



***In vitro* regeneration of double haploid line of African marigold (*Tagetes erecta*) derived from ovule culture using non-axillary explants**

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ABSTRACT

An efficient protocol has been developed for *in vitro* regeneration of double haploid line of African marigold variety Local Orange derived through ovule culture. Maximum survival (85%) of leaf explant was found in the treatment comprising of Bavistin (0.2%), Ridomil (0.2%) and 8-HQC (200 ppm). The surface sterilization treatment of explants with mercuric chloride (0.1%) for 3 minutes resulted in maximum survival (88.33%) and minimum contamination (18.33%). Three different portions of leaf explant (Tip, middle and basal) were used for regeneration. The treatment comprising MS medium supplemented with BAP (0.5 mg/l) and NAA (0.25 mg/l) resulted in maximum percent regeneration (86.25), lowest number of days taken for callus initiation (3.05), minimum number of days for shoot regeneration (13.25), maximum number of micro shoots per explant (3.50), minimum number of days to reach the stage of sub culturing (5.75) with basal portion of leaf explant. Among the different explants, basal portion of the leaf was found as the best explant for maximum regeneration. The basal portion of the leaf explant was further treated with best regeneration treatment, i. e. BAP (0.5 mg/l) and NAA (0.25 mg/l) along with different concentrations of putrescine and it was observed that maximum percent regeneration (89.83%), minimum number of days for shoot regeneration (13.50), maximum number of micro shoots per explant (6.25) and minimum number of days to reach the stage of subculturing (5.75) were found in the treatment combination of BAP (0.5 mg/l), NAA (0.25 mg/l) and Putrescine (50 mg/l).

Key words: Doubled haploid line, *In vitro* regeneration, Non axillary explant, *Tagetes erecta* L.

Marigold (*Tagetes* spp.) is one of the most important flower crop known globally for its ornamental and medicinal values. It is an important member of Asteraceae family and native to South and Central America (Mexico). Genus *Tagetes* is comprise of approximately 55 species (Godoy-Hernandez and Miranda-Ham 2007), out of which, *Tagetes erecta* L. (African marigold) and *Tagetes patula* L. (French marigold) are of commercial importance. Marigold ranks first amongst loose flower crops grown in India with respect to area and production. It is being cultivated in an area of 66,130 ha with the production of 6,03,180 metric tonnes (Anonymous 2017) and is grown almost throughout the country. Biotechnology provides powerful tools for plant

breeding, and among these, tissue culture, particularly haploid and double haploid technology, is extremely valuable in plant breeding and genetics. The potential of haploidy for plant breeding arose in 1964 with the achievement of haploid embryo formation from *in vitro* culture of *Datura* anthers (Guha and Maheshwari 1964, 1966), which was followed by successful *in vitro* haploid production in tobacco (Nitsch and Nitsch 1969). Many attempts have been made since then, resulting in published protocols for over 250 plant species belonging to almost all families of the plant kingdom (Maluszynski *et al.* 2003). Haploids are valuable for obtaining pure lines through chromosome diploidization. In double haploid production system homozygosity can be achieved in single generation hence several generations of self-pollination are not needed. Such pure lines can provide two valuable advantages; first, direct establishment of new cultivars in self-pollinated crops and second, developing pure lines that could be used to produce uniform, high yielding F₁ hybrids (Veilluex 1994). 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 and can be done under both *in vivo* and *in vitro* conditions. The major drawback of *in vivo* propagation is lack of sufficient planting material for large

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scale multiplication and season bound nature of the activity. To overcome such limitations, *in vitro* mass multiplication is one of the best options to obtain maximum population of doubled haploids under short span of time, and moreover, it is a year round activity. *In vitro* multiplication of double haploids has been successfully done in crops like tall fescue (Kasperbauer and Eizenga 1985), sugar beet (Magdalena and Baranski 2013) etc.

MATERIALS AND METHODS

The present investigation was conducted during 2016–18 at ICAR-Indian Agricultural Research Institute, New Delhi.

Plant material: The source of explant was the doubled haploid line of African marigold variety Local Orange developed through ovule culture. The plants of the doubled haploid line were maintained under net house conditions at the farm of the Division of Floriculture and Landscaping, ICAR- Indian Agricultural Research Institute, New Delhi,

Three different portions (Tip, middle and basal) of leaf were used as explants.

