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Standardization of protocol for *in vitro* hardening of gynogenically induced regenerates in African marigold (*Tagetes erecta* L.)

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Abstract
African marigold (*Tagetes erecta* L.) belongs to the Asteraceae family and is originated at South and Central America, specifically from Mexico. Gynogenesis leads to rapid development of homozygous parental lines in the shortest possible time, which will further help in development of high-yielding F1 hybrids, but their hardening is still the hardest parts of their production. The high percentage of plant loss is obtained when multiplying and transferring to an *ex vitro* environment, however, frequently limits its wider use. Therefore, the objective has been made to develop efficient protocol for *in vitro* hardening of gynogenically induced regenerates in African marigold. Well-developed healthy gynogenically induced plantlets after *in vitro* multiplication and rooting of genotypes 'DAMH-24' and 'DAMH-55' used as explants. These plants were transferred to the different pots containing hardening media *i.e.* mixture soilrite, cocopeat and perlite in the ratio of 1:1:1 (v/v) with ¼ strength MS medium. Out of the different *in vitro* hardening techniques, plastic pots covered with polythene cover gave the best results; highest percent survival (70.0%), maximum number of leaves (9.17), highest shoot length (8.24 cm) as well as best visual growth scores (4.5). Among both genotypes 'DAMH-24' performs best towards *in vitro* hardening of gynogenically induced plantlets. This protocol is highly useful for development of plants after hardening of *in vitro* gynogenically developed plantlets.

Keywords: *In vitro*, *ex vitro*, marigold, gynogenesis, hardening, pots

Introduction
Marigold "Rose of Indies" is an important flower growing in India. It belongs to the Asteraceae family and is native to South and Central America, specifically from Mexico (Kumar *et al.*, 2017[a]) [14]. Marigold is the most important flower crop among the loose flowers; it ranks first in both area and production. It has been growing in area of 66.13 thousand hectares with a production of 603.18 thousand metric tonnes (Anonymous, 2017[a]) [1]. In India, marigold flowers are in great demand all around the year for various festive occasions, marriages, religious ceremonies, and social functions. African marigold (*T. erecta* L.) is commercially grown as a loose flower in India but now a day these are also used as cut flowers (Kumar *et al.*, 2018[a]) [15]. They also have anti-inflammatory, antiseptic, antispasmodic, astringent, diaphoretic, and emmenagogue properties (Gupta, 2013) [10]. Since yellow and orange colour in marigold is due to the presence of xanthophyll particularly lutein pigment which accounts for 80-90% of xanthophyll in the form of palmitic and myristic acid, hence they are also used as value-added poultry feed which helps to intensify the yellow colour of egg yolks and broiler skin (Guerin *et al.*, 2003) [8]. Lutein is also used as a flavoring and coloring agent in the food industry and having an effective functional nutrient can benefit health by preventing age-related macular degeneration (AMD) (Chiu and Taylor, 2007; Guerin *et al.*, 2003) [4, 8], cardiovascular diseases (Dwyer *et al.*, 2001) [5] and fatal diseases such as cancer (Heber and Lu, 2002) [11]. In marigold *in-vitro* gynogenesis (ovule/ovary culture) along with induced parthenogenesis (induction of egg cell by pollination with irradiated pollen followed by *in vitro* haploid embryo rescue) is the most efficient and sustainable option for induction of haploids and doubled haploids from male-sterile hybrids/lines (Li *et al.*, 2020; Kumar *et al.*, 2020[b]) [16, 13].
The controlled conditions during *in vitro* cultures often result in the formation of tender and open environment-sensitive plantlets with abnormal anatomy, physiology, and morphology. So, if these plants are directly shifted to the field (*ex vitro*), a sudden change in the

environmental conditions may cause the death of these gynogenically developed plants. With a gradual decrease in air humidity in *in vivo* condition, acclimatization of regenerates will eliminate threat of infection and wilting of regenerates. Many abnormalities in the *ex vitro* environment, such as high levels of radiation and low humidity and their limited access to water because roots and root-stem connections have low hydraulic conductivity (Fila *et al.*, 1998) [6]. That's why, these plants need a period of acclimatization in order to get acclimatized and avoid the direct shock of biotic and abiotic components of the environment. Hence, in this study, we have optimized the efficient *in vitro* hardening techniques that will help in plant survival and reduction of plant mortality. These gynogenic plants have the potential to create diverse homozygous lines and these will further use for development of cultivars or F1 hybrid of high commerce. This novel technique of one-step hardening of gynogenically developed plants was successfully employed and established with a high degree of efficiency.

