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Development of protocol for *in vitro* rooting and hardening of doubled haploid line of *Tagetes erecta* L. derived through ovule culture

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ABSTRACT

***In vitro* regenerated doubled haploid line of *Tagetes erecta* L. (African marigold) derived through ovule culture using non axillary explants, were subjected to different rooting media wherein the elongated micro shoots were transferred individually to the rooting medium consisting of Half MS basal media supplemented with sucrose (60 g/l) and different concentrations of Auxins, IBA (0.5, 1.0 mg/l), NAA (0.5, 1.0 mg/l) and their combination IBA + NAA (0.25 mg/ l + 0.25 mg/l). The results revealed that treatment with ½ MS + IBA (0.5 mg/l) was best for parameters like number of days taken for root initiation (3.44), number of roots per shoot (33.54), *per cent* rooting (93.67). Although, the longest root length (9.64 cm) was found in treatment comprised of MS only and devoid of hormones (control) which produced roots that were light green in colour, thin, lanky and showed low performance while, roots produced in treatment with ½ MS supplemented with IBA (0.5 mg/l) medium were shortest of all treatments (3.43 cm) but were healthy, white and thick, vigorous and showed maximum survival. Thereafter, the rooted plants were transferred to the plastic pots containing different hardening media like soil, soilrite and mixture of cocopeat, vermiculite and perlite in the ratio of 1:1:1 (v/v) with ½ strength MS medium containing only macro and micro-salts. The results showed that the treatment comprising of cocopeat, vermiculite and perlite in the ratio of 1:1:1 (v/v) was best for parameters like *per cent* survival after 15 days (96.80), *per cent* survival after 30 days (95.00), *per cent* survival after 45 days 95.00, shoot length after 15 days (12.50 cm), shoot length after 30 days (15.46 cm), shoot length after 45 days (23.40 cm) and number of leaves per plant (18.60).**

Key words: *Tagetes erect*, *in vitro* rooting, hardening, doubled haploid line.

INTRODUCTION

Marigold (*Tagetes* spp.) is one of the most important flower crops owing to its various uses. It belongs to family Asteraceae and is native of Mexico. Genus *Tagetes* is reported of comprising of approximately 55 species (Godoy-Hernandez and Miranda-Ham, 3), out of which, *Tagetes erecta* L. (African marigold) and *Tagetes patula* L. (French marigold) are of commercial importance. It is being cultivated in an area of 66.13 thousand hectares with the production of 603.18 thousand metric tons (Anonymous, 1). It is gaining popularity among garden lovers on account of its characteristics like free flowering habit, short duration, attractive colour, shape, keeping quality, *etc*. Marigold flowers are extensively used as loose flower for making garlands, beautification, religious offerings, social functions and other purposes like pigment extraction (Gupta and Vasudeva, 4), mosquito and nematode repellent, as a feed additive for poultry industry, *etc*. Apart from these uses, marigold is widely grown in garden beds and pots for ornamental purpose. Tissue culture, particularly haploid and double haploid technology, can be extremely valuable in plant breeding and genetics. Haploids are valuable for obtaining pure lines through chromosome diploidization. Double haploid production systems offer several advantages like homozygosity can be achieved in one generation thus eliminating the need for several generations of self-pollination. *In vitro* multiplication of doubled haploids has been successfully done in plants like tall fescue (Kasperbaeur and Eizenga, 5); sugar beet (Magdalena and Baranski, 6) *etc*. Mass multiplication of homozygous lines mainly in highly cross pollinated crops like marigold is preferred by vegetative means as it maintains true to type nature of plants. The vegetative propagation can be done both under *in vivo* and *in vitro* conditions. The major drawback of *in vivo* propagation is lack of sufficient plant material for large scale multiplication and moreover, it is a season bound activity. Hence, to overcome such limitations, mass multiplication can be best achieved under *in vitro* conditions. Tissue culture is one of the best options to obtain maximum population of doubled haploids under short span of time. The advantage of *in vitro* multiplication of doubled haploids is that large number of quality regenerants per explant can be obtained and moreover it is a year round activity. The use of doubled haploids developed through tissue

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culture drastically reduces the time for precise and efficient selection for desirable traits.

