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Standardization of an *in vitro* regeneration protocol in gerbera (*Gerbera jamesonii* Bolus ex. Hooker F.)

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ABSTRACT

An experiment was undertaken to develop an improved *in vitro* regeneration protocol in gerbera. Murashige and Skoog (MS) medium was supplemented with various growth regulators at different concentrations for callus induction and organogenesis. Newly emerging leaves of Gerbera cv. Rosalin were used as explants. Experimental results showed that maximum rate (74.07%) of formation of callus with good growth was recorded on MS medium supplemented with 2.0mgL^{-1} 2,4-D + BAP 0.5mgL^{-1}. Best shoot regeneration (57.8 %) with maximum shoot number (12.0) was achieved on with BAP 2.0mgL^{-1} + NAA 0.5mgL^{-1} fortified MS medium. Maximum (66.7 %) and earliest (12.3 days) root formation in shoots was recorded on IBA 3.0mgL^{-1} is ½MS media. Survival rate of regenerated plantlets was maximum (73.33 %) in the potting mixture containing garden soil, sand and vermicompost (1:1:1).

Key words: *Gerbera*,*Gerbera jamesonii*, leaf explants, callus, *in vitro* regeneration

INTRODUCTION

Gerbera (*Gerbera jamesonii* Bolus ex. Hooker F.), also known as Transvaal daisy or Barberton Daisy, is one of the important commercial cut flowers in global flower business. Gerbera is propagated vegetatively by the division of suckers or clumps. Propagation through seeds is not preferred as the plants exhibit heterozygosity and non-uniformity. Also, the improved semi-double and double cultivars do not set seed. Propagation by division of suckers or clumps gives true-to-type plants, but multiplication rate is very low. Many new varieties are being introduced every year. To popularize these varieties and, also, to meet the demand for quality planting material of elite varieties, there is a need to develop a technology for rapid multiplication. Micropropagation is used mainly to to clonally propagate gerbera for commercial production of millions of plant-lets each year. Gerbera is propagated by direct or indirect *in vitro* organogenesis using various explants, including stem tips, floral buds, leaf, capitulum, etc. (Paduchuri *et al*., 2010; Akter *et al.*, 2012; Samanthi *et al*., 2013). Type of explant, different type of explant, growth regulator/ s and combinations thereof and genotype are the main factors for successful in vitro regeneration of gerbera. Organogenesis refers to production of organs, either directly from the explant or from callus culture. It relies on the inherent plasticity of plant tissues, and is regulated by altering the components in a medium. Biochemical changes that precede onset of organogenesis or embryogenesis can serve as markers for the differentiation processes that bring about morphological, developmental and functional specialization (Thorpe, 1990). During morphogenesis, certain enzymes and proteins produced are responsible for callus proliferation and differentiation into shoot buds (Chawla, 1991). As conventional methods of propagation are inadequate to meet the demand for planting material for commercial production, this experiment was set up to study the effect of some growth regulators to optimize culture media for *in vitro* regeneration. In this study, individual or combination effects of different growth regulators in MS media have been investigated for inducing indirect organogenesis.

MATERIAL AND METHODS

The experiment was conducted in Tissue Culture Laboratory of Department of Floriculture, Medicinal and Aromatic Plants, Faculty of Horticulture,

Uttar Banga Krishi Viswavidyalaya, Cooch Behar, West Bengal India, during the years 2011-2014.

Plant material and culture media

Murashige and Skoog (MS) medium was used as the base medium in all the experiments (Murashige and Skoog, 1962). For explant source, newly emerging leaves of gerbera cv. Rosalin were transferred to MS medium containing 2,4-D (1.0, 2.0 or 3.0mgL^{-1}) and NAA (1.0, 2.0 or 3.0mgL^{-1}) singly, or in combination with BAP (0.5mgL^{-1}) in for callus formation (Table 1). Callus initiation and growth were recorded to evaluate indirect organogenesis and for biochemical analysis. Three concentrations of BAP (1.0, 2.0 or 3.0mgL^{-1}) and Kinetin (1.0, 2.0 or 3.0mgL^{-1}), alone or in combination with NAA (0.5mgL^{-1}) in MS medium, were used for shoot regeneration from callus (Table 2). Various levels of NAA (1.0, 2.0 or 3.0mgL^{-1}) and IBA (1.0, 2.0 or 3.0mgL^{-1}) alone, as supplements in ½ MS medium, were used for root initiation in shoots (Table 3).

