# Micropropagation protocol for *Stevia rebaudiana* through axillary shoot proliferation

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#### ABSTRACT

The present investigation was carried out at Anand Agricultural University, Gujarat during the year 2016 to develop a micropropagation protocol for mass multiplication of medicinally rich stevia plants. Axenic culture was established by sequential application of 200 mg/l Kanamycin, 200 mg/l Carbendazim-50% and 1000 mg/l HgCl<sub>2</sub>. Out of two different basal media, viz. Gamborg (B<sub>5</sub>) and Murashige and Skoog (MS) used, full strength MS gave highest (98%) culture establishment. Out of three different basal media, viz. Gamborg's (B<sub>5</sub>), Linsmair and Skoog (LS), Murashige and Skoog (MS) and four different cytokinins, viz. 6-benzylaminopurine (BAP), Kinetin (Kn), Thiadiazuron (TDZ), Zeatin (Zn) tested for multiple shoot induction, full strength MS medium and 2 mg/l BAP, respectively, promoted maximum shoot formation. In the experiment to study the effect of varying strength of MS media and various plant growth regulators, viz. Indole-3-acetic acid (IAA), Indole-3-butyric acid (IBA), Naphthalene acetic acid (NAA) on *in vitro* rooting, ½ strength MS and 1 mg/l NAA, respectively, gave superior response. Among the 26 different combinations of potting mixtures used for primary acclimatization of *in vitro* rooted plants, cocopeat based substrates yielded highest survival rate (75.06%). Secondary acclimatization was carried out in the soil bags in the poly house. Thus, a reproducible and reliable micropropagation protocol for mass multiplication of *Stevia rebaudiana* (Bertoni) Bertoni using nodal segments has been developed.

Key words: Axillary shoot proliferation, Micropropagation, Stevia rebaudiana

Stevia [Stevia rebaudiana (Bertoni) Bertoni], belonging to the family Asteraceae, is a semi bushy perennial herb which possesses many medicinal properties. The plant is native to eastern Paraguay and has been used as a non caloric natural sweetener for many years. The plant is estimated to be 300 times sweeter than sucrose (Ahmed et al. 2007). The key ingredient of the plant is 'Stevioside' which is a diterpene glycoside and forms the largest part of sweetener molecules present in the leaves (Jahan et al. 2014). Stevioside is highly recommended for the medical conditions like diabetes, hypoglycemia, high blood pressure and obesity as it remains unmetabolized in human body (Jitendra et al. 2012). As per the estimates published by Kaveeshwar and Cornwall in the year 2014, it is assumed that about 31.7 million Indians are presently suffering from diabetes and by 2025 India's contribution to the global diabetic population would be 79.4 million. Increased availability of raw materials for production of steveoside will be the challenge for the industry in future. Further, the traditional propagation methods such as seeds and stem cuttings are inefficient for large scale plantations.

The drawbacks of seed propagation are low germination rate, small size of the seeds (1000 seeds weigh 0.3 to 1 g) and their self-incompatibility (Brandle *et al.* 1998). Additionally, seeds produce less homogeneous populations, resulting in variations in sweetening levels (Lata *et al.* 2013). Propagation by stem cutting is also limited as very low number of plants can be obtained simultaneously from a single plant (Zayed *et al.* 2017). Thus, micropropagation becomes a viable alternative for large-scale production of true to type and disease free stevia plants through tissue culture (Anbazhagan *et al.* 2010, Dey *et al.* 2013, Giridhar *et al.* 2010). The present investigation was mainly focused to develop a complete micropropagation protocol for large-scale production of stevia plant.

## MATERIALS AND METHODS

The present investigation was carried out at Center for Advanced Research in Plant Tissue Culture, Anand Agricultural University, Anand, Gujarat, India during the year 2016. The place of study lies 43 m amsl and has a tropical climate. The climate is classified as AW by the Koppen-Geiger system. The temperature averages around 27.2°C and the rainfall is 882 mm annually.

Collection and surface sterilization of the explants: The experimental material was collected from five months old

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elite plant grown at botanical garden at AAU. The mother plant selection was based on profuse type of branches at the base and having minimum half a meter height. Juvenile nodal explants were chopped to 1–2 cm in size and thoroughly washed with Tween-20. The details of the various surface sterilization treatments used in the present study are presented in Table 1.

