.5	0.2	2.78	2.00	2.44	2.41	1.24	1.10	1.13	1.16
T_2	1.0	0.2	3.56	2.55	2.89	3.00	1.73	1.20	1.42	1.45
T_3	1.5	0.2	3.11	2.67	2.44	2.74	2.42	2.00	2.08	2.17
T_4	0.5	0.5	2.56	1.89	2.33	2.26	1.09	0.90	0.90	0.96
T_5	1.0	0.5	3.22	2.33	2.78	2.78	1.63	1.10	1.33	1.36
T_6	1.5	0.5	2.33	2.11	1.96	2.13	1.88	1.54	1.69	1.71
Mean	-	-	2.71	2.13	2.30		1.57	1.24	1.32	
			C D (p=0.05)		SEm±		C D (p=0.05)		SEm±	
Genotype			0.30		0.10		0.03		0.01	
Treatment			0.45		0.16		0.04		0.01	
Genotype × Treatment			N/S		0.28		0.07		0.02	

**MS^m: MS+ PVP (125 mg/l) + GA_3 (0.2 mg/l)

minimum number of shoots and the shortest shoot length. TDZ, a substituted phenyl urea-type cytokinin has been established as an important regulator for morphogenic responses, *in vitro* adventitious bud induction and shoot organogenesis (Ahmad *et al*., 1). This hormone appears to mimic cytokinin-like activity and thus helpful in the release of lateral buds. TDZ have shown better response in terms of shoot regeneration efficiency, compared to other cytokinins. The effectiveness of TDZ have been proved in the micropropagation of *Stevia rebaudiana* (Singh and Dwivedi, 15) and *Matricaria chamomilla* L. (Ahmad *et al*., 1).

Significant genotypic variations were observed among the three apetalous male sterile lines of marigold in their *in vitro* establishment and regeneration ability from shoot tip explants (Table 1 &2). The culture establishment ranged from 55.24 % in MS-7 to 66.35 % in MS-5 line. Among the three apetalous male sterile lines, MS-5 showed better culture establishment response than MS-8 and MS-7. Such genotypic variation during culture establishment phase have been noticed earlier by Kumar *et al*., (11) in marigold. Genotypes specific response of TDZ for shoot differentiation have also been reported in *Dieffenbachia* (Shen *et al*., 14).

The culture media, genotypes and media × genotype interactions play a significant role in the mass proliferation of marigold. MS^m media enriched with different levels of TDZ, KIN and NAA significantly improved the shoot proliferation over the control T_0 in all the tested genotypes (Table 3 & Table 4). In TDZ supplement media, the earliest initiation of shoots with the highest shoot proliferation was obtained in the T_2 medium containing TDZ (0.5 mg/l) and NAA (0.1 mg/l). A linear increase in the number of shoots was observed as the level of TDZ was increased from 0.25 to 0.5. However, increasing the levels of TDZ further to 0.75 severely reduced the shoot proliferation and lead to callus formation at the basal ends of shoots. This effect was more pronounced when a higher level of TDZ was coupled with a high concentration (0.2 mg/l) of NAA. The role of TDZ is well-established for shoot induction and multiplication in *Echinacea purpurea* (Jones *et al*., 10). Guo *et al*., (7) have also noticed that as compared to other chemicals having cytokinin-like activity, TDZ promotes shoot multiplication in several plants only when used in lower amounts. TDZ has been reported to cause phytotoxicity when used at higher concentrations and thus reduce multiplication rates. TDZ at high concentrations were shown to inhibit shoot proliferation in *Spathiphyllum cannifolium* (Dewir *et al*., 5) and shoot elongation in *Aglaonema* (Chen and Wei, 4). In KIN-supplemented media, the earliest shoot initiation and the maximum number of micro shoots induced per shoot were recorded in the T_2 treatment comprising of KIN (0.5 mg/l) and NAA (0.1 mg/l) (Table 4). KIN was proved effective for multiple shoot induction only when used at lower concentrations. While supplementation with higher levels of KIN (> 0.5 mg/l) and NAA (> 0.1 mg/l) led to a significant reduction in the number of shoots and simultaneously increased callus formation at the base of micro shoots. The favourable effects of cytokinins on shoot meristem initiation, axillary

Table 3. Effects of TDZ and NAA on in *vitro* mass proliferation of apetalous male sterile lines of marigold.

