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Circumventing phenolic exudation and poor survival in micropropagation of marigold

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ABSTRACT

Marigold is one of the popular ornamental crops grown mostly for loose flower production and garden display. It is usually propagated through seeds, but some germplasm including male sterile lines (petaloid and gynomonoecious forms) can only be maintained through vegetative means of propagation. Year round production and maintenance of true-breeding lines can be possible by employing efficient tissue culture techniques. However, exudation of phenols from explants and poor *ex vitro* survival of marigold plants are the major hindrances. Therefore, the present investigation was carried out with an objective to standardize the protocol for controlling phenolic exudation from nodal explants and also enhancing the *ex vitro* survival of four marigold genotypes *viz*., Pusa Arpita, Pusa Basanti Gainda, Siracole Orange and Siracole Yellow. The exudation of phenolic compounds from nodal explants was significantly controlled by incorporating 125 mgl^{-1} ascorbic acid into the culture induction medium supplemented with BAP (2.0 mgl^{-1}) + NAA (0.1 mgl^{-1}) in all the genotypes. Marigold micro-shoots cultured on ½ MS liquid rooting medium supplemented with 0.5 mgl^{-1} IBA showed highest rooting percentage (99.00%) which was followed by ½ MS + 0.5 mgl^{-1} NAA (98.75%). Early root induction (5.88 days), longest roots (2.78 cm), moderately high number of roots (47.56) per shoot and highest *ex vitro* survival (98.75%) were observed with ½ MS + 0.5 mgl^{-1} NAA. Among the different hardening strategies employed, lowest mortality (11.55%), maximum plant height (15.15 cm) and leaf number (20.95) were noted in plants that were hardened in disposable polypropylene glasses.

Key words: *Tagetes* spp., phenol, liquid culture, rooting, hardening.

INTRODUCTION

Marigold (*Tagetes* spp.) is an Asteraceous plant and is native to Mexico. It is of the farmer's first choices for commercial cultivation on account of its easy cultivation, short duration, vast adaptability, wide spectrum of shape, size and good keeping quality. Apart from this, marigold is also highly popular in pharmaceutical and poultry industries. Conventionally, marigold is propagated by seeds, though vegetative propagation through herbaceous shoot-tip cuttings is also being successfully employed in maintaining GMS lines and ornamentally high valued petaloid male sterile varieties for commercial cultivation. However, vegetative propagation is highly season dependent, slow in multiplication and may spread phyllody like diseases rapidly. Plant tissue culture has the potential for rapid multiplication of a large number of disease-free, true-to-the type and quality plants in the shortest possible time and can be employed as an alternative tool. But, the high phenol exudation from explants in the initial culture establishment stage and high plant mortality in hardening stage are the most limiting factors in developing the commercial scale marigold micropropagation (Kumar, 4). The quinines produced due to the oxidation of phenols, inhibit the enzyme activity leading to the death of explants due to auto-toxicity. In other crops, some of the strategies like treating the explant with anti-oxidants, change of sucrose levels, use of liquid rather than agar solidified medium, use of activated charcoal or polyvinylpyrrolidone, dark incubation and frequent culture transfer helps in reducing the media staining and improved explant survival (Preece and Compton, 11). Earlier, few workers reported the *in vitro* propagation of marigold (Misra and Datta, 7; Kumar *et al.,* 5; Gupta *et al.,* 1 and Majumder *et al.,* 6). But it is noteworthy to point out that, there are no reports available on mitigating phenol exudation, efficient root induction in liquid medium and hardening strategies in *Tagetes* spp. Keeping the above problems in view in the present investigation some new techniques were employed to improve the *in vitro* multiplication of African and French marigold genotypes.

