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Standardization of rapid multiplication protocol in petaloid male sterile lines of African marigold (*Tagetes erecta*) through *in vitro* culture

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ABSTRACT

Marigold (*Tagetes erecta* L.) is one of the farmer's first choice for commercial cultivation. It is commonly propagated through seeds, but some ornamentally high valued petaloid and gynomonoecious lines can only be maintained through vegetative propagation. Therefore, the objective of the present investigation was to develop efficient *in vitro* protocol for mass multiplication of commercially high valued petaloid male sterile cultivars. Nodal segments were chosen as explant from two thermotolerant marigold cultivars, viz. Siracole Orange and Siracole Yellow. Explants were pre-treated with carbendazim (0.2%) + metalaxyl (0.2%) + 8-hydroxy quinoline citrate (200 mg/l) for 60 min followed by surface sterilization with 0.1% $HgCl_2$ for 4 min to eliminate the microbial contamination. Highest culture establishment (82.2%) and earliest bud emergence (3.88 days) was recorded in Murashige and Skoog (MS) medium supplemented with BAP (0.5 mg/l) and NAA (0.05 mg/l). Maximum (6, 28, 122 and 404 shoots/explant) proliferation with healthy shoots and free from callus was obtained on MS medium supplemented with 0.5 mg/l BAP + 0.1 mg/l NAA + 2.5 mg/l $AgNO_3$ in 30, 60, 90 and 120 days after culture respectively. Maximum elongation (2.10 cm) was observed on MS media devoid of growth regulators (control). Highest rooting percentage (96.50%), maximum number of roots (23.37), rapid root induction (5.25 days) and high *ex vitro* survival (91.25%) was noted in ½ MS medium supplemented with 0.5 mg/l IBA. Highest plant survival (98.10%) and superior plant growth was observed when rooted plants were shifted to low-cost polypropylene glasses instead of traditional glass bottle system. This protocol is highly useful for mass multiplication of true-to-type, disease free planting material as well as helpful in long term maintenance of germplasm lines.

Key words: African marigold, Male sterile lines, Micropropagation, Nodal segment, Siracole

African marigold (*Tagetes erecta* L.) is a member of the Asteraceae family. It is a native of Mexico and naturalised in India about 350 years ago. Marigold is one of the popular and commercially important ornamental crop in India on account of its easy cultivation, short duration, vast adaptability, wide spectrum of shape, size and good keeping quality. It is cultivated in an area of 56.04 thousand ha. with 501.87 thousand MT production and occupied maximum area among the loose flowers (Anonymous 2015). Apart from loose flower cultivation, it is also widely grown for extraction pigments (lutein) added to poultry feed for intensification of yellow colour of egg yolk (Hojnik *et al.* 2008). It is also endowed with other properties like insecticide (pyrethrins), antibiotic, nematicide and fungicides (thiophenes). Marigold is sexually propagated through seeds. But, seed propagation has limited application in some of the popular petaloid commercial varieties, due to poor seed set, low viability and genetic segregation of progeny. These varieties are being propagated asexually through herbaceous shoot-tip cuttings for commercial cultivation. Tejaswini *et al.* (2016) reported the vegetative propagation of marigold petaloid and gynomonoecious lines in different breeding programmes. However, vegetative multiplication is cumbersome, slow, season dependent and one of the prime causes for spread of diseases like phyllody which is caused by phytoplasma. Plant tissue culture has the potential for rapid multiplication of a large number of disease-free, true-to-type quality plants in the shortest possible time and can be employed as an alternative tool. Earlier, few workers demonstrated techniques of multiplication of marigold through shoot tip and axillary bud proliferation. (Misra and Datta 2000, Kumar *et al.* 2003, Gupta *et al.* 2013 and Majumder *et al.* 2014). However, no work was reported on the mass multiplication

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of thermo-tolerant petaloid male sterile lines for commercial use, moreover earlier results were not repeatable in nature.

Therefore, a study was conducted to develop an efficient and reproducible protocol for rapid *in vitro* propagation of commercially important petaloid male sterile marigold cultivars.

