

ASSESSING THE INFLUENCE OF BASAL MEDIUM AND HORMONAL SUPPLEMENTS ON *IN VITRO* MICROPROPAGATION OF *PLECTRANTHUS AMBOINICUS*

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Scientific J. Flowers & Ornamental Plants,
10(1):63-70 (2023).

Received:
2/2/2023

Accepted:
17/2/2023

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ABSTRACT: *Plectranthus amboinicus* plant is known for its traditional and modern pharmacological uses as well as its ornamental value. The high demand for plant harvest necessitates the existence of several alternative commercial propagation protocols. Therefore, the present paper explores the impact of various combinations of basal nutrient media and hormonal supplements on micropropagation for *P. amboinicus*. The results confirmed that White's medium at full strength was superior to MS and B₅ media with respect to the establishment and proliferation of shoot tip explants. Besides, White's medium amended with BA at 0.50 mg/l gave a higher shoot number, while hormone-free White's medium induced the best plant vigor, leaves number and shoot length. *In vitro* rooting of produced shootlets under a low concentration of IBA (0.250 mg/l) achieved the best survival and rooting percentages (90 and 100%, respectively) as well as length and number of roots (6.40 cm and 9.83 roots/shoot, respectively). The *in vitro*-raised plantlets were successfully acclimatized with the best survival percentage when cultured on a mixture of peat moss and sand.

Keywords: *Plectranthus amboinicus*, *in vitro*, White's medium, MS medium, B₅ medium, BA, IBA, acclimatization.

INTRODUCTION

Plectranthus amboinicus plant native to southern and eastern Africa and belongs to the family Lamiaceae. The genus *Plectranthus* is a large genus including around three hundred species dispersed in Australia, Tropical Africa and Asia (Paton *et al.*, 2004). Family Lamiaceae includes several other important genera, such as *Mentha*, *Salvia* and *Ocimum*, with a wealthy ethnic botanical use. Likewise, a number of species including *P. esculentus*, *P. caninus*, *P. barbatus*, *P. amboinicus*, have been recorded to have anticancer, antitumor and cytotoxic activity (Lukhoba *et al.*, 2006). The leaves of *P. amboinicus* are also used to treat indigestion, flatulence, cholera, especially in children, epilepsy, chronic asthma, hiccups, bronchitis, kidney and

bladder stones, liver disease and Malaria fever (Hertzberg and Buaer, 2000). *Plectranthus amboinicus* is a therapeutically valued herb highly sought by various industries. 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 (Arumugam *et al.*, 2020). The value of this herb has led to an indiscriminate collection of wild populations, driving this herb towards extinction. Therefore, an alternate propagation method and conservation strategy are necessary for the sustainable utilization of this herb. In some of the medicinal and aromatic plant species, vegetative propagation is the general practice

for large-scale multiplication (Swamy *et al.*, 2010). *P. amboinicus* plants rarely flower and set seeds, and hence, the preferred method of its propagation is generally achieved by stem cuttings (Arumugam *et al.*, 2016). This exemplifies the urgent need for an alternate conservation method for *P. amboinicus* wherefore, micropropagation can be a desirable alternative to efficiently produce beneficial secondary metabolites (Ruffoni *et al.*, 2010) greatly facilitating industries in providing uniformed and contamination free cultures and biochemical compounds (Arumugam, 2018 and Hole *et al.*, 2009). Most literature on *P. amboinicus* primarily focuses on its medicinal value, however, there are several investigations on the *in vitro* propagation reported in the past decade. Rahman *et al.* (2015) described the *in vitro* establishment of *P. amboinicus* 'Variegatus' from shoots and stem segments, subsequent rooting and transplantation. Previous experiments were done to propagate *P. amboinicus* by tissue culture with great differences in terms of using media, hormones and different explants such as Rahman *et al.* (2015), EL-Zefzafy *et al.* (2016) and Arumugam *et al.* (2020). It was found that *P. amboinicus* readily responded to tissue culture, but careful attention to media formulation and type of explants was necessary to avoid abnormal plant development and thus influence the composition of essential oils extracted from field-grown plants and *in vitro* shoots. Plant growth regulators, including auxins and cytokinins, have traditionally been considered an essential factor influencing differentiation, metabolic formation and cell growth in plant tissue culture (Liang *et al.*, 1991). Plant responses involve increased protein metabolism and nucleic acid. Nevertheless, no conclusive directory exists on these outgrowth-furthering phytohormones' directness on plants' protein synthesis system (Datta, 1994). MS (Murashige and Skoog, 1962), B₅ (Gamborge *et al.*, 1968), White's medium (White, 1963) and derivatives of these media have wide application to different plant species and different cultural targets

