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Thaneshwari
Ph.D. Scholar-ICAR- Indian Agricultural Research Institute, New Delhi, India

C Aswath
Principal Scientist, Division of Floriculture and Medicinal Crops-ICAR- Indian Institute of Horticultural Research, Bengaluru, Karnataka, India

Correspondence
C Aswath
Principal Scientist, Division of Floriculture and Medicinal Crops-ICAR- Indian Institute of Horticultural Research, Bengaluru, Karnataka, India

Effect of plant growth regulators and sucrose concentration on callus induction and shoot differentiation from ovary culture of marigold (*Tagetes* spp).

Thaneshwari, C Aswath

Abstract
Marigold (*Tagetes* spp) an important ornamental plant, is also recognized as potential source of carotenoids and currently being used as nutritional supplements, food colorants and poultry feed additives. Most of the hybrids and varieties of marigold have been developed by conventional method of breeding. *In vitro* androgenesis and gynogenesis is a rapid and efficient method for developing new varieties in a relatively short time. The present paper reports the effect of growth regulators and the level of sucrose on callus induction from ovary culture of marigold cultivar 'Arka Agni'. Ovaries were cultured on a MS medium supplemented with seventeen different combinations of plant growth regulators. Seven levels of sucrose were evaluated. Variable callogenic responses were expressed on induction media supplemented with varying concentration of growth hormones. Pale green colour compact calli with 89.66% callus induction rate has been reported in MS media supplemented with 4.44 µM BAP and 4.52 µM 2,4-D. 10.08% shoot differentiation rate has been reported when calluses were sub cultured on MS media supplemented with 4.44 µM BAP and 1.07 µM NAA. MS media without plant growth regulator was found to be the best rooting medium. 4% sucrose concentration on MS media was found to be optimum for callus induction. In summary, the present study shows positive result in terms of regenerating marigold plantlets from ovary culture. There is a need for testing of the plantlets produced in terms of genetic variation as well as the ploidy level. Since there is a high genotypic dependence of the ability for gynogenesis, more studies involving different marigold cultivars are required.

Keywords: ovary culture, callus induction, plant growth regulator, plant regeneration, *Tagetes erecta*

1. Introduction
Marigold (*Tagetes* spp) a potential ornamental plant, belongs to family Asteraceae. This genus is recognized as potential source of carotenoids and currently being used as nutritional supplements, food colorants, poultry feed additives and in ophthalmology for the treatment of age related ocular diseases. The essential oil from *Tagetes erecta* flowers is used in perfumery. Extract of marigold flower is used for coloration of 100% cotton, silk and wool fabrics. In India, at present the area under marigold is 55.89 thousand hectare with a production of 511.31 thousand metric tons. Its productivity varies from 2 tons per acre in Uttar Pradesh to 8.2 tons per acre in Karnataka (National Horticulture Board, 2015). Indian Institute of Horticultural Research, Bangalore, released a hybrid, Arka Agni, which yields up to18 tons per acre. This variation in marigold productivity is due to its heterozygous nature. Improvement of this valuable species is hampered by its heterozygosity. Producing homozygous lines by conventional methods is time consuming and difficult, because this species prefer cross pollination. Production of homozygous line by doubling the chromosome number of haploid line produced through androgenesis and gynogenesis is a better alternative.
In marigold, anther culture was employed for the production of androgenic haploids, but only callus mediated embryogenesis was obtained (Li *et al*., 2007; Yingchun *et al*., 2011) [26]. To the best of our knowledge, there appears to be no reports on successful production of haploids and doubled haploids through microspore culture in marigold. Ovules are a possible alternative source for haploid production in plants, particularly in species where microspores or pollens are not much responsive to manipulation *in vitro*. Gynogenesis is, therefore, one of the methods used for obtaining haploid plants through culture of unpollinated ovules, *in vitro* and successful production of gynogenic haploids has been reported in several crop species

including wheat, maize, rice, onion, sugar beet, cucumber, carnation, squash, gerbera, sunflower, wheat, barley etc (reviewed by Chen *et al*., 2011) [6].

The objective of the present study was to develop a protocol for callus induction and plant regeneration in marigold using unpollinated ovules from ray and disc florets. We studied the effects of growth regulators and sucrose concentration on *in vitro* gynogenesis and subsequent plantlet regeneration.

2. Materials and methods

Plant material

Marigold cultivar 'Arka Agni' plants were grown using standard package and practice.