Rooted plantlets, after removal from the culture bottle, were washed off any adherent agar and planted in polycups containing different types of hardening media. The plantlets were hardened for 15 days under a mist chamber (80-90% humidity). After primary hardening, the plants were transferred to polybags containing potting medium and placed in a greenhouse for 15 days before transfer to field.

Per cent callus induction, days to callus induction, intensity and morphology of the callus formed, and fresh and dry weight of the callus were recorded. *Per cent* shoot regeneration, days to shoot regeneration, number and length of shoot; and, fresh and dry weight of shoot was also recorded. Root parameters like *per cent* root formation, days to shoot initiation, number and length of roots, and, fresh and dry weight of roots was recorded. Final survivability was recorded after hardening of the plantlets in a greenhouse.

The experiments were laid out under Completely Randomized Design, with ten replications (n=10) per treatment. Data were analyzed by Fisher's analysis of variance (ANOVA) technique, and results were interpreted. Where treatments were found significant in ANOVA, individual treatment means were compared by LeastSignificance Difference (LSD) test at P = 0.05.

RESULTS AND DISCUSSION

Exogenous supply of plant growth regulators is required to disturb the established polarity of auxins in explants. Leaf explants of cv. Rosalin showed maximum rate of callus induction (74.07 %) with good growth of callus, on MS medium supplemented with 2mgL^{-1} 2, 4-D + 0.5mgL^{-1} BAP (Table 1, Fig. 1). Increase or decrease in concentrations of growth regulators affected callus formation and, subsequent, callus growth. Paduchuri *et al*. (2010) found the same result (callus formation) on MS basal medium supplemented with BAP 2mgL^{-1} + 2,4-D 2.5mgL^{-1}. Formation of callus was positively correlated with concentration of growth regulators in the nutrient media. Results presented in Table 1 illustrate that MS medium fortified with 2,4-D 3mgL^{-1} + BAP 0.5mgL^{-1} took minimum time (10.3 days) for callus induction. This may be due to a stimulated, unorganized cell-growth under higher concentrations of the auxins (2,4-D and NAA), and the cytokinins may have facilitated early cell-division and cell-elongation in the culture. Fresh weight of callus increasing concentration of auxin 2,4-D and NAA, upto 3.0mgL^{-1} (Table 1). This could be due to the higher concentration of auxins applied exogenously to induce more number of unorganized cells which, in turn, increased cell volume and weight. Dry weight of callus increased with increasing fresh weight. Similar results on callogenesis were reported by Dutta and Gantait (2016), Paduchuri *et al.* (2010), Son *et al.* (2011), Akter *et al.* (2012), Samanthi *et al*. (2013) and Bhargava *et al*. (2013).

BAP 2.0mgL^{-1} + NAA 0.5mgL^{-1} resulted in highest shoot formation (57.78 %) in callus (Table 2, Fig. 2 & 3). Hasbullah *et al*. (2011) showed that only a few calli developed shoots when transferred to MS medium supplemented with 2.0mgL^{-1} BAP + 0.5 mgL^{-1} NAA, in addition to 3% (w/v) sucrose and 0.8% (w/v) agar in the medium. Number of days required for shoot regeneration decreased as the concentration of BAP increased (Table 2). Higher dose of growth regulators could have forced the plant into early shoot initiation. Data presented in Table 2 show that number of shoots increased significantly with higher concentration of BAP, upto 2.0mgL^{-1}. Also, higher cytokinin (BAP) concentration advanced shoot regeneration. Cytokinin to auxin ratio is critical. It is clear from the above findings that cytokinin (BAP) is a potent plant growth regulator for generation of

Table 1. Effect of 2,4-D and NAA, alone or in combination with BAP, on callus induction and morphology in gerbera

