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High frequency plant production via shoot organogenesis in *Leucosceptrum canum* Smith (Lamiaceae)

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ABSTRACT

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Leucosceptrum canum Smith, the only short tree species of Lamiaceae, does not produce viable seeds and is mainly propagated vegetatively. A rapid in-vitro propagation of whole plant was achieved from axillary shoot culture in Murashige and Skoog Medium (MS) supplemented with 6-benzylaminopurine (BAP, 2 mg/l), Adenine sulphate (AdS, 40 mg/l) and thiamin HCl (4 mg/l). The multiplication rate of the explant was 90% and each explant developed 10.53± 0.5 shoots averaging 4.82± 0.3 cm length within 40 days of culture. The regenerated shoots were rooted on MS medium containing indolebutyric acid (IBA, 1 mg/l) and the explants producing 12.53± 0.3 roots per shoot within a period of 30 days. It is direct and more efficient method for ensuring trueness to type than any other in-vitro procedure. The explants were acclimatized by transferring them to earthen pots containing a mixture of autoclaved soil:sand:compost (1:1:1) and they were immediately covered with transparent polybag. This system provides high fidelity micropropagation system for efficient and rapid production of this important plant.

Key-words: *Leucosceptrum canum*, Lamiaceae, high frequency plant production, shoot organogenesis.

INTRODUCTION

Leucosceptrum canum Smith (Lamiaceae) is a tomentose or villous shrub or small tree. It is mainly distributed in temperate Himalaya from Kumaon to Bhutan at 610 to 2440 m and Khasia mountains at 1220 to 1525 m above sea level (Flora of British India 1885).

Propagation of *Leucosceptrum canum* from seed offers one possibility for large scale cultivation of the plant. But one of the constraints in this method of propagation is the very short span of seed viability. The vegetative growth of this plant is periodic. Hence, it is felt that there is a great need of large scale propagation of this plant. In-vitro techniques therefore could be of advantage. Micropropagation of *Leucosceptrum canum* would provide large amount of highly uniform plantlets suitable for further propagation in the field. Micropropagation by shoot bud proliferation has proved to be the most reliable method for large scale production of many medicinal plants. So in this paper, we describe in-vitro propagation of *Leucosceptrum canum*.

Commercial exploitation and elimination of natural habits, consequent to urbanization, have led to gradual extinction of several plants. In recent years, there has been an increased interest in in-vitro culture techniques which offer a viable tool for mass multiplication and germplasm conservation of rare, endangered and threatened plants (Ajithkumar & Seeni 1998, Prakash et al. 1999, Aastha et al. 2010). Micropropagation is an effective approach to conserve such germplasm. In-vitro propagation has proven as a potential technology for mass scale production of plant species

(Wawrosch et al. 2001, Martin 2003, Azad et al. 2005, Hassan & Roy 2005, Hassan et al. 2009). Therefore, it is important to develop an efficient micropropagation technique for *Leucosceptrum canum* to rapidly disseminate superior clones once they are identified.

MATERIAL AND METHOD

Plant material and explant preparation: The young shoot buds (both terminal and axillary) were used as explant. Shoot cuttings with the youngest two to five leaves were collected from plants grown in the experimental garden of the institute. Material is collected fresh in the morning hours while the plants are still turgid, and not during mid day when they tend to be flaccid. Material collected from the garden is invariably heavily contaminated with dust and microorganisms. After excision, the shoot tips (about 1-3 cm in length) were subjected to preliminary washing under running tap water for 10 minutes to 30 minutes which reduces the microflora to a substantial extent. Healthy and uniform explants were agitated thoroughly in 5% savlon solution for 8-10 minutes. Explants were then rinsed under running tap water. Then they were surface sterilized with 0.1% (w/v) aqueous mercuric chloride solution for 8 minutes, followed by 4-5 rinses of 3 minutes duration in sterile distilled water.