Concentration of growth regulators (mg/l)			Days to shoot initiation (days)				Average no. of shoots				Mean length of shoots (cm)			
Treatments	TDZ	NAA	MS-5	MS-7	MS-8	Mean	MS-5	MS-7	MS-8	Mean	MS-5	MS-7	MS-8	Mean
T_0	-	-	10.27	10.87	10.73	10.62	1.11	1.11	1.22	1.15	3.31	2.33	2.78	2.81
T_1	0.25	0.1	7.33	7.80	7.87	7.67	2.67	2.22	2.22	2.37	3.20	2.57	2.51	2.76
T_2	0.50	0.1	5.07	5.67	5.47	5.40	4.22	2.78	3.22	3.41	4.76	3.82	4.10	4.23
T_3	0.75	0.1	5.73	6.40	6.27	6.13	3.55	2.11	2.67	2.78	4.01	3.00	3.12	3.38
T_4	0.25	0.2	6.73	7.13	7.00	6.96	3.33	2.33	2.78	2.81	3.46	2.88	2.89	3.08
T_5	0.50	0.2	5.47	6.07	5.73	5.76	3.67	1.67	2.33	2.56	4.51	3.51	3.56	3.86
T_6	0.75	0.2	9.13	9.60	9.53	9.42	2.56	1.44	1.89	1.96	2.87	2.24	2.43	2.51
Mean			7.11	7.65	7.51		3.02	1.95	2.33		3.73	2.91	3.06	
			SEm±		C D (p=0.05)		SEm±		C D (p=0.05)		SEm±		C D (p=0.05)	
Genotype			0.11		0.3		0.10		0.28		0.02		0.07	
Treatment			0.16		0.46		0.15		0.42		0.04		0.10	
Genotype × Treatment			0.28		N/S		0.25		N/S		0.06		0.18	

*MS^m: MS+ PVP (125 mg/l) + GA_3 (0.2 mg/l)

Table 4. Effects of KIN and NAA on in *vitro* mass proliferation of apetalous male sterile lines of marigold.

Concentration of growth regulators (mg/l)			Days to shoot initiation (days)				Average no. of shoots				Mean length of shoots (cm)			
Treatments	KIN	NAA	MS-5	MS-7	MS-8	Mean	MS-5	MS-7	MS-8	Mean	MS-5	MS-7	MS-8	Mean
T_0	-	-	10.00	10.93	10.20	10.38	1.33	1.22	1.44	1.33	3.42	2.82	2.67	2.97
T_1	0.5	0.1	4.53	5.53	5.03	5.03	4.78	3.78	4.00	4.19	5.02	4.02	4.25	4.43
T_2	1.0	0.1	4.93	5.67	5.20	5.27	4.33	3.44	3.78	3.85	4.67	3.63	3.70	4.00
T_3	2.0	0.1	6.07	6.93	6.33	6.44	2.78	1.67	2.44	2.30	3.79	3.11	3.22	3.37
T_4	0.5	0.2	5.60	6.13	5.73	5.82	3.78	2.67	3.00	3.15	4.22	3.34	3.50	3.68
T_5	1.0	0.2	7.13	7.73	7.33	7.40	2.45	2.00	2.33	2.26	3.48	2.67	2.67	2.94
T_6	2.0	0.2	9.07	10.00	9.20	9.42	2.33	1.44	1.78	1.85	2.95	2.54	2.52	2.67
Mean	-	-	6.76	7.56	7.01		3.11	2.32	2.68		3.94	3.16	3.22	
			SEm±		C D (p=0.05)		SEm±		C D (p=0.05)		SEm±		C D (p=0.05)	
Genotype			0.17		0.49		0.08		0.22		0.02		0.06	
Treatment			0.26		0.75		0.12		0.33		0.03		0.10	
Genotype × Treatment			0.45		N/S		0.20		N/S		0.06		0.17	

bud bursting and multiple shoot production during shoot proliferation in marigold has earlier been demonstrated by several researchers (Gupta *et al.*, 8; Kumar *et al.*, 11; Kumar *et al.*, 12).

When the two cytokinins, namely TDZ and KIN were compared for their shoot proliferation potentials, it was observed that shoot multiplication was better in the Kinetin-supplemented medium than in the TDZ-supplemented medium. The maximum shoot proliferation was obtained on MS* medium augmented with 0.5 mg/l of KIN and 0.1 mg/l of NAA. The kind and concentration of cytokinin had a significant impact on the proliferation of shoots as reported by Bhatia *et al.* (3). Kumar *et al.* (12) also observed profuse callusing and vitrification of shoots when higher concentrations of cytokinin were used which led to a poor proliferation in marigold genotypes. Among the three male sterile lines, MS-5 exhibited better shoot proliferation than MS-7 and MS-8 (Fig. 2). Genotypic variations during mass proliferation have been reported in marigold by several workers (Kumar *et al.*, 11; Gupta *et al.*, 8; Kumar *et al.*, 12). These variations may be attributed to differences in the endogenous hormone levels among the different genotypes.

We have noticed that marigold shoots can induce roots without an extraneous supply of growth regulators. However, more aerial root induction rather than true root formation has been noticed in absence of auxins. Hence, for the induction of good-quality roots, the use of auxins particularly NAA and IBA is essential. An improvement in rooting was observed on fortification of rooting media with various levels of auxins (Fig. 1 & Fig. 3). We observed significant early rooting along with the maximum number of roots per micro shoot on ½ strength basal MS

Fig. 1. *In vitro* proliferation and rooting in apetaloid male sterile lines of marigold, **a-c**: shoot proliferation in MS-5, MS-7 and MS-8 on T_2 medium containing TDZ (0.5 mg/l) and NAA (0.1 mg/l), **d-f:** *In vitro* rooting of micro shoots MS-5, MS-7 and MS-8 on ½ MS + 0.5 mg/l IBA.