Materials and Methods

The present study was carried out during 2021–2022, in the tissue culture laboratory of the Division of Floriculture and Landscaping, ICAR-Indian Agricultural Research Institute (IARI), New Delhi, 110012. Well-developed healthy gynogenically induced plantlets after *in vitro* multiplication and rooting of two genotypes 'DAMH-24' and 'DAMH-55' of African marigold were used as explants.

First of all of soilrite, cocopeat and perlite was mixed in 1:1:1 ratio followed by keeping inside autoclavable polythene bags and tightly closed with the help of cello tape and then double sterilized in an autoclave at 121 ºC for 22 minutes at 15 $lbsinch^{-2}$ pressure. Along with them number of autoclavable glass jars, caps, disposable glasses, plastic pots, polyethylene covers and 1L solution of ¼ MS also sterilized in autoclave in a single cycle. For *in vitro* hardening of rooted plants four hardening strategies was followed *viz.* Plastic pot covered with polythene cover, Disposable poly propylene glass covered with disposable glass, Disposable poly propylene glass covered with polythene cover and glass jars with polypropylene caps. These vessels were filled with prepared sterilized media. Gynogenically developed already proliferated plantlets with 3-4 leaves and well developed roots of 20–25 days taken out from culture media and washed in running tap water to remove the traces of agar (fig.1.a). The plantlets were subsequently immersed in 1% fungicide (Bavistin and Ridomil) solution and then transplanted in different vessel in replication of 3 for each treatment (Fig. 1). Different covers over treatments vessel were applied and kept inside controlled condition for 10 days at 25±1°C temperature, 16:8 hours photoperiod of light and dark cycles under fluorescent white light (47µmol/m^2/s). After 10 day kept inside partial sunlight outside of controlled condition upto 15 day, followed by partial pricking of cover for air circulation inside vessel upto 20day, followed by removal of cover and finally all data was taken on 30th day of hardening.

The experiments were laid out on Completely Randomized Design (CRD), three replications for each treatment. Opstat software was used to analyze the recorded data and the data were subjected to standard analysis of variance (ANOVA) to test significance among different lines (Anonymous, 2022^b) [2]. The reported data's are the means of two experiments because these experiments repeated twice. The percentage related data were subjected to an Arc Sine transformation in Opstat.