Media preparation: The shoot bud explants were transferred to 20 ml Murashige and Skoog (1962) medium (MS) containing 3% sucrose supplemented with various concentrations of 6-benzylaminopurine (BAP), Adenine sulphate (AdS), indoleacetic acid (IAA), indolebutyric acid (IBA), a-naphthaleneacetic acid (NAA) and thiamin HCl either individually or in combination. The medium pH was adjusted to 5.8 prior to adding 0.7% agar (w/v, Qualigens) and was autoclaved at 121°C for 15 minutes. The cultures were incubated at 24±1°C under 16 hours daily illumination with fluorescent light (12000 lux). The medium was dispensed into 25x150 mm culture tubes containing 20 ml medium or 100 ml wide mouth conical flasks containing 50 ml medium each and cotton plugs were used to close the culture tubes.

Explants were cultured on MS medium supplemented with various concentrations and combinations of plant growth regulators for shoot bud differentiation in different experiments. The surface sterilized shoot buds were subsequently dried in a petridish containing sterile filter paper and placed on the culture medium (one shoot per culture tube). Each treatment was replicated three times, using a total of six replicates for each treatment. The cultures were maintained at culture room conditions and subcultured onto the fresh media every ten days intervals. The tissue culture raised microshoots longer than 3-4 cm were counted and harvested after 40 days and cultured on fresh medium for rooting.

Rooting of microshoots: For induction of roots, the microshoots having the length above 4 cm, regenerated from multiple shoot clusters, were cultured on hormone free MS medium as well as MS medium supplemented with different concentrations of IBA (0.5-3 mg/l). The cultures were incubated at 24±1°C under 16 hours daily illumination with fluorescent light.

Acclimatization: Rooted plants were taken from the rooting medium and washed several times with sterile distilled water. Plantlets were potted in sterile sand:loam:peat in a ratio of 1:1:1 mixture, covered with a polythene bag to maintain high humidity and were kept under controlled temperature at 22-26°C and light (12000 lux) conditions in the culture room. The bag was removed periodically for gradual hardening. After 2-3 weeks, when new leaves emerged from such plantlets, they were taken outside the culture room and kept in a shady place under normal temperature and

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Plate 1

1-6. In-vitro regeneration through multiple shoot multiplication of *Leucosceptrum canum*. 1. Shoot bud explant of *Leucosceptrum canum* cultured on MS + BAP (2.0 mg/l) + AdS (40 mg/l) + thiamin HCl (4.0 mg/l). 2. Formation of multiplied shoots on MS + BAP (2.0 mg/l) + AdS (40 mg/l) + thiamin HCl (4.0 mg/l) after two weeks of culture. 3-4. Rooting of in-vitro raised shoots on MS + IBA (1.0 mg/l). 5. Potted in-vitro raised plantlets. 6. Peroxidase isozyme pattern.