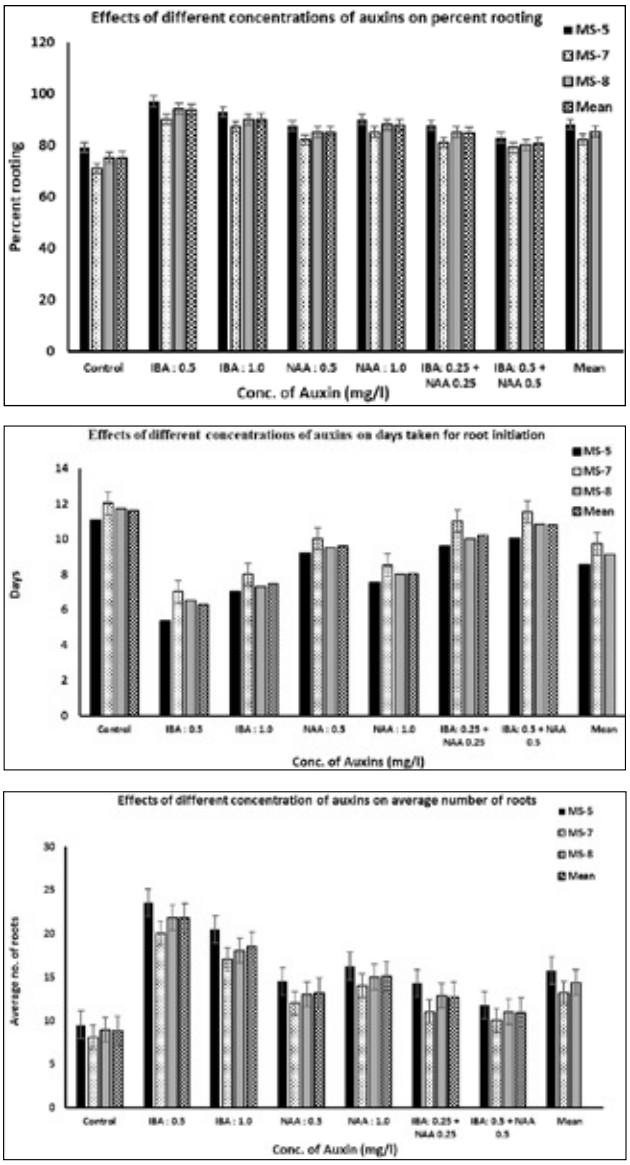

Fig. 2. Effects of different concentrations of auxins on percent rooting, days to root initiation and number of roots in apetalous male sterile lines of marigold.

media fortified with 0.5 mg/l IBA. The micro shoots cultured on TR_0 medium exhibited delayed rooting and induced thread-like roots mostly from the aerial portion than the basal section of the shoots. Further, it was observed that auxins were more effective in inducing good-quality roots at lower concentrations only. While more callusing was noticed when higher concentration of auxins was used which drastically reduced the number of roots per micro shoots. Among the two different auxins used, IBA showed better results when compared to NAA for inducing well-formed, uniform and quality roots. The increase in the percentage of rooting in auxin-rich media might be due to the mobilization of reserve food materials, elongation of meristematic cells and differentiation of cambial initials into root primordial. The effectiveness of IBA in inducing quality roots has earlier been proved by Kumar *et al*., (12) in marigold and Bhatia *et al.* (3) in gerbera.

The three male sterile lines showed a varied response when compared for their *in vitro* root induction ability. MS-5 genotype showed early root initiation and produced more roots per micro shoot when compared to MS-8 and MS-7 (Fig. 2). The genotypic variations during *in vitro* rooting have been reported previously by several workers (Kumar *et al*., 12; Gupta *et al*., 8). The ultimate success of any commercial tissue culture enterprise is determined by the survival of tissue-cultured plants under *ex vitro* environments, such as in the field or greenhouse. The *in vitro* raised plants were successfully hardened in plastic pots covered with polyethene cover in sterilized peat perlite and vermiculite (3:1:1)-based media.

This study has proved the effectiveness of thidiazuron for *in vitro* establishment and mass proliferation of apetalous male sterile lines marigold. The results provided the evidence of the role of genetic factors in determining tissue culture responses of different male sterile genotypes of marigold, highlighting the importance of genotype screening in tissue culture and in determining the model or pioneer genotype. This protocol can be efficiently utilized for the maintenance of male sterile parental lines for hybrid seed production in marigold.

AUTHORS' CONTRIBUTION

Conceptualization of research (NG, RB, KPS), Designing of the experiments (NG, RB), Contribution of experimental materials (RB, KPS, SP), execution of field experiments/ lab analysis, data collection (NG, KD, RA), Analysis of data and interpretation (NG, RB, KPS, SP, HB, SJ); Preparation of the manuscript (RB, NG, KD).

DECLARATION

There is no conflict of interests among any authors.

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