Culture establishment and multiplication: Two different basal media, i.e. MS and B<sub>5</sub> at their half and full strength were used for successful establishment of the axenic cultures. For multiplication phase three different media, viz. Gamborg's (B<sub>5</sub>) (Gamborg et al. 1968), LS (Linsmaier and Skoog 1965), MS (Murashige and Skoog 1962) were used at their half and full strength. Different concentrations (0.1, 0.5, 1, 2 and 5 mg/l) of cytokinins, viz. BAP, Kn, TDZ and Zn were evaluated for shoot multiplication (Table 2). All the cultures were incubated at  $25 \pm 1^{\circ}$ C under alternate light and dark regimes of 16 and 8 hr, respectively. The photoperiod was provided by cool, white fluorescent tubes (36 W; Phillips, India) having a light intensity of 36.8 μmol/ m<sup>2</sup>/s. The cultures were regularly subcultured at the interval of 21 days. Observation on number of shoots, nodes, leaves and length of shoots and branching at base was recorded.

Rooting phase: Varying strengths of MS medium (1/4, 1/3, 1/2 and full) were checked for *in vitro* root induction. Effects of various auxins (IAA, IBA and NAA) with their

different concentrations (0.5, 1, 2 mg/l) on root induction were studied to identify the best rooting response (Table 3). Observation on days to root initiation, number of roots per shoot, average length of roots, length of the longest root and number of roots intervened by callus was recorded.

Acclimatization: Twenty six different potting substrates, viz. cocopeat, farmyard manure (FYM), sand, soil, vermiculite and their mixtures were evaluated for primary acclimatization of plantlets (Table 4). The plants were then acclimatized and maintained in poly house until their transplantation to field.

Statistical Analysis: The data obtained from the various experiments conducted for culture establishment, multiplication and rooting phases were statistically analyzed using completely randomized design. The data were subjected to one way analysis of variance (ANOVA) in the excel sheet followed by Duncan multiple range test (DMRT) in DSAASTAT software (Onofri and Pannacci 2014).

### RESULTS AND DISCUSSION

Surface sterilization: Neither antibiotic nor mercuric chloride treatments were found effective in establishment of reasonably healthy and satisfactorily contamination free cultures. Among the different antibiotics used for surface sterilization, the minimal contamination per cent (90.40%) was recorded in  $S_1$  but all the antibiotics failed to control

Table 1 Contamination and axenic culture percentage recorded for various surface sterilization treatments

Treatment	Surface sterilizing agent	Concentration (mg/l)	Time (min)	Contamination (%)	Axenic culture (%)
$\overline{S_1}$	Cefotaxime	200	10	90.40	9.60
$S_2$	Kanamycin	200	10	100.00	0.00
$S_3$	Streptomycin	200	10	100.00	0.00
$S_4$	Tetracycline	200	10	100.00	0.00
$S_5$	$\mathrm{HgCl}_2$	100	10	100.00	0.00
$S_6$	$\mathrm{HgCl}_2$	1000	10	50.00	50.00
$S_7$	$\mathrm{HgCl}_2$	5000	10	40.00	60.00
$S_8$	$\mathrm{HgCl}_2$	100	20	60.00	40.00
$S_9$	$\mathrm{HgCl}_2$	1000	20	20.00	80.00
S <sub>10</sub>	$\mathrm{HgCl}_2$	5000	20	10.00	90.00
S <sub>11</sub>	Streptomycin	200	10	75.00	25.00
	$\mathrm{HgCl}_2$	1000	10		
S <sub>12</sub>	Kanamycin	200	10	72.00	28.00
	$\mathrm{HgCl}_2$	1000	10		
S <sub>13</sub>	Cefotaxime	200	10	10.00	90.00
	$\mathrm{HgCl}_2$	1000	10		
S <sub>14</sub>	Carbendazim-50%	200	10	9.00	91.00
	$\mathrm{HgCl}_2$	1000	10		
S <sub>15</sub>	Carbendazim-50%	200	10	80.00	20.00
	Cefotaxime	200	10		
S <sub>16</sub>	Carbendazim-50%	200	5		
	Kanamycin	200	10	5.00	95.00
	HgCl <sub>2</sub>	1000	10		