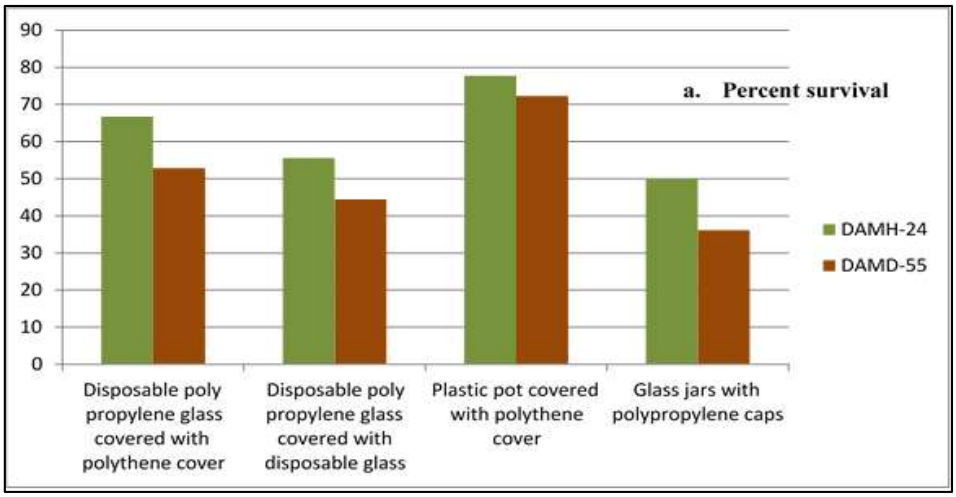
Results and Discussion

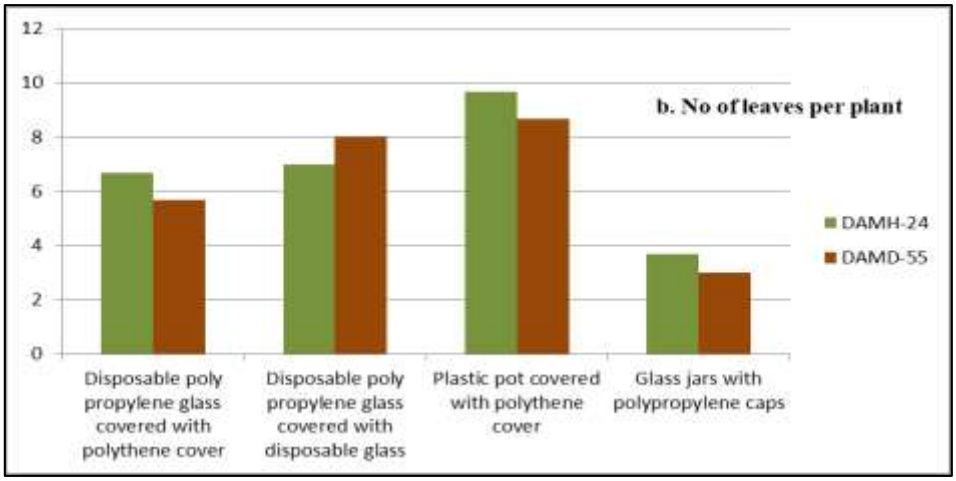
The controlled conditions during *in vitro* cultures often result in the formation of tender and open environment-sensitive plantlets with abnormal anatomy, physiology, and morphology. So, if these plants are directly shifted to the field (*ex vitro*), a sudden change in the environmental conditions may cause the death of these gynogenically developed plants. Hence, these plants need a period of acclimatization in order to get acclimatized and avoid the direct shock of biotic and abiotic components of the environment. Hence, in this study, we have optimized the efficient *in vitro* hardening techniques that will help in plant survival and reduction of plant mortality. It was noticed that percent survival was found to be the highest in treatment having Plastic pot covered with polythene cover (75.00%) (fig.2.a). This treatment was followed by disposable polypropylene glass covered with polythene cover (59.72%). *In vitro* hardening in glass jars covered with polypropylene caps resulted in the lowest survival percent (43.06%). While, when the survival of *in vitro* raised plants from two different genotypes was compared, it was observed that plants of DAMH-24 showed a higher survival percentage (62.5%) over the DAMH-55 (51.39%) after *in vitro* hardening (fig.1, Table 1.a). The genotypes × treatment interactions were found non-significant for the survival of gynogenically developed plants during *in vitro* hardening. These findings are supported by Kumar *et al.* (2017) [14] they reported that low-cost polyethylene plastic cups had the highest survival rate (98.1%), while a glass jar with a polypropylene cap had the lowest (29.3%). It was noticed that the maximum number of leaves (9.17) were recorded in plastic pot with polythene cover (fig.1.e) which was higher than disposable poly propylene glass covered with disposable glass and disposable poly propylene glass covered with polythene cover which recorded 7.5 and 6.17 number of leaves, respectively (fig.2.b). Among both genotypes, it was observed that DAMH-24 produced a significantly higher (6.75) number of leaves than DAMH-55 (6.33) (Table 1.b). The genotypes × treatment interactions were non-significant for the number of leaves per plant during *in vitro* hardening.