Sl.No.	Medium composition	% Callus formation (Mean ± SE)	Days to callus initiation (Mean ± SE)	Fresh weight of callus (g) (Mean ± SE)	Dry weight of callus (g) (Mean ± SE)	Intensity of callus formation	Callus morphology (structure & colour)
1.	MS + 1mgL^{-1} 2,4-D	59.26 ± 1.08	14.0 ± 0.0	2.28 ± 0.22	0.64 ± 0.11	C^{++}	Green, compact
2.	MS + 2mgL^{-1} 2,4-D	68.52 ± 4.83	13.0 ± 0.0	3.11 ± 0.22	0.86 ± 0.07	C^{+++}	Greenish-white, compact
3.	MS + 3mgL^{-1} 2,4-D	55.56 ± 1.85	12.3 ± 0.3	3.79 ± 0.02	1.05 ± 0.11	C^{++}	Greenish-white, compact
4.	MS + 1mgL^{-1} NAA	37.04 ± 1.11	16.7 ± 0.3	1.77 ± 0.09	0.49 ± 0.07	C^{+}	Brown,friable
5.	MS + 2mgL^{-1} NAA	40.74 ± 2.18	15.0 ± 0.0	1.95 ± 0.16	0.55 ± 0.07	C^{++}	Brown,compact
6.	MS + 3mgL^{-1} NAA	31.48 ± 1.15	13.7 ± 0.7	2.62 ± 0.10	0.72 ± 0.05	C^{+}	Brown-white, fragile
7.	MS + 1mgL^{-1} 2,4-D +	66.67 ± 3.33	13.0 ± 0.0	1.91 ± 0.14	0.54 ± 0.07	C^{++}	Green, nodular
8.	0.5mgL^{-1} BAP MS + 2mgL^{-1} 2,4-D +	74.07 ± 2.38	11.7 ± 0.3	2.40 ± 0.04	0.67 ± 0.06	C^{++}	Green-brown, nodular
9.	0.5mgL^{-1} BAP MS + 3mgL^{-1} 2,4-D +	7.41 ± 1.08	10.3 ± 0.3	2.65 ± 0.29	0.73 ± 0.09	C^{++}	Green-brown, nodular
10.	0.5mgL^{-1} BAP MS + 1mgL^{-1} NAA + 0.5mgL^{-1} BAP	40.74 ± 2.18	15.0 ± 0.0	1.25 ± 0.19	0.34 ± 0.03	C^{+}	Brown, fragile-rooting callus
11.	MS + 2mgL^{-1} NAA + 0.5mgL^{-1} BAP	44.44 ± 1.85	13.7 ± 0.3	1.64 ± 0.15	0.46 ± 0.06	C^{+}	Light-brown, nodular
12.	MS + 3mgL^{-1} NAA + 0.5mgL^{-1} BAP	35.19 ± 1.11	12.0 ± 0.0	1.96 ± 0.15	0.54 ± 0.05	C^{+}	Brown, nodular
	$LSD_{(P=0.05, n=10)}$	6.70	0.85	0.48	0.22		

† MS (Murashige and Skoog, 1962), 2,4-D (2,4-Dicloropheoxy acetic acid), BAP (6- Benzylamino-purine), NAA ("-Naphthalene acetic acid), IBA (Indole-3-butyric acid)
*Observation: C^{+} Poor callus; C^{++} Moderate callus; C^{+++} Profuse callus
Values are means ± SE at $P = 0.05$ as per LSD test (n = 10)

Table 2. Effect of BAP and Kinetin alone or in combination with NAA, on different shoot regeneration in gerbera