MATERIALS AND METHODS

The present experimentation was carried out at the Central Tissue Culture Laboratory, National Research Centre on Plant Biotechnology, New Delhi during 2014-2017. Two petaloid male sterile marigold cultivars, viz. Siracole Orange (SO) and Siracole Yellow (SY) were used for the study. In this research work, axillary shoots containing dormant buds were selected as explants. The explants were collected in early hours from the actively growing mother plants before the commencement of reproductive phase. The availability and quality of explants were observed to be low during flowering stage. Nodal segments of 2.0-2.5 cm length were excised and the leaf primordia removed with a sterile scalpel blade. Well prepared nodal segments were washed with Teepol®(0.1%) solution for 5 min followed by washing under running tap water for 10 min to remove the residue of the detergent. The explants were pre-treated with carbendazim (0.2%) + metalaxyl (0.2%) + 8-hydroxy quinoline citrate (200 mg/l) on a horizontal shaker (100 rpm) for 60 min followed by surface sterilization using $HgCl_2$ (0.1%) for 4 min under laminar air-hood. The sterilised nodal segments were thoroughly washed with sterile double distilled water for 3 to 4 times to remove the chemical residues. The above treatments were used on the basis of initial experiments conducted by using different pre-treatment and surface sterilisation combinations. The nodal segment was inoculated in each test tube (150 mm × 25 mm) with 15 ml of modified Murashige and Skoog (MS) medium, supplemented with 3% sucrose, 0.8% agar and various concentrations of BAP (0 - 3.0 mg/l) with NAA (0.05 mg/l) for culture initiation.

Thereafter, the micro-shoots were excised from aseptic cultures and subcultured at 30 days interval on proliferation media containing BAP (0 (T0), 0.5 (T1), 1.0 (T2), 1.5 (T3), 2.0 (T4) and 3.0 (T5) mg/l), kinetin (0.5 (T6) and 1.0 (T7) mg/l) individually and in combination (0.5 + 0.5 (T8), 1.0 + 0.5 (T9) mg/l) with NAA (0.1 mg/l). On the basis of initial experiment results, silver nitrate (2.5 mg/l) was tested with 0.5 mg/l BAP and 0.1 mg/l NAA as one of the proliferation treatment (T10). As $AgNO_3$ is a thermolabile compound it was added to autoclaved medium after filter sterilisation with 0.22 µM filters. To test the efficiency of different proliferation media and to determine the rate of proliferation, the experiment was continued up to 120 days. Thereafter, the multiplied shoots of 2.0 to 2.5 cm length were excised from proliferation media and individual micro-shoots were transferred onto different elongation medium comprising basal MS medium supplemented with various concentrations of GA_3 (0.0, 0.5, 0.1 and 2.0 mg/l) to standardise the best elongation media.

For standardisation of rooting, elongated micro-shoots were then excised individually and sub-cultured onto full and ½ strength MS medium supplemented with 60 g/l sucrose and 0.8% agar. Four auxin concentrations IBA (0.5 and 1.0 mg/l) and NAA (0.5 and 1.0 mg/l) were tested both in full- and ½ strength MS media to adjudge best basal media and growth regulator combination.

For hardening of rooted plantlets, four types of strategies were tested, i.e. earthen pots (4.5') with polythene cover, plastic pot (4.5') with polythene cover, glass jars with polypropylene caps and disposable transparent polypropylene glasses with same covering. The rooted plantlets were washed with sterile distilled water to remove the adhering media and agar before hardening. The potting media consisting of 1:1 ratio of sterilised peat and soilrite mixture saturated with ½ strength MS medium containing only macro- and micro-salts. The plants were gradually hardened by loosening of caps and puncturing of polythene covers and plastic glasses after 10 days. After 20 days, the caps were uncovered completely to acclimatise the hardened plants to external environmental conditions. After 30 days, fully hardened plants were transferred to polyhouse for further growth and maintenance.

The cultures were maintained at 24 ± 2°C under fluorescent white light (47 mol/m^2/s) at a photoperiod of 16/8 hours light and dark cycles. All cultures were examined periodically and observations on any morphological changes were recorded.

Twenty-five explants were inoculated per treatment and each treatment was replicated thrice, and the reported data are mean of three replications. The data was statistically analysed employing completely randomised design. The percentage data were subjected to angular transformation before analysis.