Pretreatment, surface sterilization and explant preparation: Explants were collected from the healthy and disease free plants in the morning and washed with liquid detergent Teepol (0.1%) for 5 min. followed by washing under running tap water for 10 min. Different pre treatments were given comprising of Bavistin, Ridomil and 8-HQC for duration of 30 and 60 min. in a horizontal shaker at 120 rpm (Table 1). After pretreatment, the explants were sterilized with $HgCl_2$ for different durations (Table 1). The explants were washed 4–5 times with sterilized double-distilled water. Twenty explants were cultured per replication. The explants were cut into small sections and placed aseptically in test tubes containing culture media.

Culture establishment and multiplication: Leaf explants (tip, middle and basal) were used to establish aseptic shoot culture on MS (Murashige and Skoog 1962) basal media supplemented with various concentrations of plant

Table 1 Effect of pretreatment and surface sterilization on contamination (%) and survival (%) of the leaf explant

Treatment	Bacterial (%)	Fungal (%)	Total Contamination (%)	Survival (%)
<i>Pretreatment</i>				
T ₀ - Control (Distilled water) for 60 min.	30.00 (33.20)*± 0.00	70.00 (56.77) ±0.00	100.00 (90.00) ± 0.00	00.00 (0.00) ± 0.00
T ₁ - Bavistin (0.1%) + Ridomil (0.1%) + 8-HQC (200 mg/l) for 30 min.	12.35 (20.57) ± 0.06	70.46 (57.06)± 0.06	82.80 (65.52) ± 0.87	17.20 (24.45) ±0.87
T ₂ - Bavistin (0.1%) + Ridomil (0.1%) + 8-HQC (200 mg/l) for 60 min.	9.33 (17.77) ± 0.14	44.68 (41.93) ±0.07	54.00 (47.28) ± 1.08	46.00 (42.68) ±1.08
T ₃ - Bavistin (0.2%) + Ridomil (0.2%) + 8-HQC (200 mg/l) for 30 min.	8.71 (17.15) ± 0.09	69.29 (56.32) ±0.15	78.00 (62.04) ± 0.84	22.00 (27.93) ±0.84
T ₄ - Bavistin (0.2%) + Ridomil (0.2%) + 8-HQC (200 mg/l) for 60 min.	4.40 (12.10) ± 0.13	10.64 (19.03) ±0.12	15.00 (22.55) ± 1.82	85.00 (67.42) ±1.82
CD (P≤ 0.05)	0.34	0.21	3.24	3.24
<i>Surface sterilization</i>				
T ₀ - distilled water	31.00 (33.81)*±1.00	69.00 (56.15) ±1.00	100.00 (90.00)±0.00	00.00 (00.00)±0.00
T ₁ - $HgCl_2$ (0.1%) for 3 min.	7.07 (15.41) ±0.09	11.26 (22.26) ±0.07	18.33 (25.30) ± 1.67	88.33 (70.67)±4.41
T ₂ - $HgCl_2$ (0.1%) for 4 min.	7.30 (15.67) ±0.15	14.36 (19.60) ±0.02	21.67 (27.70) ± 1.67	68.33 (55.75)±1.67
T ₃ - $HgCl_2$ (0.1%) for 5 min.	6.47 (14.72) ±0.22	26.93 (31.25) ±0.09	33.33 (35.24) ± 1.67	56.67 (48.82)±1.67
T ₄ - $HgCl_2$ (0.1)% for 6 min.	6.93 (15.26) ±0.09	33.14 (35.13) ±0.09	40.00 (39.22) ± 0.00	48.33 (44.03)±1.67
T ₅ - $HgCl_2$ (0.1%) for 7 min.	6.17 (14.37) ±0.09	35.57 (36.60)± 0.18	41.67 (40.18) ±1.67	46.67 (43.07)±1.67
T ₆ - $HgCl_2$ (0.1%) for 8 min.	5.70 (13.81) ±0.15	45.97 (42.67) ±0.12	51.67 (45.94) ± 1.67	45.00 (42.10)±2.89
CD (P≤ 0.05)	1.13	1.26	3.49	6.26