Table 2 Effect of different cytokinins on shoot multiplication

Treatment	BAP (mg/l)	Kn (mg/l)	TDZ (mg/l)	Zn (mg/l)	No. of shoots	No. of nodes	No. of leaves	Length of shoots (cm)	Branching at base
$\overline{\mathrm{SM}_1}$	-	-	-	-	2.31 <sup>fg</sup>	8.40 <sup>bc</sup>	2.23 <sup>k</sup>	3.14 <sup>a</sup>	2.46 <sup>d</sup>
$SM_2$	0.1	-	-	-	4.14 <sup>de</sup>	7.01 <sup>cd</sup>	$14.00^{f}$	3.02 <sup>a</sup>	4.08 <sup>cd</sup>
$SM_3$	0.5	-	-	-	7.18 <sup>b</sup>	10.00 <sup>b</sup>	20.00 <sup>cd</sup>	2.86 <sup>ab</sup>	7.21 <sup>ab</sup>
$SM_4$	1	-	-	-	5.11 <sup>cd</sup>	8.30bc	16.60 <sup>ef</sup>	2.92 <sup>ab</sup>	5.17 <sup>bcd</sup>
$SM_5$	2	-	-	-	9.20a	12.20 <sup>a</sup>	24.40 <sup>b</sup>	2.82 <sup>ab</sup>	8.46 <sup>a</sup>
$SM_6$	5	-	-	-	7.06 <sup>b</sup>	7.50 <sup>c</sup>	$15.00^{\rm f}$	2.77 <sup>ab</sup>	7.10 <sup>ab</sup>
$SM_7$	-	0.1	-	-	5.03 <sup>cd</sup>	4.30 <sup>ef</sup>	8.62gh	2.65ab	5.15 <sup>bcd</sup>
$SM_8$	-	0.5	-	-	5.20 <sup>cd</sup>	4.61 <sup>ef</sup>	9.22gh	2.73 <sup>ab</sup>	5.29 <sup>bcd</sup>
$SM_9$	-	1	-	-	5.32 <sup>cd</sup>	5.01 <sup>de</sup>	10.00 <sup>g</sup>	2.68 <sup>ab</sup>	8.14 <sup>a</sup>
$SM_{10}$	-	2	-	-	5.05 <sup>cd</sup>	3.01 <sup>ef</sup>	60.20 <sup>a</sup>	2.55 <sup>ab</sup>	4.14 <sup>cd</sup>
$SM_{11}$	-	5	-	-	3.30 <sup>ef</sup>	$2.20^{\rm f}$	$4.42^{jk}$	2.50 <sup>ab</sup>	5.19 <sup>bcd</sup>
$SM_{12}$	-	-	0.1	-	7.03 <sup>b</sup>	13.40 <sup>a</sup>	22.80 <sup>bc</sup>	2.44 <sup>ab</sup>	7.14 <sup>ab</sup>
$SM_{13}$	-	-	0.5	-	6.02 <sup>bc</sup>	7.61 <sup>c</sup>	14.60 <sup>f</sup>	2.27 <sup>ab</sup>	6.39abc
$SM_{14}$	-	-	1	-	5.13 <sup>cd</sup>	9.02 <sup>bc</sup>	18.00 <sup>de</sup>	2.66 <sup>ab</sup>	5.14 <sup>bcd</sup>
SM <sub>15</sub>	-	-	2	-	5.23 <sup>cd</sup>	5.08 <sup>de</sup>	10.20 <sup>g</sup>	2.22 <sup>ab</sup>	5.31 <sup>bcd</sup>
$SM_{16}$	-	-	5	-	6.11 <sup>bc</sup>	4.51 <sup>ef</sup>	6.32hij	2.71 <sup>ab</sup>	4.15 <sup>cd</sup>
$SM_{17}$	-	-	-	0.1	$2.19^{fg}$	4.10 <sup>ef</sup>	5.22 <sup>ijk</sup>	2.00 <sup>ab</sup>	3.14 <sup>d</sup>
$SM_{18}$	-	-	-	0.5	4.06 <sup>de</sup>	5.11 <sup>de</sup>	6.20hij	1.80 <sup>ab</sup>	4.21 <sup>cd</sup>
$SM_{19}$	-	-	-	1	3.09ef	9.17 <sup>bc</sup>	7.10 <sup>ghij</sup>	2.20 <sup>ab</sup>	5.14 <sup>bcd</sup>
$SM_{20}$	-	-	-	2	1.30 <sup>g</sup>	4.20 <sup>ef</sup>	8.42ghi	1.70 <sup>ab</sup>	4.07 <sup>cd</sup>
$SM_{21}$	-	-	-	5	2.06 <sup>fg</sup>	8.52 <sup>bc</sup>	8.20ghi	1.50 <sup>b</sup>	5.21 <sup>bcd</sup>

