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Efficient *in vitro* micropropagation and regeneration of *Artemisia vulgaris* L.

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ABSTRACT - *This paper describes efficient propagation of* Artemisia vulgaris *using shoot tip explants isolated from 35 days old* in vitro *grown seedlings. Optimum proliferation was obtained on Murashige and Skoog's salts and B_5 vitamins supplemented with 3% sucrose, 4.44 µM BA, and 0.7% agar. Shoot proliferation was maximal (99.8%) with 14-23 shoots per explant after 6 weeks of culture. Shoots with a minimum length of 1.5 cm were transferred to shoot elongation medium supplemented with 0.44 µM BA and 1.44 µM GA_3. The successfully elongated shoots with a height of 7.2-12.1 cm were transferred to rooting medium augmented with 8.56 µM IAA. Rooted plantlets were transferred to plastic cups containing autoclaved garden soil, farmyard soil and sand (2:1:1) for hardening. Plantlets were initially maintained under culture room conditions (5 weeks), followed by normal laboratory conditions (4 weeks) and finally transferred to a Botanical Evaluation Garden and maintained there.*

Key words: shoot organogenesis, rooting *in vitro* establishment, hardening, *ex vitro*.

INTRODUCTION

Artemisia vulgaris L. (mugwort) belongs to the family Asteraceae and is a tall aromatic perennial herb, which grows throughout a hilly district (Kodaikanal) of India in areas up to 2,400 m altitude. In traditional medicine, this plant is being widely used for the treatment of diabetes and extracts of the whole plant are used for epilepsy and, in combined forms, for psychoneurosis, depression, irritability, insomnia and anxiety stress (Walter et al. 2003). The plant is also useful in the treatment of uterine cancer (Shaik and Hussain 2004). An infusion of the leaves is given as a vermifuge against intestinal parasites (George and Roger 2000). Moreover, the plant is a highly effective antidote to insect poison (Asima and Satyesh 1997). Several medicinally active components of *A. vulgaris* have been identified including vulgarin, quercetin, coumarins, sesquiterpene lactones, volatile oils and Inulin (USDA-ARS-NGRL 2004). The dry leaves and flowers in infusion are emmenagogues. The essential oil of the plant was reported to present 90% mosquito repellency against *Aedes aegypti*, a mosquito that transmits yellow fever (Ram and Mehrotra 1995). A paste or powder of the leaves is applied over skin diseases (Kapoor 2000); leaves are used as inferior substitutes for cinchona in fevers.

For further research into the biochemical compositions and potential medicinal values of this plant, an *in vitro* regeneration system for the production of plants is required because field-grown plants may be subjected to seasonal and somatic variations, infestation of bacteria and fungi as well as environmental pollutions that can

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affect the medicinal value of the harvested tissues (Geng et al. 2001). We have found a large variation in volatile oil contents among mugwort grown in different parts of India . We therefore believe that there is a strong need to genetically improve and cultivate mugwort with high and stable contents of volatile oil.

In vitro propagation offers many advantages over conventional propagation methods. True-to-type multiplication provides uniform plants with genetic identity. Since the harvest of medicinal plants on a mass scale from their natural habitats is leading to a depletion of plant resources, the conservation of these valuable genotypes is imperative. The plant is at low risk of extinction, according to the first Red Data List of south Indian medicinal plants (IUCN version) prepared by the Conservative Assessment and Management Plan (CAMP), India. Micropropagation via shoot culture, often utilized to maintain clonal fidelity would be a special advantage in this respect (Sen and Sharma 1991). In recent years, there has been an increased interest in *in vitro* techniques, which offers powerful tools for germplasm conservation and the mass multiplication of many threatened plant species (Murch et al. 2000). Further, genetic improvement is another approach to augment the drug-yielding capacity of the plant (Tejavathi and Shailaja 1999). Therefore it is important to develop an efficient micropropagation technique for *Artemisia vulgaris* to rapidly disseminate superior clones once they are identified. Though there are reports on other species of the genus *Artemisia* (Liu et al. 2003, Mackay and Kitto 1988, Mathe and Laszloffy 1991, Mozetti and De-Donato 1998, Nin et al. 1996, Saxena 2001, Sharief et al. 1997), there is no systematic cultivation of this plant and no published reports on tissue cultures of *Artemisia vulgaris* are available. Therefore, there is a need to develop a means for rapid plantlet regeneration.