(Kumar, 1999). EL-Zefzafy *et al.* (2016) found that peat moss and sand interaction as a growing media recorded the best result in the acclimatization of rooted plants and Arumugam *et al.* (2020) reported that acclimatization was achieved by transferring the rooted plants onto sterile peat moss moistened with MS medium and exposed to the glasshouse environment gradually over a period of 2 months for *P. amboinicus*. Vegetative media containers for transplanting rooting plants are essential for perfect healthy plants; inhibitors or radical pH changes at the media impair root growth and, therefore, transplant success (Jones, 1982; McCown, 1986). The majority of labs and plantations are cultivated to consistent media which is sufficiently supportive for the plant, has the proper pH, multiply, pored enough to permit proper drain and jagged (Miller, 1983). Sand is a fine material because of its physical characteristics, the coarse sand particles or being medium provide optimal beneficial modifications at media texture. Surely, the low price of sand among whole inorganic adjustments makes it a worthy modifier for propagation media and potting mixture (Wilson and Stoffella 2006). Many studies showed that peat provided high macropore volumes (45–50%) and also greater water volumes (40–45%) at low tensions (<1 kPa) (Ercisli *et al.*, 2005 and Sahin *et al.*, 2004). Moreover, the mixed mediums containing peat have more water holding capacity in the root zone and create a more aerated environment. Peat moss was selected because it is inexpensive and easy to use (Rasmussen *et al.*, 2002).

Therefore, the present study explores the impact of various combinations of basal nutrient media and hormonal supplements on the micropropagation of *P. amboinicus*.#

MATERIALS AND METHODS

The current work has been executed in the Tissue Culture Lab and Farm of the Egyptian Drug Authority (EDA) (National Organization for Drug Control and Research, formerly NODCAR), Kafr El-Gabal, Pyramid, Giza, Egypt.

Shoot tip explants (2 cm long) were collected from healthy *P. amboinicus* plants and kept in a solution of equal parts of PVP (polyvinylpyrrolidone), ascorbic acid and citric acid under continuous shaking for two hours. After cleaning the explants with a detergent for 10 min, they were rinsed under running tap water for a half-hour to ensure the removal of dust and surface contaminants. The explants were then transferred to the laminar flow cabinet for surface sterilization with 25% commercial bleach (0.625% sodium hypochlorite) for 5 min, and then washed three times with sterile distilled water. The explants were then cultured on different growing media tested. All media contained sucrose (40 g/l) and the macro- and micro-nutrients of the corresponding basal medium. The pH of the media was adjusted to 5.7 ± 0.1 before the addition of the agar (7 g/l). The media were dispensed into culture jars (40 ml/jar) and sterilized by autoclaving at 121-125 °C for 20 min at 15 lbs. Cultures were incubated for eight weeks at 25 ± 2 °C and cool white fluorescent lamps light of 2000 lux for 16 h.

The establishment and proliferation of shoot tip explants were examined on three different basal media; B₅, MS and White's medium at full strength. The experiment was arranged in a completely randomized design (CRD) with three replicates, four jars for every replicate, and every jar contained one explant. Data were collected after 8 weeks on survival %, number of shoots/explant, shoot length (cm), number of leaves/explant, rooting %, number of roots/explant and root length (cm). In addition, shoot vigor was estimated according to the method described by Bottino (1981); as a score from 1 to 5, where passively grow score = 1, below average grow = 2, average grow = 3, above average grow=4, and superior grow=5.

Another experiment was set up to test the effect of benzyladenine (BA) and kinetin (Kin), on the growth of shootlets. The explants (nodal segments of 1-1.25 cm in length with two axillary buds) from the in vitro-raised shootlets were subcultured on

White basal medium supplemented with different concentrations of BA or Kin (0.125, 0.250, 0.50, 1.0 and 2.0 mg/l). The basal medium lacking any hormonal treatment served as a control. Thus, the experiment consisted of eleven treatments arranged in CRD with three replicates, four jars per replicate and two explants in each jar. After 8 weeks of incubation, data were collected on number of shoots/explant, shoot length (cm), number of leaves/explant and plant vigor as previously mentioned (Pattin, 1981).