2.1 Effect of plant growth regulators on callus induction from in vitro ovary culture of marigold-Preparation of induction media, sterilization procedure of bud and induction of callus

MS media (Murashige & Skoog, 1962) [16] as induction media supplemented with seventeen varying concentrations of growth regulators keeping 3% sucrose, 0.75% agar as constant (I_1–I_{17}) was used to determine optimal conditions supporting *in vitro* callus formation from cultured ovary (Tab. 1). The media was adjusted to pH 5.75 - 5.80 and autoclaved at 121 °C for 15 minutes. 25 ml aliquots of media were poured into 100×15 mm diameter Petri dishes. 20–25 mm long flower bud were collected from field. Under aseptic conditions, these buds were surface sterilized with 0.1% bavistin for 12-15 min, followed by rinsing with sterile distilled water. These buds were then disinfected by dipping in 70% ethanol for 30 s and in 1% NaOCl for a min. The disinfected buds were then rinsed with sterilized water for 3 times and blotted dry with aseptic filter papers. Ovaries were excised from sterilized flower buds and 70 ovaries were placed in a single plate. Seventeen varying hormone combinations were tested using complete random design, with 3 replicates for each type of induction medium. Ovary cultures were incubated at 25±1 °C in darkness until callus formed, then incubated under fluorescent 20 W daylight lamps (30–40 mol m^{-2} s^{-1}) for 16/8 h (light/dark). Callus induction rates and size of calli was recorded 25 day after ovary culture. Days to callus initiation was recorded when 50% of the ovary callused per plate.

2.2 Effect of plant growth regulators on shoot differentiation from in vitro ovary culture of marigold-Preparation of differentiation media and plant regeneration

MS media supplemented with seventeen varying concentrations of hormones were tested (labeled as D_1– D_{17}) as differentiation media (Tab. 2). Differentiation media was prepared similarly to induction media. In order to easily identify the origin of calli in this phase of the experiment, only calli produced in induction medium supplemented with 4.44 µM BAP and 4.52 µM 2,4-D were used for differentiation. Fifty callus were transferred to each Petri dishes of differentiation media. These Petri dishes were incubated under a 16 h photoperiod at 30–40 mol m^{-2} s^{-1} provided by fluorescent 20 W day light lamps at 25±1 °C for 30 days. A complete randomized design was used, with three replicates for each combination.

2.3 Effect of sucrose concentration on callus induction and shoot differentiation rate

Flower buds were collected from the field early in the morning. They were surface sterilised as given in 2.1 and then ovary were inoculated on MS media supplemented with 4.44 µM BAP and 4.52 µM 2, 4-D with different concentrations of sucrose (0%, 2%, 4%, 6%, 8%, 10% and 12%). These cultured ovaries and induced calluses from them are maintained and sub-cultured as given in 2.1.

2.4 Plantlet rooting and transplanting

Healthy shoots differentiated from green calli with 3 - 4 true leaves were excised from the culture and transferred to one of the four types of rooting media. All rooting media consisted of MS salts, 3% sucrose, 0.8% agar, and pH adjusted to 5.75–5.80. Each type was supplemented with four levels of IBA (0, 0.49, 0.98 and 1.47 µM) and subjected to autoclaving as previously described. The well-developed plantlets were acclimatized for ten days. Subsequently, the plantlets were washed with tap water to remove traces of the medium and transferred to pots containing coco peat.

3. Results

3.1 Effect of plant growth regulators on callus induction from in vitro ovary culture of marigold

Ovaries of marigold cultivar 'Arka Agni' were cultured on MS media with different plant growth regulator combination (Tab. 1; I_1-I_{17} induction media). Highest callus induction rate (89.66%) was recorded when ovaries were cultured on MS media supplemented with 4.44 µM BAP + 2.26 µM 2, 4-D or 4.44 µM BAP + 4.52 µM 2, 4-D or 4.44 µM BAP + 2.68 µM NAA or 4.44 µM BAP + 5.37 µM NAA. Minimum days to callus initiation (15 days) was reported in MS media supplemented with 2.22 µM BAP + 2.68 µM NAA, which was found to be statistically at par with treatment where MS media was supplemented with 1.99 µM Ki + 2.68 µM NAA (15.67 days). Varying calogenic response was recorded in different treatment (Tab.1 and Fig 1). Pale green colour compact calli of size 8.00 mm was recorded in MS media supplemented with 4.44 µM BAP and 4.52 µM 2,4-D (I_{10}). Combination of Ki and 2,4-D (I_1-I_4) produced brown coloured small calli (1.83-4.00 mm) and Combination of Ki and NAA (I_5-I_8) produced yellow coloured small and compact calli (1.00 - 4.50 mm). Combination of BAP and NAA (I_{13}-I_{16}) produced calli with white fibrous root.

