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IN VITRO PLANT PROPAGATION FOR RAPID MULTIPLICATION OF *MELICOPE LUNU-ANKENDA*: A PLANT SPECIES OF HIGH MEDICINAL VALUE

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ABSTRACT

In vitro micropropagation of *Melicope lunu-ankenda* was carried out by shoot regeneration from explants followed by rooting of the multiplied shoots. Two types of culture media, MS and WPM media, supplemented with the cytokinin 6-benzylaminopurine (BAP) (0, 0.5, 1.0, 3.0 and 5 mg/l) were used for shoot initiation. For shoot propagation, BAP was tested in combination with kinetin (KIN). Explants from lateral shoots responded better to shoot initiation as compared with those derived from shoot apices, when cultured on MS medium supplemented with 1 mg/L BAP. The combination of BAP (3.0 mg/l) and KIN (1 mg/L) was superior to all other hormonal treatments for shoot proliferation with a maximum of 25 shoots per explants being obtained. The auxins indole-3-butyric acid (IBA) and indole-3-acetic acid (IAA) were used in six concentrations (0.1, 0.2, 0.5, 1.0 and 2.0 mg/l) for root induction from the regenerated shoots. MS supplemented with IBA (0.5 mg/l) was the best medium to induce rooting from regenerated shoots. A plantlet survival rate of greater than 85% was achieved during acclimatization. The availability of this protocol is a key step towards large scale production of *Melicope lunu-ankenda* for the exploitation of its pharmaceutical potential in Malaysia.

KEY WORDS: Micropropagation, *Melicope lunu-ankenda*, 6-benzylaminopurine, kinetin, cytokinin, auxin

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INTRODUCTION

Historically, traditional medicines have played important roles in many cultures worldwide. Today, such native medicines and dietary supplements are also used widely. In Malaysia, herbs have been used in the treatment of many different ailments for more than 1,000 years. In traditional medicine, there is widespread belief that foods have medicinal properties that influence the internal metabolic balance essential for the maintenance of wellness. Among the herbs consumed for this purpose is *Melicope lunu-ankenda* (Gaertn.), a tree species belonging to the family Rutaceae. Locally known as "Tenggek burung", it is a popular *ulam* (salad) consumed raw in Malaysia. Leaves of *M. lunu-ankenda* are traditionally used to revitalize the body as well as to prevent hypertension. It is also used in Indian traditional medicine to relieve fevers, and as a tonic and for improving complexion. Previous phytochemical studies showed that isolates of this plant have bacteriostatic and fungicidal activity, besides being a natural antioxidant. *M. lunu-ankenda* has also been found to be an effective anti-inflammatory and immunomodulatory agent (Lal *et al*., 2005). The normal propagation through conventional method for this uncommon species is inefficient due to low propagation rate and its lengthy period to maturity. In addition, sufficient planting materials is often a problem for the plantation sector. Therefore, there is a need to develop methodologies for mass multiplication of this plant. For this reason, the tissue culture technique could be the best alternative to overcoming such problems. Vegetative propagation through micropropagation techniques is hence important in *M. lunu-ankenda* tree improvement and breeding programmes, as with several other tropical hardwood species where microporpagation has played important roles in the multiplication of planting materials (Pijut *et al*., 2012). Moreover, *in vitro* shoot multiplication would also result in the production of more uniform stocks with high genetic stability. This present study is aimed at developing an efficient mass micropropagation protocol for *M. lunu-ankenda* using shoots as explants, a methodology that has never been previously reported.

MATERIALS AND METHODS

Plant Material and Aseptic Culture

Melicope lunu-ankenda plants were grown in pots for 5 months (Figure 1a), and maintained in a glass house for two weeks prior to explant excision and establishment *in vitro*. Shoot apices and segments of lateral shoots were used to initiate shoot cultures. These explants were cut into pieces of about 2 - 3 cm before being kept in a conical flask. They were then washed in a detergent (Teepol) solution for 20 min before being rinsed in distilled water. Next, the explants were transferred to a laminar air flow chamber where they were sterilized with 20% Clorox® containing several drops of Tween-20 for 5 - 20 min, followed by 5% of Clorox® for another 20 min inoculation onto culture media.

