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Mass Production of *Plectranthus amboinicus:* A valuable medicinal and aromatic plant with a future value

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Abstract
Plectranthus ambonicus (Loureiro) Sprengel is a medicinal and an aromatic herb in family Lamiaceae. It is known to contain strong aromatic chemicals as other members of the Plectranthus genus. This herb is highly sought by various industries like pharmaceutical industry, perfumery industry and flavoring industry, for its therapeutical value. This has led to its uncontrolled harvest from the wild leading to the risk of extinction. As an alternative, commercial propagation of *P. amboinicus* is desirable to avoid further depletion of wild populations. This paper describes the establishment of an efficient protocol for the multiplication, rooting and acclimatization of *P. amboinicus*. Shoot tips and nodal segments were selected as explants, collected from well-maintained shade house grown one-month old mother plants. Murashige and Skoog (MS) medium was used throughout the experiment. For shoot induction and proliferation, MS medium supplemented with combination of 1-Naphthalene acetic acid (NAA) and different levels of N6-benzylaminopurine (BAP) were used. *In vitro* rooting was achieved to 50% strength MS basal medium containing different levels of Indole Acetic Acid (IAA) and NAA. Such *in vitro* produced plants were acclimatized and survival percentages were obtained. The explants from nodal segments gave better results in shoot initiation compared to those from the shoot tips on all the media combinations tested. The highest rate of shoot induction (92.6%) and highest number of shoots per explant (8.7) were obtained in MS medium supplemented with 2.0 mg/l BAP from nodal explants and was significantly different at 5% significant level. The highest rate of shoot proliferation (75%) and number of shoots per explant (7.8) was obtained on media supplemented with 2.0 mg/L BAP + 0.1 mg/L NAA after six weeks of nodal culture and shoot tip cultures supplemented with 2.0 mg/L BAP + 0.1 mg/L NAA resulted highest number of shoots per explant (5.5) with 68% shoot proliferation, after 6th week of culture initiation. The shoot proliferation was more effective in nodal segments than from shoot tip cultures resulting from observations and analysis. Therefore 6th week is the best period to get optimum number of shoots from nodal segments. It was observed that with 0.5mg/L IAA with 0 mg/L NAA resulted highest number of roots/explant and the longest roots were recorded with the control treatment. Therefore, it is possible to deduce that the current protocol is promising for *in vitro* mass propagation of *Plectranthus ambonicus*, a valuable medicinal plant with promising future in aromatic and pharmaceutical industry.

Keywords: *Aromatic, In vitro, medicinal plant, micropropagation,* Plectranthus ambonicus*, pharmaceutical industry*

1. Introduction

Plectranthus amboinicus, widely known by Indian borage belongs to the family Lamiaceae. It is a perennial fleshy plant with a distinctive aromatic character. This herb is widely distributed, naturalized or cultivated in the warm and tropical regions of Asia, Africa and Australia. *P. amboinicus* is a commercially valuable herb, primarily because of its essential oils (EO) which comprises of high amounts of carvacrol [1] or thymol [2]. Various bioactivities of EOs extracted from *P. amboinicus* have been reported previously, including antimicrobial [3], larvicidal [4], antioxidant [5], anti-feedant [6], anti-cancer [7] and anti-inflammatory activity which potentially used in the treatment of a wide range of diseases [8]. Moreover, *P. amboinicus* is a famous culinary herb reported to have a high nutritional value [9] and can be used as natural preservative [10]. The value of this herb has led to excessive collection of wild populations, driving this herb towards extinction. Therefore, an alternate propagation method and conservation strategy is necessary for the sustainable utilization of this herb. Many of the medicinal and aromatic plant species, vegetative propagation is the general practice for large-scale multiplication [11]. The problem with sexual propagation of *P. amboinicus* plants is that they rarely flower and set seeds and their propagation is generally achieved by stem cuttings [8]. Anyhow, this vegetative propagation method is slow and inadequate for commercial-scale propagation and is highly susceptible to the disease and pest incidences. Besides, when such propagated plants are to be used as a source of metabolites, a number of factors may affect and limit yield, including tissue maturity, geography of the cultivated region, environmental conditions, water accessibility and influence of diseases [12]. As a remedy and an alternative, micropropagation techniques can be adopted to propagate genetically and biochemically uniform planting materials on a large scale in a short period under controlled environment [13]. There are several studies on *in vitro* propagation of *P. amboinicus*. Some of them are; *in vitro* establishment of *P. amboinicus* ‘Variegatus’ from shoots and stem segments subsequent rooting and transplantation [14] and *in vitro* establishment of *P. amboinicus* from nodal cuttings subsequent rooting and transplantation [15].