Rooting and acclimatization are the crucial steps for the success of *in vitro* regenerated plants. Adventitious root regeneration of *in vitro* shoots is generally induced by the addition of certain auxins to the rooting medium. Hence, rooting of marigold plants depends on growth regulators and sugars provided in the media. The special conditions during *in vitro* cultures result in the formation of plantlets of abnormal morphology, anatomy and physiology. After transfer to *ex vitro* conditions, these plantlets might easily be impaired by sudden changes due to change in environmental conditions, and so need a period of acclimatization to correct the abnormalities. When *in vitro* plantlets are transferred from the culture room to external conditions, they may desiccate or wilt rapidly and can die due to changes in the environment, unless substantial precautions are taken so that the plantlets are adapted to new environment. The acclimatization of *in vitro*-grown plantlets to an *ex vitro* environment by gradually weaning them towards ambient relative humidity and light levels ensures better survival of young and sensitive plantlets. The *in vitro* raised plantlets must thus undergo a period of acclimatization to correct anatomical abnormalities and hence to enhance their physiological performance to ensure survival under *ex vitro* conditions. Under such conditions, plants convert to an autotrophic growth, develop a fully functional root system, and better control of their stomatal and cuticular transpiration. Hence, present study was focused to develop an efficient *in vitro* protocol for rooting and hardening which will help in maximum plant survival and reduction of plant mortality in marigold.

MATERIALS AND METHODS

The present investigations were conducted during 2016-18 in Central Tissue Culture Laboratory, ICAR-Indian Agricultural Research Institute, New Delhi. The *in vitro* raised doubled haploid plants of *Tagetes erecta* L. (African marigold) variety Local Orange were maintained under net house of the Division of Floriculture and Landscaping, ICAR-IARI, New Delhi. After generating a successful protocol for *in vitro* regeneration of doubled haploid line of African marigold variety Local Orange derived through ovule culture, the regenerated and elongated microshoots were transferred individually to the rooting medium consisting of Half MS (Murashige and Skoog, 10) basal media supplemented with sucrose (60 g/l) and different concentrations of auxins *i.e*. IBA (0.5, 1.0 mg/l), NAA (0.5, 1.0 mg/l) and their combination IBA + NAA (0.25 mg/ l + 0.25 mg/l). Different media were transferred to glass bottles and then autoclaved at 121°C for 20 min. at 15-20 psi (1.05-1.40 Kg cm^{-2}) pressure in laminar air flow cabinet. The media was allowed to cool down and then the microshoots were transferred in glass bottles and the transferred bottles were further kept in culture room having 24 ± 2°C temperature and 16/8 hrs (light/dark) photoperiod with a photosynthetic light at a flux rate of 3000 Lux and a relative humidity of 80-90% was maintained. Different observations like *per cent* rooting, days to root initiation, average number of roots per shoot and mean length of longest root were recorded at different time intervals.

Twenty days old rooted plants were delicately removed from culture vessels and washed with sterilized double distilled water. Then, plants were transferred to the plastic pots containing hardening media (soil, soilrite and a mixture of cocopeat, vermiculite and pertlite, 1:1:1 v/v) with ½ strength MS medium containing only macro and micro-salts. After filling the pots, the pots were covered with aluminum foil and autoclaved at 121°C temperature for 20 min. at 15-20 psi (1.05-1.40 Kg cm^{-2}) pressure. After autoclaving the pots containing the mixture were shifted to laminar air flow cabinet and allowed to cool down and were enriched with ½MS medium. Further, rooted micro shoots were transferred to the pots which then were covered with polythene in order to maintain high humidity. For the acclimatization process, the polythene was removed gradually as the day's progressed and different observations like per cent survival after 15, 30 and 45 days, shoot length after 15, 30 and 45 days, number of leaves per plant were recorded at different time intervals.

Statistical analysis

The experiments were laid out in completely randomized design (CRD). Each treatment had four replications and each replication had 20 units/plants. Each experiment was repeated at least twice and the reported data are the means of two experiments. Recorded data were analyzed statistically using analysis of variance technique (ANOVA). All the percentage data was subjected to angular transformation before calculating ANOVA.

RESULTS AND DISCUSSION

The role of auxins (IBA and NAA) in inducing early rooting over treatment with MS devoid of hormones (control) was observed (Table 1). Significant early rooting (3.44 days) was observed on the treatment with ½MS supplemented with IBA (0.5 mg/l) followed by treatment containing ½MS supplemented with IBA (1.0 mg/l) and treatment with only MS medium (control) in which number of days to root induction was recorded as 4.43 days and 5.10 days, respectively. Maximum number of days to rooting (7.38) was observed in treatment with ½MS supplemented with IBA (0.25 mg/l) and NAA (0.25 mg/l). Lower concentrations of auxins resulted in early rooting as compared to higher concentration. It was elucidated from the observation