3.2 Effect of plant growth regulators on shoot differentiation from in vitro ovary culture of marigold

After 25 days of culture on differentiation media (D_1-D_{17}), calli showed various responses (Tab. 2). In particular, highest shoot induction rate (10.08%) was observed on MS medium containing 4.44 µM BAP and 1.07 µM NAA (Fig 1). This treatment was found to be significantly superior then all other treatments. In contrast, calli grown on medium D_9-D_{12} produced no shoots. Callus grown on media D_1-D_4 became brown and died within 7 days of transfer. Callus grown on media D_5-D_8 gradually became brown and died after a week or two.

3.3 Effect of sucrose concentration on callus induction and shoot differentiation rate

MS medium supplemented with 4.44 µM BAP and 4.52 µM 2, 4-D having 0%, 2%, 4%, 6%, 8%, 10% and 12% sucrose showed significance result in term of callus induction rate and shoot differentiation rate (Tab. 3). Highest callus induction rate (89.66%) was reported in media having 4% and 6% sucrose followed by in media having 2% sucrose (87.48%). The treatment (4% and 6% sucrose) was found to be significantly superior from all other treatment in terms of

callus induction rate and size of calli. Maximum calli size was found in media with 4% sucrose but as the concentration of sucrose increased from 4%, calli size decreased. There was no callus induction in media with 0% sucrose. Highest shoot differentiation rate had been reported in media having 4% sucrose and it was found to be statistically superior to other treatment.

3.4 Rooting of the regenerated plants and transplanting

Regenerated shoots were excised and transferred for rooting in four different media. The pattern of rooting differed in each medium. In PGR free MS medium, the roots were long and extensive. With increases in IBA concentration, rooting rate decreased, and roots became shorter and thicker. Long root (7.06 cm) was reported in MS media without any plant growth regulators. As the concentration of IBA increases in rooting media from 0 to 1.47 µM, we observed a reduction in length of root. Smallest root of length 0.66 cm was reported in MS media having 1.47 µM IBA. As the concentration of IBA increases in rooting media from 0 to 1.47 µM, thickness of roots also increased. 2.48 mm and 0.50 mm thick root was observed in MS media supplemented with 1.47 µM IBA and 0.00 µM respectably (Tab. 4). After 20-25 days of culture on rooting medium, the plantlets were transferred to coco peat in growth chamber.

4. Discussion

In gynogenesis different explants may be used: whole flowers inoculated directly onto the medium were used for onion (Martinez, 2003) [13], the ovaries were efficient in tomato (Bal and Abak, 2007) [2] wheat (Salma-Ayed and Slim-Amara, 2007) [19], carnations (Sato *et al*., 2000) [21] and chrysanthemum (Miler and Muszczyk, 2015) [15] while in gerbera (Sitbon, 1981; Meynet and Sibi, 1984; Tosca *et al*., 2000) [22, 14, 24] and hibiscus (Ibrahim, 2015) [10] only naked ovules were used. In our research we had used ovaries as explants.

Medium composition and PGR concentrations have a key role in the formation and differentiation of calli. It is found that the key step in the culture procedure for obtaining haploid plants is the induction of high quality callus from the ovary, and the main strategy is the use of plant growth regulators in appropriate concentrations. Growth regulators, especially auxins are commonly used for induction of gynogenesis, and their optimum concentrations have been reported to vary considerably from species to species (San Noeum and Gelebart, 1986) [20]. In sunflower, gynogenesis occurred only when 2,4-D or NAA was added to the medium (Gelebart and San Noeum, 1987) [7]. Whereas, gynogenic haploids in mulberry could only be produced on media supplemented with BA or kinetin (Lakshmi Sita and Ravindran, 1991) [12]. In our experiment the most efficient induction medium for callus regeneration was supplemented with 4.44 µM BAP + 4.52 µM 2,4-D (Tab. 1). Similarly Burbulis *et al*., 2007 [5] reported that ovaries of flax cultivar Szaphir showed better response for callus induction when these were cultured on MS media supplemented with 1.0 mg l^{-1} BAP+2.0 mg l^{-1} 2,4 D. Bhat and Murthy, 2007 [3] also found that indirect callus-mediated gynogenesis occurred in cultivar JNC-6 and Ootacamund of niger when the ovules were cultured on MS medium supplemented with 2,4–D either alone (0.5–2.0 mM) or in combination (2.0 mM) with different cytokinins, such as adenine, BA, 2iP and kinetin (0.5–2.0 mM). Burbulis *et al*., 2011 [4] also found that combination of 1 mg L^{-1} BAP with 2 mg L^{-1} 2,4-D promoted callus development in ovaries of cultivar 'Norman'. Combination of 2, 4-D and BAP has also been reported good for callus induction in lily (Ramsay *et al*., 2003) [18], chrysanthemum (Miler and Muszczyk, 2015) [15] and onion (Yarali and Yanmaz, 2017) [25].