Our investigations were aimed at applying tissue culture techniques to generate high-quality somaclones of mugwort with an increased, constant level of volatile oils for phytomedicine production. In the present work, we have, for the first time, established a rapid and reproducible method for shoot multiplication from shoot tip explants, followed by successive establishment of regenerated plantlets in soil. We further examined the morphological growth characteristics and survival efficiency of the plant. The protocol reported here could be used for the conservation, large-scale propagation and for further research on the biochemical composition and medicinal importance of this valuable medicinal herb.

MATERIAL AND METHODS

Seed germination and explant establishment

Seeds of *Artemisia vulgaris* L. were collected from the company Johnny's Selected Seeds, USA located at Winslow, Maine, in September 2004. The botanical identity of these seeds was confirmed by comparison to reference standards used by Johnny's Selected Seeds, USA. The seeds were surface-disinfected with 10% (v/v) dettol disinfectant (Reckitt Benckiser, Kolkata, India) for 5 min, followed by rinsing three to five times in sterile distilled water. The seeds were then surface-sterilized with 0.1% (w/v) aqueous mercuric chloride ($HgCl_2$) for 4-5 min and finally rinsed with autoclaved distilled water (three to five changes). Surface-sterilized seeds were inoculated into culture flasks containing 25 ml MS medium supplemented with 10% (v/v) filter-sterilized coconut water and incubated in a dark chamber for 5-7 d at a temperature of 23 °C to facilitate germination. Later they were transferred to photoperiodic conditions for another 28-32 d for seedling growth. Shoot tips with one or two leaf primordia excised from 35 d old *in vitro* grown seedlings were used as the explant source for the present study.

Medium composition and culture conditions

Shoot tips (5-6 mm) were cultured on a basal medium containing MS salts (Murashige and Skoog 1962), B_5 vitamins (Gamborg et al. 1968), 30 g l^{-1} sucrose (Himedia, Mumbai, India) and 0.1 g l^{-1} myo-inositol. The pH of the medium (supplemented with growth regulators) was adjusted to 5.7 with 1N NaOH or 1N HCl before gelling with 0.7% agar (Himedia, Mumbai, India). In all experiments the chemicals used were of analytical grade (Himedia, Mumbai, India, Sigma-Aldrich, USA and E. Merck, Germany). The medium was dispensed into culture vessels (Borosil, India) and autoclaved at 105 K Pa and 121 °C for 15 min. The explants (shoot tip) were implanted vertically on the culture medium (test tubes (150x25 mm) containing 15 ml medium) and fastened tightly with non-absorbent cotton. All cultures were incubated at 25±1 °C under a 16/8 (light/dark) photoperiod of 45-50 μ mol m^{-2} s^{-1} irradiance provided by cool white fluorescent tubes (Philips, India) and at 55-60% relative humidity (RH). All subsequent subcultures were initiated at 3 week intervals.

Multiple shoot induction and plantlet regeneration

Shoot tips (5 - 6 mm) with one or two leaf primordia excised from 35 d old *in vitro* grown seedlings were

inoculated vertically on MS medium containing 3% (w/v) sucrose, 0.7% (w/v) agar, 0.1 g L^{-1} myo-inositol and supplemented with different BA (N^6 – Benzyl adenine) concentrations (0.44 – 13.32 μM) for multiple shoot induction. For each treatment, a total of 10 replicates with 2 explants each were inoculated, i.e., 20 explants per treatment were tested. The initiated multiple shoots were subcultured on MS medium containing 4.44 μM BA at every 3 weeks for 28-34 d. A control group was maintained (MS basal medium without BA supplementation) to record the frequency of response.

Shoot elongation

After 45 d, multiple shoots induced in BA supplemented medium were excised individually and subcultured on MS medium supplemented with 0.44 μM BA and varying concentrations of GA_3 (Gibberellic acid) (0.29-4.33 μM) for shoot elongation.

Rooting

To induce rooting, individual elongated shoots (7.2-12.1 cm long) were isolated from the elongation medium after 2-3 weeks and individually transferred to MS medium containing different types of auxins viz., NAA (α- Naphthalene acetic acid) (2.69 – 16.11 μM), IAA (Indole - 3 - acetic acid) (2.85 – 17.13 μM) and IBA (Indole – 3 – Butyric acid) (2.46 – 14.76 μM). One set of cultures were inoculated in basal MS medium without the addition of auxins as control.