*Values in parenthesis are angular value

growth regulators. Before autoclaving at 121°C for 20 min. at 15-20 psi (1.05-1.40 kg/cm²) pressure, pH of the medium was adjusted to 5.8. Putrescine was incorporated into autoclaved medium (after filter-sterilization using 0.22 µm filters). The cultures were maintained at 24±2°C temperature under 16/8 h (light/dark) photoperiod with a photosynthetic light at a flux rate of 3000 Lux and a relative humidity of 80-90%, maintained inside the closed vessels and that of culture rooms was automatically maintained by air conditioner. Optimum concentration of growth regulators was standardized for efficient shoot morphogenesis by testing different concentrations of BAP and NAA (Table 2). Subculturing of micro shoots was done on shoot multiplication media as standardized by Ravindra (2018). Different concentrations of Putrescine were tested in combination with best regeneration medium for further improvement of shoot regeneration (Table 3). The basal MS media was supplemented with nicotinic acid (0.9 mg/l), thiamine HCL (0.9 mg/l), pyridoxine (0.9 mg/l), ascorbic acid (0.4 mg/l), citric acid (0.4 mg/l), polyvinylpyrrolidone (200 mg/l), silver nitrate (2.5 mg/l), glutamine (80 mg/l) and plant preservative mixture (1 ml/l).

Statistical analysis: The experiment was laid out in completely randomized design (CRD). Each treatment had four replications and each replication had 20 units/plants. Each experiment was repeated 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

Effect of different pre-treatmentson the survival of leaf explant: The effect of different pre-treatments on leaf explant of doubled haploid line of marigold variety Local Orange derived through ovule culture is presented in Table 1. Bavistin (0.2%), Ridomil (0.2%) and 8-HQC (200 mg/l) for 60 min. recorded minimum total contamination of 15% including bacterial (4.40%) and fungal (10.64%) contamination as compared to control which recorded 100% total contamination including bacterial (30%) and fungal (70%). Maximum survival of explants (85%) was observed in treatment combination with Bavistin (0.2%) + Ridomil (0.2%) + 8-HQC (200 mg/l) for 60 minutes, as compared to control (00.00%). It was observed that total contamination decreased by increasing the concentration of both the fungicides in combination, i. e. Bavistin and Ridomil from 0.1 to 0.2% at constant level of 8-HQC (200 mg/l) thereby increasing survival percentage of explant. It was also observed that duration of treatment was also important as duration of treatment increased from 30–60 min., it resulted in less contamination and maximum survival percentage. The combination of Bavistin and Ridomil aids in controlling the fungal contamination whereas

Table 2 Effect of growth regulators on direct shoot regeneration from basal portion of leaf explant

Treatment	Days for initial callus formation	Regeneration (%)	Days required for shoot regeneration	No.of micro-shoots/ explants	Days required by micro shoots to reach the stage of sub culturing	No.of vitrified shoots (percentage)
T ₀ - control only MS ,devoid of hormone	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00 (0.00)± 0.00
T ₁ - MS + BAP(0.25 mg/l) + NAA (0.25 mg/l)	5.45±0.19	65.00 (53.71)*±0.00	18.50±0.29	1.00±0.00	7.25±0.25	2.25 (8.38)± 0.63
T ₂ - MS + BAP (0.5 mg/l) + NAA (0.25 mg/l)	3.05±0.09	86.25 (68.27)±1.25	13.25±0.25	3.50±0.29	5.75±0.48	0.75 (3.47) ±0.48
T ₃ - MS + BAP (1.0 mg/l) + NAA (0.25 mg/l)	4.98±0.56	56.25 (48.58)±2.39	17.00±0.41	2.85±0.03	7.00±0.41	3.00 (9.90)± 0.41
T ₄ - MS + BAP (2.0 mg/l) + NAA (0.25 mg/l)	6.50±0.20	47.50 (43.54)±2.50	19.50±0.29	2.48±0.03	6.50±0.65	3.50 (10.64)± 0.25
T ₅ - MS + BAP (0.25 mg/l) + NAA (0.5 mg/l)	7.30±0.19	42.50 (40.67)±1.44	19.75±0.25	1.00±0.00	6.75±0.25	4.25 (11.83) ±0.48
T ₆ - MS + BAP (0.5 mg/l) + NAA (0.5 mg/l)	6.50±0.21	42.50 (40.66)±1.44	20.50±0.29	1.00±0.00	6.50±0.29	1.75 (7.53)± 0.25
T ₇ - MS + BAP (1.0 mg/l) + NAA (0.5 mg/l)	7.58±0.17	33.75 (35.47)±2.39	21.25±1.25	2.35±0.12	6.00±0.00	3.25 (10.36)± 0.25
T ₈ - MS + BAP (2.0 mg/l) + NAA (0.5 mg/l)	7.90±0.32	38.75 (38.48)±1.25	25.25±0.25	2.23±0.08	6.75±0.25	2.50 (8.84)± 0.64
CD (P≤0.05)	0.76	4.86	1.43	0.31	1.01	1.37