RESULTS AND DISCUSSION

Pre-treatments

Aseptic culture establishment is first and foremost step for the successful development of micropropagation protocol on a commercial scale. In this study, various fungicides and bactericides were tried in different combinations and durations to eliminate the microbial contamination from the nodal explants. Among the different fungicidal treatments tried, explants agitation in carbendazim (0.2%) + metalaxyl (0.2%) + 8-hydroxy quinoline citrate (200 mg/l) for 60 min gave significantly higher survival (65.6%) over other treatments (Table 1). In comparison between the two genotypes, per cent survival was significantly high in Siracole Orange (33.8%) over Siracole Yellow (29.4%). The two-way interaction between the pre-treatment and genotype was found to be non-significant. Under our experimental conditions, significantly lowest contamination (31.1%) was observed in explant treated with carbendazim (0.2%) + metalaxyl (0.2%) + 8-hydroxy quinoline citrate (200 mg/l) for 90 min, which was statistically at par with 60 min duration (34.4%) of treatment. However, the survival

Table 1 Effect of different pre-treatments in the sterilization of nodal explants in marigold cv. Siracole Orange (SO) and Siracole Yellow (SY)

Treatment	Treatment details	Duration (minutes)	Survival (%)			Contamination (%)		
			SO	SY	Mean	SO	SY	Mean
T0	Control (Distilled water shake)	60	0 (0)	0 (0)	0 (0)	100 (88.15)	100 (88.15)	100 (88.15)
T1	Carbendazim (0.1%) + Metalaxyl (0.1%) + 8-HQC (200 mg/l)	30	32.2 (34.55)	28.8 (32.40)	30.6 (33.48)	67.7 (55.41)	71.1 (57.55)	69.4 (56.48)
T2	Carbendazim (0.1%) + Metalaxyl (0.1%) + 8-HQC (200 mg/l)	60	51.1 (45.61)	46.6 (43.06)	48.9 (44.34)	48.8 (44.35)	53.3 (46.89)	51.1 (45.62)
T3	Carbendazim (0.1%) + Metalaxyl (0.1%) + 8-HQC (200 mg/l)	90	22.2 (28.07)	17.7 (24.78)	20.0 (26.42)	44.4 (41.75)	48.8 (44.32)	46.6 (43.04)
T4	Carbendazim (0.2%) + Metalaxyl (0.2%) + 8-HQC (200 mg/l)	30	48.8 (44.35)	41.1 (39.84)	45.0 (42.09)	51.1 (45.61)	58.8 (50.12)	55 (47.86)
T5	Carbendazim (0.2%) + Metalaxyl (0.2%) + 8-HQC (200 mg/l)	60	66.6 (54.73)	64.4 (53.40)	65.6 (54.06)	33.3 (35.23)	35.5 (36.56)	34.4 (35.89)
T6	Carbendazim (0.2%) + Metalaxyl (0.2%) + 8-HQC (200 mg/l)	90	15.5 (23.03)	6.6 (12.12)	11.1 (17.57)	28.8 (32.49)	33.3 (35.11)	31.1 (33.80)
Mean			33.8 (32.90)	29.4 (29.375)		53.47 (49.00)	57.28 (51.24)	
CD (P<0.05)								
Treatments					4.857			3.738
Genotype					2.596			1.998
T × G					NS			NS

*Figures given in parentheses are angular transformed values

percentage (11.1%) was significantly low when explants were treated for 90 min. This might be due to the toxic effect of chemicals under prolonged duration of treatment (Table 1). All pre-treatments gave significantly better response compared to control, where 100 per cent contamination was noted. Microbes such as bacteria and fungi were responsible for culture contamination and can completely spoil the cultures. These findings were in confirmation with earlier results reported by Singh *et al.* (2011) in grape, Verma *et al.* (2012) in chrysanthemum and Sen *et al.* (2013) in *Achyranthes aspera* L. Most of these findings proved the usefulness of carbendazim (0.1 - 3.0%) and metalaxyl (0.1 - 3.0%) as effective fungicides. Fungicide dosage and treatment duration depend on the type and tenderness of explant. But higher concentrations of these disinfectants and prolonged durations of treatment became toxic and were responsible for poor growth and low establishment of cultures particularly in herbaceous crops.