Acclimatization and transfer of plantlets to soil

Plantlets with well-developed roots were removed from the culture medium and after washing the roots gently under running tap water to remove the adhering medium, plantlets were transferred to plastic cups (10 cm diameter) containing autoclaved garden soil, farmyard soil and sand (2:1:1). Each potted plantlet was irrigated with distilled water every 2 d for 3 weeks followed by tap water for 2 weeks. The cups were initially maintained at culture room conditions (5 weeks) and afterwards transferred to normal laboratory conditions (4 weeks). After 65 d the plantlets were transplanted to the Botanical Evaluation Garden and kept under shade for further growth and development. The morphological characters, growth characteristics and survival efficiency were assessed.

Statistical analysis

The experiments were set up in a randomized block design (RBD) and each experiment usually had 10 replications and was repeated at least 3-5 times. Ten to twenty explants were used per treatment in each replication. Observations on the frequency (number of cultures responding with multiple shoot proliferation and root development) and the number of shoots per explant, shoot length, number of roots per shoot, and root length were recorded, respectively. The appropriate analysis of variance (ANOVA) for the design was carried out to detect the significance of differences between the treatment means; the treatment means were compared using Duncan's Multiple Range Test (DMRT) at 5% significance (Gomez and Gomez 1976).

RESULTS AND DISCUSSION

Surface-sterilized seeds were germinated on MS medium in the presence of 10% filter-sterilized coconut water. 95% of the seeds germinated after 5-7 d of dark exposure at 23 °C. Fully grown seedlings were observed within 30 d, after transferring to photoperiodic conditions (16/8 h light/dark) at 25±1 °C. Shoot tips (5-6 mm) (35 d old) with one or two leaf primordia of *A. vulgaris* were cultured on MS medium supplemented with B_5 vitamins and various concentrations of BA (0.44 – 13.32 μM) for shoot regeneration. After 2 weeks of culture in the MS basal media containing different BA concentrations, the explants cultured in all concentrations showed shoot bud initiation. Figure 1 demonstrates the effect of BA on shoot multiplication of *A. vulgaris* shoot tip explants. After 45 d, the shoot multiplication rates were between 46.1% and 99.8% compared to that of control (15.3%) (Figure 1). The number of shoots ranged between 14.4 and 23.3 compared to thecontrol (4.1) (Figure 2). Of all different BA concentrations tested, 4.44 μM BA was found to be most suitable in shoot sprouting and multiple shoot proliferations.

The cytokinin BA has been commonly used for the induction of organogenesis in many plants (Zilis et al. 1979, Vijaya Kumar and Ranjitha Kumari 2005, Baskaran and Jayabalan 2005). A comparison of the relative effectiveness for multiple shoot ranked the different cytokinins as follows: BA > KN > Zeatin > Adenine (Shiva Prakash et al. 1994). In agreement with our report, BA has been used in preference to other cytokinins to induce multiple shoots in *Artemisia pallens* (Umer Sharief and

Jagadish Chandra 1991). Similarly, Shamsudeen Varisai et al. (1999) reported that BA alone or in combination with other cytokinins induced shoot organogenesis from shoot tips and cotyledonary nodes of *Macrotyloma uniflorum*. Shoot tip cultures are well established in a wide range of plant species (George and Sherrington 1984) and can be used for clonal propagation. It is stated that BA is most effective for meristem, shoot tip and bud cultures (Kartha 1978). Regeneration through shoot buds is also reported in *Azadirachta indica* and *Atropa belladonna* (Shekawat et al. 1995).

The duration of exposure to BA affected the regeneration induction in shoot tip cultures. Cultures grown on medium supplemented with 4.44 µM BA for 45 d produced a maximum number of shoots (23) per shoot tip, and there was no significant increase in the number of shoots formed when the exposure to BA was extended (data not shown). A similar response was observed in *Artemisia judaica* (Liu et al. 2003). In the present study, higher BA concentrations (6.66 – 13.32 µM) reduced the shoot numbers as well as percentage of response (Figure 1 and 2). Hu and Wang (1983) reported that higher cytokinin concentrations reduced the number of micropropagated shoots. A similar response was observed by Indhra and Dhar (2000).