The combination of BAP and NAA played an important role in regulating shoot regeneration from marigold ovary calli in our study. Subculture of these calli onto regeneration medium containing BAP and NAA promoted shoots formation. Most shoots regenerated from ovaries which had been transferred from the medium containing 4.44 µM BAP+4.52 µM 2,4-D. Callus regenerated on the media containing BAP and 2,4-D was pale green, compact, semi hard and uniform, however, it became green after a subculture onto 4.44µM BAP+0.54/1.07 µM NAA containing MS medium. 10.08% of the ovary-derived callus produced shoots when subcultures on to MS media containing 4.44 µM BAP+1.07 µM NAA. Bhat and Murthy, 2007 [3] found that in niger gynogenic embryo differentiated and mature on media supplemented with 0.5 mM NAA+1.0 mM kinetin, and 0.5 mM ABA, respectively. Similar to our result, Burbulis *et al*., 2011 [4] also reported highest rate of shoots per ovary-derived callus when ovaries of linseed cultivar 'Mikael' were sub cultured on medium supplemented with 2 mg L^{-1} BAP with 1 mg L^{-1} NAA. Similar result has also been reported in flax (Burbulis *et al*., 2007) [5] and chrysanthemum (Miler and Muszczyk, 2015) [15].

We also reported that MS media without PGRs is suitable for rooting of marigold plantlets. Simlar results has been reported in Tagetes patula (Yingchun *et al*., 2011) [26] and baby primerose (Jia *et al*., 2014) [11].

The concentration of sucrose in the nutrient medium is also an important predetermining factor in the induction of callus or embryo from ovary culture. The present investigation has shown influence of varying sucrose concentrations on callus induction in ovary culture of marigold cultivar 'Arka Agni'. Increased levels of sucrose from (4% to 6%) induced induction of ovaries however, with increase in level of sucrose from 8% to 12% the number of ovaries producing callus significantly reduced. There was no callus induction in media having 0% sucrose. Similar results has been reported in lily (Ramsay *et al*., 2003) [18], flax (Burbulis *et al*., 2007) [5] and leek (Alan *et al*., 2016; Toprek *et al*., 2017) [1].

In conclusion, the present experiment results show that gynogenic haploid induction in marigold is possible through culture of unpollinated ovules *in vitro*. MS medium supplemented with 30 g l^{-1} sucrose, 4.52 µM 2,4-D and 4.44 µM BAP is suitable for induction of gynogenesis. Shoot differentiation can be achieved on MS medium (30 g l^{-1} sucrose) supplemented with 4.44 µM BAP+1.07 µM NAA. Until now, this is the first report on *in vitro*-culturing of non-pollinated ovaries of marigold. In summary, the present study shows positive result in terms of regenerating marigold plantlets from ovary culture. There is a need for testing of the plantlets produced in terms of genetic variation as well as the ploidy level. Since there is a high genotypic dependence of the ability for gynogenesis, more studies involving different marigold cultivars are required.