Sl No.	Medium composition	% Shoot regeneration (Mean ± SE)	Days to shoot initiation (Mean ±SE)	Shoot length (cm) (4 weeks after inoculation) (Mean ±SE)	Shoot length (cm) (6 weeks after inoculation) (Mean ±SE)	No. of shoots/ culture (4 weeks after inoculation) (Mean ±SE)	No. of shoots/ culture (8 weeks after inoculation) (Mean ±SE)	Fresh weight of shoot (mg) (Mean ± SE)	Dry weight of shoot (mg) (Mean ± SE)
1.	MS + 1mgL^{-1} BAP	15.56 ± 1.716	18.0 ± 0.0	1.0 ± 0.0	2.0 ± 0.0	4.0 ± 0.6	6.000 ± 0.8	43.33 ± 1.76	14.44 ± 0.59
2.	MS + 2mgL^{-1} BAP	33.33 ± 0.000	17.7 ± 0.3	1.3 ± 0.1	2.5 ± 0.1	3.3 ± 0.3	8.000 ± 0.6	48.33 ± 1.67	16.11 ± 0.56
3.	MS + 3mgL^{-1} BAP	20.00 ± 0.000	16.0 ± 0.0	1.6 ± 0.9	2.9 ± 0.1	2.0 ± 0.0	4.667 ± 0.3	55.33 ± 2.91	18.45 ± 0.97
4.	MS + 1mgL^{-1} BAP + 0.5mgL^{1} NAA	37.78 ± 1.323	16.7 ± 0.3	1.8 ± 0.15	3.0 ± 0.1	4.3 ± 0.3	10.667 ± 0.7	51.67 ± 4.41	17.22 ± 1.47
5.	MS + 2mgL^{-1} BAP + 0.5mgL^{1} NAA	57.78 ± 1.286	15.3 ± 0.3	2.4 ± 0.08	3.5 ± 0.3	4.7 ± 0.3	12.000 ± 1.6	64.33 ± 2.33	21.45 ± 0.78
6.	MS + 3 mgL^{-1} BAP + 0.5mgL^{1} NAA	44.44 ± 1.286	13.7 ± 0.3	2.6 ± 0.15	4.1 ± 0.1	2.7 ± 0.3	7.667 ± 0.7	72.67 ± 1.76	24.22 ± 0.59
	LSD $_{(P= 0.05, n=10)}$	2.40	0.57	0.24	0.18	1.7	0.6	5.52	1.84

†Values are means ±SE at *P= 0.05* as per LSD test (n = 10)

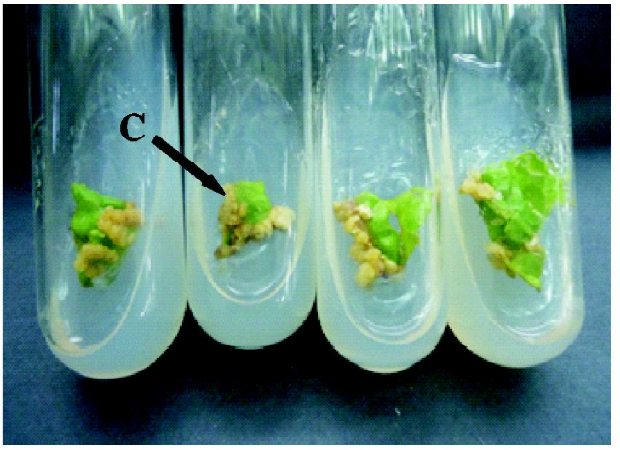

Fig. 1. Callogenesis on MS medium supplemented with 2,4-D 2.0mfL^{-1} + BAP 0.5mgL^{-1} (C, callus)

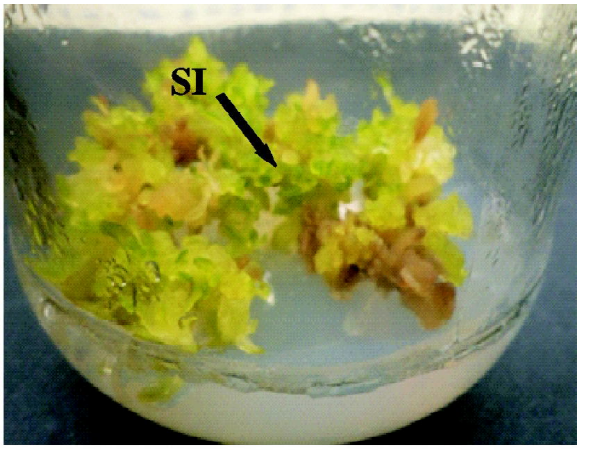

Fig. 2. Shoot initiation from callus on MS medium supplemented with BAP 2.0mgL^{-1} + NAA 0.5mgL^{-1} (Sl, shoot initiation)

Fig. 3. Shoot multiplication from callus on MS medium fortified with BAP 2.0mgL^{-1} + NAA 0.5mgL^{-1} (SM, shoot multiplication)

