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In vitro propagation of *Hemidesmus indicus* (L.) R. Br. (*Iramusu)* through nodal culture

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ABSTRACT. *The effects of different nodal positions and 6-benzylaminopurine (BAP) concentrations on shoot development from nodal explants of Hemidesmus indicus (L.) Br. (Iramusu) were investigated. The highest number of shoots per explant (2.57±0.97) was observed in 4th nodal position, while the longest shoot length (3.23± 0.52 cm) was achieved in 5th nodal position at 2 mg/L BAP with 0.1 mg/L Naphthalene Acetic Acid (NAA). Reduction of total shoot length and number of shoots were observed when BAP concentration was gradually increased from 5 to 15 mg/L. Half-strength semi solid MS medium with 1.5 mg/L indole-3-butyric acid (IBA) exhibited the best in vitro rooting. Hundred percent of the rooted shoots survived during the acclimatization.*

INTRODUCTION

Hemidesmus indicus (L.) R. Br. (*Iramusu),* of the family Asclepiadacea is distributed in India, Bangladesh and Sri Lanka. In Sri Lanka, it thrives well only up to an elevation of 2500 m. It is common in deciduous scrubland and deciduous forest of the dry regions as well as rubber, coconut and *pinus* plantations (Gunatilleke *et al*., 2002). The plant is a perennial, semi-shrubby tawnier with a woody rootstock. The stem is very long, prostrate or ascending and slightly twining. Internodes are 1.5 - 7.2 cm in length. Leaves are simple, opposite and variable from oblong-oval to linear. Flowers are regular, bisexual and contain numerous bracts (Jayaweera, 1982).

In ancient ayurvedic practices in India and Sri Lanka, *H. indicus* has been commonly utilized because of its strong fragrance, aromatic taste and medicinal properties due to presence of 2-hydroxy 4-methoxy benzoic acid in roots. It mainly acts as a blood purifier, antipyretic and antioxidant (Sreekumar *et al.*, 2000). Roots are commonly used for the preparation of several decoctions, syrups, sherbet and medicinal mixtures to recover from venereal diseases, plumpness, asthma, bronchitis, chronic skin disorders and rheumatoid arthritis (Siddique *et al*., 2003). High quantities of plant materials such as stem, leaf and root extracts are used in ayurvedic, unani and homeopathic medicines in India and Bangladesh (Siddique *et al*., 2003). Even in such countries where the commercial level manufacturing systems exist, establishment of commercial plantations of this herb has been difficult due to unavailability of sufficient desirable quality planting materials. Therefore, wild population is exploited for the extraction purposes (Soma *et al*., 2003). This has been a

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heavy threat for the existence of the plant species. In addition, field grown plants have shown high seasonal variations. To overcome these problems, *in vitro* propagation techniques can be applied for the rapid large-scale clonal propagation, germplasm conservation and production of multiple shoots with homogenous chemical composition of the *in vitro* populations. In addition to that, different *in vitro* protocols have been developed for the large scale production of antioxidants such as iupeol, vanillin and rutin in shoot cultures, callus cultures and root cultures (Soma *et al*., 2003).

Even though *H. indicus* is a well known common ancient medicinal plant species in Sri Lanka, no sufficient attention has been given for the efficient utilization and conservation of the plant species in their natural habitat. Therefore, development of clonal propagation techniques will be an essential tool for the rapid multiplication of the plant species and to utilize in the ancient ayurvedic medicines in Sri Lanka. Furthermore, this plant species can be modified through several genetic and epigenetic techniques such as genetic transformation and mutation treatments in order to change its phenotype as an ornamental plant species and popularize among the nation. Therefore, this study was conducted with the objective of developing a suitable protocol for shoot regeneration of *H. indicus* through single nodal culture.