MATERIALS AND METHODS

The present studies were carried out at the Central Tissue Culture Laboratory, ICAR-National

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Research Centre on Plant Biotechnology, New Delhi during 2014-2017. Nodal segments having dormant buds were chosen as explants and collected from three African marigold cultivars, *viz*., Pusa Basanti Gainda (PBG), Siracole Orange (SO) and Siracole Yellow (SY) and one French marigold cv. Pusa Arpita (PA). The explants were collected in early hours from the actively growing mother plants before the commencement of reproductive phase. Explants were washed with Teepol®(0.1%) solution for 5 minutes followed by washing under running tap water for 10 minutes to remove the residue of the detergent. The explants were pre-treated with Bavistin (0.2%) + Ridomil (0.2%) + 8-hydroxy quinoline citrate (200 mgl^{-1}) on a horizontal shaker (100 rpm) for 60 minutes followed by surface sterilization using $HgCl_2$ (0.1%) for 4 minutes under laminar air-hood. The sterilised explants were thoroughly washed with sterile double distilled water for 3 to 4 times to remove the chemical residues. The pre-treatment and surface sterilization treatments were employed as per the previously standardized protocols. After that, the nodal segments were cultured on modified MS medium supplemented with 2.0 mgl^{-1} BAP + 0.05 mgl^{-1} NAA, 3% sucrose and six different concentrations of ascorbic acid (0, 25, 50, 75, 100 and 125 mgl^{-1}) were used. Sprouted axillary buds were transferred to MS media devoid of growth regulators for further maintenance. Based on visual observation, the extent of media discolouration was assessed. Data was recorded as per the rating scale 1 to 5 (1 implies no discoloration, 5 implies extreme discoloration) given by Ziv and Halevy (14) and interpreted the results.

To enhance the quality and for more number of functional roots, an experiment was carry out by placing the micro-shoots in liquid half strength MS media supplemented with 60 g/l sucrose and four different auxin concentrations *i.e*., IBA (0.5 and 1.0 mgl^{-1}) and NAA (0.5 and 1.0 mgl^{-1}) to adjudge the best rooting media. The pH of the medium was adjusted to 5.8 before autoclaving at 121 ºC for 20 minutes at 15 $lbs/inch^2$ pressure. 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. For hardening of rooted plantlets, four types of strategies were tested, *i.e*., glass jars with polypropylene caps, plastic pot (4.5') with polythene cover, earthen pots (4.5') with polythene cover and disposable transparent polypropylene glasses with same covering. The plants were gradually hardened by loosening of caps and puncturing of polythene covers and plastic glasses.

Fifteen explants were inoculated per treatment and each treatment was replicated five times. The data was statistically analysed employing completely randomised design. The percentage data were subjected to analysis of variance.

RESULTS AND DISCUSSION

In this study, explants cultured on MS medium supplemented with various concentrations of ascorbic acid significantly reduced the phenolic exudation and enhanced the survival as compared with control (Fig. 1). Among the different treatments tested, complete elimination of browning (1.01) was observed when the medium was supplemented with 125 mgl^{-1}