*Values in parenthesis are angular value

Table 3. Effect of Putrescine on direct shoot regeneration from basal portion of leaf explant

Treatment	Days for initial callus formation	Regeneration percentage	Days required for shoot regeneration	No. of micro shoots/ explant	Days required by micro shoots to reach the stage of sub culturing (from shoot emergence)
T ₀ . control MS + BAP (0.5 mg/l) + NAA(0.25 mg/l)	3.05± 0.09	85.62 (67.70)*± 0.44	13.75± 0.48	3.50± 0.29	6.75 ± 0.25
T ₁ . MS + BAP (0.5 mg/l) + NAA (0.25 mg/l) + Putrescine (10 mg/l)	3.05±0.09	86.38 (68.39)±1.37	13.75± 0.50	3.75± 0.25	6.5 ± 0.65
T ₂ . MS + BAP (0.5 mg/l) + NAA (0.25 mg/l)+ Putrescine (30 mg/l)	3.57± 0.16	88.25 (69.99)± 1.18	13.50± 0.29	4.00± 0.41	6.25 ± 0.48
T ₃ . MS + BAP (0.5 mg/l) + NAA (0.25 mg/l) + Putrescine (50 mg/l)	3.72± 0.24	89.83 (71.82)± 0.28	13.50± 0.29	6.25± 0.48	5.75 ± 0.48
T ₄ . MS + BAP (0.5 mg/l) + NAA (0.25 mg/l) + Putrescine (70 mg/l)	3.82± 0.21	78.00 (62.11)± 2.71	14.50 ±0.29	3.75± 0.25	7.00 ± 0.41
T ₅ . MS + BAP (0.5mg/l) + NAA (0.25 mg/l)+ Putrescine (100 mg/l)	4.15± 0.31	71.82 (57.93)±4.08	15.25± 0.85	4.00± 0.41	7.25 ± 0.25
CD (P≤0.05)	0.51	4.29	6.97	1.07	N/A

*Values in parenthesis are angular value

8-HQC reduces the bacterial infection. The results are in confirmation with earlier (Ravindra 2018).

Effect of surface sterilant on the survival of leaf explant:

After the pre-treatment, the leaf explants were surface sterilized with 0.1% HgCl₂ for different durations (Table 1). It was reported that surface sterilization of leaf explant in treatment with HgCl₂ (0.1%) for 3 min exhibited minimum total microbial contamination (18.33%) which included bacterial infection (7.07%) and fungal infection (11.26%) as compared to treatment with distilled water only (control) which exhibited total contamination (100%) that included bacterial infection (31%) and fungal infection (69%). No survival was reported in control. Prolonged exposure (5, 6, 7 and 8 min.) of leaf explants to HgCl₂ (0.1%) although recorded the reduced microbial contamination of (33.33%, 40%, 41.67% and 51.67%) but the survival was affected adversely (56.67%, 48.33%, 46.67% and 45%). Moreover, it caused browning of the explants followed by subsequent drying within a week. It was also observed that exposure of leaf explants to HgCl₂ (0.1%) for more than 3 minutes resulted in mortality. This may be due to the toxicity caused by increased duration of sterilant exposure on leaf explants. Similar results are reported by Kothari and Chandra (1984, 1986), Misra and Datta (1999, 2000), Nikam and Khan (2014), Soudamini (2017) and Ravindra *et al.* (2018) in marigold.

Effect of basal media and growth regulators on direct shoot morphogenesis from different portions of leaf explant:

