Natural Science, 2015, 7, 81-87
Published Online February 2015 in SciRes. http://www.scirp.org/journal/ns
http://dx.doi.org/10.4236/ns.2015.72009

Direct Shoot Regeneration from Callus of *Melicope lunu-ankenda*

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Received 22 January 2015; accepted 11 February 2015; published 12 February 2015

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Abstract

***Melicope lunu-ankenda* is commonly used in traditional medicine. The conventional propagation method for this species is inefficient due to low propagation rate and its lengthy period to maturity. In addition, insufficient planting materials often pose a problem for the plantation sector. The tissue culture technique is best alternative to overcome the problems. The callus induction and direct shoot regeneration protocols for *M. lunu-ankenda* were established. Callus was successfully initiated from leaves explants cultured in MS medium added with 2,4-D at concentrations 0.5 to 5.0 mg/L singly or in combination with NAA at concentrations 1.0 to 10 mg/L. Shoot was regenerated from callus in phytohormone-free medium, BAP at concentrations 0.5 - 5.0 mg/L singly or in combination of BAP with NAA or 2,4-D at concentration 0.5 and 1.0 mg/L, respectively. BAP at 1.0 mg/L induced the highest shoot regeneration rate (80%) and number of plantlet per calli. The established methods might be used for production of phytochemicals and plantlets in large scale.**

Keywords

Melicope lunu-ankenda*, Regeneration, Plant Growth Regulator, *In Vitro

1. Introduction

Melicope lunu-ankenda (Gaertn.), a tree species belonging to the family Rutaceae. It endemic plant to Malaysia and locally known as "Tenggek burung", a popular ulam (salad) consumed raw by Malays. Leaves of this plant 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. Extracts of the plant were exhibited

How to cite this paper: Rahman, Ab.Z., Othman, A.N., Kamaruddin, F.L.I. and Ahmad, A.B. (2015) Direct Shoot Regeneration from Callus of *Melicope lunu-ankenda*. *Natural Science*, **7**, 81-87. http://dx.doi.org/10.4236/ns.2015.72009

bacteriostatic and fungicidal activity, natural antioxidant and effective anti-inflammatory and immunemodulatory agent [1]. The plant could also serve as leads in the search for anti-quorum sensing compounds [2]. Phytochemical screening shows that the *Melicope* plant containing alkaloid; melicarpine, semecarpine, 8-methoxyplatydesmine [3], dictamnine, confusameline and chromenes [1]. Production of such compounds requires a huge amount of plant materials, at the same time, the resource must be sustained. The conventional propagation method for this uncommon species is inefficient due to low propagation rate and its lengthy period to maturity. In addition, insufficient planting materials is often a problem for the plantation sector. Therefore, there is a need to develop methodologies for mass multiplication of this plant.

Advances in biotechnology have generated new opportunities for *Melicope* genetic improvement and metabolites production. The tissue culture technique is best alternative to overcome the problems. Vegetative propagation through *in vitro* culture techniques of *M. lunu-ankenda* had been previously reported [4]. The genetic improvements of the plant also feasible through somaclonal variation [5]. Such technique is a helpful tool to reduce the time for improvement of woody plant often takes many years using traditional plant-breeding methods [6]. The somaclonal variations techniques were reported to improve citrus against different a biotic stresses, low yield and conserve important citrus genotypes though exploiting somatic cell hybridization [7] and transformation of high yielding cultivars [8] disease free plants. Somaclonal variation exhibited in a wide range of traits including plant height, overall growth habit, flower, fruit and leaf morphology, juvenility, maturity date, disease resistance, yield, virus-free and biochemical characteristics. However, all these highly sophisticated technique requires the presence of highly responsive regeneration protocol. Nonetheless, most reports generally deal with either Solanaceous or cereal crops and limited information has been reported in woody trees.

The present study was to develop an efficient callus initiation system of *M. lunu-ankenda* through tissue culture, which might be used in genetic transformation system and/or efficient and suitable regeneration protocol of the plant in future. 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. The cell culture would also providing unlimited sources for metabolites production and genetic modification. Furthermore, shoot regeneration from cell culture is the prerequisite for the success in genetic modification. To our knowledge, this is the first report on the shoot regeneration from callus culture on *Melicope*.