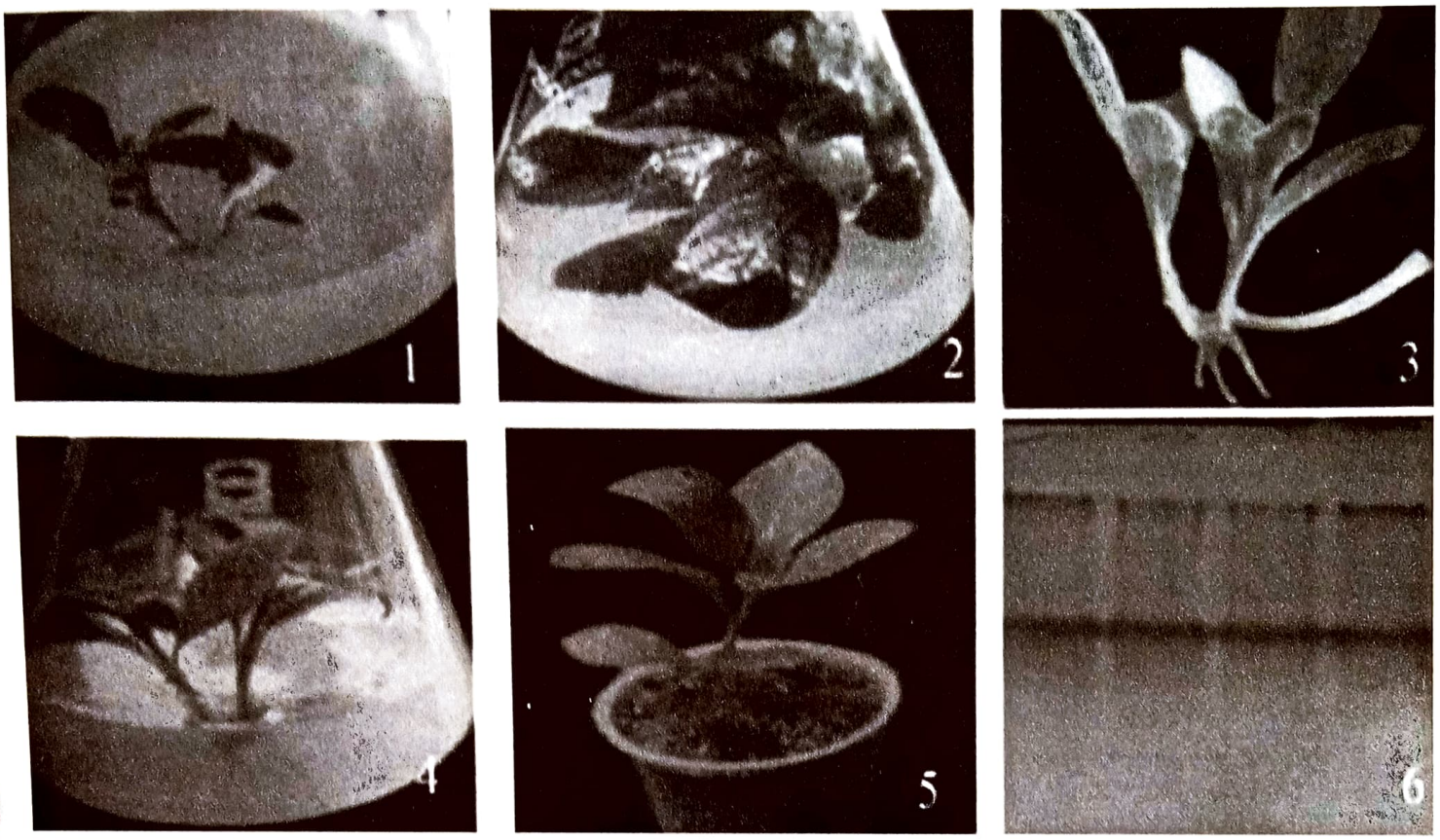

Plate 1

light. They were watered every two days for 15 days.

Isozyme analysis: Isozyme (peroxidase) analysis was performed by macerating 1 gm leaf material collected from mature plants and two months old tissue culture raised plants growing in the same environment. The leaves were excised and kept in -70°C for some days to avoid chlorophyll interference. The materials were crushed in an ice-cold mortar pastle with ice-cold PEB buffer (the composition of PEB buffer were 0.1 M Tris-HCl, 0.25 M Sucrose, 1 % Polyvinylpyreledon (PVP), 1 % Ascorbic acid, 0.1% Cystein HCl, 1 mM EDTA, 0.4 mM $MgCl_2$, 0.4 mM DTT, 1% β-marcaptoethanol. All the chemicals were dissolved in double distilled water and the pH was adjusted to 6.8).

The homogenate was collected and centrifuged in a RC 5B Sorval Refrigerated centrifuge for 45 minutes at 12000 rpm. The clear supernatant was collected and again centrifuged in a Beckman L7-55 Ultracentrifuge at 40000 rpm for 2 hours. After centrifugation, the pellet was discarded and the supernatant was lyophilized for 10-12 hours as required. SDS-PAGE was performed using 12% polyacrylamide gels. After polymerization, 150 mg of protein samples were loaded to each well with the help of a micropipette fitted with multiflex tips (MULTI, USA).