The establishment of a tissue culture system for *P. amboinicus* will greatly facilitate the provision of uniform and microbial contamination free tissue to various industries and compliment conservation strategies [16]. With the accelerating demand for natural products worldwide, the demand for this valuable herb is sky rocketing proving that harvest from the wild is not sufficient and will lead to the extinction. Hence, this study was aimed to establish an efficient micropropagation system for *P. amboinicus* for commercial purpose. Further, this study will serve as a means of facilitating the propagation of *P. amboinicus* on a large-scale and serve as a support system for other future biotechnological advances like synthetic seed production and cryopreservation while being a valuable tool in conservation.

2. Materials and methods

2.1. Plant Materials

Mother plants were maintained in shade house conditions, Department of Crop Science, Faculty of Agriculture, University of Ruhuna, Mapalana, Kamburupitiya, Sri Lanka. Watering was done regularly and all other agronomic practices were practiced as required. Shoot tips and nodal segments were used as explants. Mother plants were sprayed the fungicide solution (Daconil 1 ml/L) prior to the collection of explants and after 1-hour explants were collected from freshly emerged sprouts. The collected explants were washed with liquid detergent (2-3 drops of Teepol). Then the explants were kept under running tap water for 1-hour. This was followed by dipping in a fungicide solution (Daconil-concentration 1ml/L) for 30 minutes. Then the explants were transferred to a laminar air flow chamber where they were surface sterilized with 10% Clorox® containing drops of Tween-20 for 15 minutes. After rinses with sterile distilled water for 3 times, the explants (shoot tips and nodal segments) were cut into 2 - 3 cm pieses.

2.2. Shoot induction and proliferation

Basal MS medium with 30 g/l sucrose and gelled with 0.8% (w/v) agar and the pH was adjusted to 5.8 and sterilized in an autoclave under 15 psi and 121℃. The medium was supplemented with combinations of BAP (0.5, 1.0, 1.5 and 2.0 mg/L) and NAA (0.1 mg/L) Explants were inoculated on to 40 mL of medium contained in 150 mL flasks. The cultures were incubated in a plant growth room at a temperature of 25˚C ± 1˚C with a 16/8 h photoperiod provided by cool-white fluorescent lamps (1000 - 2000 lux) and were checked regularly for contamination. Percentage of shoot induction, number of shoots per explant was obtained after 15 days and shoot proliferation rate was recorded at weekly intervals for ten weeks. Twenty replicated flasks were used in each treatment to compare the effects of the growth regulators in Complete Randomized Design (CRD) and data were analyzed using Minitab 17 statistical software using ANOVA and means were separated using Duncan's Multiple Range Test (DMRT).

2.3 *In vitro* rooting and acclimatization of Plantlets

Shoots (>2.0 cm long) from the best treatment were individually placed inside the flasks for root initiation. The explants were cultured on 50% MS basal medium with glucose (15g/L) and gelled with 0.8% (w/v) agar supplemented with IAA and NAA with different combination levels as 0.25 mg/L IAA and NAA, 0.5 mg/L IAA and NAA, 0.5 mg/L IAA with 0mg/L NAA and 0 mg/L IAA with 0.25, 0.5 & 1.0 mg/L NAA. Twenty replicated flasks were used in each treatment. The number of shoots that produced roots, as well as the number and length of the induced roots were recorded after 30 days. Complete plantlets produced *in vitro* were removed from the culture medium and the roots were washed to remove the agar. The plantlets were then transferred into pots containing top soil: compost: sand in 1:1:1/2 ratio and placed in the shade house under controlled conditions with 75% shading and temperature at 28˚C-32˚C. To maintain humidity, the plants were watered periodically twice a day. Observations were recorded on the percent survival of rooted and acclimatized plants.

3. Results & Discussion

3.1 Explants establishment and shoot initiation

A simple and effective protocol was developed for the *in vitro* micro propagation of *P. ambonicus*. Two different types of explants (shoot tips and nodal segments) were cultured on MS media containing different concentrations of BAP and NAA to evaluate their effects on shoot initiation. Explants showed varying success in shoot initiation depending on the type of explant and the growth regulators added. The response of explants cultured in MS media supplemented with BAP and NAA are shown in Table 1.