2. Material and Methods

2.1. Plant Material and Culture Condition

The *M. lunu-ankenda* plants grown and maintained in a glass house was used as explant. Glass-house conditions were maintained at temperatures 29˚C ± 3˚C with a 12L:12D photoperiod and the relative humidity fluctuated between 50% - 70%. Lateral shoots segments were used to initiate shoot cultures. Explants were cleaned in a detergent (Teepol) solution for 20 min and rinsed in distilled water. Surface sterilization was preceded by immersed in 20% (v/v) Clorox® containing several drops of Tween-20 for 20 min and repeated for another 20 min in 5% (v/v) concentration. Subsequently, rinsed with sterilized distilled water few times. The death tissue were excised and shoot explants were inoculated onto MS medium supplemented with 3% sucrose and 1.0 mg/L BAP. The medium was adjusted to pH 5.8 prior solidified with 0.3% (w/v) gelrite and sterilized by autoclaving at 121˚C and 104 kPa for 15 min. All cultures were incubated in culture room under white light provided with white fluorescent light at intensity of 3000 lux at a photoperiodic 16 h. The room temperature was maintained at 25˚C ± 2˚C. The established *in vitro* plantlets were sub-cultured every three month interval **Figure 1(h)**. These plantlets was used as explant for callus induction.

2.2. Callus Induction

Under aseptic condition, stem of the *in vitro* plantlets were excised in 1 - 2 cm length and inoculated onto callus initiation medium. The medium consisted of MS [9] basal medium, 3% (w/v) sucrose, 3 g/L phytogel and 2,4-D at concentrations of 0, 0.5, 1.0, 3.0 or 5.0 mg/L or NAA at concentrations of 0, 1.0, 3.0, 5.0 or 10 mg/L, or combination of both phytohormones (**Table 1**). All cultures were maintained under light (1200 lux) and monitored weekly. The percentage of explants produced callus and day of callus formed were scored as amount of callus produced at every seven days intervals. Callus induction success was expressed as Percentage of explants induce