Surface sterilization

Standardisation of surface sterilisation treatment followed by efficient pre-treatment is a vital process for axenic culture establishment. It is clear from the Table 2 that significantly higher survival (75.0%) was recorded when the explants were pre-treated with carbendazim (0.2%) + metalaxyl (0.2%) + 8-hydroxy quinoline citrate (200 mg/l) for 60 min followed by 4 min $HgCl_2$ (0.1%) treatment over all other treatments. It was also observed that explants were killed when treatment duration was increased beyond 4 min in $HgCl_2$ (0.1%). This might be due to the toxic effect of surface sterilant on explants (Table 2). It was clearly evident from the data, that NaOCl (4%) treatment for 15 and 20 min was less efficient than $HgCl_2$ (0.1%) for 4 min in controlling the microbial contamination. Among the two genotypes, per cent survival was significantly highest in Siracole Orange (38.8%) over Siracole Yellow (36.5%). The two-way interaction between the surface sterilant and genotype was found to be non-significant. Our research finding revealed that explants treated with $HgCl_2$ (0.1%) for short duration (< 3 min) failed to kill the microbes effectively, whereas longer durations (5 to 8 min) resulted in complete or partial tissue killing. Treating the explants with $HgCl_2$ (0.1%) for 4 min resulted in higher survival of explants with low contamination (22.2%). Our results were in tantamount to Singh *et al.* (2011) in grape and Verma *et al.* (2012) in chrysanthemum. But these results were in contrast with Majumder *et al.* (2014), where they reported only 2 min treatment with $HgCl_2$ (0.1%) resulted in highest culture establishment in Pusa Narangi Gainda and the variation might be due to change in the genotype.

Culture initiation

Different BAP concentrations (0, 0.5, 1.0, 2.0 and 3.0 mg/l) were tried along with NAA (0.05 mg/l) for culture establishment (Table 3). Under our experimental conditions, among the different growth regulators tested, the highest

Table 2 Effect of different surface sterilisation treatments of nodal explants in marigold cv. Siracole Orange (SO) and Siracole Yellow (SY)

Treatment	Treatment details	Survival (%)			Contamination (%)		
		SO	SY	Mean	SO	SY	Mean
T0	Control (Distilled water shake)	0 (0)	0 (0)	0 (0)	100 (88.15)	100 (88.15)	100 (88.15)
T1	0.1% $HgCl_2$ for 3 min	64.4 (53.37)	62.2 (52.04)	63.3 (52.70)	35.5 (36.59)	37.7 (37.92)	36.6 (37.25)
T2	0.1% $HgCl_2$ for 4 min	77.7 (61.87)	72.2 (58.23)	75.0 (60.05)	20.0 (26.50)	24.4 (29.58)	22.2 (28.04)
T3	0.1% $HgCl_2$ for 5 min	55.5 (48.18)	51.1 (45.61)	53.3 (46.9)	16.6 (24.01)	15.5 (23.20)	16.1 (23.60)
T4	0.1% $HgCl_2$ for 6 min	18.8 (25.36)	13.3 (21.30)	16.1 (23.33)	12.2 (20.39)	13.3 (21.38)	12.7 (20.88)
T5	0.1% $HgCl_2$ for 7 min	3.3 (10.46)	2.2 (6.97)	2.7 (8.71)	11.1 (19.17)	12.2 (20.32)	11.6 (19.75)
T6	0.1% $HgCl_2$ for 8 min	1.1 (3.48)	0 (0)	0.55 (1.74)	8.8 (16.52)	10.0 (18.03)	9.4 (17.27)
T7	4.0% NaOCl for 15 min	61.1 (51.39)	62.2 (52.07)	61.6 (51.73)	35.5 (36.59)	35.5 (36.56)	35.5 (36.57)
T8	4.0% NaOCl for 20 min	66.6 (54.73)	65.5 (54.05)	66.1 (54.39)	27.7 (31.74)	31.1 (33.78)	29.4 (32.76)
Mean		38.8 (34.32)	36.52 (32.25)		29.8 (33.3)	31.1 (34.32)	
CD (P<0.05)							
Treatments				3.697			3.631
Genotype				1.743			NS
T × G				NS			NS

*Figures given in parentheses are angular transformed values

culture establishment (82.2%) was noted with 0.5 mg/l BAP + 0.05 mg/l NAA, followed by 1.0 mg/l BAP + 0.05 mg/l NAA (70.0%), which were significantly different. The culture establishment was higher in the genotype Siracole Orange (54.6%) followed by Siracole Yellow (54.0%) both were at par with each other. The interaction between treatment and genotype was insignificant.

Early bud sprouting (3.88 days) was observed on MS medium supplemented with 0.5 mg/l BAP + 0.05 mg/l NAA, followed by 1.0 mg/l BAP + 0.05 mg/l NAA (4.12 days), which were statistically significant with each other. Explants cultured on MS medium devoid of any growth regulators took longer duration (6.34 days) for axillary bud sprouting. Among the genotypes, significantly earlier axillary bud sprouting (4.54 days) was recorded in Siracole Orange compared to Siracole Yellow (5.05 days). The interaction between growth regulator and genotype was also found significant. Duration for bud sprouting was earlier in Siracole Orange (3.83 days) than Siracole Yellow (3.93 days) when they were cultured on MS medium supplemented with 0.5 mg/l BAP + 0.05 mg/l NAA treatment.