Table 1: Effect of plant growth regulators on shoot induction from different explants after 15 days of culture.

Explant segment	Growth regulator Concentration (mg/L)		Shoot induction (%)	Average number of shoots/explant
	BAP	NAA		
Shoot tips	0.5	0.1	65.3	3.8 ± 0.02[a]
	1.0	0.1	71.0	4.1 ± 0.01[a]
	1.5	0.1	75.6	4.2 ± 0.04[a]
	2.0	0.1	80.2	4.9 ± 0.06 [a]
Nodal segments	0.5	0.1	61.6	3.1 ± 0.04 [c]
	1.0	0.1	78.2	4.7± 0.07 [b]
	1.5	0.1	80.0	5.5± 0.02 [b]
	2.0	0.1	92.6	8.7± 0.02 [a]

The explants from nodal segments gave better results in shoot initiation compared to those from the shoot tips on all the media combinations tested. Generally, the increased level of BAP resulted in significantly higher shoot initiation and the number of shoots at the initiation stage, with constant level of NAA. Although bud break was dependent on BAP supply, the synergistic combination of BAP and NAA induced the optimum frequency of shoot formation as well as shoot number. The highest rate of shoot induction (92.6%) and highest number of shoots per explant (8.7) were obtained in MS medium supplemented with 2.0 mg/l BAP + 0.1 mg/l NAA which was significantly different from all other hormonal combinations and type of explant used at 5% significant level (Table 1). Similar results were reported [17] in their studies on the medicinal plant *Hybanthus enneaspermus*, that supplementation of MS medium with 2.0 mg/L BAP give better results than when the growth regulator was added in higher or lower concentrations. According to [18], BAP concentrations of up to 2.0 mg/L were effective in inducing shoots of *Grand naine*.

3.2. Shoot proliferation and *in vitro* rooting

Different concentrations of BAP or NAA added to MS medium in combination has affected shoot proliferation rate, the number of shoots produced and the average length of the shoots. The highest rate of shoot proliferation (75%) and number of shoots per explant (7.8) was obtained on media supplemented with 2.0 mg/L BAP + 0.1 mg/L NAA after six weeks of nodal culture (Fig. 1). Same way, shoot tip cultures supplemented with 2.0 mg/L BAP + 0.1 mg/L NAA resulted in highest number of shoots per explant (5.5) with 68% shoot proliferation, after 6th week of culture initiation.

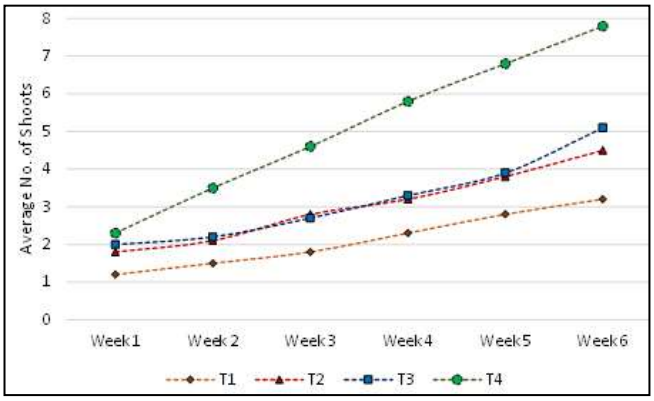

Fig 1: Plantlet proliferation rate of *Plectranthus ambonicus* from nodal segments

But, the shoot proliferation was more effective in nodal segments than from shoot tip cultures (Plate1- Below).

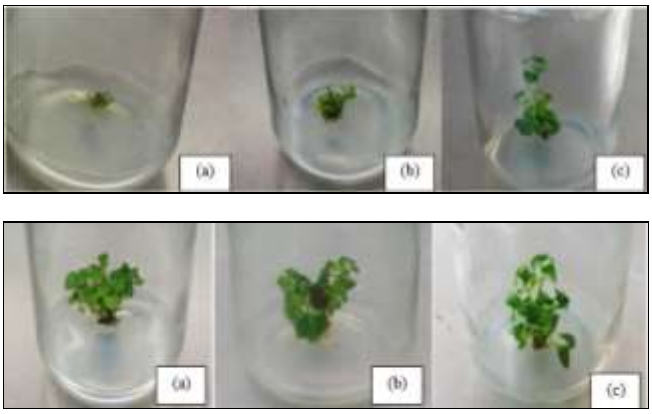

Plate 1: Effect of different treatments for shoot tip proliferation from shoot tip explants (Top) and nodal segments (Below); (a) BAP 0.5mg/L + NAA 0.5mg/L NAA, (b) BAP 1mg/L + NAA 0.5mg/L NAA, (c) BAP 1.5mg/L + NAA 0.5mg/L NAA, (d) BAP 2mg/L + NAA 0.5mg/L NAA.