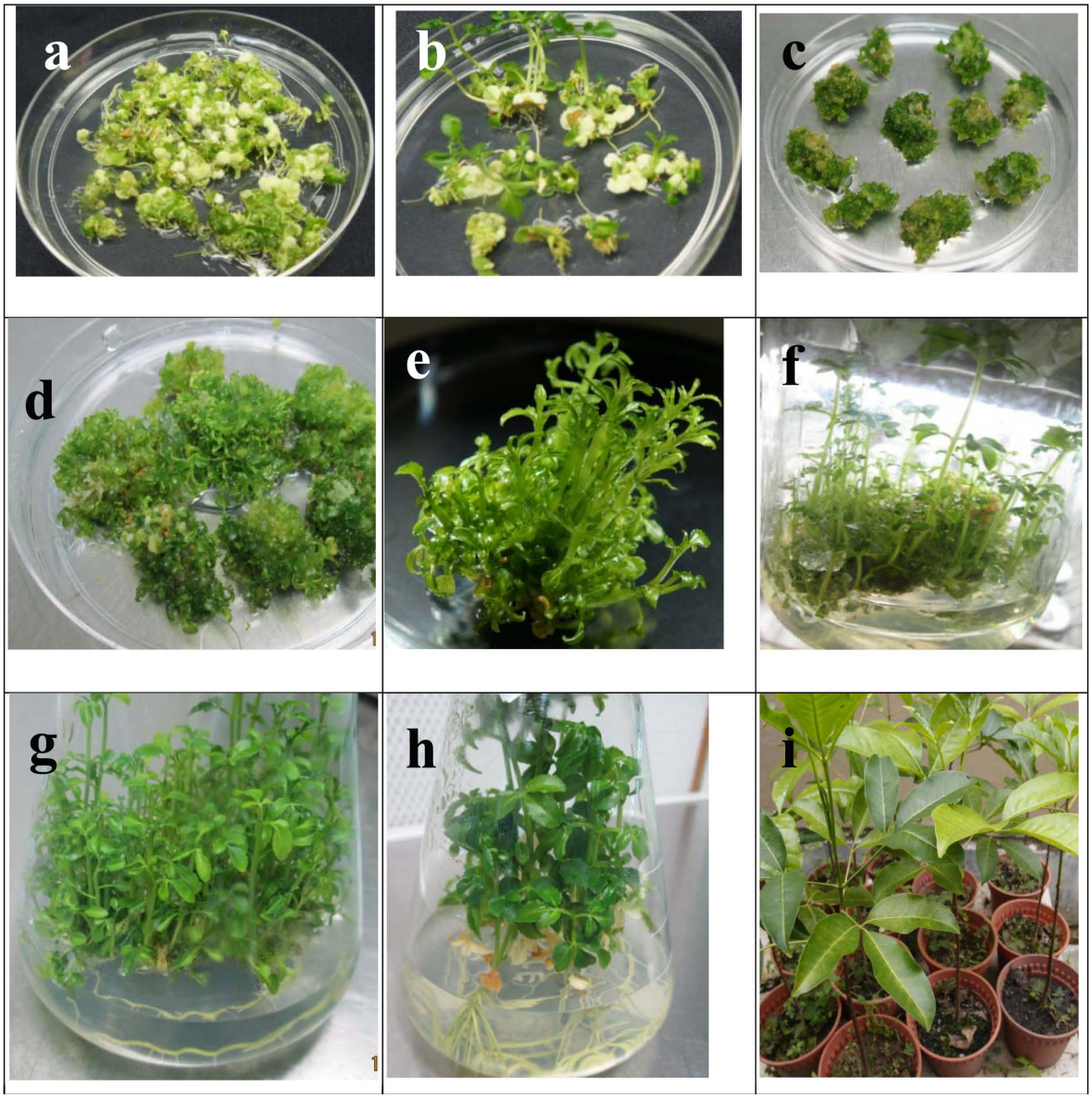

Figure 1. The direct shoots regeneration of *Melicope lunu-ankenda*. Callus were produced from the wounded side of the stems within a month of culture on 2,4-D alone and in combination with NAA (a)-(c); greenish callus were successfully regenerated to plantlets (d)-(g); rooted plantlets (h) *in vitro* plant acclimatization, growth in the net house (i).

callus and weight of green callus after second sub-culture (two months).Any further changes in the coloursof the callus were also recorded.

2.3. Plant Regeneration and Rooting

The yellow-green and dark-green calli were used for induction of shoot and plant regeneration. A small pieces of callus (app.3 gram) were aseptically transferred onto regeneration medium consisted of MS [9] basal medium, 3% (w/v) sucrose, 0.3% (w/v) phytogel and phytohormones; BAP at concentrations of 0, 0.5, 1.0, 3.0 or 5.0 mg/L singly or in combination with NAA at concentrations of 0.5 or 1.0 mg/L, or with 2,4-D at concentrations of 0.5 or 1.0 mg/L, respectively (**Table 2**). All cultures were maintained under a 16 h photoperiod with lighting provided by cool-white fluorescent lamps (100 - 2000 lux). The percentage of calli regenerated to plant and number of plantlets or shoots per clump of green callus were recorded after 45 days of culture. The completely regene-

Table 1. Effect of different combination of 2,4-D and NAA on callus induction of *Melicope lunu-ankenda* on MS medium after 45 days.

Plant growth regulator (mg/L)		Percentage of explants induce callus (%)	Weight of green callus after second sub-culture	Colour of callus
NAA	2,4-D			
0	0	0	-	-
	0.5	25 ± 4.1	0.16 ± 0.01	Yellow-green
	1.0	30 ± 2.2	0.33 ± 0.12	Yellow-green
	3.0	15 ± 3.3	2.11 ± 0.11	Yellow-green
	5.0	10 ± 0.9	0.97 ± 0.23	Yellow-green
1.0	0	0	-	-
	0.5	10 ± 1.1	0.34 ± 0.04	Yellow-green
	1.0	10 ± 1.4	0.56 ± 0.06	Yellow-green
	3.0	54 ± 4.5	4.98 ± 0.87	Yellow-green
	5.0	34 ± 3.1	2.12 ± 0.56	Yellow-green
3.0	0	0	-	-
	0.5	0	0.45 ± 0.05	Yellow-green
	1.0	45 ± 6.7	0.97 ± 0.12	Yellow-green
	3.0	35 ± 2.3	7.81 ± 1.23	Dark-green
	5.0	30 ± 1.1	2.34 ± 0.45	Yellow-green
5.0	0	0	-	-
	0.5	12 ± 2.1	4.56 ± 0.89	Dark-green
	1.0	65 ± 7.2	5.49 ± 1.21	Dark-green
	3.0	50 ± 6.7	6.45 ± 1.24	Dark-green
	5.0	20 ± 2.3	1.89 ± 0.40	Necrotic-green
10.0	0	0	-	-
	0.5	34 ± 3.3	7.50 ± 2.41	Dark-green
	1.0	75 ± 9.5	8.45 ± 0.98	Dark-green
	3.0	55 ± 3.5	6.54 ± 0.89	Necrotic-green

rated plantlets were separated to single plantlet and transferred to MS basal medium containing 0.5 mg/L IBA for root induction. After shoots, the rooted and healthy plantlets were individually transplanted to plastic pots containing hardening medium consisted of top soil:compost mixture (2:1). The plantlets were kept under controlled environment in a net house with 75% shading for 60 days. The survival rate of plantlets was recorded during the period of acclimatization.