After the loading, the apparatus was attached to an electric power supply. The gel was run at constant current of 60-80 voltage. The entire operation was performed in an air-conditioned room. Ice bags and cubes were placed all around the apparatus to maintain low temperature.

When the run was completed, i.e. as the tracking dye reached the anodic end, the power supply was switched off and the glass plates having the gels were removed from the apparatus. The stacking gel part was cut off and the rest was incubated in buffer and substrate solution of Isozyme. The gel was incubated in the staining solution containing 20 ml of 0.15 (M) acetate buffer and then were added 50 ml of Guaicol and 50 ml of H_2O_2 for 5-10 minutes at 4°C. When the blue coloured bands appeared, the reaction was arrested by immersing the gel in distilled water and finally the gel was stored in 1% acetic acid.

RESULTS

The surface sterilization procedure followed in the present study yielded 90% of the plant (Plate 1, figure 1) free of microbial contamination. Multiple shoot development could not be induced from the axillary bud of *Leucosceptrum canum* on a growth regulator free MS medium (M 1). The present experiment revealed that addition of cytokinin to the medium was essential to induce multiple shoot formation in the explant. In a series of media (M2-M15) supplemented with BAP, AdS, IAA and vitamin thiamin HCl were tested to induce multiple shoot formation (Table 1). MS medium containing only BAP as a cytokinin at a concentration of 1.0 mg/l (M2) induced shoot multiplication within 10-15 days. Initially, 1-2 shoot buds were developed. After that, number of shoot bud development was increased gradually when the cultures were maintained in the same medium up to 2-3 passages (10 days duration of each passage). In presence of only BAP, 5.23±0.3 shoots were formed per explant at the end of 4th passage. Number of shoot buds production did not increase even after increasing the concentration of only BAP up to 2 mg/l. Addition of AdS and thiamin HCl along with BAP improved the rate of multiplication. The best multiplication rate (M8) was achieved (Plate 1, figure 2) in MS medium containing BAP (2 mg/l), AdS (40 mg/l) and excess thiamin HCl (4 mg/l) (actual concentration in medium 4.1 mg/l because the MS medium contains 0.1 mg/l of thiamin HCl). The multiplication rate of the explant was 90% and each explant developed 10.53±0.5 shoots averaging 4.82±0.3 cm length within 40 days of culture (Table 1). The other media (M3, M4, M6 and M7) containing lower concentration of AdS (20 mg/l) showed lower multiplication rate. The use of higher concentration of BAP (3-4 mg/l) along with AdS and thiamin HCl resulted in poor multiplication rate (M 9 and M10). Low multiplication rate was also found in absence of thiamin HCl (M6). Addition of IAA (0.5 mg/l) in combination with BAP, AdS and thiamin HCl containing medium (M11 and M12) also exhibited comparatively lower multiplication. The use of IAA (1mg/l) with only BAP (1 mg/l) did not show any advantage rather the multiplication rate became lowest. In order to increase the rate of shoot multiplication, all cultures in M8 medium were maintained up to 4th passage. After 5th passage, all cultures gradually developed into dense cluster of shoots but the growth of individual shoot became stunted. The number of shoots was so high that it was very difficult to count and to separate individual shoot from the cluster. In such cases, the microshoots were separated individually and subcultured on M4 medium.

Table 1. Nutrient media used for micropropagation of axillary shoot bud of *Leucosceptrum canum* (MS basal medium with 3% sucrose, 0.7% agar and pH=5.8). Results are the mean of 6 replicates ± SE (after 40 days of culture).