After pre-treatment and surface sterilization, the tip, middle and the basal portion of leaf explant were cultured on MS medium supplemented with different concentrations of growth regulators (BAP, NAA) (Table 2). Best results were shown by the basal portion of the leaf explant in terms of various parameters as recorded. With the basal portion as

leaf explant, best establishment was observed in treatment with MS supplemented with BAP (0.5 mg/l) and NAA (0.25 mg/l), which resulted in minimum number of days (3.05) taken for initial callus formation, maximum per cent regeneration (86.25), minimum number of days for regeneration (13.25), maximum number of micro shoots (3.5) per explant, minimum number of days (5.75) to reach the stage of subculturing and minimum percent of vitrified shoots (0.75) as compared to control (Table 2). Auxins and cytokinins are compatible to each other and play a very important role in affecting regeneration. Hence it was recorded that Lower (basal) portion of leaf responded well to the best treatment for the above mentioned parameters. The composition of the culture media largely affects the *in vitro* growth and morphogenesis of plant tissue. MS (Murashige and Skoog 1962) medium is widely used to induce direct or indirect shoot organogenesis/regeneration from different explants inoculated on to culture media. However, requirements of plants vary greatly; medium composition is often devised considering the specific need of a particular plant system. Hence, alterations are to be made in media composition suiting to their best growth and development. Misra and Datta (2001) observed direct regeneration from leaf explants of African marigold when cultured on MS + GA₃ (14.43mM) and BA (4.44mM). Ying-Chun *et al.* (2005) obtained maximum regeneration capacity in *Tagetes patula* leaf explants cultured on BA (1 mg/l) and NAA (1.5 mg/l). Ravindra reported that maximum establishment of leaf explants was observed on EMS supplemented with 2 mg/l BAP and 0.5 mg/l NAA.

The results showed the importance of basal media along with hormones for direct regeneration from leaf explants of African marigold. The enhanced regeneration is observed with optimum level of cytokinin and auxin

along with addition of organics, viz. coconut water (5%), amino acids and vitamins. The use of coconut water for regeneration was reported by several workers, Ravindra *et al.* (2018), Soudamini (2018) in marigold and Khatun *et al.* (2018) in carnation.

Optimization of best portion of leaf explant for in vitro mass multiplication: The source of explant was standardized on the basis of number of shoots produced per explant. It was found that basal portion of leaf produced highest number of shoots per explant (3.2) when cultured on MS supplemented with BAP (0.5 mg/l) and NAA (0.25 mg/l) followed by 2.77 in treatment with MS supplemented with BAP (1 mg/l) and NAA (0.25 mg/l). Similar results were observed by Ravindra *et al.* (2018). They showed that best regeneration was observed in matured leaf bases of marigold which was statistically at par with young leaf base explant.

Effect of Putrescine on multiple shoot induction and shoot proliferation: After standardizing the best combination of BAP and NAA, the best explant portion (basal) was established on MS supplemented with BAP (1 mg/l) and NAA (0.25 mg/l) with added Putrescine in different concentrations. From the results (Table 3), it was recorded that Putrescine in the concentration of 50.0 mg/l showed best results in parameters like number of micro shoots per explant, days required by micro shoots to reach the stage of subculturing and percent regeneration wherein Putrescine (50 mg/l) in combination with BAP (0.5 mg/l) and NAA (0.25 mg/l) produced maximum number of micro shoots (6.25) per explant, minimum number of days (5.75) to reach the stage of sub culturing and a maximum per cent regeneration (89.82%). However days required for regeneration was found minimum (13.50) equally in two combinations, i.e BAP (0.5 mg/l), NAA (0.25 mg/l) and Putrescine (30 mg/l) and BAP (0.5mg/l), NAA (0.25 mg/l) and Putrescine (50mg/l). Data also showed that minimum number of days (3.05) taken for initial callus formation was observed equal in treatment with MS supplemented with BAP (0.5 mg/l) and NAA (0.25 mg/l), i. e. control and treatment with MS supplemented with BAP (0.5 mg/l), NAA (0.25 mg/l) and Putrescine (10 mg/l). The results are in confirmation with the findings of Soo CheonChae (2016) who cultured leaf explants of *Echinacea angustifolia* for initial shoot regeneration in media with different concentrations of Putrescine. They found that increase in the concentration of Putrescine resulted in increased regeneration capacity and shoot growth. They observed best treatment of Putrescine was 70 mg/l. Similarly Rohilla *et al.* (2016) found that Putrescine at 50 mg/l promoted culture response, number of shoots regenerated per explant and mean shoot length. Kim *et al.* (2016) also evaluated the effect of various concentrations of Putrescine on shoot regeneration and shoot length of *Polygonum tinctorium* and found that culture media supplemented with Putrescine 50 mg/l recorded longest and highest number of shoots and further increase in concentration reduced the shoot regeneration and elongation. Similarly, Park *et al.* (2012) also reported the improved shoot organogenesis of gloxinia

(*Sinningia speciosa*) with Putrescine @ 50 mg/l.

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