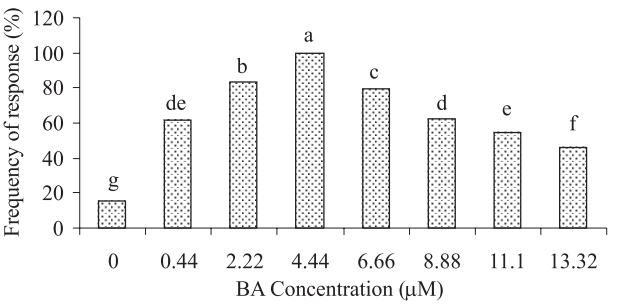

Figure 1. Effect of BA on *in vitro* shoot sprouting frequency of shoot tip explants of *A. vulgaris*
Evaluation after 45 d of culture
Treatment means followed by different letters are significantly different from each other at 5% significance; comparison by Duncan's Multiple Range Test (DMRT)

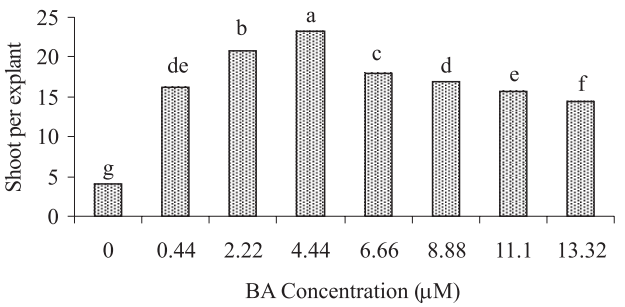

Figure 2. Effect of BA on number of shoots induced per shoot tip explant of *A. vulgaris*
Evaluation after 45 d of culture
Treatment means followed by different letters are significantly different from each other at 5% significance; comparison by Duncan's Multiple Range Test (DMRT) 0

Although a maximum multiplication rate of 14-23 shoots per explant was observed, it was necessary to establish a shoot elongation protocol. Shoots with a minimum length of 1.5 cm and with at least three to four leaves were used for shoot elongation to maintain the endogenous GA_3 supply, as young leaves have the highest levels of active gibberellins (Taiz and Zeiger 1998). Excised shoots that were cultured in the same minimal multiplication medium (0.44 µM BA) and in the presence of GA_3 at different concentrations for 2 weeks were evaluated for shoot length. The results suggest that small amounts of GA_3 (1.44 µM) were effective at stimulating *A. vulgaris* shoot elongation (Figure 3). Shoots attained heights of 7.2 to 12.1 cm during GA_3 treatments (Figure 4). Jamison and Renfroe (1998) also observed that GA_3 with BA or 6-γ-γ- (dimethylallylamino) purine (2iP) obtained the same response in *Betula* cultures. GA_3 stimulates shoot elongation by inhibiting auxin action in meristematic regions (Taiz and Zeiger 1998).

To induce rooting, elongated shoots were cultured on MS medium augmented with different types of auxins viz., NAA (2.69 – 16.11 µM), IAA (2.85 – 17.13 µM) and IBA (2.46 – 14.76 µM) individually. A control group was also maintained. Among the three different auxins tested, root length and number of roots varied (Table 1). Plantlets significantly developed lengthy roots and root induction was strengthened within 20 d of culture. IAA was found to be more adequate than NAA and IBA. Roots were visible within 5-10 d following transfer of elongated shoots to the rooting medium. After 2 weeks, the plantlets had developed the primary and secondary root system. The frequency of rhizogenesis was almost 98%. In agreement, IAA was reported as potential auxin for rooting in *Arachis stenosperma* and *A. villosa* (Vijayalakshmi and Giri 2003). Increasing the concentration of IAA to over 8.56 µM gradually led to a decrease in the frequency of root regeneration. A similar response was observed in *Sesbania drummondii* (Cheepala et al. 2004). The average number of roots per

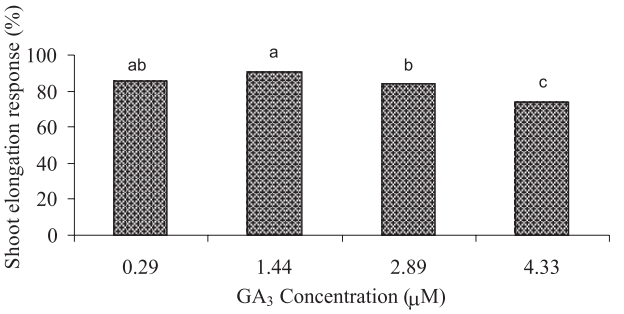

Figure 3. Effect of GA_3 in combination with 0.44 µM BA on shoot elongation response of *A. vulgaris*
Evaluation after 2 weeks of culture
Treatment means followed by different letters are significantly different from each other at 5% significance; comparison by Duncan's Multiple Range Test (DMRT)