Perusal of data from Table 3 revealed that, maximum number of micro-shoots per explant (1.66) was recorded in 2.0 mg/l BAP + 0.05 mg/l NAA, which was statistically at par with 0.5 mg/l BAP + 0.05 mg/l NAA (1.5). The genotype response to different BAP concentrations and the interaction between treatment and genotype was insignificant in terms of number of micro-shoots per explants. Among the treatments, significantly longest micro-shoots (1.38 cm) were obtained with 0.5 mg/l BAP + 0.05 mg/l NAA treatment followed by 1.0 mg/l BAP + 0.05 mg/l NAA (1.05 cm), which were significant over each other. The genotype effect and interaction between treatment and genotype was insignificant in terms of micro-shoot length. Under our experimental condition, low and moderate callusing was observed nodal segments in treated with 2.0 and 3.0 mg/l BAP along with 0.05 mg/l NAA respectively. It is well known that cytokinins are essential for axillary bud formation, growth and shoot multiplication. Among the cytokinins reported, BAP is widely used in tissue culture as it is synthetic and stable in nature. Earlier, Kumar *et al.* (2003) and Gupta *et al.* (2013) reported the use of 2.0 mg/l BAP alone or in combination of 0.5 mg/l NAA for better culture establishment of different marigold varieties and apetalous male sterile lines. Majumder *et al.* (2014) reported that a lower concentration of BAP (1.0 mg/l) along with GA_3 was found to be most effective in marigold cv. Pusa Narangi Gainda in culture establishment. They also reported the formation of callus along with micro-shoot when was NAA added along with BAP and GA_3. These conflicting results could be attributed to the use of different species as well as the possible effects of different genotypes. Our results also showed that the higher concentrations of BAP along with NAA lead to callus formation resulted in the poor establishment of cultures in marigold. However, a lower concentration of BAP (0.5 mg/l) along with the low level of NAA (0.05 mg/l) was beneficial in early culture establishment, micro-shoot growth and cultures were free from callusing.

Effect of different growth regulators on shoot proliferation

It is clearly evident from the Table 4 that, maximum (100%) proliferation of micro-shoots was recorded on MS medium devoid of any hormones (control), following by MS medium supplemented with 0.5 mg/l BAP + 0.1 mg/l NAA, 0.5 mg/l Kinetin + 0.1 mg/l NAA and 0.5 mg/l BAP + 0.1 mg/l NAA + 2.5 mg/l $AgNO_3$. Under our experimental conditions, both the genotypes were unable

Table 3 Effect of BAP and NAA on *in vitro* culture establishment (%), days to bud sprouting, no. of shoots/explant, avg. shoot length (cm) and callusing after 25 days after culture initiation in cv. Siracole Orange (SO) and Siracole Yellow (SY)

Treatment	Growth regulators (mg/l)		Culture establishment (%)			Days to bud sprouting			Shoots per explant			Av. shoot length (cm)			Callusing	
	BAP	NAA	SO	SY	Mean	SO	SY	Mean	SO	SY	Mean	SO	SY	Mean	SO	SY
T0	0.0	0.00	54.4 (47.5)	55.5 (48.1)	55.0 (47.8)	6.00	6.69	6.34	1.00	1.00	1.00	0.86	0.63	0.75	-	-
T1	0.5	0.05	83.3 (66.5)	81.1 (64.4)	82.2 (65.4)	3.83	3.93	3.88	1.33	1.66	1.50	1.33	1.43	1.38	-	-
T2	1.0	0.05	68.8 (56.11)	71.1 (57.4)	70.0 (56.7)	4.03	4.20	4.12	1.33	1.33	1.33	1.03	1.06	1.05	+	+
T3	2.0	0.05	40.0 (39.1)	36.6 (37.1)	38.3 (38.1)	4.30	5.11	4.70	1.66	1.66	1.66	0.93	0.66	0.80	++	++
T4	3.0	0.05	26.6 (31.0)	25.5 (31.0)	26.1 (30.6)	4.56	5.31	4.93	1.33	1.33	1.33	0.70	0.53	0.61	+++	+++
Mean			54.6 (48.0)	54.0 (47.5)		4.54	5.05		1.33	1.40		0.97	0.86			
CD (P<0.05)																
Treatments					5.231			0.092			0.211			0.066		
Genotype					NS			0.173			NS			NS		
T × G					NS			0.387			NS			NS		