Therefore 6th week is the best period to get optimum number of shoots from nodal segments. Furthermore, it was reported that the cotyledonary node explants of Yeheb *(Cordeauxia edulis*) cultured on MS medium supplemented with 2.0 mg/L BAP resulted in the highest rate of shoot initiation (89%) and the highest number of shoots per culture after nine weeks [19]. Consistent with this result [20], reported that 2.5 mg/L BAP + 0.15 mg/L NAA in MS medium produced the highest number of Aloe vera plantlets (up to 28.47 plantlets per explants). The maximum shoot regeneration and maximum number of regenerated shoots in Datura stramonium were obtained in the treatment containing 2 mg/L BAP + 1 mg/L NAA [21].

3.3 Rooting and Acclimatization

Roots were produced in all media. However, supplementation with IBA and NAA affected rooting in *P. ambonicus* shoots differently (Table 2). Visual observations during the study revealed that roots of micro-shoots grown on medium containing high NAA levels were morphologically different from roots of other shoots supplemented with high IBA with or without NAA. These roots appeared thicker and short. Among the tested treatment combinations 0.5mg/L IAA with 0.5mg/L NAA and 0.5mg/L IAA with 0 mg/L NAA showed no significant difference resulting the highest number of roots per explant (P>0.05).

Table 2: Effect of different concentrations of IAA and NAA on the rate of explant rooting, number of roots per plant and average root length.

IAA	NAA	No. of Roots/Explant	Average Root Length (cm)
0	0	5.8± 0.39^{e}	2.6 ± 0.9^{a}
0.25	0.25	10.5 ± 0.91^{b}	1.5 ± 0.4^{b}
0.5	0.5	11.9 ± 0.72^{a}	1.5 ± 0.3^{c}
0.5	0	12.2 ± 1.09^{a}	1.9 ± 0.1^{c}
0	0.5	9.6 ± 0.34^{c}	1.0 ± 0.4^{d}
0	0.25	6.7 ± 0.51^{d}	0.3 ± 0.04^{e}
0	1	5.5 ± 0.44^{e}	0.2 ± 0.03^{f}

Means with same letters are not significantly different at P>0.05 level

But, with 0.5 mg/L NAA, callus production was high and it is not a desirable observation during rooting stage. Therefore, 0.5mg/L IAA without NAA is the suitable hormone level for rooting of *P. ambonicus*. According to the findings of [22] the percentage of rooted explants (100%) and the number of roots (10 - 12.5 roots/explant) produced were highest in media containing IAA 1.0mg/L with 0.5mg/L NAA. The lower response of NAA in promoting root formation of *in vitro* grown banana explants was also reported by Arun *et al*., 2012. They found that the culture medium containing 1.0 mg/L NAA was less efficient than IAA in the promotion of rooting, with the former inducing only 1.64 roots/explant. As in the present study, it was recorded that maximum root length (3.1 cm) was attained on growth regulator free MS medium [22, 23].

The acclimatization of rooted plants in ex vitro conditions was carried out with the plants bearing well developed roots transferred to small pots containing potting mixtures (top soil: compost: sand in 1:1:1/2). They were maintained at about 70% relative humidity in the greenhouse with 75% shading. A survival rate 100% was achieved after 6 weeks.

4. Conclusions

This study provides an efficient *in vitro* propagation method for *Plectranthus ambonicus* using a simple and efficient protocol for producing true to type plants in a relatively short period and with high multiplication rate using nodal tips and shoot tips. Hence, this protocol, culturing of nodal segments in MS medium supplemented with 2.0 mg/l BAP + 0.1 mg/l NAA for shoot induction and proliferation and for rooting ½ MS supplemented with 0.5 mg/l IAA is found to be optimal for *in vitro* mass production of the plant *P. ambonicus*. To solve the commercialization and conservation bottle necks of this economically valuable aromatic and medicinal plant.

5. References

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