MATERIALS AND METHODS

Establishment of nodal cultures

The nodal explants were taken from the plants grown in the plant house, two days after spraying of 0.1% Bavastin™ (carbendazim) solution. The nodal explants were surface sterilized by washing 3 times with Teepol™, then dipping in 0.1% Bavastin™ solution for 30 min vacuum sterilized with 10% Clorox™ (sordium hypochlorite) and two drops of Tween 20 (Tween 20-polyoxyethelene sorbian monolaurate) for 5 min, then thoroughly shaked with 10% Clorox for 5 min. This was followed by three to four times washing with sterile distilled water to make the explant material free from chemicals. After the sterilization process 1-2 cm single nodal segments were inoculated on Murashige and Skoog (1962) (MS) basal medium supplemented with 1 mg/L, 6-benzylminopurine (BAP) and 0.5 mg/L Naphthalene Acetic Acid (NAA), 3% Sucrose, 100 mg/L Myoinositol, 15 mg/L Adenine sulphate, 0.1% streptomycin, 0.1g/L ascorbic acid and solidified with 8.0 g/L agar.

Those cultures were incubated at 25±2^{o}C under the warm fluorescent light with intensity varying from 900 to 1500 lux and 16:8 hrs (day:night) photoperiod. Each treatment consists of 120 culture tubes. Performances of the plants were observed at weekly intervals. The success rate, percentage of shoot initiation and growth measurements such as shoot length and number of shoots/explant were recorded at the end of the sixth week.

The effects of nodal positions of the explants on shoot induction and multiplication

Surface sterilized nodal explants from five nodal positions from the apical bud (Plate 1), were inoculated on MS medium containing different combinations of BAP (0, 2, 5, 10, 15 mg/L) with 0.1 mg/L NAA. Cultures were kept under the same conditions mentioned above. Treatment combinations were replicated for six times. Performances of the plants

were observed at weekly intervals and shoot length and number of shoots/explant were recorded three weeks after culture establishment.

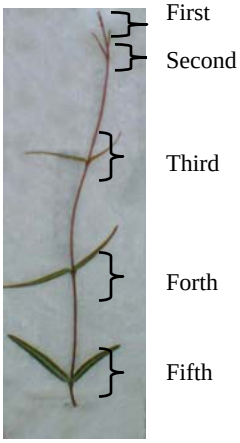

Plate 1. Nodal positions of the shoot explant.

Note: Growing shoot tip is considered as the first node and the node immediately below is the second node. This continues as third, forth and fifth towards the base of the plant.

The effects of different BAP concentrations on shoot induction and multiplication

Nodal explants (1 - 2 cm) taken from 4^{th} and 5^{th} nodal positions were surface sterilized and inoculated on MS medium supplemented with different combinations of BAP (0, 1, 2, 5, 10 and 15 mg/L) with 0.1 mg/L NAA. Those cultures were incubated at 25±2^{o}C under the warm fluorescent light with intensity varying from 900 to 1500 lux and 16:8 hrs (day:night) photoperiod. Each treatment consists of 120 culture tubes. Performances of the plants were observed at weekly intervals. Shoot length and number of shoots/explant were recorded three weeks after culture establishment.

***In vitro* shoot multiplication**

The shoots produced from MS basal medium supplemented with different BAP concentrations were multiplied on MS basal medium with 2 mg/L BAP and 0.1 mg/L NAA for three months. Then the nodal segments (1 - 2 cm) from in vitro grown shoots were excised and cultured on MS basal medium with different concentrations of BAP (0, 2, 5, 10, 15, 20 mg/L) with 0.1 mg/L NAA. Subculturing on to the same medium composition was conducted at monthly intervals. Adenine sulphate (15 mg/L) was incorporated onto the medium in the subsequent subculturing stages only when it is necessary to prevent abscission of leaves and shoot tips. Cultures were kept under the conditions mentioned above. Growth measurements such as shoot length and number of shoots/explant were recorded six weeks after subculturing.

In vitro rooting

Well developed *H. indicus in vitro* shoots (4 - 5 cm) produced on MS basal medium supplemented with different concentrations of BAP, were transferred to MS basal medium, with 4 mg/L Indole-3-Butyric Acid (IBA) and 1 mg/L Kinetin (Siddique *et al.*, 2003) and to half strength MS medium containing 1.5 mg/L IBA (Soma *et al*., 2003) for rooting, respectively. Performances of the root development were observed at weekly intervals. The percentage of root initiation and root length and number of roots/explant were recorded at the end of the twelve weeks. When the roots were fully developed, plants were transferred to the pots containing soil and sand (1:3) (Soma *et al*., 2003) and kept in the propagator box for acclimatization.