Establishment and Micropropagation of Shoot Cultures

All sterilized explants derived from shoot apices and lateral shoots were cultured individually under aseptic conditions on basal MS or WPM medium containing 3.0% sucrose and 0.3% gelrite agar for gelling. The media were supplemented with five concentrations of the cytokinin 6-benzylaminopurine (0, 0.5, 1.0, 3.0 and 5.0 mg/L BAP) for shoots initiation. Medium sterilization was performed by autoclaving at 121°C for 20 min. The pH of the medium was adjusted to 5.7 - 5.8 before agar was added. Explants were inoculated into 150 ml flasks containing 40 mL of the desired medium. The cultures were placed in a plant growth room at a temperature of 25°C ± 1°C. A photoperiod provided by cool-white fluorescent lamps (1000 - 2000 lux) for 16 hrs. Observations were recorded at weekly intervals. Results were expressed as percentage of shoot initiation, number of shoots per explant, and shoot length after 30 days of culture. The effect of the cytokinins BAP and kinetin (KIN) on shoot micropropagation was examined in a separate experiment. Shoot initials derived from lateral shoot explants were cultured on solid MS media supplemented with different concentrations of BAP (0.5, 1.0, 3.0 or 5.0

mg/l), either alone or in combination with KIN (0.5, 1.0, 3.0 or 5.0 mg/l). Results were expressed as percentage of explants showing shoot regeneration, number of shoots per explant, and shoot length after 30 days of culture.

Rooting of Regenerated Shoots and Transplantation of Plantlets

Regenerated shoots (>3.0 cm long) were cultured on MS medium supplemented with either IBA (0.1, 0.2, 0.5, 1.0 and 2.0 mg/L), or IAA (0.1, 0.2, 0.5, 1.0 and 2.0 mg/L). The percentage of explants rooted, number of shoots producing roots, as well as the number and length of the induced roots were recorded after 30 days. *In vitro*-raised plantlets were removed from the culture medium and roots were washed under running tap water to remove the agar. Regenerated shoots with well-developed roots were transferred to plastic pots containing hardening medium and a top soil-compost mixture (2:1). The plantlets were maintained at about 70% relative humidity in the greenhouse where the plantlets were hardened for 60 days. Observations were recorded with respect to the survival rate of plantlets rooted during the period of acclimatization.

Statistical Analysis

All statistical analyses were performed using SPSS soft-ware. The experimental layout employed a completely randomized design, using 15 flasks (each containing two samples) for each treatment. The means and standard errors (indicated as ± values) were calculated for the measurements made.

RESULTS AND DISCUSSION

Effects of BAP on shoot induction

In cultures maintained on BAP-containing media, explants from lateral shoots were more successful in producing multiple shoots than those derived from shoot apices. The mean percentage shoot induction, number of shoots per explants, and mean shoot length in each tested medium are shown in Table 1. Other than WPM containing 5 mg/L BAP, all experimental media supported the induction of shoots, *viz.* 57-89% for MS, 56-76% for WPM and 56-70% in controls where cytokinins were not supplemented (Figure 1b). With the explants from lateral shoots, MS medium containing 1 mg/L BAP gave the highest in percentage of shoot induction (89%), followed by treatments containing 0.5 and 3 mg/L BAP (80%). The highest number of shoots obtained per explants (6 shoots) was recorded on MS medium containing 1 and 3 mg/L BAP, where the explants were sourced from the lateral shoots. The longest induced shoots from lateral shoot explants were obtained in the presence of 0.5 – 1.0 mg/ L BAP. The shoots ranged in length from 3.1 to 3.9 cm (MS medium) and 3.2-3.3 cm (WPM medium). Further increase in BAP concentration in the culture medium resulted in a decline in the shoot length. With explants from shoot apices, MS medium containing 1 mg/L BAP concentration induced the greatest mean shoot length of 3.7 cm, while the lowest length of shoots was obtained with 5 mg/L BAP in MS medium (1.9 cm).