Parameters have been recorded after 12 weeks of transfer in different media. Means with the same letter along the column are not significantly different at P=0.05

Table 3 Effect of ½ strength of MS supplemented with various concentrations of IAA, IBA and NAA on rooting

Treatment	IAA (mg/l)	IBA (mg/l)	NAA (mg/l)	Days to root initiation	No. of roots per shoot	Average length of roots (cm)	Length of longest root (cm)	No. of roots intervened by callus
$\overline{SR_1}$	-	-	-	7.00	2.00°	4.00 <sup>a</sup>	6.80 <sup>a</sup>	0.00 <sup>d</sup>
$SR_2$	0.5	-	-	4.00	3.90 <sup>bc</sup>	3.00 <sup>ab</sup>	5.10 <sup>ab</sup>	$0.00^{d}$
$SR_3$	-	0.5	-	4.00	4.10 <sup>bc</sup>	2.80 <sup>ab</sup>	4.30 <sup>ab</sup>	$0.00^{d}$
$SR_4$	-	-	0.5	4.00	4.00 <sup>bc</sup>	2.00 <sup>ab</sup>	4.20 <sup>ab</sup>	$6.00^{a}$
$SR_5$	1	-	-	5.00	4.90 <sup>bc</sup>	3.50 <sup>ab</sup>	5.50 <sup>ab</sup>	0.70 <sup>cd</sup>
$SR_6$	-	1	-	3.00	6.30 <sup>b</sup>	3.10 <sup>ab</sup>	4.80 <sup>ab</sup>	2.40 <sup>bc</sup>
SR <sub>7</sub>	-	-	1	4.00	11.08 <sup>a</sup>	3.30 <sup>ab</sup>	6.00 <sup>ab</sup>	$0.00^{d}$
$SR_8$	2	-	-	4.00	3.50 <sup>bc</sup>	2.00 <sup>ab</sup>	4.60 <sup>ab</sup>	$0.30^{d}$
$SR_9$	-	2	-	3.90	3.10 <sup>bc</sup>	1.50 <sup>ab</sup>	5.00 <sup>ab</sup>	$4.00^{b}$
$SR_{10}$	-	-	2	5.50	2.40 <sup>c</sup>	1.00 <sup>b</sup>	2.30 <sup>b</sup>	$3.50^{b}$

Parameters have been recorded after 12 weeks of transfer in different media. Means with the same letter along the column are not significantly different at P=0.05

Table 4 Effect of different potting substrates on survival percentage of plantlets during acclimatization

Treatment	Potting mixture	Survival (%)
$\overline{H_1}$	Cocopeat	84.74
$H_2$	Farmyard manure ( FYM)	0.00
$H_3$	Sand	50.48
$H_4$	Soil	38.46
$H_5$	Vermiculite	18.67
$H_6$	Cocopeat: Sand (1:1)	72.18
$H_7$	Cocopeat : Soil (1:1)	52.19
$H_8$	Cocopeat: FYM (1:1)	25.67
$H_9$	Cocopeat : Vermiculite (1:1)	45.34
$H_{10}$	FYM : Sand (1:1)	11.11
$H_{11}$	FYM : Soil (1:1)	10.15
$H_{12}$	FYM: Vermiculite (1:1)	9.83
$H_{13}$	Sand: Soil (1:1)	35.12
$H_{14}$	Sand : Vermiculite (1:1)	37.83
H <sub>15</sub>	Soil : Vermiculite (1:1)	30.42
$H_{16}$	Cocopeat: FYM: Sand (1:1)	36.74
H <sub>17</sub>	Cocopeat: FYM: Vermiculite (1:1:1)	32.50
$H_{18}$	Cocopeat : Soil : Vermiculite (1:1:1)	67.01
$H_{19}$	Cocopeat: Sand: Soil (1:1:1)	70.83
$H_{20}$	FYM : Sand : Soil (1:1:1)	16.02
$H_{21}$	Sand : Soil : Vermiculite (1:1:1)	51.67
H <sub>22</sub>	Sand : Soil : FYM : Vermiculite (1:1:1:1)	14.50
$H_{23}$	Cocopeat :FYM : Sand : Soil (1:1:1:1)	17.53
H <sub>24</sub>	Cocopeat : Sand : Soil : Vermiculite (1:1:1:1)	75.06
H <sub>25</sub>	Cocopeat : FYM : Sand : Vermiculite (1:1:1:1)	14.33
H <sub>26</sub>	Cocopeat: FYM: Sand: Soil: Vermiculite (1:1:1:1)	15.85