Table 1: Effect of various induction media on callus induction from cultured ovary of marigold cv. 'Arka Agni'

PGR (µM)					Callus induction rate (%)1	Days to callus induction	Callus size (mm)	Callus growth pattern
Induction medium	Ki	BAP	2,4-D	NAA				
I_1	3.98	-	2.26	-	78.90 [c]	30.33 [a]	1.83[i]	Very small brown calli
I_2	3.98	-	4.52	-	81.22 [b]	22.33 [d]	3.00[g]	Brown calli
I_3	1.99	-	2.26	-	66.27[ef]	30.67 [a]	4.00[f]	Brown soft calli
I_4	1.99	-	4.52	-	68.19[de]	28.67 [b]	4.00[f]	Brown soft calli
I_5	3.98	-	-	2.68	78.90[c]	16.33 [g]	4.33[e]	Yellowish calli
I_6	3.98	-	-	5.37	79.52[bc]	18.67 [f]	1.00[j]	Yellowish calli
I_7	1.99	-	-	2.68	40.6 [h]	15.67[gh]	4.50[e]	Yellowish calli
I_8	1.99	-	-	5.37	53.87[g]	16.33[g]	1.00 [j]	Yellowish calli
I_9	-	4.44	2.26	-	89.66[a]	23.33[c]	6.00[d]	Pale green friable calli
I_{10}	-	4.44	4.52	-	89.66[a]	19.33[f]	8.00[c]	Pale green compact calli
I_{11}	-	2.22	2.26	-	64.8[f]	21.67[d]	2.00[i]	Pale yellow friable calli
I_{12}	-	2.22	4.52	-	69.0[d]	20.3 [e]	2.33[h]	Pale yellow friable calli
I_{13}	-	4.44	-	2.68	89.66[a]	16.00 [g]	8.00[c]	White friable calli
I_{14}	-	4.44	-	5.37	89.66[a]	16.33[g]	10.00[b]	White calli with roots rroots
I_{15}	-	2.22	-	2.68	39.78[h]	15.00 [h]	12.00[a]	White friable calli
I_{16}	-	2.22	-	5.37	54.45[g]	16.00 [g]	4.00[f]	White calli with roots
I_{17}	0	0	0	0	0.34[i]	0.00 [i]	0.00[k]	No callus

70 ovary were inoculated in one petri dish; 3 replicates of each medium were performed
1Callus induction rate (%) = (No. of anther formed callus / No. of ovary inoculated) ×100.
Data followed by different letters are significantly different at 1% level of significance

Table 2: Effect of various differentiation media on shoot regeneration from calli derived from marigold cv. 'Arka Agni' ovary.

PGR (µM)					Numbers of calli cultured	Shoot differentiation rate (%)1
Induction medium	Ki	BAP	2,4-D	NAA		
I_1	3.98	-	0.45	-	50	0.41[c]
I_2	3.98	-	0.91	-	50	0.41[c]
I_3	1.99	-	0.45	-	50	0.41[c]
I_4	1.99	-	0.91	-	50	0.41[c]
I_5		4.44	0.45	-	50	0.41[c]
I_6		4.44	0.91	-	50	0.41[c]
I_7		2.22	0.45	-	50	0.41[c]
I_8		2.22	0.91	-	50	0.41[c]
I_9	3.98	-	-	0.54	50	0.41[c]
I_{10}	3.98	-	-	1.07	50	0.41[c]
I_{11}	1.99	-	-	0.54	50	0.41[c]
I_{12}	1.99	-	-	1.07	50	0.41[c]
I_{13}	-	4.44	-	0.54	50	8.13[b]
I_{14}	-	4.44	-	1.07	50	10.08[a]
I_{15}	-	2.22	-	0.54	50	0.41[c]
I_{16}	-	2.22	-	1.07	50	0.41[c]
I_{17}	-	-	-	-	50	0.41[c]

[1]Shoot differentiation rate (%) = (No. of callus that generating shoots in differentiation medium / No. of callus inoculated) × 100
Data followed by different letters are significantly different at 1% level of significance

Table 3: The effect of different concentration of sucrose on callus induction and shoot differentiation from ovary culture of marigold cv. 'Arka Agni'

Sucrose concentration	Callus induction rate (%)[1]	Days to callus induction	Callus size (mm)	Callus growth pattern	Shoot differentiation rate (%)[2]
0%	0.34[e]	0.00[e]	0.00[f]	ovary dried and became black	0.41[d]
2%	87.48[a]	23.67[d]	3.10[b]	yellowish green callus	9.74[b]
4%	89.66[a]	24.00[d]	4.43[a]	Pale green compact callus	10.31[a]
6%	89.66[a]	25.00[c]	3.00[b]	white calli	9.28[c]
8%	75.05[b]	26.00[b]	1.93[c]	White calli	0.41[d]
10%	70.68[c]	26.33[b]	1.40[d]	White calli	0.41[d]
12%	62.09[d]	30.00[a]	1.00[e]	White calli	0.41[d]