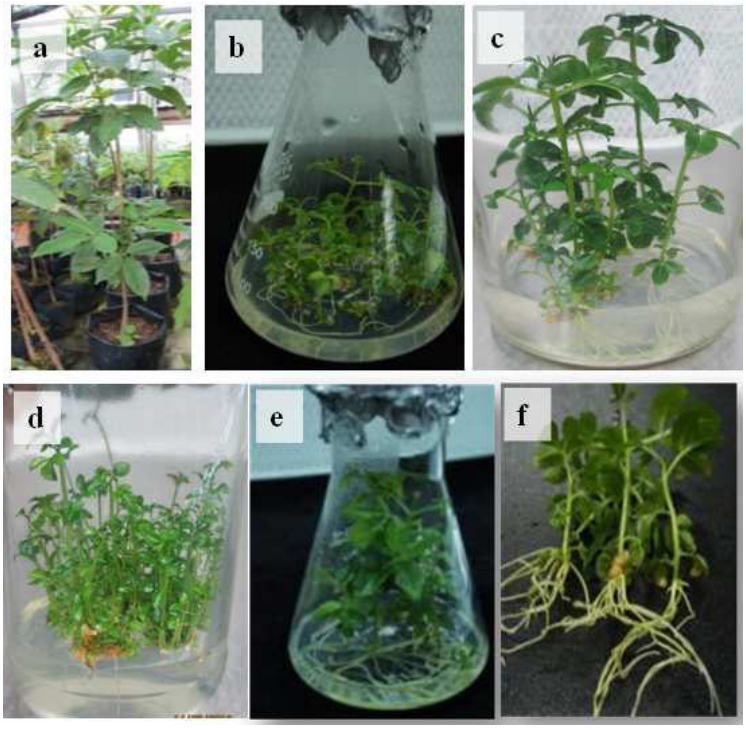

Figure 1: ***In vitro micropropagation of Melicope lunu-ankenda. (a) Mother plant, five month-old used as source of explants. (b) Shoot induction. (c) Development of shoots on control MS medium without cytokinins. (d) Development of shoots on MS medium containing BAP (3 mg/L)+KIN (1 mg/L). (e) Root formation on medium containing 1 mg/L IBA. (f) In vitro plantlets that ready for transferred into soil medium.***

We were successful in developing a protocol for the micropropagation of *M. lunu-ankenda* using two different culture media, MS and WPM. The former produced cultures with better vegetative growth characteristics, such as shoot induction rate and shoot length, as compared with cultures on WPM medium. Similar results were reported by Patil *et al.* (2011) in their studies on pomegranate. They observed 10 to 15 shoots per explant on MS medium, as compared to the six to eight shoots per explant on WPM medium. In contrast, Samir *et al*. (2009) found WPM superior to MS medium for vegetative growth. The addition of BAP to the culture medium promoted shoot initiation of *M. lunu-ankenda,* the optimal concentration being 1 mg/L BAP. This finding was agreement with Zuraida *et al.* (2013) who found that this dosage of BAP maximized the rate of *Vanilla* shoot induction and the length of shoots that developed. They also observed that media containing BAP at concentrations greater than 1 mg/L produced a smaller number of shoots as well as shorter shoot length. According to Tan *et al.* (2011) also, the highest number of shoots per explant was observed from medium supplemented with 1.0 mg/l BAP; the shoot induction rate decreased when BAP concentrations exceeded 3 mg/L. The same trend was observed by Mackay *et al.*(1995) in *Cercis canadensis.* George *et al.* (2008) noted that elevated high of cytokinin caused many small shoots to be produced, but such shoots typically failed to elongate. In contrast to observations in the present study, there have also been reports stating that the number of induced shoots paralleled increasing BAP concentrations. This was the case for *Sesbania drummondii* (Cheepala *et al.*, 2004), and also for the axillary shoots of *Quercus semecarpifolia* (Tamta *et al.*, 2008).

Table 1
Effect of different BAP concentrations on shoot initiation from explants derived from shoot apices and lateral shoots (after 30 days of culture)

Explant	*Culture medium*	*BAP conc. mg/L*	*Shoot induction (%)*	*No.of shoot/ Explant*	*Mean shoot length (cm)*
Shoot apices	MS	0	57	1±0.08	2.9±0.05
		0.5	67	1±0.10	3.1±0.12
		1	78	3±0.45	3.7±0.14
		3	77	3±0.65	2.1±0.31
		5	70	4±0.24	1.9±0.62
	WPM	0	56	1±0.05	2.1±0.02
		0.5	59	1±0.19	2.1±0.11
		1	60	2±0.11	2.3±0.04
		3	67	1±0.05	2.2±0.06
		5	0	0	-
Lateral shoots	MS	0	70	1±0.03	2.5±0.11
		0.5	80	2±0.09	3.1±0.06
		1	89	6±1.23	3.9±0.26
		3	80	6±1.14	2.4±0.21
		5	75	3±0.21	2.2±0.03
	WPM	0	70	2±0.05	2.1±0.11
		0.5	73	3±0.11	3.2±0.05
		1	68	2±0.05	3.3±0.10
		3	76	1±0.05	2.6±0.06
		5	56	1±0.45	2.1±0.12