Data analysis

The experiments were set up in a completely randomized design. Fifteen replicates were used for each treatment. The individual effect of BAP and nodal position on shoot length and the effect of different medium composition on root length were analyzed by using one way ANOVA with SAS statistical software. The interaction effect of BAP and nodal position on total shoot length was analyzed by using two-way ANOVA with SAS. The effect of BAP and nodal position on number of shoots per explant and the effect of medium composition on number of roots per explant were analyzed by chi-square contingency test with MINITAB. Mean comparisons were done with Duncan's multiple range test (DMRT).

RESULTS AND DISCUSSION

Establishment of nodal cultures

The sterilization procedure used for the establishment of *H. indicus* was successful and the success rate was 76.7%. Due to the low contamination percentage, this sterilization process was practiced throughout the study to sterilize the nodal explants.

Shoot buds appeared 7 - 8 days after culture establishment in the MS medium supplemented with 1.0 mg/L BAP and 0.5 mg/L NAA. Six weeks after nodal culture establishment, a great variation in shoot initiation and shoot growth was observed. Some axenic cultures produced no shoots despite of swelling. In some explants only basal callus formation was observed. Among all the explant, which showed proliferation, slow growth was observed in 52.3% of cultures. Rest of the explants (15.9%) showed shoot growth of 1 - 4.5 cm. Due to the higher variation at the end of six week period, it was decided to investigate the effect of nodal position on multiplication and elongation of *Hemidesmus indicus* plants. The average number of shoots produced by the nodal explants was 1.57±0.64 while shoot length was 1.99±1.36 cm.

In previous research on *H. indicus*, it has been revealed that the explants both in MS medium and MS with cytokinin alone, failed to show any shoot multiplication despite the swelling. (Soma *et al*., 2003). High concentrations of cytokinin stimulate shoot development. Among various cytokinins, BAP and kinetin are stronger than others. However, efficiency of BAP over kinetin has been reported in *H. indicus* (Soma *et al*., 2003). When a low concentration of NAA with a high concentration of BAP or kinetin was

added, shoot bud multiplication was stimulated (Soma *et al*., 2003). However, high level of NAA caused unnecessary callus formation. The same synergetic effect of BAP in combination with auxin has been demonstrated in medicinal plants of family *Asclepiadaceae*, namely *Gymnema sylvestre* (Komalavalli and Rao, 2000) and *Holostemma ada-kodien* Schult (Martin, 2002). Therefore, in the present study, MS medium containing combination of BAP and NAA were tested for the shoot proliferation of nodal explants.

In the present study, abscission of the leaves and shoot tips was observed. Abscission of leaves and shoot tips in shoots developed through axillary bud multiplication and via indirect organogenesis has been reported in *H. ada-kodien* Schult (Martin, 2002) and in *Psoralea corylifolia* (Saxena *et al.*, 1998). In agreement with the result of Patnaik and Debata, 1996 it was observed that the incorporation of adenine sulphate was highly effective to prevent immature leaf fall. According to the Mhatre *et al*. (1998) this phenomenon is due to the accumulation of ethylene and it has been successfully alleviated in cucumber by using ethylene inhibitors such as $AgNO_3$ and $CoCl_2$. However, due to reduction of number of shoots, the addition of $AgNO_3/CoCl_2$ has not been advantageous.

The effect of nodal position of the explants on shoot induction and multiplication

There was a significant difference between the treatment effects of BAP and nodal positions and also between the interaction effect of BAP and nodal positions at 0.05 probability level (Tables 1 and 2). Nodal positions 1 and 2 did not produce any shoots and 3rd nodal position produced only basal callus (Plate 2: N1, N2, N3, Tables 1 and 2). Responses of shoot initiation to different concentrations of BAP were shown only in 4th and 5th nodal positions (Plate 2: N4, N5, Tables 1 and 2). At 0 mg/L BAP level, no shoot initiation was observed (Plate 2-A, Tables 1 and 2). At 2 mg/L BAP level, the highest number of shoots per explant (2.57±0.97) was observed in 4th nodal position (Plate 2: B-N4, Table 1), while the longest shoot length (3.23±0.52 cm) was achieved in 5th nodal position (Plate 2: B-N5, Table 1). No significant difference on shoot length and number of shoots per explant was observed between 4th and 5th nodal positions. Further, reduction in the number of shoots per explant and total shoot length was observed when BAP concentration was gradually increased from 5 mg/L to 15 mg/L (Plates 2-3, 4, 5, Tables 1 and 2). Repeated experiments also justified the same results.