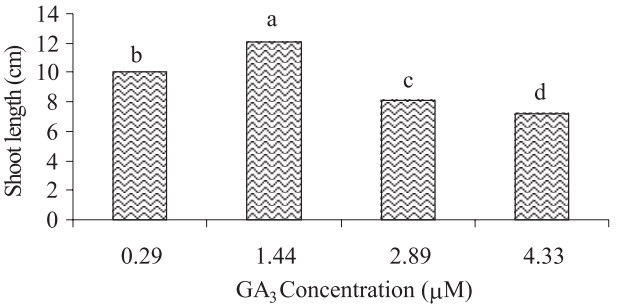

Figure 4. Effect of GA_3 in combination with 0.44 µM BA on induction of shoot length of *A. vulgaris*
Evaluation after 2 weeks of culture
Treatment means followed by different letters are significantly different from each other at 5% significance; comparison by Duncan's Multiple Range Test (DMRT)

Table 1. Effect of different auxins on rooting of *in vitro*-grown shoots of *A. vulgaris*

GR (µM)	PS	NR/S (Mean ± SE)	RL (cm) (Mean ± SE)
Control	10.4^{e}	4.1±0.22^{c}	3.4±0.29^{d}
NAA			
2.69	65.6^{c}	12.6±0.21^{d}	8.8±0.19^{c}
5.37	76.2^{b}	13.8±0.13^{c}	10.8±0.18^{bc}
8.06	82.1^{a}	16.3±0.20^{a}	12.7±0.20^{a}
10.74	59.9^{cd}	15.3±0.28^{b}	11.2±0.18^{b}
16.11	41.2^{d}	13.2±0.28^{cd}	8.2±0.19^{cd}
IAA			
2.85	73.4^{c}	19.9±0.36^{c}	12.4±0.20^{cd}
5.71	86.7^{b}	21.6±0.32^{b}	13.3±0.21^{b}
8.56	98.2^{a}	25.1±0.22^{a}	15.5±0.16^{a}
11.42	84.1^{bc}	18.7±0.28^{d}	12.6±0.15^{c}
17.13	62.6^{d}	16.7±0.24^{e}	10.9±0.22^{d}
IBA			
2.46	67.6^{bc}	10.6±0.21^{d}	7.50±.16^{cd}
4.92	72.8^{a}	15.2±0.18^{a}	11.5±0.16^{a}
7.38	68.4^{b}	12.8±0.19^{c}	8.6±0.15^{bc}
9.84	53.2^{c}	13.3±0.20^{b}	8.9±0.22^{b}
14.76	37.9^{d}	12.0±0.20^{cd}	8.1±0.22^{c}

GR = Growth regulators; PS = Percentage of shoots developing roots; NR/S = Number of roots / shoot; RL = Root length
Evaluation after 2 weeks of culture
Treatment means followed by different letters are significantly different from each other at 5% level of significance; comparison by Duncan's Multiple Range Test (DMRT)

shoot ranged from 16-25 and the maximum root length observed was 15.5 cm (Table 1). However, NAA and IBA formed slender roots. In contradiction to our report, IBA was used for rooting in *Artemisia judaica* (Liu et al. 2003) and NAA in *Morus alba* (Anuradha and Pullaiah 1992). Shoots also produced roots when transferred to basal medium containing no growth regulators, but the rooting was higher with IAA (2.85 – 17.13 µM).

The successfully rooted plantlets were transferred to plastic cups containing sterile garden soil, farmyard soil and sand (2:1:1) for hardening. Plantlets were initially maintained in the culture room (25±1ºC) conditions for 5 weeks and thereafter transferred to normal laboratory conditions for about 4 more weeks. Finally the plantlets were transferred to the Botanical Evaluation Garden and maintained there. The initial growth rates of plant height were 14.9 to 25.3 cm during the first 5 weeks of acclimatization. However, in the following 8-20 weeks, substantial increases in plant height were observed (Figure 6). The survival rate decreased to 97.3 and 80.0%, respectively after 10-20 weeks of acclimatization. (Figure 5). Initially, two to three healthy leaves were developed adjacent to the main shoot. The stem was very slender without branching and growth of minute hairs on the stem and on the leaf underside was observed. The number of leaves increased after 8-20 weeks of acclimatization. There was no detectable variation among the acclimatized plants with respect to morphological and growth characteristics. All micropropagated plants were free of external defects.