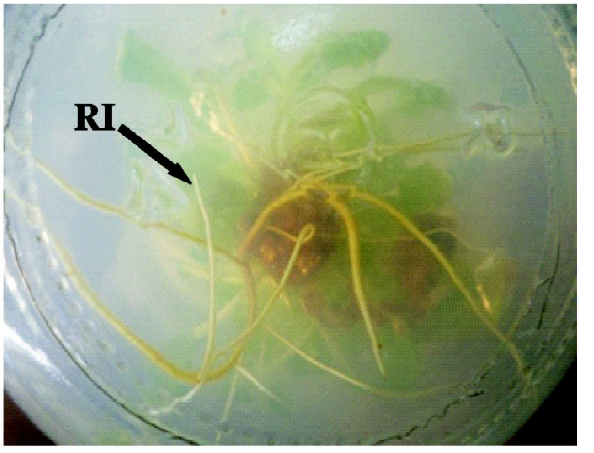

Fig. 4. Root initiation and generated from shoots gerbera on half-MS medium supplemented with IBA3.0mgL^{-1} (Ri, root initiation)

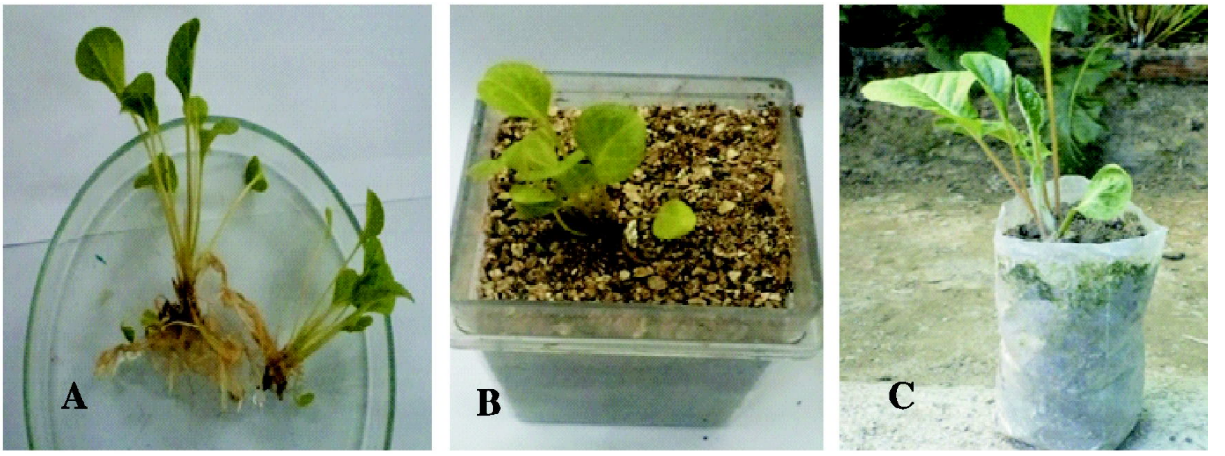

Fig. 5. Hardening of *in vitro* regenerated gerbera plantlets: A. *In vitro* rooted plantlets ready for transfer to hardening medium, B. Primary hardening of regenerated plantlets one week after transfer, C. Secondary hardening of plantlets under green-house

Table 3. Effect of NAA and IBA on rooting in gerbera

Sl. No.	Medium composition	% Root regeneration (Mean ± SE)	Days to root initiation (Mean ± SE)	No. of roots/ shoot (Mean ± SE)	Root length (cm) (Mean ± SE)	Fresh weight of root (mg) (Mean ± SE)	Dry weight of root (mg) (Mean ± SE)
1.	½ MS + 1.0mgL^{-1} NAA	38.89 ± 3.25	16.3 ± 0.3	2.0 ± 0.0	1.3 ± 0.3	7.00 ± 0.58	2.11 ± 0.11
2.	½ MS + 2.0mgL^{-1} NAA	44.44 ± 6.49	15.0 ± 0.0	4.0 ± 0.0	2.0 ± 0.0	7.67 ± 0.33	2.33 ± 0.19
3.	½ MS + 3.0mgL^{-1} NAA	50.00 ± 5.62	14.0 ± 0.0	3.3 ± 0.3	2.5 ± 0.2	8.00 ± 1.16	2.44 ± 0.44
4.	½ MS + 1.0mgL^{-1} IBA	55.56 ± 3.25	14.3 ± 0.3	3.0 ± 0.0	2.0 ± 0.0	8.67 ± 1.33	2.61 ± 0.39
5.	½ MS + 2.0mgL^{-1} IBA	61.11 ± 6.97	13.7 ± 0.3	4.7 ± 0.3	2.7 ± 0.3	10.67 ± 0.67	3.28 ± 0.43
6.	½ MS + 3.0mgL^{-1} IBA	66.67 ± 6.04	12.3 ± 0.3	4.0 ± 0.0	3.0 ± 0.0	12.00 ± 0.00	3.67 ± 0.33
	LSD $_{(P=0.05, n=10)}$	5.47	0.9	0.6	0.7	2.54	1.06