Figures given in parentheses are angular transformed values

to establish and proliferate where the concentrations of BAP and Kinetin were more than 0.5 mg/l. Vitrified shoots and profuse callusing was frequently observed in higher cytokinin concentrations which led to poor establishment of cultures in proliferation media. It is also evident from the data (Table 4) that significantly higher proliferation with healthy micro-shoots was observed when the media was supplemented with 0.5 mg/l BAP + 2.5 mg/l $AgNO_3$ + 0.1 mg/l NAA compared to all other growth regulators. Senescence of leaves, shoot tip death and longer intermodal lengths were observed in MS media supplemented with 0.5 mg/l BAP + 0.1 mg/l NAA devoid of $AgNO_3$.

Among the different treatments, maximum (6, 27.5, 122.2 and 403.7 shoots/explant) shoots were obtained on MS medium supplemented with BAP (0.5 mg/l) + NAA (0.1 mg/l) + 2.5 mg/l $AgNO_3$ (Fig. 1) which was statistically significant with BAP (0.5 mg/l) + NAA (0.1 mg/l) (5.5, 20.0, 64 and 179 shoots/ explant) in 30, 60, 90 and 120 days respectively. The two way interaction between growth regulator and genotype was found to be significant in 30, 60, 90 and 120 days. The interaction between treatment and genotype revealed that maximum (427.66) shoots were obtained from Siracole Yellow which was significantly different from Siracole Orange (379.66 shoots) when cultured on MS medium supplemented with BAP (0.5 mg/l) + NAA (0.1 mg/l) + $AgNO_3$ (2.5 mg/l) after 120 days.

Misra and Datta (1999) reported that addition of kinetin, 2, 4-D and higher levels of BAP in proliferation media of white marigold culture produced undesirable callus, hyperhydrated leaves and vitrified shoots. GA_3 causes browning of shoot tips in proliferation media. Misra and Datta (2001) also reported the use of low concentration of BAP (1.1 mM) along with $AgNO_3$ (29.41 mM) for better shoot proliferation. Silver nitrate is known to promote multiple shoot formation in different plants. *In vitro* shoot formation was improved by incorporating silver nitrate in the culture medium (Kumar *et al.* 2009). The present findings lend support from the previous work done by Misra and Datta (1999, 2000 and 2001).

Effect of different concentrations of GA_3 on micro-shoots elongation

Amongst the treatments, significantly highest elongation of shoots (2.10 cm) was observed in control i.e.

Fig 1 Effect of BAP, Kinetin, NAA and $AgNO_3$ on mean number of shoots per explant after 30, 60, 90 and 120 days after inoculation (DAI) in proliferation media

Table 4 Effect of BAP, Kinetin, NAA and $AgNO_3$ on micro-shoot proliferation in cv. Siracole Orange (SO) and Siracole Yellow (SY)

Treatment	Treatment details (mg/l)				Proliferation (%)			Callusing	
	BAP	Kinetin	NAA	$AgNO_3$	SO	SY	Mean	SO	SY
T0	0	0	0	0	100 (88.15)	100 (88.15)	100 (88.15)	-	-
T1	0.5	0	0.1	0	100 (88.15)	100 (88.15)	100 (88.15)	-	-
T2	1.0	0	0.1	0	90.0 (74.3)	86.6 (68.8)	88.3 (71.5)	++	++
T3	1.5	0	0.1	0	63.3 (53.0)	53.3 (46.9)	58.3 (49.9)	+++	+++
T4	2.0	0	0.1	0	53.3 (46.9)	46.6 (43.06)	50.0 (44.9)	+++	+++
T5	3.0	0	0.1	0	20.0 (26.06)	16.6 (23.8)	18.3 (24.9)	+++	+++
T6	0	0.5	0.1	0	100 (88.15)	100 (88.15)	100 (88.15)	-	-
T7	0	1.0	0.1	0	96.6 (82.6)	93.3 (77.0)	95.0 (79.8)	+	+
T8	0.5	0.5	0.1	0	73.3 (58.9)	70.0 (56.7)	71.6 (57.8)	++	++
T9	1.0	0.5	0.1	0	66.6 (55.0)	66.6 (54.9)	66.6 (55.01)	+++	+++
T10	0.5	0	0.1	2.5	100 (88.15)	100 (88.15)	100 (88.15)	-	-
Mean					78.4 (68.1)	75.7 (65.8)			
CD (P<0.05)									
Treatments							6.828		
Genotype							NS		
T × G							NS		