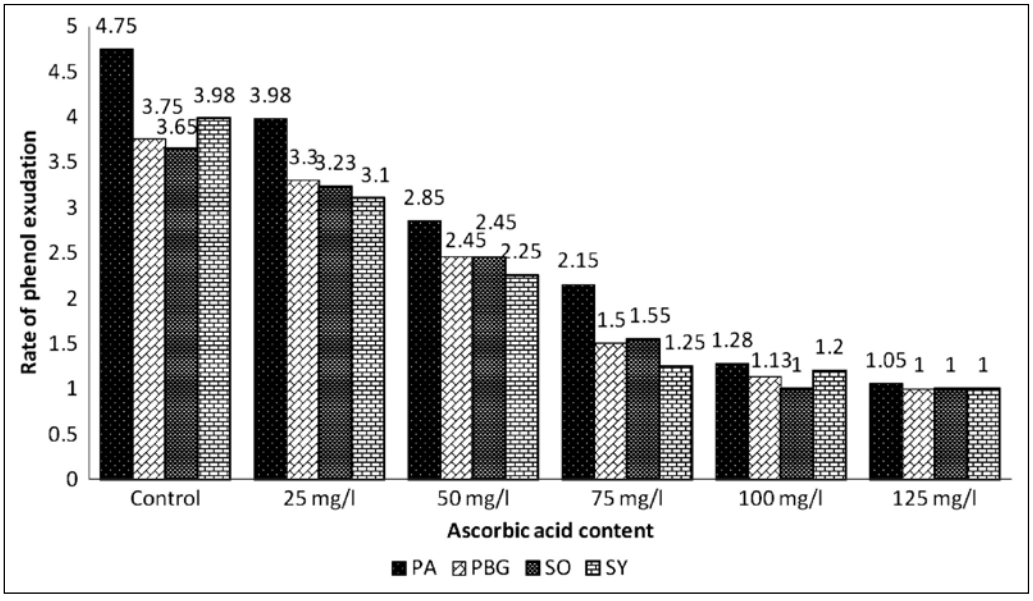

Fig. 1. Effect of ascorbic acid on controlling the phenolic exudation and lethal browning in marigold genotypes

ascorbic acid (Fig. 3b) followed by 100 mgl^{-1} (1.15), whereas maximum (4.03) phenol exudation was recorded in control (Fig. 3a). Among the genotypes, lowest browning (2.13) of explants was recorded in SY followed by SO (2.15) and PBG (2.19) and were significantly superior to PA (2.68). It is also clearly evident from the data that French genotype (PA) showed maximum phenolic exudation and browning as compared to African genotypes (PBG, SO and SY). These results corroborate with the investigations carried out by Singh *et al*. (12) in pomegranate, Kariyana and Nisyawati (3); Ngomuo *et al.* (10) in *Musa* spp., Ndakidemi *et al.* (9) in *Brahylaena huillensis*. All the studies reported the excessive phenolic compound exudation from wounded explant. Browning in plants occurs mainly due to oxidation of phenolic compounds by phenol oxidase. Welsh *et al.*, (13) suggested the use of media supplements like ascorbic acid to limit the production of these lethal substances. The ascorbic acid protects the plant tissues from oxidative injury by scavenging oxygen radicals produced when the explant tissue is wounded.

Poor *ex vitro* survival of marigold tissue culture plants is a major hindrance for its micropropagation. The poor survival might be attributed to poor quality roots and excessive root damage while transferring from solid rooting medium to hardening. To avoid the root damage and for maximum quality root induction, in this study, shoots were supported by filter paper bridges and cultured on ½ MS liquid medium supplemented with two auxins (NAA and IBA). Highest per cent rooting in all the four genotypes was observed in medium supplemented with either 0.5 mgl^{-1} IBA or NAA (Table 1). The per cent rooting was decreased with increased auxin levels in the medium. Misra and Datta (7) reported the rooting of marigold shoots in medium devoid of growth regulators. However, such poor quality roots did not help the establishment of plants in soil. Maximum number of roots per shoot (54.19) was noted in the treatment supplemented with 1.0 mgl^{-1} IBA followed by medium supplemented with 0.5 mgl^{-1} NAA (47.56), which were significant with each other (Table 1). Among the four genotypes maximum number of roots (71.25) was produced by Pusa Basanti Gainda (Fig. 4) followed by Pusa Arpita (50.13). These results were similar to Gupta *et al*. (1) where they reported best rooting on 6 µM IBA.

Perusal of data from Table 1 reveals significant differences among the treatments and in between the genotypes for length of longest root. Among the different treatments, longest root (2.78 cm) was observed in the media supplemented with 0.5 mgl^{-1} NAA followed by 0.5 mgl^{-1} IBA (1.98 cm). Among

Table 1. Effect of different auxins on rooting, number of roots per micro-shoot and length of longest root in liquid half MS medium in marigold genotypes.