Medium	Growth regulators (mg/l)			Vitamin	Mean number of shoots	Mean length of shoot (cm)
	BAP	AdS	IAA	Thiamin		
M1	0.0	0.0	0.0	0.0	00	00
M2	1.0	0.0	0.0	0.0	5.23 ± 0.3	1.42 ± 0.3
M3	1.0	20	0.0	0.0	6.33 ± 0.4	1.52 ± 0.5
M4	1.0	20	0.0	4.0	6.42 ± 0.5	2.66 ± 0.3
M5	1.0	40	0.0	4.0	6.33 ± 0.5	2.36 ± 0.6
M6	2.0	20	0.0	0.0	2.85 ± 0.1	2.84 ± 0.4
M7	2.0	20	0.0	4.0	7.15 ± 0.3	3.55 ± 0.5
M8	2.0	40	0.0	4.0	10.53 ± 0.5	4.82 ± 0.3
M9	3.0	40	0.0	4.0	6.52 ± 0.6	2.33 ± 0.4
M10	4.0	40	0.0	4.0	6.42 ± 0.6	2.34 ± 0.8
M11	1.0	40	0.5	4.0	3.31 ± 0.8	2.45 ± 0.5
M12	1.0	40	1.0	4.0	3.81 ± 0.3	2.85 ± 0.4
M13	1.0	40	1.0	0.0	4.65 ± 0.5	2.55 ± 0.6
M14	1.0	0.0	1.0	0.0	2.65 ± 0.5	2.43 ± 0.5
M15	2.0	4.0	2.0	4.0	2.42 ± 0.5	2.44 ± 0.5

After 20 days of subculture, the stunted microshoots were grown normally. Among them, shoots longer than 4 cm were harvested and remaining microshoots were transferred to the fresh multiplication medium (M4) and this process was repeated in every subculture.

Multiple shoot proliferation and elongation: The shoot length increased in response to increasing BAP concentration, reaching the highest growth with 2.0 mg/l depending on AdS (40 mg/l) and thiamin HCl (4 mg/l) treatments and declining when BAP concentration reached 3 mg/l. The best combination for *Leucosceptrum canum* shoot elongation consisted of BAP (2 mg/l), AdS (40 mg/l) and thiamin HCl (4 mg/l) (Table 1). The medium supported effective enhancement in shoot length and recorded a maximum length of 10.53±0.5 cm within 40 days.

Rooting of microshoots: Microshoots recovered from axillary bud explant cultures had no roots. Rooting could not be induced in the excised shoots in an auxin free MS medium even after 30 days. For rooting 3-4 cm long shoots were separated as minicutting, cultured on MS medium supplemented with IBA (0.5-3 mg/l) and no cytokinin for the development of a proper root system (Plate 1, figures 3, 4). Roots were produced within 3 weeks of culture. Of all the concentrations tried for rooting on in-vitro raised shoots, the best results were obtained in IBA at 1.0 mg/l (12.53±0.3 roots per explant) (Table 2).

Table 2. Effect of IBA on root regeneration of *Leucosceptrum canum* after 30 days incubation. Results are the mean of 6 replicatesSE.

IBA (mg/l)	No. of roots	Root length (cm)
0.0	00	00
0.5	10.23 ± 0.2	4.76 ± 0.6
1.0	12.53 ± 0.3	7.46 ± 0.4
1.5	10.33 ± 0.4	6.66 ± 0.7
2.0	8.50 ± 0.2	3.50 ± 0.7
2.5	3.73 ± 0.2	2.55 ± 0.5
3.0	2.23 ± 0.4	2.11 ± 0.6

The explants showing root initiation after 8-10 days of starting the culture were transferred to medium of the same composition to promote further proliferation (with the increase of number of root laterals, length of roots, etc.) with the lapse of 25 days. The results indicate that IBA concentrations supplied to MS medium significantly influenced root proliferation and shoot growth. The best rooting treatment were 1 mg/l, since it gave the highest percentage of root induction. Higher levels of IBA reduced root number. When other auxins like IAA and NAA were added to the medium, callus was formed from the shoot base, which did not favour root formation.