The overall objective of the current study was to develop a system for mass propagation and aseptic growth of *A. vulgaris*. Shoot tip cultures are well established in a wide range of plant species (George and Sherrington 1984) and can be used for clonal propagation. In the present study, shoots were regenerated from excised shoot tips of

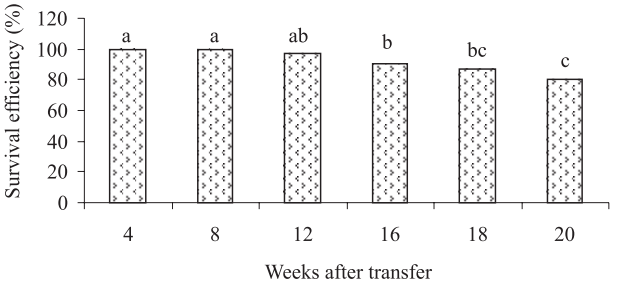

Figure 5. Frequency of *ex vitro* growth of acclimatized microplants of *A. vulgaris*
Treatment means followed by different letters are significantly different from each other at 5% significance; comparison by Duncan's Multiple Range Test (DMRT)

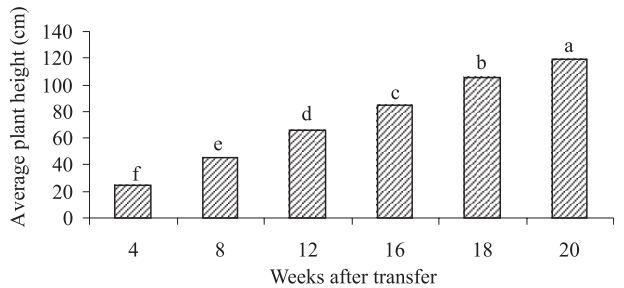

Figure 6. Frequency of *ex vitro* survival efficiency of acclimatized *A. vulgaris* microplants
Treatment means followed by different letters are significantly different from each other at 5% significance; comparison by Duncan's Multiple Range Test (DMRT)

mugwort on BA-supplemented medium. The presence of BA was obligatory for induction and proliferation of shoot buds. George and Rao (1982) also reported the necessity of BA for shoot induction in safflower cotyledons. The mechanism by which it initiates shoot organogenesis is not clear but it has been suggested that BA interrupts the chromosomal DNA replication and reprograms the developmental fate of certain cells (Buising et al. 1994).

The literature describes different regeneration systems for mass propagation of many *Artemisia* plantlets for different purposes. But this is the first report on *in vitro* culture of *A. vulgaris*. Plantlets derived from intact seedling systems can be a prolific tissue for the biochemical characterization of medicinally active components and for the selection and cloning of superior individual genotypes. Plants produced from *de novo* regeneration on excised tissues will be useful for crop improvement through genetic engineering and cell culture techniques. In combination, these approaches may result in novel uses for this valuable species.

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Eficiência da propagação e regeneração *in vitro de Artemisia vulgaris* L.

RESUMO - *Este estudo descreve a eficiência da propagação* in vitro *de* Artemisia vulgaris *com explantes isolados de brotos apicais de plântulas com 35 dias pós-germinação. Obteve-se ótima multiplicação em sais de Murashige e Skoog e vitaminas B5 com suplemento de sacarose 3%, BA 4,44 µM e àgar 0,7%. A brotação foi máxima (99,8%) com 14-23 hastes por explante depois de 6 semanas de cultivo. As brotações com comprimento mínimo de 1,5 cm foram transferidos para meio de crescimento suplementado com BA 0,44 µM e GA_3 1,44 µM. As brotações que se desenvolveram com sucesso e atingiram o cumprimento de 7,2-12,1 cm foram transferidas para meio de enraizamento acrescido de IAA 8,56 µM. Para a aclimatação em recipientes plásticos, as plantas enraizadas foram estabelecidas em solo de jardim, solo comum e areia (2:1:1) autoclavados. Inicialmente as plantas foram mantidas nas condições da sala de propagação (5 semanas); posteriormente nas condições normais de laboratório (4 semanas) e por fim, transferidas para o campo, onde permaneceram para avaliação botânica.*

Palavras-chave: organogênesis de caule, enraizamento, estabelecimento *in vitro*, aclimatação, *ex vitro*.

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