Treatments	Rooting (%)				Mean	Number of roots per micro-shoot				Mean	Length of longest root (cm)				Mean
	PA	PBG	SO	SY		PA	PBG	SO	SY		PA	PBG	SO	SY	
½ MS + IBA (0.5 mgl^{-1})	98.00 (62.46)*	99.00 (85.72)	100.00 (88.15)	99.00 (88.15)	99.00 (81.12)	35.25	59.00	41.75	23.00	39.75	1.08	2.88	2.10	1.88	1.98
½ MS + IBA (1.0 mgl^{-1})	98.00 (80.86)	96.00 (79.63)	92.00 (77.45)	90.00 (71.62)	94.00 (77.40)	51.00	80.25	54.00	31.50	54.19	2.20	3.48	1.40	1.45	2.13
½ MS + NAA (0.5 mgl^{-1})	99.00 (82.07)	100.00 (88.15)	98.00 (85.72)	98.00 (83.29)	98.75 (84.81)	55.75	80.75	35.00	18.75	47.56	1.30	5.28	2.30	2.25	2.78
½ MS + NAA (1.0 mgl^{-1})	95.00 (78.42)	97.00 (80.86)	96.00 (79.63)	92.00 (75.98)	95.00 (78.73)	58.50	65	24.75	15.00	40.81	2.25	3.78	1.38	1.63	2.26
Mean	97.50 (75.95)	98.00 (83.59)	96.50 (82.74)	94.75 (79.76)		50.13	71.25	38.88	22.06		1.71	3.85	1.79	1.80	
	SEm±	CD (p=0.05)				SEm±	CD (p=0.05)				SEm±	CD (p=0.05)			
Treatment (T)	2.905	8.424				0.852	2.423				0.072	0.204			
Genotype (G)	2.905	8.424				0.852	2.423				0.072	0.204			
T × G	5.809	16.846				1.704	4.850				0.143	0.407			

*Figures given in parentheses are angular transformed values

the genotypes, Pusa Basanti Gainda produced the longest (3.85 cm) roots as compared to other genotypes. More rooting percentage and shortest root system in liquid medium were attributed to easy availability of nutrients, sugars through capillary action of filter paper and direct shoot contact to hormones (Nazki *et al*., 8). Earliest (5.88 days) root induction was noted in half MS liquid medium supplemented with 0.5 mgl^{-1} NAA (Table 2). Among the genotypes, significantly earliest (5.48 days) root initiation was observed in Pusa Basanti Gainda followed by Seracole Orange (5.50 days) whereas, Seracole Yellow took maximum (8.94) number of days for root induction. Earlier, Gupta *et al.* (1) reported earliest (3.75 days) rooting on MS solid medium supplemented with 6.0 µM IBA. Abundant nutrient availability and absence of stress stimulation may also be one of the reasons for delayed root initiation in liquid medium as compared to solid medium. It is clearly evident from the data presented in Table 2 that among the treatments, highest *ex vitro* survival (98.75%) was recorded in medium supplemented with 0.5 mgl^{-1} IBA, followed by 0.5 mgl^{-1} NAA (97.5%). This is the first report on rooting of marigold micro-shoots in liquid medium and the effect of *in vitro* rooting on *ex vitro* acclimatization of plants. Irrespective of auxin treatments, *ex vitro* survival percentage was very high in plantlets rooted in liquid MS medium. Imtiyaz *et al.* (2) also reported the similar observations in gerbera and our results are in agreement with them. Earlier, Gupta *et al*. (1) observed 73 % survival in apetalous male sterile line rooted in solid medium. Majumder *et al*. (6) observed only 68.10% survival. The per cent survival was significantly enhanced in liquid medium-produced shoots as compared to solid medium. Marigold roots bear abundant root hairs due to which traces of sucrose and other nutrients along with agar medium tends to stick to them and it is practically impossible to remove completely and roots are going to be damaged during the washing. These associated agar media residues attract more microbial (bacterial and fungal) contamination and further spread in highly humid hardening chamber/ vessel which leads to high mortality of the plants. The lowest per cent survival of plants in solid medium could be attributed to considerable level of root damage while isolating the rooted shoots from the solid agar medium.