Figures given in parentheses are angular transformed values

devoid of any growth regulator followed by 2.0 mg/l GA_3 (1.42 cm), which were significant over each other. There was a significant difference in the response by the two marigold genotypes for micro-shoot elongation. Among the two genotypes, significantly highest shoot elongation (1.52 cm) was observed in Siracole Yellow followed by Siracole Orange (0.88 cm) (Fig. 2). The interaction effect of treatment and genotype was found to be non-significant. Sekioka and Tanaka (1981) were of the opinion that gibberellic acid (GA_3) can act as a replacement for auxin in shoot induction, and thus a ratio of cytokinin - GA may be decisive for differentiation in certain plant tissues. Through our experimental results, it can be concluded that marigold shoot tissues might be having high levels of endogenous auxins. Thereby adding additional GA resulted in negative effect on shoot elongation. Our research findings were in agreement with the results reported by Qi *et al.* (2011).

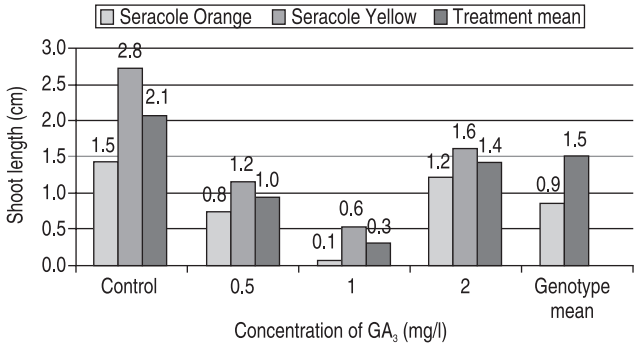

Fig 2 Effect of GA on micro-shoot elongation in African marigold cultivars

Effect of different strengths of basal media and auxins on in vitro *rooting of micro-shoots*

Among the growth regulator treatments, significantly maximum rooting (96.50%) was recorded in the treatment where ½ strength MS medium was supplemented with IBA (0.5 mg/l). The two-way interaction between treatment and genotype was found to be non-significant. Among the treatments, lengthiest roots were observed in control (7.90 cm) followed by full MS + 0.5 mg/l IBA (5.76 cm), which were significantly different from each other. Shortest roots (1.55 cm) were observed in ½ MS media supplemented with 1.0 mg/l NAA. The response of the two genotypes and the interaction effect of treatment and genotype towards length of longest root was found to be insignificant (Table 5).

Maximum number of roots (23.37) per shoot was observed in ½ strength MS medium supplemented with 0.5 mg/l IBA followed by full MS + 0.5 mg/l IBA (19.25), which was significant with each other. Earliest (5.25 days) root initiation was observed in case of the treatment where ½ MS was supplemented with 0.5 mg/l IBA, whereas full MS supplemented with 1.0 mg/l NAA took maximum number of days (9.25 days) for root induction. The rooting response of the two cultivars was found to be insignificant. Under our experimental conditions, highest survival (91.25%) was recorded in ½ strength MS medium supplemented with 0.5 mg/l IBA followed by ½ MS + 0.5 mg/l NAA (86.25%) which were significant over all other treatments and insignificant amongst themselves. Poor survival rates were noted in control (23.75%). The survival response of the two genotypes and the interaction effect of treatment × genotype was found to be insignificant.

Table 5 Effect of basal media strength and auxin(s) on *in vitro* rooting of marigold cv. Siracole Orange (SO) and Siracole Yellow (SY) micro-shoots