Establishment of plants in soil: After rooting, the plants were transferred to vermicompost (Plate 1, figure 5) and acclimatized to green house condition. Regenerated plants were very sensitive to changes in the physical environment. They were grown in very high humidity and respond to decreased relative humidity too slowly to prevent desiccation of the rooted plants. Regenerated plants must be acclimatised to increased light intensity in much the same manner as acclimatisation to decreased relative humidity.

For detection of peroxidase, the Guaicol -H_2O_2 method has been used as the staining substrate. Both, the mother plant as well as regenerated plants, show bands in the upper (+ve) region of the gel. There was no difference in intensity of bands between the mother plants as well as regenerated plants (Plate 1, figure 6) of *Leucosceptrum canum*.

DISCUSSION

This paper provides protocols for rapid rooted shoot production in *Leucosceptrum canum* using axillary shoot bud explants. Axillary shoot buds have the potential for unlimited shoot proliferation because of the presence of apical meristem constantly undergoing cell division and cell differentiation. This potentiality was not expressed when the excised explants were grown in culture medium containing no growth regulators. The presence of cytokinin in the medium is obligatory for shoot proliferation. In *Leucosceptrum canum*, higher number of shoots per explant was recorded on a medium containing BAP, AdS and excess amount of vitamin like thiamin HCl. Therefore, BAP was the most effective cytokinin in shoot proliferation in the explant, but the rate of multiplication was augmented in presence of AdS and thiamin HCl. So, BAP alone is not adequate for the production of an acceptable rate of proliferation. Similar results were reported by Kaur et al. (1998),

Eeswara et al. (1998) and Maity et al. (2001). AdS and thiamin HCl along with BAP were also found necessary for higher rate of proliferation. The presence of adenine in the medium is reported to promote axillary bud differentiation in many cases (Sivakumar & Krishnamurthy 2000, Chetia & Handique 2000, Maity et al. 2001). Furthermore, increase in BAP concentration (3-4 mg/l) and keeping the same concentration of AdS and thiamin HCl, caused decrease in shoot bud proliferation. So, higher concentration of BAP is not effective for increasing shoot bud proliferation in case of *Leucosceptrum canum*. This is in agreement with the observation of banana-Lal Kela by Ganapathi et al. (1998). It has been observed that the shoot bud proliferation was reduced in absence of thiamin HCl in the medium. There are several reports which suggest that thiamin is known to stimulate cell division and the completion of differentiation occurred only in a culture medium and thiamin HCl was considered to be a growth factor (Cramer & Bridgen 1997, Maity et al. 2001). Auxin (IAA) in low concentration in the medium along with BAP, AdS and thiamin HCl stimulated the shoot proliferation but at higher level caused a suppression of shoot proliferation. Highest number of shoots per explant was noted in M8 medium in absence of IAA. So, IAA was not necessary for shoot proliferation. Similar results were reported by Marwani and Sarosa (2003) in *Papuacalia versteegii* and Anita and Pullaiah (1999) in *Sterculia* species.

IBA (1 mg/l) was found to be the ideal concentration for root initiation, root length and number of root laterals. IBA is an effective auxin in root induction in a wide range of plants from herbs to tree (Jasrai et al. 1999). IBA was reported to have favoured root initiation in several plant species in culture (Maity et al. 2001, Nandwani & Myazoe 2002). The efficiency of auxins alone for root induction on microshoots in the present system is similar to the report on *Artocarpus altilis* (Nandwani & Myazoe 2002), *Scutellaria integrifolia* (Joshee & Yadav 2002), *Solanum trilobatum* (Alagumanian et al. 2004), etc.

When other auxins like IAA and NAA were added to the medium, callus was formed from the shoot base, which did not favour root formation. Root developments were inhibited by BAP, in the presence of either IBA or NAA. This observation was at per with those of Furmanowa and Olszowska (1992) and Saez et al. (1994).

Isozyme profile (peroxidase) showed no variation between donor and in-vitro raised plants through multiple shoot formation. Although, isozymes have been used extensively for genomic modification and for identification of varieties and cultivars (Livneh & Vardi 1998).

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