2.4. Statistical Analysis

The data (20 replicates per treatment) were subjected to one way analysis of variance (ANOVA) to assess treatment differences and interaction using SPSS version 11.0 software. Significance of differences between means was tested by Duncan's Multiple Range Test ($p < 0.5$).

3. Results and Discussion

3.1. Callus Induction

The effect of different phytohormone on callus induction from stem of *M. lunu-ankenda* were successfully

Table 2. Effect of different combination of NAA, 2,4-D and BAP percentage of regenerating and multiple shoot formation of *Melicope lunu-ankenda* that initiated from green callus.

Plant Growth regulator (mg/L)			Percentage of callus produced shoot (%)	Number of shoot per clump green callus (3 gram)
NAA	2,4-D	BAP		
-	-	0	30 ± 4.5	11 ± 1.1
	-	0.5	70 ± 8.7	27 ± 3.4
	-	1.0	80 ± 9.6	31 ± 6.1
	-	3.0	50 ± 3.4	17 ± 2.3
	-	5.0	35 ± 5.7	12 ± 2.1
0.5	-	0	10 ± 1.3	7 ± 0.9
	-	0.5	35 ± 3.4	18 ± 4.5
	-	1.0	45 ± 6.6	19 ± 1.3
	-	3.0	30 ± 3.2	18 ± 4.5
	-	5.0	15 ± 3.1	6 ± 1.0
1.0	-	0	10 ± 2.1	15 ± 2.2
	-	0.5	35 ± 6.7	18 ± 4.1
	-	1.0	35 ± 8.1	15 ± 2.1
	-	3.0	25 ± 2.3	15 ± 2.4
	-	5.0	0	0
-	0.5	0	0	0
-		0.5	20 ± 2.1	9 ± 0.9
-		1.0	15 ± 2.3	12 ± 2.1
		3.0	0	0
		5.0	5 ± 1.5	8 ± 0.5
-	1.0	0	5 ± 0.9	0
-		0.5	15 ± 0.9	8 ± 0.5
-		1.0	15 ± 1.3	11 ± 1.3
		3.0	10 ± 1.2	0
		5.0	0	0

established (**Table 1**). Yellow to green, friable, non-embryogenic callus were produced from the wounded side of the stems within a month of culture on 2,4-D alone and in combination with NAA (**Figures 1(a)-(c)**). The best response of callus induction (75%) was observed in 1.0 mg/L of 2,4-D + 10 mg/L of NAA with dark-green colour of calli with the highest percentage (30%) in 1.0 mg/L 2,4-D. This was followed with 1.0 mg/L of 2,4-D + 5.0 mg/L of NAA (dark-green callus) and 3.0 mg/L of 2,4-D + 1.0 mg/L of NAA (yellow-greenish), which 65 and 55%, respectively. These results are in conformity with some of the earlier studies on different Rutaceae, which showed good callus induction response under the influence 2,4-D in combination with other phytohormone [10] [11]. Interestingly, 2,4-D combined with NAA at high concentrations (>3 mg/l) was enhanced the size or biomass the green colour of the calli. Green colour normally due to the formation of photosynthetic apparatus, the chlorophyll. A similar phenomena was previously reported in few plant species [11].

3.2. Plant Regeneration

The greenish callus were successfully regenerated to plantlets (**Figures 1(d)-(g)**). The highest percentage of plant regeneration (80%) and the highest number of shoot per calli clump (31 shoots per 3 gram callus) were

obtained in 1.0 mg/L BAP (**Table 2**). In BAP (1.0 mg/L) + NAA or 2,4-D with increasing concentrations (0.5 - 1.0 mg/L), shown a decreases in the plant regeneration as well of number of shoot produced. A similar response was reported [10] on shoot regeneration of citrus callus a member of Rutaceae family. The *in vitro* rooted plantlets transplanted into plastic pots in 75% shading net house were exhibited 98% survival rate after 60 days of transplanting (**Figures 1(h)-(i)**).

4. Conclusion

The callus and plant regeneration protocol were successfully established for *M. lunu-ankenda*. The size, colour and biomass of the calli produced were varies between the induction medium used. Combination of 3.0 mg/L 2,4-D and 1.0 mg/L NAA was the best for callus formation. BAP at concentration 1.0 mg/L was the best for induction of direct shoot regeneration using callus explant. The easily direct shoot regeneration of callus is useful for genetic modification and *in vitro* metabolites production. The protocol developed may aid the sustainable production of the phytochemical and planting material.

Acknowledgements

Authors wish to thank MARDI for providing the research grant and platform for the study.

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Abbreviations

NAA = 1-naphthaleneacetic acid;
2,4-D = 2,4-dichlorophenoxy acetic acid;
BAP = benzylamino purine;
IBA = indole-3-butyric acid.

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