fungal contamination. In case of  ${\rm HgCl_2}$  based treatments,  ${\rm S_{10}}$  yielded higher % of (90.00%) axenic culture but sprouting of explants has failed. Recommendation of long  ${\rm HgCl_2}$  exposures in the present study was in contradiction to the investigations of Ghauri *et al.* (2013), Mitra and Pal (2007) and Tadhani *et al.* (2006) where they had used  ${\rm HgCl_2}$  for shorter duration (1, 2 and 4 minutes, respectively). Of the six sequential combinations of various surface sterilants tested,  ${\rm S_{16}}$  resulted in complete elimination of surface contaminants (Table 1).

Establishment and multiplication: Among the two different media tested for establishment, the % establishment and growth of cultures was highest on full MS, however, the minimum number of days (7d) to sprout induction was recorded in case of full  $B_5$ . Percentage response of cultures was satisfactory on both MS and  $B_5$  media (40% and 15%, respectively) when used at full strength, whereas decreased

growth was observed on half B5 which hints the higher nutritional requirement of explants for the establishment phase. Multiple shoot formation varied significantly with different types of media. Among the three different media tested for shoot multiplication, full MS was found to be the best basal medium for multiplication in terms of number of shoots (14.06), nodes (43.07), leaves (85.86) and length of shoots (5.65 cm) recorded. Use of MS medium for the establishment and multiplication phase in this study is well supported by the work of Jain et al. (2009), Kalpana et al. (2009) and Mathur and Begum (2015), where they have used full strength MS for culture establishment. The higher levels of calcium in MS than B<sub>5</sub> and LS may have promoted the active growth of newly forming shoots in stevia. Sha et al. (1985) reported stunting of shoots and dieback of growing apices due to calcium deficiency in Lewisia cotyledons. The obvious reason for poor response on B<sub>5</sub> was the moderate concentrations of various macro and micro elements as compared to MS or LS (Bhojwani and Razdan 1997). Among various treatments tested to check the effects of four different cytokinins on multiplication, highest degree of shoot proliferation was recorded on SM5 which produced 9.20 shoots, 12.20 nodes and 24.40 leaves. No significant increase in shoot number and shoot development was observed on Kn augmented media as compared to BAP and TDZ supplemented media. Number of shoots, nodes, and shoot length were found to decrease considerably in case of Kn containing media when concentration was raised from 2 mg/l to 5 mg/l. These findings were contradictory to the reports of Morini et al. (2003) wherein, better performance of stevia shoots was observed by replacing BAP with Kn in the medium. Mitra and Pal (2007) also favored Kn over BAP for production of higher number of healthy shoots. However, the present results were in agreement with the findings of Yadav et al. (2016) who reported maximum shoot multiplication response on 2.0 mg/l BAP. In case of number of nodes and leaves, the response of TDZ was almost comparable with BAP. The highest number of nodes recorded was 13.40 with 22.80 leaves on SM<sub>12</sub>, whereas 12.20 nodes with 24.40 leaves on SM<sub>5</sub>. The branching at base was higher (7.14) in  $SM_{12}$  than on  $SM_2$ . This indicated the higher persistence time of TDZ in media than BAP. Response of explants on Zn supplemented media was poorest as compared to other cytokinins. The number of shoots was in the range of 1 to 4 while the maximum number of nodes was 9.17 with 7.10 leaves observed on SM<sub>19</sub>. The poor response on Zeatin supplemented media might be related to its natural occurrence and therefore more prone to degradation by action of enzymes (Bhojwani 1990). The detailed results are presented in Table 2.