that the maximum number of roots (33.54) was observed in the treatment with ½ MS supplemented with IBA (0.5 mg/l) followed by 31.61 and 30.26 days in treatment with ½MS supplemented with IBA(1.0 mg/l) and treatment with ½MS supplemented with NAA (0.5 mg/l), respectively. However, minimum number of roots (5.32) was found in treatment with MS Medium only (control). Lower amount of auxins resulted in vigorous rooting as compared to higher concentration. Moreover, data showed that maximum *per cent* rooting (93.67) was observed in treatment with ½MS with supplemented IBA (0.5 mg/l) followed by treatment with MS with supplemented NAA (0.5 mg/l) and treatment with ½MS supplemented with ½ NAA (1.0 mg/l) which recorded 93.00% and 90.23% rooting respectively. However, minimum rooting (74.17%) was found in treatment with MS medium only (control) Hence it was concluded that treatment with ½ MS supplemented with IBA (0.5 mg/l) was found best in various parameters (Table 1, Fig 1). However, it was noticed that treatment in which only MS medium was used and was devoid of hormones (control) produced longer roots (9.64 cm) but the roots were slight light green in colour, thin, lanky and showed low performance while roots produced in treatment with ½ MS supplemented with IBA (0.5 mg/l) medium were shortest of all treatments (3.43 cm) but were healthy, white, thick and vigorous which showed easy holding of the hardening media and maximum survival. Similar results were also found by Pierik and Sergers (12) who stated that IBA (0.5 mg/l) showed better results as compared to NAA for inducing good quality and well developed roots in gerbera. Similar findings were observed in marigold by Satyajit *et al.* (13) who reported that rooting of the differentiated shoots was best in MS medium supplemented with combination of IBA (1.5 mg/l) and IAA (1.5 mg/l). However, Majumdar *et al.* (7) in their studies showed high rhizogenesis on half strength MS medium with 1.0 mg/l NAA and 1.0 mg/l IBA. Tyagi and Kothari (16) observed maximum rooting of *in vitro* shoots on MS medium supplemented with 0.5 mg/l IAA in *Gerbera jamesonii*. Misra and Datta (8, 9) employed a lower IBA concentration (0.05 mg/l) or NAA (0.1 mg/l) and found 100% rooting response with both IBA and NAA in marigold.

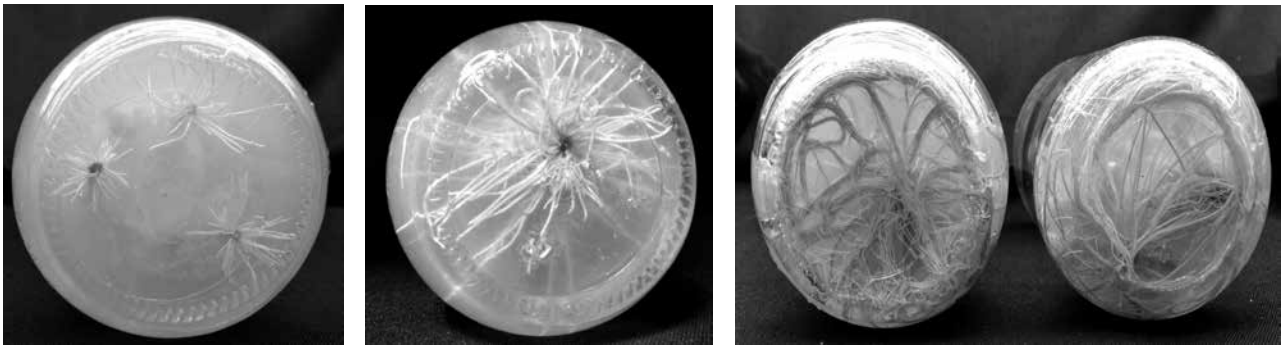
Data recorded in the *in vitro* hardening experiment (Table 2), concluded that there was significant difference among different hardening techniques with respect to survival of *in vitro* raised plantlets. It was observed that per cent survival after 15, 30 and 45 days was found highest (96.80% 95.00% and 95.00 %, respectively) in treatment with a mixture of cocopeat, vermiculite and perlite in the ratio of 1:1:1 (v/v). The treatment, comprised of Soilrite, showed a survival percentage of 78.00, 75.00 and 75.00 after 15, 30 and 45 days, respectively. While treatment in which only soil was used showed lowest survival of 62.00%, 50.00% and 50.00% after 15, 30 and 45 days after transfer of plantlets, respectively. Further in treatment comprised of only soil showed highest desiccation and mortality. Data showed that increase in the shoot length was found highest in treatment with mixture of cocopeat: vermiculite : perlite in the ratio of 1:1:1 (v/v) in which the shoot length increased from 12.50 cm through 15.46 cm to 23.40 cm in 15, 30 and 45 days, respectively. Treatment with Soilrite showed length increment from 9.62 cm through 10.55 cm to 16.80 cm at 15, 30 and 45 days, respectively whereas treatment with only soil showed lowest length increment from 6.04 cm through 8.10 cm to 12.80 cm in 15, 30 and 45 days, respectively. From the results obtained, it was observed that treatment with cocopeat: vermiculite: perlite in ratio of 1:1:1(v/v) showed highest number of leaves per plant (18.60) followed by treatment with Soilrite, which showed 12.60 leaves per plant. The lowest number of leaves per plant (5.68) was observed in treatment with soil only. The plants in treatment with soil only, showed pale green

Table 1. Influence of auxins on rhizogenesis in plantlets raised from leaf explant of doubled haploid line of African marigold variety Local Orange.