Shoot proliferation generally depends upon the size of the explant cultured and physiological stage of the explant as well. The smaller the explant, the lesser the regenerative ability due to lack of stored carbohydrates. In many plant species faster shoot bud initiation ability has been reported in the larger explants, irrespective of the auxin to cytokinin concentration (Reinert and Bajaj, 1977). Differences of shoot regeneration, observed in the present study, may be due to the differences of the size and physiological stage of the explant cultured. Callus formation at the basal cut ends of nodal explants has been reported in *G. sylvestre* (Komalavalli and Rao, 2000) and *H. ada-kodien* Schult (Martin, 2002) and *Tylophora indica* (Chandrasekhar *et al*., 2006). It may be due to the action of accumulated auxin at the basal cut ends, which stimulates cell proliferation, specially in the presence of cytokinin.

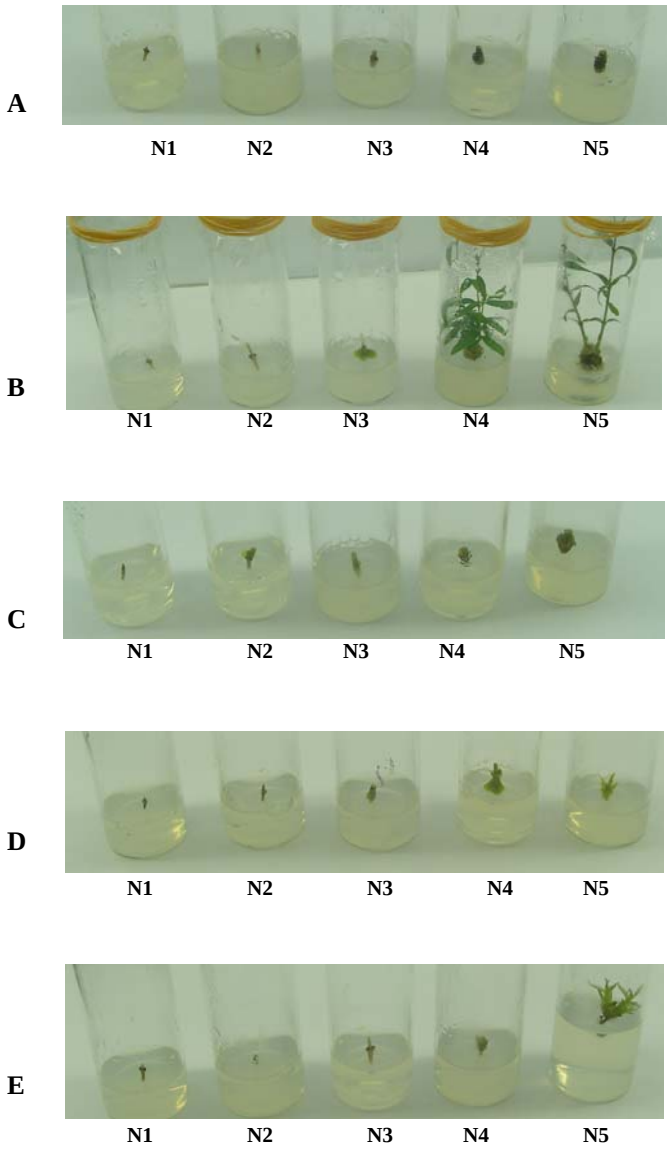

Plate 2. Variations of shoot initiation and shoot growth from different nodal positions cultured on different concentrations of BAP three weeks after culture establishment. Responses of nodal cultures on **(A)** MS + 0 mg/L BAP + 0.1 mg/L NAA **(B)** MS + 2 mg/L BAP + 0.1 mg/L NAA **(C)** MS + 5 mg/L BAP + 0.1 mg/L NAA **(D)** MS + 10 mg/L BAP + 0.1 mg/L NAA **(E)** MS + 15 mg/L BAP + 0.1 mg/L NAA. One to five nodal positions are indicated as N1, N2, N3, N4, and N5.

Table 1. Responses of different concentrations of BAP on shoot induction and multiplication of 4th and 5th nodal positions at 3 weeks after culture establishment.