The third experiment was devoted to studying the effect of IBA at different concentrations (0.0, 0.125, 0.250, 0.50, 1.0 and 2.0 mg/l) on the rooting of in vitro-raised shootlets. The experimental design of this experiment was also CRD with three replicates and four jars per treatment with three shootlets per jar. Data were collected after 8 weeks on survival %, rooting %, number of roots/explant and root length (cm).

Finally, the acclimatization of the in vitro-rooted shoots was examined on mixtures of peat moss and sand at different ratios (1:0, 0:1, 1:1, 1:2, 1:4, 1:8, 2:1, 4:1 and 8:1 v:v). Before culturing, the rooted shoots were washed with distilled water to remove the agar medium and then soaked in a fungicide (Benlate at 2.0 g/l) for 5 min. Afterwards, the plantlets were cultured in plastic pots (14 cm diameter) containing the corresponding potting mixture. The nine tested treatments were arranged in CRD with three replicates, where four pots with one plant each were assigned for each treatment. Data were collected on survival %, shoot length (cm), root length (cm) and number of roots/plant.

Statistical analysis:

All experiments were arranged in a completely randomized design (CRD) with three replicates. The data were subjected to the statistical analysis of variance procedures and all the means were compared using the LSD at 5% level of significance, according to Gomez and Gomez (1984).

RESULTS AND DISCUSSION

Influence of basal medium on the establishment stage:

The impact of different basal media (MS, White's and B₅ media) on the establishment stage of the explants is shown in Table (1). The highest rates of survival (94%), rooting (86%) and shoot vigor (4.82) were recorded when shoot tips were cultured on White's medium. The explants cultured on B₅ medium recorded inferior results (91%, 67% and 4.10, respectively), while those grown on MS medium attained the lowest results (75%, 58% and 2.01, respectively). In addition, the explants showed the best response regarding shoots number/explant, leaves number/explant and roots number/explant when cultured on White's medium (3.40, 5.70 and 3.81, respectively), while on MS medium they showed the lowest results (1.20, 2.40 and 1.60, respectively). At the same time, the uppermost significant values for shoot and root lengths (4.14 and 3.30 cm, respectively) were observed on White's medium, while the lowest values were recorded on MS medium (1.34 and 1.11 cm, respectively). In this regard, White's medium was shown to promote growth compared with MS or B₅ basal media, possibly due to the high content of magnesium sulfate of White's medium. Magnesium is a core element in the chlorophyll molecule and acts as an essential enzyme activator, which could explain the better vegetative growth rate as a result of accelerating photosynthesis rate (Kumar, 1999).

Impact of BA and Kin on growth and proliferation of explants:

The effects of BA and Kin on the growth of nodal segment explants of *P. amboinicus* are shown in Table (2). In general, BA was more potent than Kin in encouraging shoot proliferation. Fortifying White's medium with 0.50 mg/l BA led to a significant increase in number of shoots/explant (4.04), but higher BA concentrations resulted in a significant reduction in the number of shoots/explant. Meanwhile, hormone-free

White's medium showed the highest values of shoot length (2.10 cm), leaves number/explant (9.61) and plant vigor (3.10). These values significantly declined with the increase in the concentration of either BA or Kin recording the lowest shoot length (3.60 cm), and leaves number/explant (2.60) and plant vigor (2.00) when BA at 2.0 mg/l was applied. The gradual decrease of the leaves number and shoot length with the increase in the concentration of BA and Kin agrees with the findings of EL-Zefzafy *et al.* (2016), who found that BA at 1.0 mg/l increased shoots number/explant of *P. amboinicus*. In the same context, shoots number of all explants cultured on the media supplemented with Kin was lower than that on BA.

Effect of indole-3-butyric acid (IBA) on rooting of shootlets:

Effects of different concentrations of IBA on rooting performance of *P. amboinicus* shootlets were estimated as survival %, rooting %, number of roots/explant and root length (cm). According to data presented in Table (3), IBA at a low concentration (0.250 mg/l) induced the highest survival and rooting rates (90% and 100%, respectively), number of roots (9.83) and root length (6.40 cm). Whereas White's medium without hormones (control) showed the lowest survival and rooting rates (70 and 75%, respectively), number of roots (8.40) and root length (4.30 cm). Increasing the concentration of IBA from 0.250 to 2.0 mg/l resulted in a gradual decrease in the recorded measurements. In this regard, Rahman *et al.* (2015) came up with that IBA at 1.0 mg/l is more efficient in different media tested for rooting of *P. amboinicus*. The root quality obtained was better than that obtained with NAA or IAA.