[1]Callus induction rate (%) = (No. of anther formed callus / No. of anthers inoculated) × 100.
100 anthers were inoculated in one petri dish
[2]Shoot differentiation Rate (%) = (No. of callus generating shoots in differentiation medium / No. of callus inoculated) × 100
Data followed by different letters are significantly different at 5% level of significance

Table 4: Effect of different concentration of IBA on rooting

IBA concentration (µM)	Root length (cm)	Root thickness (mm)
0 µM	7.06[a]	0.50[d]
0.49 µM	2.96[b]	1.00[c]
0.98 µM	1.42[c]	2.02[b]
1.47 µM	0.66[d]	2.48[a]

Data followed by different letters are significantly different at 1% level of significance

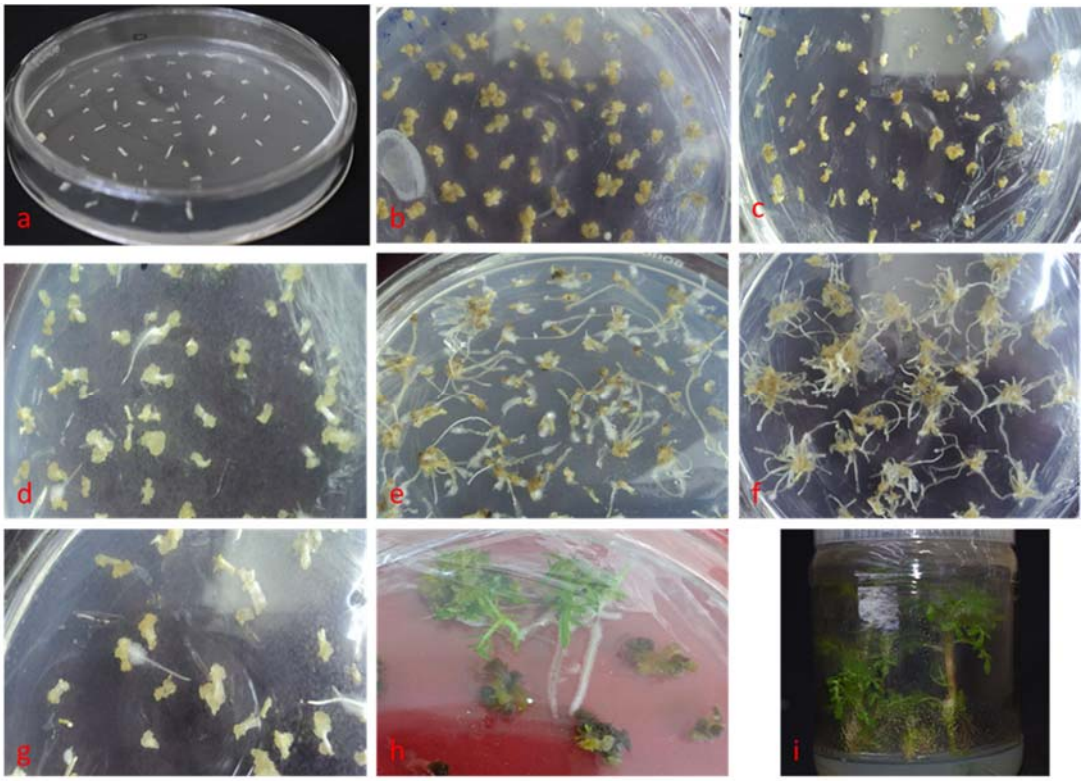

Fig 1: Callus induction and shoot regeneration of marigold *via* ovary culture; a) ovary culture on induction media; b) Callus on MS media supplemented with 3.98 µM Kinetin + 4.52 µM 2,4-D; c) Callus on MS media supplemented with 3.98 µM Kinetin + 2.68 µM NAA; d) Callus on MS media supplemented with 4.44 µM BAP + 2.26 µM 2,4-D; e) Callus on MS media supplemented with 2.22 µM BAP + 2.68 µM NAA; f) Callus on MS media supplemented with 4.44 µM BAP + 5.37 µM NAA; g) Callus on MS media supplemented with 4.44 µM BAP + 4.52 µM 2,4-D; h) Shoot differentiation (from callus derived from ovary culture) on MS media supplemented with 4.44 µM BAP + 1.07 µM NAA; i) Plantlet in growth regulator free MS media.

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