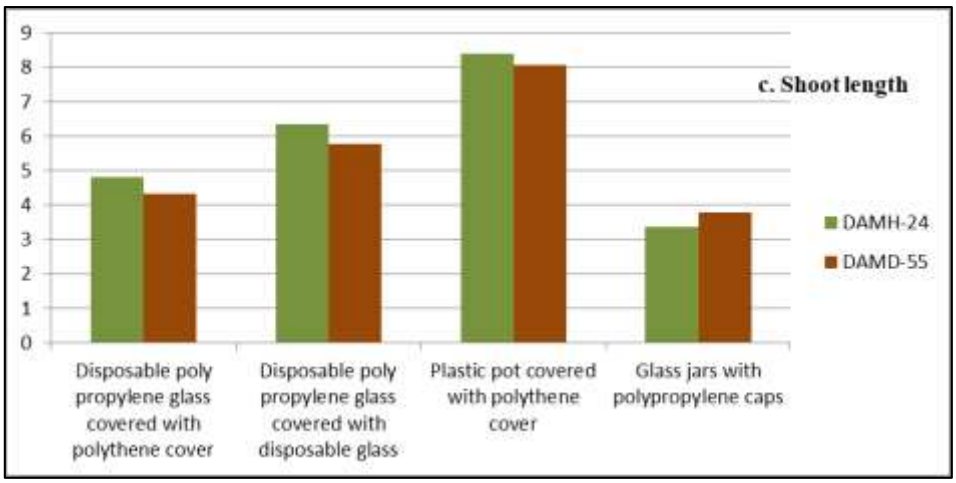
The shoot length under the Plastic pots covered with polythene covers was found highest (8.24 cm) and is significantly higher than the disposable poly propylene glass covered with disposable glass (6.06cm). Among the African marigold genotypes, DAMH-24 showed a shoot length of 5.73cm which was non-significantly higher than the DAMH-55 genotype (5.49cm) (fig.2.c, Table 2.a). The genotypes × treatments interactions were non-significant for shoot length during *in vitro* hardening. It was noted that the various hardening strategies had a significant impact on the visual growth of *in vitro* raised plantlets, the visual score was better in Plastic pots covered with polythene covers and a score of 4.5 was assigned to this treatment. The visual score of Plastic pots covered with polythene covers was non-significantly higher than the disposable polypropylene glass covered with polythene cover (3.83) (fig.2.d). Among the genotypes, DAMH-24 was assigned a score of 3.33 which was relatively higher than DAMH-55 genotype (3.00) (fig.1.f). The interactions between genotypes × treatments were significant for visual score during *in vitro* hardening. Based on the growth, the maximum score was assigned to DAMH-24 when

its gynogenically developed plants were hardened in the plastic pots covered with polythene cover (4.67), which was significantly higher than DAMH-55 when hardened on the same treatment (4.33). The lowest score of 1.00 was assigned to DAMH-24 when the *in vitro* raised plants were hardened in glass jars with polypropylene caps (Table 2.b). This high success during hardening in the plastic pots covered with polythene covers can be attributed to the minimum contact of leaves of plantlet to the outer surface of the container or cover used for *in vitro* hardening. As marigold plantlets have a larger canopy hence, the gynogenically developed plants are more susceptible to any direct contact with the surface/ or to the water droplets dropping from the surface of the covering. Hence, the larger diameter of plastic pots might have given more area and hence plants would have hardened more freely. These findings are contradictory to the results reported by Nazki *et al*. (2015) [17] in gerbera. These contradictions might have resulted due to the morphological differences between the two crops *i.e.* gerbera and marigold. The other factor of higher survival is larger size plastic pot covered with polyethylene had higher CO_2 concentration along with constant and higher humidity level, thus, improving the vegetative growth and survival of plantlet, this theory supported by Gribaudo *et al*. (1995) [7]. Spraying of potting mixture with ¼ MS salts (macro and micro salts) improved the acclimatization of plants, similar reported were given by Khawale *et al.* (2006) [12] in grape (*Vitis vinifera*).

Fig 1: *In vitro* hardening of gynogenically developed plants in African marigold genotype; a (plant ready for hardening); b, c, d (left container having DAMH-24 & right having DAMH-55); b (disposable poly propylene glass covered with polythene cover), c (disposable poly propylene glass covered with disposable glass), d (glass jars with polypropylene caps), e-best container for hardening (Plastic pot covered with polythene cover), f (plant after hardening ; DAMH-24