Treatment	Treatment details		Rooting (%)			Length of longest root (cm) after 20 days			Average no. of roots per shoot			No. of days required for root initiation			Survival (%)		
	MS	Hormone	SO	SY	Mean	SO	SY	Mean	SO	SY	Mean	SO	SY	Mean	SO	SY	Mean
T0	Full	D. water	69 (56.1)	65 (53.7)	67 (54.9)	8.30	7.50	7.90	7.25	8.50	7.87	6.00	6.25	6.13	25.00 (29.3)	22.50 (28.2)	23.75 (28.78)
T1	Full	0.5 IBA	94 (76.2)	90 (71.2)	92 (73.9)	5.65	5.87	5.76	19.50	19.00	19.25	5.50	5.25	5.38	42.50 (40.5)	45.00 (42.0)	43.75 (41.34)
T2	Full	1.0 IBA	88 (70.3)	86 (68.1)	87 (69.2)	5.50	5.07	5.29	14.25	15.75	15.00	6.50	6.25	6.38	47.50 (43.5)	42.50 (40.50)	45.00 (42.06)
T3	Half	0.5 IBA	97 (82.0)	96 (80.8)	96.5 (81.4)	5.50	5.75	5.63	20.00	26.75	23.37	5.00	5.50	5.25	92.50 (75.6)	90.00 (73.6)	91.25 (74.67)
T4	Half	1.0 IBA	91 (74.4)	91 (74.4)	91 (74.4)	5.10	4.75	4.93	15.00	16.25	15.62	5.50	6.25	5.88	82.5 (65.4)	80.00 (63.7)	81.25 (64.61)
T5	Full	0.5 NAA	91 (72.8)	90 (71.6)	90.5 (72.2)	2.30	2.42	2.36	16.75	16.00	16.37	7.50	7.75	7.63	37.5 (37.6)	40.00 (39.1)	38.75 (38.4)
T6	Full	1.0 NAA	74 (59.4)	76 (60.6)	75 (60.0)	1.85	1.95	1.90	9.25	9.00	9.12	9.75	8.75	9.25	50 (45.0)	52.50 (46.4)	51.25 (45.76)
T7	Half	0.5 NAA	93 (75.0)	91 (72.5)	92 (73.8)	2.75	2.37	2.56	16.75	18.50	17.62	6.50	8.25	7.38	87.5 (69.5)	85.00 (67.4)	86.25 (68.48)
T8	Half	1.0 NAA	78 (62.1)	76 (60.6)	77 (61.4)	1.57	1.52	1.55	8.50	9.75	9.15	9.25	9.00	9.13	77.5 (61.7)	75.00 (60.0)	76.25 (60.91)
Mean			86.1 (69.8)	84.5 (68.2)		4.28	4.14		14.14	15.50		6.83	7.03		60.27 (52.06)	59.16 (51.28)	
CD (P<0.05)																	
Treatments					5.131			0.469			3.403			1.289			6.124
Genotype					NS			NS			NS			NS			NS
T × G					NS			NS			NS			NS			NS

Figures given in parentheses are angular transformed values

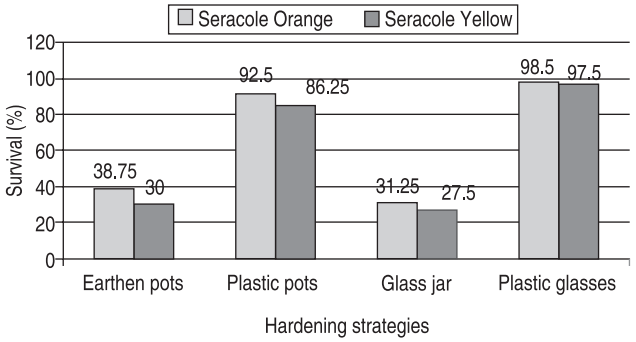

Fig 3 Effect of different hardening strategies on plant survial of *in vitro* raised marigold cultivars

Rhizogenesis was observed in all the auxin treatments including control. The roots formed in blank media devoid of any auxin did not help in the establishment of the plants in soil. This might be due to the formation of non-functional root system. Hence, auxin supplementation is mandatory for enhancing the rooting as well as root quality. These findings confirmed the results reported by Misra and Datta (2001), whereas Kumar *et al.* (2003) and Majumder *et al.* (2014) reported the combined use of 0.5 mg/l each IBA and NAA for better rooting. Reports regarding the effect of *in vitro* rooting medium on *ex vitro* establishment of rooted plantlets in marigold were not found.

Effect of different hardening strategies on plant survival (%)

It was observed that use of low-cost polyethylene plastic glasses was effective means of *in vitro* plantlet hardening in marigold which gave the highest survival (98.1%) among the four treatments tested. Poor survival (29.3%) was noted in the glass jar covered with poly propylene cap (Fig 3). This might be due to excess water and salt accumulation in jam bottle over the time and have no chance to drain out excess moisture from these bottles unlike polypropylene glasses, where holes can be made at the bottom of the glass. Marigold plants cannot tolerate excess water unlike other ornamental crops like chrysanthemum. These findings confirmed the results reported by Nazki *et al.* (2015) in gerbera.

From the present studies, it can be concluded that ornamentally high valued petaloid male sterile lines can be taken up for the production of true-to-type, disease free quality planting material in large scale by using this efficient protocol. This can also be helpful for long term maintenance of germplasm, valuable breeding lines and other biotechnological related works.

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