Effects of BAP and KIN on shoot multiplication

The effects of different concentrations of BAP on shoot multiplication from explants derived from lateral shoots, in the presence or absence of KIN, were investigated. Table 2 shows the percentage of shoot regeneration from explants, number of shoots per explants, and mean shoot length obtained after 30 days of culture on the media tested (Figures 1c and 1d). For most of the tested media, BAP alone or combined with KIN showed up to 100% shoot regeneration. However, MS medium supplemented with BAP and KIN was more effective than BAP alone in the number of shoots induced. When BAP was applied on its own, the number of shoots per explant increased from 6 to17 in tandem with the increase in concentration of BAP in MS medium. Moreover, the combined effects of BAP and KIN significantly increased the number of shoots induced in M. *lunu-ankenda*. The highest number of shoots (25 shoots per explants) (Figure 1d) was produced by the combination of 3 mg/L BAP and 1 mg/L KIN. Shoot proliferation was reduced when KIN concentration in the culture medium was increased to 5.0 mg/L for all concentrations of BAP. The mean shoot length was 5.6 cm in the plant growth regulator-free medium, whereas the greatest length (5.7 cm) was achieved in MS medium containing 0.5 mg/L BAP without addition of KIN. A combination of BAP and KIN has been shown to be useful for *in vitro* shoot induction in various species (Shukla, 1994; Mackay *et al*., 1995; Pattnaik *et al*., 1996; Geeta, 2001; Dhillon, 2002; Hosseini and Parsa, 2007; Mohammad *et al.*, 2007). Rocha and Quoirin (2004) and Fotso *et al.* (2007) stated that they used kinetin in micropropagation of *Ricinodendron heudelotii* and *Swietenia macrophylla,* respectively. Amir Ali *et al.* (2008) reported that 0.5 mg/l BAP with 0.25 mg/l kinetin promoted good shoot formation in sugar cane. Nevertheless, the use of cytokinins, especially, KIN, has been less effective in shoot induction in certain plant species when used in excessive concentrations. For example, Sun *et al.* (2010) found that varying concentrations of kinetin did not stimulate shoot proliferations in *Ilex glabra*. Ahmad (1989), Arinaitwe *et al.* (2000) and Madhulatha *et al*. (2004) also found high concentrations of cytokinins disadvantageous in this respect. The results in the present study showed that shoot regeneration and multiplication in MS medium were not strongly dependent on BAP/KIN concentration; shoots were induced even in the absence of cytokinin supplementation (Table 1). Nevertheless, as noted above, the inclusion of BAP, with or without the addition of KIN, was advantageous

(Tables 1 and 2). Increasing the concentration of BAP applied alone increased the number of shoots produced, with 5 mg/L BAP producing the higher number of shoots. The beneficial effect of BAP in *in vitro* shoot proliferation in several medicinal plant species has been documented (Bohidar *et al*., 2008; Kalimuthu *et al*., 2010; Kamstaityte and Stanys, 2004). According to Tavares *et al.*(1996), Meszaros *et al.*(1999), Da Silva *et al.(2005)* and Ghiorghita *et al.*(2005), the addition of BAP to the culture medium enhanced multiple shoot production in lemon balm. Similarly, BAP had a greater effect on Aloe shoot proliferation than other cytokinins. (Hashemabadi and Kaviani, 2008; Velcheva *et al*., 2005; Budhiani, 2001).

Table 2
Effect of BAP and KIN on in vitro shoot regeneration from lateral shoot explants cultured on MS medium (after 30 days of culture)

BAP (mg/L)	*KIN (mg/L)*	*Shoot regeneration in explants*	*Number of shoots/explant*	*Mean shoot length (cm)*
0	-	87	2±0.91	5.6±2.11
0.5	-	100	6±1.23	5.7±2.21
1.0	-	100	9±2.11	4.6±0.91
3.0	-	100	13±3.41	3.9±0.24
5.0	-	81	17±2.16	3.5±0.56
	0.5	67	4±0.67	4.1±1.21
0.5	1.0	56	6±1.26	4.2±0.41
	3.0	100	21±3.51	3.6±0.62
	5.0	91	15±2.14	2.7±0.17
	0.5	100	7±1.67	3.9±0.60
1.0	1.0	100	13±3.45	3.5±0.16
	3.0	100	21±3.26	3.1±0.56
	5.0	87	11±1.74	2.5±0.51
	0.5	100	12±2.46	2.6±0.02
3.0	1.0	100	25±4.21	2.6±0.09
	3.0	100	19±3.11	2.3±0.67
	5.0	89	11±2.56	2.1±0.07