Treatment	Number of days taken for root initiation	Number of roots per shoot**	Mean length of longest root**	Per cent Rooting
T_0 (control, MS Medium)	5.10 ± 0.10	5.32 ± 0.09	9.64 ±0.16	74.17 (59.43)* ± 0.44
T_1 (½MS + IBA 0.5 mg/l)	3.44± 0.08	33.54 ± 0.41	3.43 ±0.11	93.67 (75.46) ± 0.88
T_2 (½MS + IBA 1.0 mg/l)	4.43 ± 0.12	31.61 ± 0.55	4.39 ±0.03	86.00 (68.01) ± 0.58
T_3 (½MS + NAA 0.5 mg/l)	5.28 ±0.07	30.26 ± 0.02	3.65 ±0.20	93.00 (74.65) ± 0.58
T_4 (½MS + NAA 1.0 mg/l)	6.39± 0.18	24.03 ± 0.58	5.17 ±0.35	90.23 (71.76) ± 0.14
T_5 (½MS + IBA 0.25 mg/l + NAA 0.25 mg/l	7.38 ± 0.13	14.50± 0.51	5.26±0.09	80.27 (63.60) ± 0.15
C.D. (P≤0.05)	0.37	1.32	0.59	1.65

*Values in parenthesis are angular values

**After 15 days of transfer to rooting media



Rooting on 10th day
T_1 (½MS + IBA 0.5 mg/l)

Rooting on 15th day
T_1 (½MS + IBA 0.5 mg/l)

Rooting on 30th day
T_1 (½MS + IBA 0.5 mg/l)

Fig. 1. Effect of IBA and NAA on rooting of microshoots in doubled haploid line of African marigold variety Local Orange.

Table 2. Effect of *in vitro* hardening media on survival, shoot length and leaf number of plantlets raised from leaf explant of doubled haploid line of African marigold variety Local Orange.

Treatment (s)	Percent survival after 15 days	Percent survival after 30 days	Percent survival after 45 days	Shoot length after 15 days	Shoot length after 30 days	Shoot length after 45 days	Number of leaves per plant
T_0 - soil only (control)	62.00 (51.93)±1.22	50.00 (44.98)±0.00	50.00 (44.98)±0.00	6.04±0.02	8.10±0.08	12.80±0.74	5.68±0.29
T_1 - Soilrite	78.00 (62.04)±1.22	75.00 (60.02)±1.58	75.00 (60.02)±1.58	9.62±0.25	10.55±0.91	16.80±0.66	12.60±1.10
T_2 - cocopeat: vermiculite: perlite (1:1:1)	96.80 (79.92)±0.73	95.00 (77.05)±0.00	95.00 (77.05)±0.00	12.50±0.61	15.46±0.95	23.40±1.08	18.60±1.03
C.D. (P≤0.05)	3.08	2.84	2.84	1.19	2.37	2.63	2.76

*Values in parenthesis are angular values.

leaves with stout and shriveled appearance. While those in treatment with mixture of cocopeat: vermiculite: perlite in the ratio of 1:1:1 (v/v) showed vigorous growth, dark green leaves and healthy appearance. Plants in treatment with soilrite showed vigorous growth with tall and thin appearance (Fig. 2). Similar results were found by Carmelita and Prabhuling (2) who reported that the media mix of perlite + vermiculite + cocopeat (1:1:1 v/v) and cocopeat alone proved superior for primary hardening of Jamun (*Syzygium cuminii* L.). Sharma *et al*. (14) showed 100% survival rate on autoclaved cocopeat in *Gentiana kurroo*. Pawan and Ratnesh (11) reported the primary hardening of *Spathiphyllum floribundum* plants kept in 90% humidity for one month in the Pro tray filled with cocopeat. Afterwards plants were transferred to the secondary hardening in poly bags with different substrate combinations, out of them (cocopeat + sand + vermicompost) in the ratio of 2:1:1 was found the best. Singh *et al.* (15) reported that combined use of sand, cocopeat, vermicompost and vermiculite gives excellent result for gerbera micropropagation techniques but alone they failed to give desirable results.

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T_0 - Soil (control) T_1 - Soilrite T_2 - Cocopeat + Vermiculite + Perlite (1:1:1)

Fig. 2. *In vitro* hardening of plantlets raised from leaf explant of doubled haploid line of African marigold variety Local Orange.

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