A successful *in vitro* protocol needs an efficient hardening strategy with which tissue culture plants are acclimatized in the un-favourable external environmental conditions. The *ex vitro* mortality could be attributed to the fact that the micro-shoots developed *in vitro* have several anatomical abnormalities like poor cuticle, less palisade, more air space, poor vascular bundles and poor root system. Hence, the hardening strategy needs to be standardized to address all the problems in mind. In the present investigation, low-cost polyethylene plastic glasses were found to be effective means of *in vitro* plantlet hardening in marigold which gave the highest mean (88.45%) plant survival as compared

Table 2. Effect of different auxins on days required for root initiation and *ex vitro* survival of plants in liquid half MS medium in marigold genotypes.

Treatments	Days to root initiation				Mean	Survival (%)				Mean
	PA	PBG	SO	SY		PA	PBG	SO	SY	
½ MS + IBA (0.5 mgl^{-1})	6.38	6.12	5.25	8.50	6.56	95.00 (77.88)*	95.00 (77.88)	100.00 (84.23)	97.50 (81.05)	96.88 (80.26)
½ MS + IBA (1.0 mgl^{-1})	5.88	4.78	5.25	9.25	6.29	97.50 (81.05)	100.00 (84.23)	97.50 (81.05)	92.50 (75.85)	96.88 (80.55)
½ MS + NAA (0.5 mgl^{-1})	4.50	5.00	5.50	8.50	5.88	100.00 (84.23)	100.00 (84.23)	100.00 (84.23)	95.00 (77.88)	98.75 (82.64)
½ MS + NAA (1.0 mgl^{-1})	5.63	6.03	6.00	9.50	6.79	100.00 (84.23)	97.50 (81.05)	90.00 (72.68)	85.00 (68.61)	93.13 (76.64)
Mean	5.59	5.48	5.50	8.94		98.13 (81.85)	98.13 (81.85)	96.88 (80.55)	92.50 (75.85)	-
	SEm±	CD (p=0.05)				SEm±	CD (p=0.05)			
Treatment (T)	0.147	0.419				1.54	4.386			
Genotype (G)	0.147	0.419				1.54	4.386			
T × G	0.295	0.838				3.08	8.772			

*Figures given in parentheses are angular transformed values

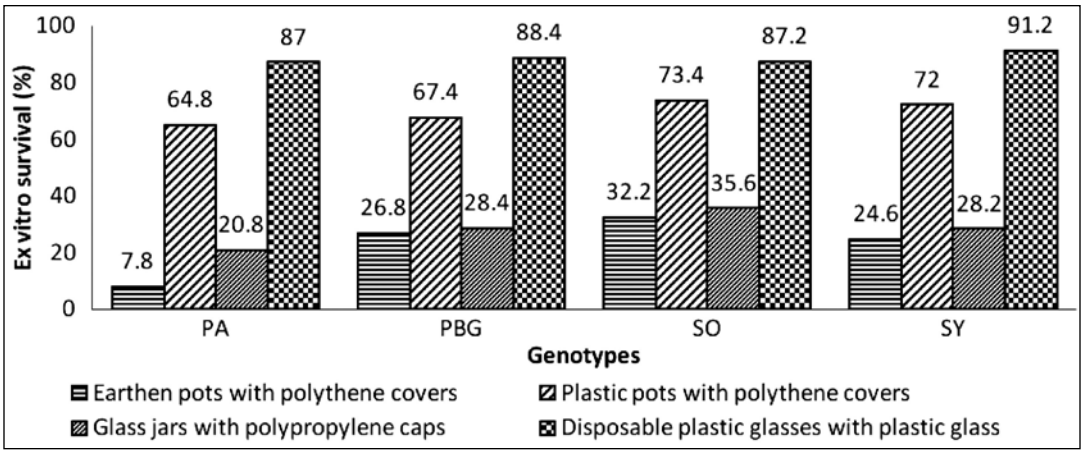

Fig. 2. Effect of various hardening strategies on acclimatization of *in vitro* raised plantlets in marigold genotypes.