†Values are means ±SE at *P= 0.05 as per* LSD test (n = 10)

Table 4. Effect of various hardening media on plantlet survival in gerbera

Sl. No.	Hardening medium composition (v/v)	Mean survivability ± SE (%)
1.	Garden soil + Perlite (1:1)	46.67 ± 6.67 d
2.	Garden soil + Vermiculite (1:1)	53.33 ± 6.67 c
3.	Garden soil + Sand (1:1)	66.67 ± 6.67 a,b
4.	Garden soil + Sand + Vermicompost (1:1:1)	73.33 ± 13.33 a
	LSD $_{(P=0.05, n=10)}$	8.82

†Mean values in each column, followed by the same lower-case letter, are not significantly different at *P= 0.05* as per LSD test (n = 10)

multiple shoots. These results are in conformity with those of Shailaja (2002) who obtained highest multiplication rate in gerbera with 3.0mgL^{-1} BAP. Results presented in Table 2 show that average length of shoots decreased significantly with higher concentration of cytokinin (BAP) when in combination with NAA. Length of shoots appeared to be inversely proportional to the number of multiple shoots. On MS medium supplemented with BAP 3.0mgL^{-1} + NAA 0.5mgL^{-1}, maximum length (2.6 cm) of shoot was observed at 4 weeks after inoculation on shoot induction media. At higher cytokinins levels, shoot of length may get reduced. Fresh weight of shoot increased with increasing concentration of the cytokinin BAP upto 3.0mgL^{-1} . Similarly, dry weight to increases in relation to an increase in fresh weight. Similar result on organogenesis was obtained by Paduchuri *et al.* (2010), Son *et al*. (2011), Bhatia *et al*. (2012) and Samanthi *et al*. (2013).

Results presented in Table 3 reveal that root formation in microshoots increased with higher concentrations of NAA or IBA in ½ MS media, upto 3.0mgL^{-1}. This enhancement with higher concentrations of NAA or IBA is expected. Akter *et al.* (2012) observed root induction with IBA at the base of *in vitro* regenerated shoots using full- and half- strength MS medium. In our study, ½ MS medium with IBA 3.0mgL^{-1} recorded maximum response in rooting (66.67%), followed by IBA 2.0mgL^{-1} (Table 3, Fig. 4); ½ MS medium with 3.0mgL^{-1} IBA produced longest roots 3.0cm. Fresh weight of root increased with concentration of auxins (IBA and NAA) upto 3.0mgL^{-1}. Similar result on rhizogenesis was reported by Dutta and Gantait (2016), Hasbullah *et al*. (2011), Bhatia *et al.* (2012) and Kadu (2013).

Significantly high survival percentage (73.33%) was recorded in garden soil, sand and vermicompost medium (1:1:1. v/v) over the other hardening potting mixtures tested,while, garden soil and perlite (1:1, v/v) mixture gave a poor result (Table 4, Fig. 5). Medium with vermicompost proved its superiority in a hardening it provided optimum conditions of aeration and a higher water-holding capacity. The primary-hardened plants were transferred to polybags containing potting media and were kept under greenhouse for 15 days. Similar result on rhizogenesis was shown by Hasbullah *et al*. (2011) and Kadu (2013).

Our results indicated that auxin (2,4-D) facilitated good amount of callus formation from leaf explants, and, cytokinin (BAP) along with NAA was required for shoot regeneration in the callus. The auxin, (IBA), facilitated root formation. Induction of callus was maximum on MS medium supplemented with 2.0mg L^{-1} 2,4-D + BAP 0.5mg L^{-1}, with good callus-growth. MS medium fortified with 2.0mg L^{-1} BAP + 0.5mg L^{-1} NAA regenerated maximum number of shoots. Highest rooting was recorded on ½ MS medium fortified with 3.0mg L^{-1} IBA. Regenerated plantlets were recorded as surviving best on a potting mixture containing garden soil, sand and vermicompost (1:1:1).

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