	Nodal positions			
	4		5	
BAP (mg/L)	Number of shoots/explant	Shoot length (cm)	Number of shoots/explant	Shoot length (cm)
0	-	-	-	-
2	2.57 ± 0.97 [a*]	2.85 ± 1.58 [a]	2.18 ± 0.75 [a]	3.23 ±0.52 [a]
5	2.00 ± 1.41 [b]	0.76 ± 0.21 [b]	1.00 ± 0.15 [b]	0.50 ±0.16 [b]
10	2.00 ± 0.95 [b]	0.40 ± 0.13 [b]	1.00 ± 0.23 [b]	0.50 ±0.26 [b]
15	-	-	1.00 ± 0.29 [b]	1.0 ±0.32 [b]

Note: Data expressed as Mean ± SE from 15 replicates. Within columns, values followed by the same letter are not significantly different at the P = 0.05.

The effect of different BAP and NAA concentrations on shoot induction and multiplication

When sterilized nodal explants were cultured on MS basal medium supplemented with different concentrations of BAP and NAA, no shoot induction was observed at 0 mg/L BAP despite nodular and basal callus formation. Three weeks after culture establishment, the highest shoot initiation and growth were achieved on MS medium supplemented with 1 and 2 mg/L BAP with 0.1 mg/L NAA and this was in agreement with the results of the earlier experiment.

Shoot induction occurred 3 - 4 days after culture establishment at 2 mg/L BAP level, while in 1 mg/L BAP level it was in 6 - 8 days after inoculation of explants. Repeated experiments also justified these results. However, a gradual reduction of shoot induction and growth were identified from 5 mg/L to 15 mg/L BAP. It took 10 - 15 days to induce shoots at high BAP levels.

***In vitro* shoot multiplication**

When the nodal segments were excised from the *in vitro* grown shoots and subcultured on to the medium with the different concentrations of BAP, it facilitated the development of increased number of shoots at all the BAP concentrations within 6 weeks of subculturing. At the 1st subculturing stage, the highest shoot length and the highest number of shoots per explant were achieved at 5 mg/L BAP with 0.1 mg/L NAA. A reduction in shoot length and number of shoots per explant, with the increases of BAP concentration from 10 to 20 mg/L was also observed at the 1st subculturing stage (Plate 3, Table 2).

This higher shoot multiplication at subsequent cultures is in agreement with *G. Sylvestre* (Komalavalli and Rao, 2000). Enhanced shoot multiplication in subsequent cultures has also been reported in *H. indicus* (Sreekumar et al, 2000). However, a reduction in shoot number has been observed by Patnaik and Dabata (1996) in the same species.

Plate 3. Effect of different concentrations of BAP on shoot induction and multiplication at 6 weeks after 1st subculturing **(A)** 0 mg/L BAP + 0.1 mg/L NAA **(B)** 2 mg/L BAP + 0.1 mg/L NAA **(C)** 5 mg/L BAP + 0.1 mg/L NAA **(D)** 10 mg/L BAP + 0.1 mg/L NAA **(E)** 15 mg/L BAP + 0.1 mg/L NAA **(F)** 20 mg/L BAP + 0.1 mg/L NAA.

Table 2. Responses of different concentrations of BAP on shoot induction and multiplication.

BAP (mg/L)	At initial culture establishment stage		At 2nd subculturing stage	
	Number of shoots/ explant	Shoot length (cm)	Number of shoots/explant	Shoot length (cm)
0	-	-	1.09 ± 0.57 [c]	2.32 ±0.68 [b]
2	2.33±0.84 [a]	1.04 ± 1.40[a]	2.69 ±1.06 [a]	3.66 ±2.50 [a]
5	1.40±0.89 [b]	3.06 ± 1.84 [b]	4.47 ±1.64 [a]	3.72 ±2.57 [a]
10	1.50±0.20 [c]	0.12 ± 0.28 [c]	3.36 ±2.21 [a]	3.09 ±2.60 [a]
15	1.00±0.21 [c]	0.10 ± 0.21 [c]	2.47 ±1.28 [b]	2.19 ±1.55 [b]
20	-	0.13 ± 0.36 [c]	2.42 ±1.09 [b]	1.74 ±1.55 [b]

Note: Data expressed as Mean ± SE from 15 replicates. Within columns, values followed by the same letter are not significantly different at the P = 0.05.