Effects of peat moss-sand potting mixtures on acclimatization of in vitro-raised plantlets:

The influence of potting soil mixtures between peat moss and sand on the acclimatization of in vitro-rooted plants is shown in Table (4). Survival % recorded the maximum response (100%) with peat moss:

Table 1. Influence of basal medium types on the establishment of *Plectranthus amboinicus* shoot tips.

Media type	Survival (%)	Shoots number/ explant	Shoot length (cm)	Leaves number/ explant	Shoot vigor	Rooting (%)	Root number/ explant	Root length (cm)
MS	75	1.20	1.34	2.40	2.01	58	1.60	1.11
White	94	3.40	4.14	5.70	4.82	86	3.81	3.30
B _s	91	2.30	2.21	4.30	4.10	67	2.53	2.10
LSD at 5%	-	1.021	0.830	1.310	0.710	-	0.832	0.811

Table 2. Impact of benzyladenine (BA) and kinetin (Kin) on the growth and proliferation of *Plectranthus amboinicus* explants.

	Cytokinins mg/l	Shoots number/ explant	Shoot length (cm)	Leaves number/ explant	Plant vigor
Control	0.0	2.00	2.15	9.61	3.10
	0.125	2.60	1.60	7.13	2.70
	0.250	2.65	1.40	6.00	2.22
BA	0.50	4.04	1.04	5.42	2.10
	1.0	1.40	1.00	4.40	2.02
	2.0	1.13	0.60	2.60	2.00
	0.125	1.44	2.10	8.74	3.03
	0.250	1.50	1.90	8.60	2.65
Kin	0.50	1.70	1.80	8.30	2.20
	1.0	2.11	1.21	5.40	2.70
	2.0	1.75	1.02	4.63	2.20
LSD at 5%		0.240	0.174	0.154	0.051

Table 3. Effect of indole-3-butyric acid (IBA) on rooting performance of *Plectranthus amboinicus* shootlets.

IBA mg/l	Survival (%)	Rooting (%)	Roots number/ explant	Root length (cm)
Control	70	75	8.40	4.30
0.125	84	98	8.90	4.60
0.250	90	100	9.83	6.40
0.50	82	96	9.53	5.80
1.0	80	93.33	9.30	5.41
2.0	74	92.33	9.10	5.00
LSD at 5%	-	-	0.150	0.251

Table 4. Effects of peat moss-sand potting mixtures on acclimatization of in vitro-raised plantlets of *Plectranthus amboinicus*.

Potting mixtures (v/v)		Survival (%)	Shoot length (cm)	Root length (cm)	Root number/ explant
Peat moss	Sand				
1	0	64	4.10	3.70	2.00
0	1	96	7.10	6.02	5.10
1	1	100	8.40	7.30	6.14
1	2	100	9.63	8.10	7.04
1	4	100	15.70	9.80	8.75
1	8	100	14.82	9.00	8.00
2	1	85	12.40	5.84	4.40
4	1	75	10.20	5.04	3.65
8	1	70	5.00	4.80	2.60
LSD at 5%		-	0.462	0.173	0.710

sand mixtures of 1:1, 1:2, 1:4 and 1:8 v/v. Meanwhile, the lowest survival rate (64%) was registered when peat moss alone was used. Furthermore, the highest results of shoot length, root length and number of roots (15.70, 9.80 cm and 8.75, respectively) were recorded with peat moss:sand mixture at 1:4 v/v, whereas the lowest values (4.10, 3.70 cm and 2.00, respectively) were recorded with the sole treatment of peat moss. Previously published data have shown that the addition of peat moss to sand are very important for *P. amboinicus* plant acclimatization. These data are consistent with those of Rasmussen *et al.* (2002), who showed that peat moss blended with sand reduces the likelihood of microbes decomposing organic matter to create anaerobic conditions and optimizes hydraulic conditions.

CONCLUSION

The current study provides valuable information about a refined micropropagation protocol for *P. amboinicus* as an important medicinal, aromatic and ornamental plant (Fig., 1). The best performance of the explants

during the establishment stage was observed on White's medium compared to MS and B5 basal media. White's medium containing 0.50 mg/l BA induced the best growth and proliferation of shoot explants. The microshoots showed superior rooting performance when cultured on White's medium supplemented with IBA at 0.250 mg/l. Sand and peat moss mixture at 1:4 v/v was the optimal mixture for the acclimatization of *P. amboinicus* in the greenhouse.