Rooting: Excellent rooting per cent (100%) was obtained on all the strengths of MS tested. The highest number of roots per shoot (10.50) was obtained on ½ MS while secondary root formation was observed at lower strengths (1/3 and ¼ MS). It was also observed that the transfer of rooted shoots from high strength medium to less concentrated one enhanced rooting. These results were in agreement with the

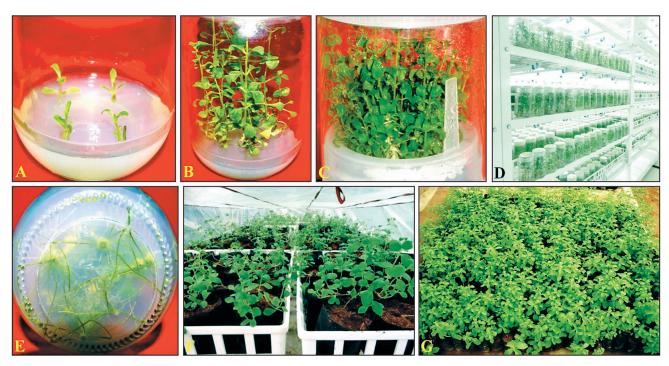


Fig 1 Complete micropropagation protocol of *Stevia rebaudiana* A. Initiation of explants, B. Shoot multiplication in glass jar, C. Shoot multiplication in polypropylene container, D. Growth shelves filled with stevia cultures, E. Root induction on fully grown shoots, F. Primary acclimatization, G. Secondary acclimatization.

reports of Majumder and Rahman (2016) and Liaei et al. (2016) where they obtained rooting on ½ strength of MS. Days to root initiation, number of roots, length of roots and presence of callus were significantly influenced by different concentrations and types of auxins (IAA, IBA and NAA) used. Out of three PGRs tested for rooting, best response (11.08 roots) was obtained on SR<sub>7</sub> followed by SR<sub>6</sub> (6.30 roots) and SR<sub>5</sub> (4.90 roots). The longest root (6.80 cm) and maximum average length of roots (4 cm) were observed within SR<sub>1</sub> (Table 3). The days to root initiation varied considerably from 3 days in case of SR<sub>6</sub> to 7 days on SR<sub>1</sub>. Moktaduzzam and Rahman (2009) have also suggested the use of 1.0 mg/l NAA for root induction but contradictorily Tiwari et al. (2013) emphasized the use of IBA instead of NAA for *in vitro* rooting. The formation of callus in place of root at higher levels of IBA or NAA could be related to the synthetic nature of hormone or supplementation of higher amount than required or accumulation just above the wound site because of polar transport (Taiz and Zeiger 1991) or due to the presence of light which is considered to involve growth inhibitors produced by root tip/cap (Golaz and Pilet 1995).

Acclimatization: Among the 26 different potting mixtures tested, the highest survival per cent (84.74%) was recorded in cocopeat based substrates. The survival per cent for various potting mixtures ranged from 0 to 84%. The lowest survival per cent (from 0 to 36%) was recorded with the FYM containing potting mixtures. Vermiculite alone failed to achieve higher survival rate but in combination with other potting substrates it gave satisfactory results except with FYM. In case of substrates like sand and soil,

moderate to good results were obtained (Table 4). These results were contradictory to the findings of Das *et al.* (2011) who reported maximum survival rates on a potting mixture containing sand:soil:vermicompost (1:1:1). Anbazhagan *et al.* (2010) also used sand:soil:FYM (1:1:1) for primary hardening.

Thus, a complete micropropagation protocol for *Stevia rebaudiana* 'Bertoni' through axillary shoot proliferation has been developed (Fig 1).

Present investigation has covered almost every aspect of the micropropagation which can be helpful to stevia growers and tissue culturist across the world for large scale production of stevia plantlets. The present study derives following conclusions: (1) Combinational use of antibacterial, antifungal and heavy metal for surface sterilization can be the most effective treatment for establishment of higher percentage of axenic cultures. (2) Initiation of explants on full strength MS media can yield higher sprouting response where as maximum shoot multiplication can be achieved by using BAP as a cytokinin. (3) *In vitro* root induction can be achieved on half strength MS supplemented with NAA. (4) Plants produced in this manner can be best hardened in cocopeat based mixtures.

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