In vitro rooting

In agreement with Soma *et al.* (2003) no root initiation was observed until 10 weeks after transferring to hormone free MS medium. However, root initiation was observed 10 - 12 weeks after transferring on to hormone free MS medium. Rapid and profuse root formation of *H. indicus,* in half strength MS medium with 1.5 mg/L Indole-3-Butyric Acid (IBA) after 8 weeks of transferring is in agreement with the results obtained by Soma *et al*. (2003). Shoots on MS basal medium supplemented with 4 mg/L IBA and 1 mg/L Kinetin, failed to initiate roots even after 10 weeks of transferring despite basal callus formation (Table 3). Callus formation at the basal cut end has also been reported in *H. ada-kodien* Schult when *in vitro* grown shoots were transferred to MS medium with NAA alone or IBA with Kinetin/BAP (Martin, 2002).

The effectiveness of IBA, over NAA and Indole-3-Acetic Acid (IAA) in root induction has been reported for medicinal plants such as *Cunila galioides* (Fracaro and Echeverrigary, 2001) and *Aloe polyphylla* (Abrie and Van Staden, 2001). According to Nickell (1982), the better function of IBA in root induction is due to the slow movement and slow degradation of IBA, which facilitates its localization near the site of application.

Rooted plantlets transferred to pots containing sterile soil and sand (1:3), showed 100% survival rate and the plantlets exhibited the similar morphological characters as the mother plants (Plate 4).

The protocol described here for the micropropagation of *H. indicus* through nodal culture facilitates the rapid propagation of the plant by reducing the higher variations arise in the *in vitro* adventitious shoot regeneration systems and the studies aimed at improving the plant by genetic and epigenetic means such as genetic transformation.

Table 3. Effect of medium composition in vitro rooting of *Hemidesmus indicus* after 12 weeks of culture.

Treatment	Rooting (%)	Number of roots per explant	Root length (cm)
MS	15.3	2.75 ± 2.87 [b]	1.41 ± 0.94 [b]
1/2 MS+1.5 mg/L IBA	38.1	3.70 ± 2.41 [a]	3.72 ± 1.38 [a]
MS+4 mg/L IBA+ 1 mg/L Kn	-	-	-

Note: Data expressed as Mean ± SE from 20 replicates. Within columns, values followed by the same letter are not significantly different at the P = 0.05.

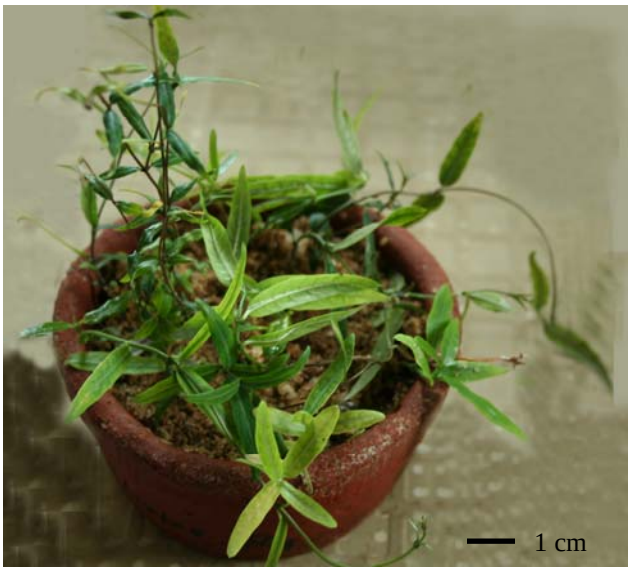

Plate 4. *In vitro* rooted plantlets of *Hemidesus indicus* at the stage of acclimatization.

CONCLUSIONS

The 4th and 5th nodal positions cultured on MS medium supplemented with 2 mg/L BAP in combination with 0.1 mg/L NAA can be recommended for multiplication of *H. indicus* plants. Reduction of total shoot length was observed at higher BAP levels (5 and 10 mg/L with 0.1 mg/L NAA). Multiplied shoots can be successfully rooted on half strength MS medium supplemented with 1.5 mg/L IBA. Rooted plants were successfully (100%) acclimatized on sterile soil and sand (1:3) medium.

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