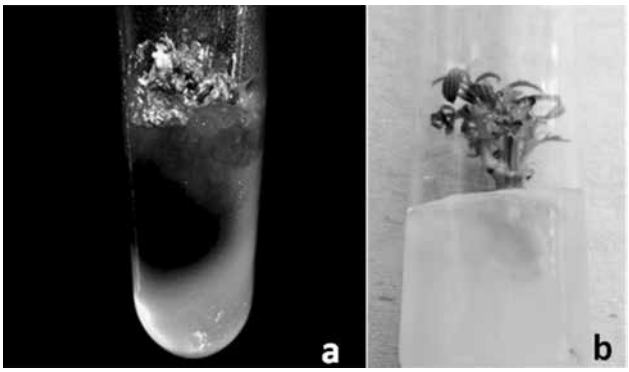

Fig. 3. Culture initiation stage (a) Phenol exudation and lethal browning of marigold nodal segment in control, (b) Pusa Basanti Gainda nodal explant cultured on MS medium supplemented with 125 mg/l ascorbic acid.

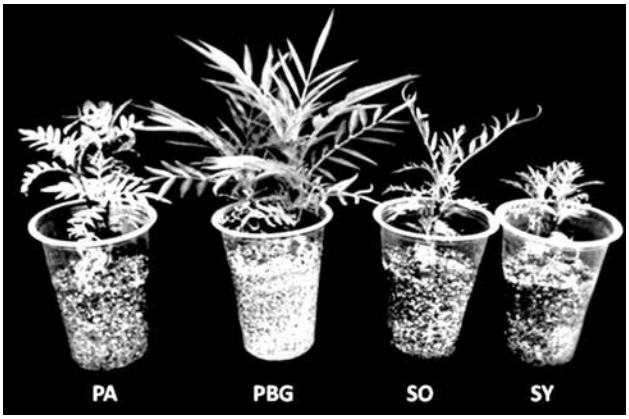

Fig. 5. Hardened plants in disposable plastic glasses

to other three hardening strategies (Fig. 2). This high success in plastic glass could be attributed to optimum moisture retention, constant maintenance of relative humidity and maximum transparency to

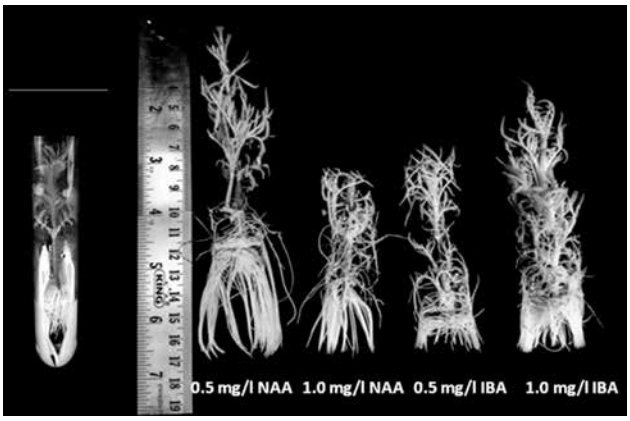

Fig. 4. Rooting of Pusa Basanti Gainda micro-shoots in liquid ½ MS medium supported with filter paper bridges and rooting of micro-shoots on various treatment combinations.

light compared to other strategies (Fig. 5). Higher mortality of plants was observed in earthen pots and glass jars. The poor plant survival in earthen pots could be attributed to the constant loss of moisture from the pots which lead to plant desiccation. Poor survival was also recorded in glass jars. This might be due to excess water and salt accumulation in glass jar over the time and have no chance to drain out excess moisture from these jars unlike polypropylene glasses, where holes can be made at the bottom of the glass. These findings confirmed the results reported by Imtiyaz *et al*. (2) and Nazki *et al.* (8) in gerbera. The plants hardened in disposable plastic glasses put forth maximum (15.15 cm) plant height and maximum (20.95) number of leaves as compared to other hardening strategies. The standardized *in vitro* protocols of above African and French marigold genotypes can be used for commercial multiplication and year round maintenance of breeding lines.

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