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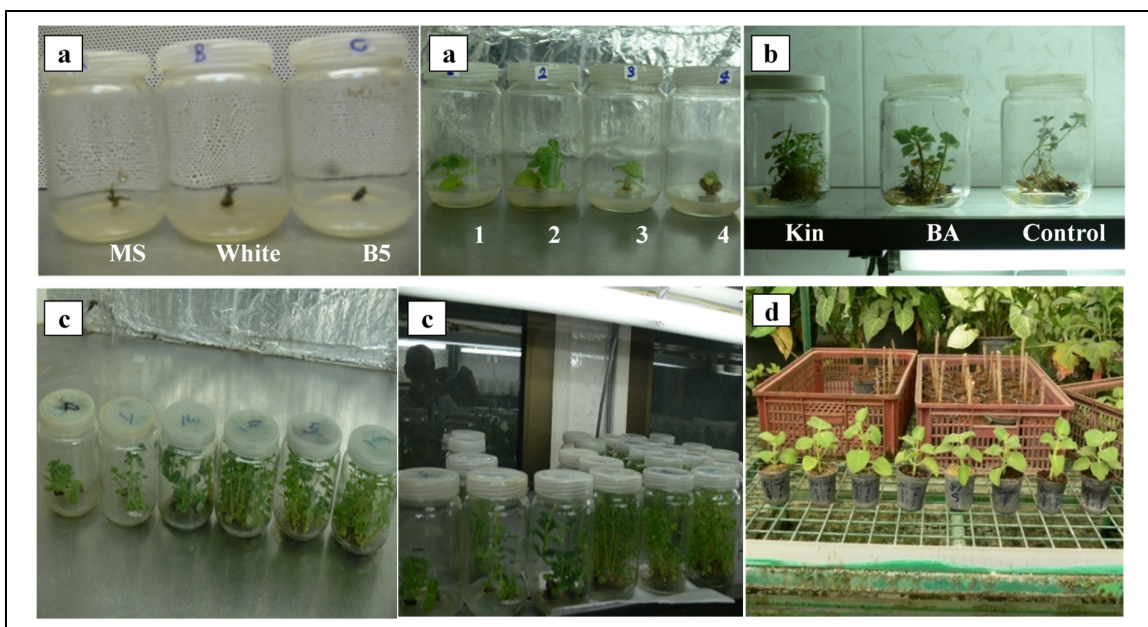


Fig. 1. Stages for the micropropagation protocol of *Plectranthus amboinicus* plants: (a) Establishment of explants on various basal media, (b) Multiplication of explants on White's medium with BA or Kin at different concentrations, (c) Rooting of micro-shoots on White's medium with IBA at various concentrations, (d) acclimatization of rooted shoots on mixtures of peat moss and sand at different ratios.

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تقييم تأثير البيئة الأساسية والإضافات الهرمونية على الإكثار الدقيق في المعمل للبلكترونسس امبينكيس

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يشتهر نبات البلكترونسس امبينكيس باستخداماته الدوائية التقليدية والحديثة بالإضافة إلى قيمته التزينية. يستلزم ارتفاع الطلب على النباتات وجود عدة بروتوكولات تكاثر تجارية بديلة. لذلك، يدرس هذا البحث تأثير التوليفات المختلفة للبيئات المغذية الأساسية والإضافات الهرمونية على التكاثر الدقيق للبلكترونسس امبينكيس. أكدت النتائج أن بيئة وايت كاملة القوة كانت متفوقة على بيئات (موراشيجي وسكوج) وجامبورج فيما يتعلق بزراعة وإكثار أجزاء النباتية القمة النامية النباتية. بالإضافة إلى أنه، أعطت بيئة وايت المدعمة بالبنزويل ادنين عند 0,50 مجم/لتر أعلى عدد من الأفرع، في حين أن بيئة وايت الخالية من الهرمونات أدت إلى أفضل قوة نباتية، عدد الأوراق وطول الفرع. حقق التجذير في المعمل للنباتات المنتجة تحت تركيز منخفض من إندول حامض البيوتريك 0,250 مجم/لتر أفضل نسب بقاء حية وتجزير (90 و 100٪ على التوالي) بالإضافة إلى طول وعدد الجذور (6,40 سم و 9,83 جذور/فرع على التوالي). نجحت النباتات التي تمت زراعتها في المعمل في تأقلمها مع أفضل نسبة بقاء حية عند زراعتها على خليط من البيتموس والرمل.