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In vitro Propagation of *Leucas biflora* (Vahl) Sm. - A Vulnerable Medicinal Herb

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Abstract

A rapidly well-developed *in vitro* regeneration system has been established for *Leucas biflora* using nodal segments as explants and MS medium supplemented with various concentrations and combinations of plant growth regulators (PGR_S). MS medium containing 2.0 mg/l BAP and 0.5 mg/l NAA showed the highest response (95.99%) in induction of shoot buds. This media combination also produced the maximum number (11.20 ± 0.49) of multiple shoot bud per explant after 3 subcultures at an interval of 14-days. Microshoots showed the maximum elongation (4.70 ± 0.07 cm) in the same medium along with subcultures. These elongated shoots were further grown on rooting media, producing vigorous and healthy root systems. The best response (95.99 ± 1.63%) for rooting was obtained when half-strength MS medium was supplemented with 0.5 mg/l IAA + 0.5 mg/l IBA in 21 days of culture. In this combination, the average number and length of roots were 5.20 ± 0.33 and 4.80 ± 0.30 cm, respectively. The seedlings were removed from culture tubes and transplanted into the pots *via* several stages of acclimatization. Finally, 98% of the seedlings were found to survive under natural condition.

Introduction

Leucas R. Br. (Lamiaceace), a genus of 80 species (Hedge 1990), has two primary centers-one in Africa, particularly tropical Africa and other in Asia. After comprehensive and critical examination of a large number of specimens, eight species of the genus *Leucas* have been identified from Bangladesh. Among them, *L. biflora* is a vulnerable species and has only been recorded from Chattogram region (Khanam and Hassan 2005)

It is a slender procumbent herb with nodal roots, produce branches from the woody root-stock, stem square, much pubescent with deflexed hairs at the ribs, leaves 0.7-3×0.5-15 cm, ovate, flowers whorl, 1-4 flowered, axillary, white in colour, bilabiate (Khanam 2009, Majumdar and Dutta 2011). Leaves and stem extracts of this plant contained alkaloids, carbohydrates, saponins, phenols, tannins, proteins, according to preliminary

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phytochemical screening and GC-MS results indicated that it contains a variety of chemical ingredients, which may be responsible for the pharmacological activity (Dhivya et al. 2022). The Tripuri community is reported to use this plant to treat eye conjunctivitis, nosebleeds and white discharge (Majumdar and Dutta 2011).

In vitro propagation opens up new opportunities for mass scale propagation of various important plants including many threatened plant species. Natural propagation of plants sometime time consuming and depends on various natural factors. *In vitro* propagation is independent of natural calamities and can grow all the year round. Various plant species including many medicinal plants have been successfully propagated by *in vitro* methods (Rout and Das 2000, Babich et al. 2020).

Traditional methods of cultivation are insufficient and sometimes are not fast enough to fulfil the demand. Micropropagation ensures an adequate, consistent supply of plant source while using the least amount of space and time (Prakash and Van Staden 2007). So far, there is no detailed report on micropropagation of *L. biflora,*. In this article *in vitro* propagation of *L. biflora* has been described in detail.