Effect of IBA and NAA on rooting

To obtain complete plantlets that would be viable when planted out in soil, *in vitro* plantlets were separated and transferred to the rooting media. MS mediums fortified with different concentrations of auxins (IBA and IAA) were used for the rooting experiment. The regenerated shoots produced roots when transferred to media supplemented with various concentrations of IBA and IAA. The presence of the auxin in the medium was not mandatory for root induction in *M. lunu-ankenda*; root formation (79%) was also observed on the control medium (Figure 1e). IBA was more effective than IAA for root induction. One hundred per cent rooting success was observed when elongated shoots were cultured on MS medium containing 0.5-1.0 mg/L IBA (Table 3). Increasing IBA concentration to 2 mg/L resulted in a reduction of rooting (67%). The highest number of roots per explant (7.4 and 7.1) was obtained on MS medium containing 0.5 and 1 mg/L IBA respectively, while the longest roots were obtained with 0.2 mg/L IBA. On the other hand, the addition of IAA to any of the concentrations tested resulted in a reduction of rooting as compared with controls that did not receive auxins. The effectiveness of IBA in root induction is in agreement with studies carried out on other plant species such as Dendrobium orchid (Rafique *et al*., 2012), *Dalbergia sissoo* (Ali *et al*. 2012) and *Plumbago zeylanicai* (Sivanesan and Jeong , 2009). Majumder *et al.* (2011) reported that the response of shoots to rooting was very much dependent on the concentrations and combination of auxins used. They found that the combination of 1.0 mg/l IBA + 0.5 mg/l IAA was the best medium for rooting. Ali *et al*. (2012) observed that *in vitro* cultures of *Dalbergia sissoo* rooted well on MS medium infused with 1.0 mg/l of IBA. Joshi *et al*. (2003) found that among three auxins tested, *viz.* IBA, NAA and IAA, the most effective for the *in vitro* rooting of *D. sissoo* was 1.0 mg/l IBA, although concentrations of IBA higher than 1.0 mg/l decreased the rooting response. Similarly,

Thirunavoukkarasu *et al*. (2010) obtained better results, applying 0.35 µ*M* IBA as compared to IAA. Up to 1.3 roots per plant were obtained with an average root length of 2.40 cm. These observations notwithstanding, where NAA is known as auxins have an inducing effect on rooting under tissue culture conditions (Werbruck and Debergh,1994). Moreover, according to Vijay et al (2013), the rooting of *in vitro Taverniera cuneifolia* developed shoots was best in MS medium containing 2 mg/l NAA. The influence of IBA and IAA for root proliferation has been also reported in many medicinal plants (Biswas *et al*, 2007; Jawahar *et al*., 2008). Liu *et al*. (2002) note that auxins induce the complicated process of lateral root formation through repetitive cell division, while George *et al*. (2008) suggest that auxins are essential for the maintenance of polarity of the plants. With regard to the effect of auxins on root induction, the deleterious effect of overdosing has been reported previously. According to Ozel *et al*. (2006), application of IBA with higher concentration inhibited the shoot buds formation, and this could stop the production of roots.

Table 3
Effect of different concentrations of IBA and IAA on the rooting of explants

IBA (mg/L)	*IAA (mg/L)*	*% of explants rooted*	*No. of roots/explant*	*Average root length (cm)*
0	0	79	5.3±1.11	5.7±1.21
0.1	0	81	5.3±1.24	5.2±0.66
0.2	0	83	6.9±1.23	5.6±1.12
0.5	0	100	7.4±0.14	3.4±0.76
1.0	0	100	7.1±0.24	4.5±0.56
2.0	0	67	5.4±0.16	3.6±0.43
0	0.1	45	2.1±0.08	4.5±0.65
0	0.2	48	2.5±0.07	3.2±0.43
0	0.5	56	2.1±0.56	3.4±0.21
0	1.0	43	3.2±0.34	3.5±0.34
0	2.0	39	2.3±0.11	3.4±1.01

Plant hardening and transplantation

The *in vitro* grown complete plantlets (Figure 1f) were transferred from the culture room to pots outside through successive phases of acclimatization. The plantlets were transplanted to pots filled with medium and a top soil- compost mixture (2:1). They were maintained at about 70% relative humidity in the greenhouse. A survival rate 85% was achieved during acclimatization, and the surviving plants have grown to a mature stage at the time of writing.

CONCLUSION

In the present study, a protocol of *M. lunu-ankenda* micropropagation was established. The results from the study demonstrated that a high rate of shoot regeneration from explants of *Melicope lunu-ankenda* could be achieved by modifying nutrient and growth regulator concentration in the culture medium. The presence of BAP and KIN in the medium was important for the development of shoots, and an average of 25 shoots per explant was obtained with 3 mg/L+1 mg/L KIN. Root formation could be induced with treatment of 0.5 mg/L IBA. *In vitro* regenerated shoots were able to acclimatize to an outdoor environment, achieving an 85% survival rate. These results on *in vitro* shoot regeneration from explants in *Melicope lunu-ankenda* are reported here for the first time, paving the way for *in vitro* commercial propagation.

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