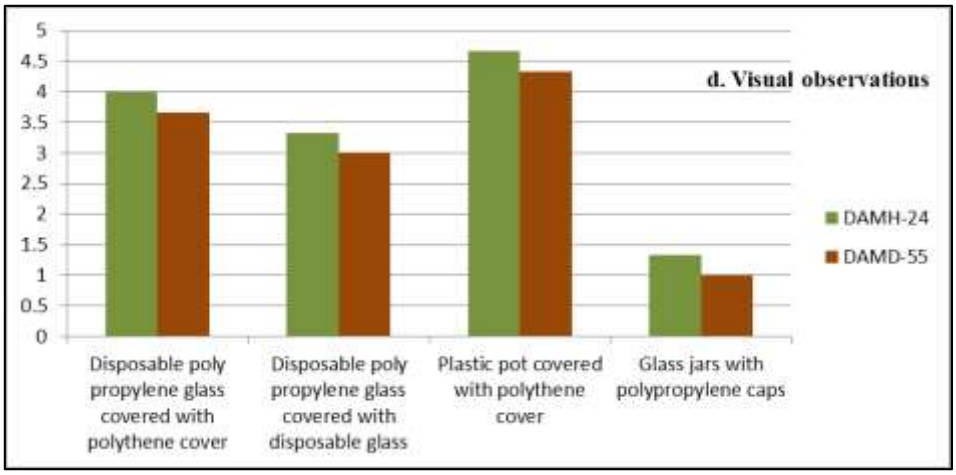


Fig 2: Graphical representation of *in vitro* hardening of gynogenically developed plants in African marigold genotypes; a. Percent survival, b. No. of leaves per plant, c. Shoot length, d. Visual observations.

Table 1: *In vitro* hardening (a) percent survival and (b) no. of leaves per plant of gynogenically developed plants in African marigold genotype.

Treatments*	Containers	Percent survival			No. of leaves per plant		
		DAMH-24	DAMD-55	Mean	DAMH-24	DAMD-55	Mean
T_1	Disposable poly propylene glass covered with polythene cover	66.67	52.78	59.72	6.67	5.67	6.17
T_2	Disposable poly propylene glass covered with disposable glass	55.56	44.44	50.00	7.00	8.00	7.50
T_3	Plastic pot covered with polythene cover	77.78	72.22	75.00	9.67	8.67	9.17
T_4	Glass jars with polypropylene caps	50.00	36.11	43.06	3.67	3.00	3.33
Mean		62.50	51.39		6.75	6.33	
		C D (p=0.05)	SEm±		C D (p=0.05)	SEm±	
Genotype		N/A	2.942		N/A	0.761	
Treatment		12.583	4.161		3.256	1.077	
Genotype × Treatment		N/A	5.885		N/A	1.523	

Table 2: *In vitro* hardening (a) percent survival and (b) no. of leaves per plant of gynogenically developed plants in African marigold genotype.

Treatments*	Containers	Shoot length			Visual observations		
		DAMH-24	DAMD-55	Mean	DAMH-24	DAMD-55	Mean
T_1	Disposable poly propylene glass covered with polythene cover	4.80	4.33	4.56	4.00	3.67	3.83
T_2	Disposable poly propylene glass covered with disposable glass	6.36	5.77	6.06	3.33	3.00	3.17
T_3	Plastic pot covered with polythene cover	8.41	8.07	8.24	4.67	4.33	4.50
T_4	Glass jars with polypropylene caps	3.35	3.78	3.57	1.33	1.00	1.17
Mean		5.73	5.49		3.33	3.00	
		C D (p=0.05)	SEm±		C D (p=0.05)	SEm±	
Genotype		N/A	0.553		N/A	0.283	
Treatment		2.366	0.783		1.208	0.4	
Genotype × Treatment		N/A	1.107		1.709	0.565	

Conclusion

In the final conclusion we can say that among different *in vitro* hardening techniques, plastic pots covered with polythene cover gave the best results; highest percent survival, the maximum number of leaves, highest shoot length as well as best visual growth score of the gynogenically induced plants. It was observed that plants of genotype DAMH-24 showed non-significantly higher survival percent, higher number of leaves, and more length of the shoots as well as best visual growth score when compared to DAMH-55. Further based on their hardening potentials suitability of these varieties on different potting vessel will be assessed and will use in future.

Future Scope

As Marigold is herbaceous flowering plant means comparatively very tender and sensitive to harsh environment. Specially, in vitro gynogenic regenerates of marigold are lacking of cutinized skin and well developed roots. So by this experiment we are developing technique to ease in plant to avoid *ex vivo* environment, which will help in multiplication of gynogenic plant of African marigold cultivars to avoid problems faced during lab-to-land transfer. And finally these plants will help in basic and strategic breeding in marigold for F1 hybrids production.

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