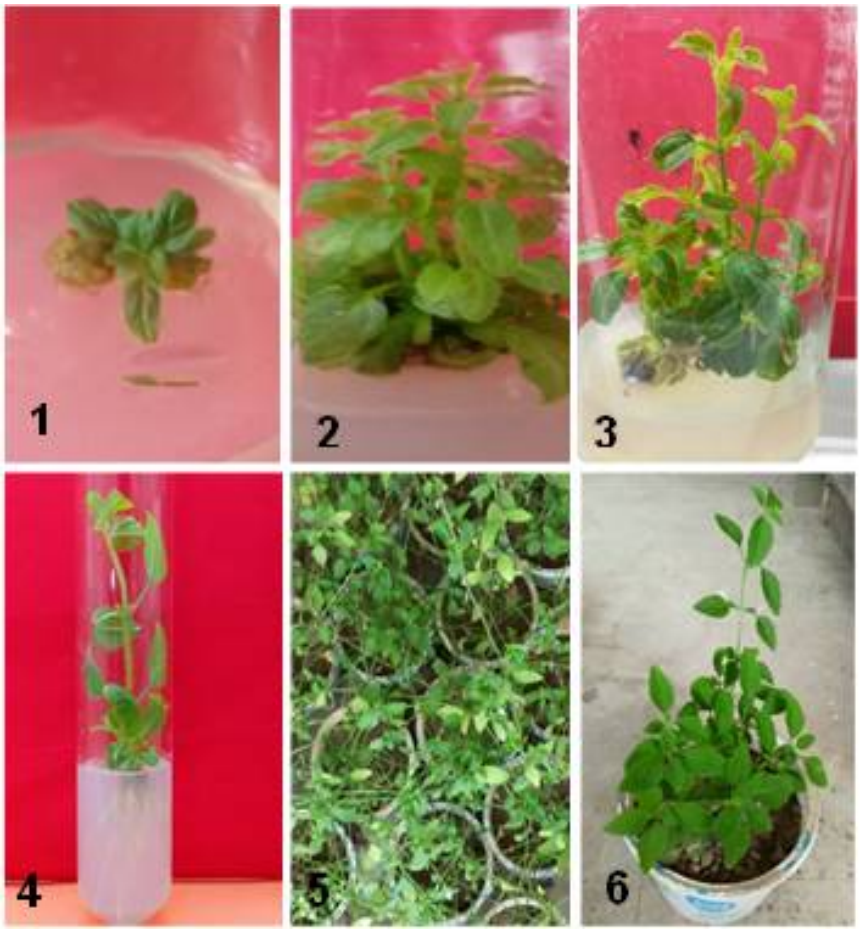
Materials and Methods

Field-grown plants were selected, and their nodal segments used as explants were carefully cleaned with flowing water. After cutting into small pieces nodal segments were washed with 1% Savlon and liquid soap for 10 to 15 minutes with continuous shaking. Afterwards, the explants were transferred to a 500-ml sterilized conical flask and rinsed three or four times with distilled water to remove all detergent. Materials were rinsed with 70% ethanol in less than 60 seconds and surface sterilized by submerging the nodal segments in 0.1% of $HgCl_2$ for 45 seconds. To remove every trace of the sterilant, the materials were then washed 4-5 times with double distilled water. Explants were aseptically cultured on a 0.8% (w/v) agar solidified MS medium that was 3% (w/v) sucrose-fortified and contained different concentrations and combinations of PGR_S. There were noticeable differences in the tested conditions regarding induction frequency, development of multiple shoot buds (MSBs) and seedlings development. For the MS medium, pH was adjusted to 5.8 using 0.1N NaOH or HCl. Agar was dissolved in the mixture by boiling it in a water bath. Then, roughly 50 ml of medium was distributed into each 100 ml culture vessel, and the containers were autoclaved (Hisense, South Korea) at 121°C for 30 min at 15 psi pressure. The cultures received 14 hours of 3500 lux illumination while kept at a constant temperature of 25 ± 2°C. The cultures were monitored at periodic intervals (7 days), and the responses were recorded. After 2 weeks, subculturing was continued in fresh medium. Through a series of acclimation phases, healthy seedlings with roots were gradually hardened. To remove the agar, the hardened seedlings were completely rinsed in sterile distilled water, and then the seedlings were moved to small pots containing moistened cocopeat and vermicompost pot in 1:1 ratio.

Statistical analysis was done using the statistical software OPSTAT (14.139.232.166/opstat). The difference among the treatments was tested applying one-way ANOVA. The level of significance was accepted at $p \leq 0.05$.

Results and Discussion

The medium containing 2.0 mg/l BAP + 0.5 mg/l NAA had the highest percentage (95.99%) of MSBs induction and it also produced the highest number (11.20 ± 0.49) of MSBs per explant after three subcultures at 14 days interval (Figs 1-2, Table 1). Similar positive responses to BAP combined with NAA on the induction of MSBs have also been reported in other medicinal plant species (Biswas et al. 2009, 2011, Gopinath et al. 2014, Jamal et al. 2016).



Figs. 1-6. Micropropagation of *L. biflora* through direct organogenesis. 1. Initiation of MSBs in nodal segments after 7 days of culture. 2-3. Proliferation and elongation of MSBs on MS medium containing MS + 1.5 mg/l BAP + 0.5 mg/l NAA; 4. Rooting if individual shoot on ½ MS + 0.5 mg/l IAA + 0.5 mg/l IBA. 5. *In vitro* grown plants acclimatized in pots. 6. *In vitro* developed complete plant in natural habitat.

The multiple shoots were proliferated and elongated in the same medium along with the subcultures. After 3 subcultures at 14 days interval the microshoots attained at an average length of 4.70 ± 0.37 cm (Fig. 3, Table 1). The BAP + NAA combination was better in terms of promoting elongation according to overall observation employing several combinations of growth regulators at varied concentrations. The elongation of *in vitro* produced shoot buds was similarly enhanced in *Rauvolfia serpentina* (Roy et al. 1995).

Table 1. Effects of BAP and Kn individually and BAP in combination with NAA on induction and proliferation of MSBs in nodal segment of *Leucas biflora*.

PGRs supplement in the mediun			Percentage* of explants gave response $\bar{x}$ ± SE	Average* number of MSBs per explant $\bar{x}$ ± SE	Average* length of MSBs $\bar{x}$ ± SE
BAP	Kn	BAP + NAA	% response	Number of root	Length of root
0.5			70.66 ± 2.66^{de}	2.87 ± 0.33^{def}	1.6 ± 0.24^{fgh}
1.0			76 ± 3.40^{cd}	3.4 ± 0.24^{def}	2.5 ± 0.38^{cdef}
1.5			84 ± 4.52^{bc}	4.4 ± 0.24^{cde}	2.8 ± 0.33^{cd}
2.0			58.66 ± 4.89^{f}	2.4 ± 0.51^{ef}	2 ± 0.15^{defgh}
2.5			53.33 ± 2.10^{fg}	1.8 ± 0.37^{f}	1.8 ± 0.33^{efgh}
	0.5		56 ± 2.66^{fg}	2.6 ± 0.40^{ef}	1.5 ± 0.22^{gh}
	1.0		60 ± 5.96^{ef}	2.4 ± 0.51^{ef}	1.4 ± 0.18^{gh}
	1.5		62.66 ± 4.98^{ef}	2.6 ± 0.40^{ef}	2.3 ± 0.20^{cdefg}
	2.0		53.33 ± 2.10^{fg}	2.2 ± 0.49^{ef}	1.6 ± 0.18^{fgh}
	2.5		45.33 ± 3.88^{g}	1.6 ± 0.24^{f}	1.2 ± 0.12^{h}
		1.5 + 0.1	85.33 ± 2.49^{abc}	4.2 ± 0.37^{de}	3.07 ± 0.24^{bc}
		1.5 + 0.5	95.99 ± 1.63^{a}	11.2 ± 0.49^{a}	4.7 ± 0.37^{a}
		2.0 + 0.1	87.99 ± 3.26^{ab}	7 ± 1.70^{b}	3.8 ± 0.37^{b}
		2.0 + 0.5	79.99 ± 5.16^{bcd}	6.4 ± 1.25^{bc}	2.8 ± 0.33^{cd}
		2.5 + 0.1	85.33 ± 2.49^{abc}	5 ± 0.89^{bcd}	2.6 ± 0.40^{cde}

*Values represent Mean ± SE of each experiment consist of five replicates with 15 explants. All test values with different superscripts in the same column are significantly different ($p \leq 0.05$).

The highest rooting percentage (96%) was noted in half-strength MS medium supplemented with 0.5 mg/l IAA + 0.5 mg/l IBA within 21 days of inoculation (Fig. 4, Table 2). After 30 days of culture, the average length of roots in this medium was 4.80 ± 0.30 cm and the average number of roots per micro shoot was 5.20 ± 0.33. The response to the root development was significantly influenced by the kind and concentration of

auxins in the media. It was observed that IBA + IAA as a good root inducer which is supported by the early reports (Majumder and Rahman, 2016, Hassan et al. 2010, Abul et al. 1998).

Table 2. Effects of different concentrations and combinations of plant growth regulators (PGRs) on the development of roots in *Leucas biflora*.

½ MS+IAA	½ MS+IBA	½ MS+IBA+IAA	% response	Number of root	Length of root
MS0 Without PGRs			72 ± 5.73^{c}	2.60 ± 0.40^{d}	2.56 ± 0.22^{e}
0.5			86.66 ± 2.10^{ab}	4.10 ± 0.24^{bc}	3.52 ± 0.43^{bcde}
1.0			88 ± 3.88^{ab}	3.98 ± 0.34^{bc}	3.6 ± 0.55^{bcde}
1.5			83.99 ± 4.00^{abc}	3.9 ± 0.51^{bc}	3.4 ± 0.29^{cde}
	0.5		82 ± 3.59^{bc}	3.66 ± 0.20^{bcd}	4.2 ± 0.25^{abc}
	1.0		90.66 ± 1.63^{ab}	4.7 ± 0.20^{ab}	4.56 ± 0.27^{ab}
	1.5		78.66 ± 2.49^{bc}	3.26 ± 0.31^{cd}	3.86 ± 0.18^{abcd}
		0.5 + 0.5	96 ± 1.63^{a}	5.20 ± 0.33^{a}	4.80 ± 0.30^{a}
		0.5 + 1.0	88 ± 3.88^{ab}	4.20 ± 0.37^{abc}	3.30 ± 0.43^{cde}
		1.0 + 1.0	85.33 ± 3.26^{ab}	3.40 ± 0.24^{cd}	3.00 ± 0.54^{de}

*Values represent Mean ± SE of each experiment consist of five replicates with 15 explants. All test values with different superscripts in the same column are significantly different ($p \leq 0.05$).

Complete plantlets were removed from the culture vessels and hardened in the lab condition and transfer to the seedling tray. After acclimatization plants were transfer to the pots with 98% survival rate (Figs. 5-6).

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