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In vitro Multiple Shoot Regeneration from Stem Explant of Commercially Important Medicinal Herb *Labisia pumila* var. *pumila*

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Labisia pumila is a popular medicinal herb species attributed with several traditional uses especially for women healthcare. In this study, a protocol for multiple shoots regeneration from stem explants was established for *Labisia pumila* var *pumila*. The adventitious shoot formation from stem explant in response to various plant growth regulators (PGRs) was investigated. Stem explants excised from *in vitro* plantlets were cultured on Murashige and Skoog (MS) medium supplemented with various concentrations of 1-Napthaleneacetic acid (NAA), Kinetin, 6-Benzylaminopurine (BAP), Zeatin (ZEA) and 2, 4-Dichlorophenoxyacetic acid (2, 4–D). Direct shoot or root regeneration started within 4-6 weeks in most of the PGRs treatments. Highest responsive explant (88.90±19.2%) with 6-7 shoots per explant was obtained from MS medium containing 20 µM ZEA after 6 weeks of culture. Combinations of NAA and ZEA promote formation of compact callus and followed by rhizogenesis. After 12 weeks, the regenerated shoots were further elongated on MS medium without plant growth regulators. The individual elongated shoots were then transferred to rooting media containing 0.05 - 2.5µM NAA. All treatments responded well on rooting formation, but treatment with 2.5µM of NAA promoted the highest number of roots per explant (6.7 ± 1.2). The rooted plantlets were acclimatized successfully on peat moss or soil media with a survival rate of 70%-90%. The micropropagation method described in this study has a potential as an alternative for sustainable propagation material of this commercially important medicinal plant.

Keywords: Kacip fatimah, micropropagation, medicinal plant, organogenesis, plant growth regulators, shoot regeneration, Zeatin

I. INTRODUCTION

Labisia pumila(Bl.) F.-Vill also known as Kacip Fatimah is a small herbaceous plant belonging to family Myrsinacea. It is widely distributed in lowland and hills of Malaysia, Thailand, Indochina, Phillipines and Papua New Guinea(Stone, 1988; Wiart, 2002).*L. pumila* is traditionally used to promote the health of female reproductive system, facilitate childbirth, post-partum medication, to regain body strength and also for regulation of the menstrual cycle (Burkill, 1966; Ong, 2004). Scientific studies revealed that *L. pumila* possess the biological activities such as antioxidant, antimicrobial, anti-fungal, anti-

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inflammatory, anti-carcinogenic, anti-proliferative, phytoestrogenic effectand prevent postmenopausal osteoarthritis(Pihie *et al.,* 2012; Karimi *et al.,* 2013;Mohd Hanafi *et al.,* 2016; Ahmad *et al.,* 2017;Madzuki *et al.,* 2018).

Nowadays, there is an increasing demand of *L. pumila* for manufacturing of various commercial health products such as drink and food supplements in the form of tonics or tablets. As a result, the plant is exposing to scarcity and their existence in natural habitat become limited.(Norhaiza *et al.,* 2009; Kadir *et al.,* 2012). *Labisia pumila* is normally propagated through seeds or by means of vegetative cutting(Rozihawati *et al.,* 2003).But, growth rate is slow in their natural habitat (Mohd Noh *et al.,* 2002) hence, limits their potential for large-scale propagation. Thus, to ensure a sustainable supply, there is a need to mass propagate *L. pumila* using plant tissue culture techniques. Several studies has reported the *in vitro* propagation of *L.pumila* such as *in vitro* seed germination (Hartinie & Jualang, 2007) and plant regeneration from leaf and stem explants of *L. pumila* var. *alata* using different types and concentrations of PGRs (Ling *et al.,* 2013; Syafiqah Nabilah *et al.,* 2016). Although the previous findings reported frequencies of shoot regeneration was up to 100%, however, low number shoots multiplication (~ 3 shoots per explant) remain a major restriction for mass propagation. Thus, the aim of this study was to evaluate the effect of different types and concentration of PGRs on *in vitro* shoot multiplication from stem explant as well as rooting of *L. pumila* var. *pumila*.

II. MATERIALS AND METHODS

Plant materials were collected from 3 months old *in vitro* plant developed from previous study. (Hartinie & Jualang, 2007). Stem nodes were cut into 0.5-1 cm segments and placed vertically on MS (Murashige & Skoog, 1962) culture medium supplemented with 3 % (w/v) sucrose, 0.8% (w/v) agar. The medium treated with single PGRs, that are, NAA (Napthaleneacetic acid), KN (Kinetin), BAP (6-Benzylaminopurine), 2, 4–D (2, 4-Dichlorophenoxyacetic acid) and Zeatin (ZEA) using concentration of 5, 10, 15 and 20 µM; or combinations of NAA (0, 10, 20, 30 µM) with ZEA (0, 5, 10, 20 µM). Medium without PGRs served as control. The pH of medium was adjusted to 5.7 - 5.8 and autoclaved at 121°C for 15 minutes. All cultures were grown in a plant growth chamber (SANYO, MLR-350H) at 25 ± 2° C with 16 hours photoperiod provided by 36µmol m^2 s^{-1} cool light fluorescent tube (SANYO, Japan). After 10 weeks, the regenerated shoots were transferred to PGR-free medium for elongation and maintained in the same culture condition. Regeneration capacity from stem segment was evaluated based on the percentage of stem segments forming shoot and number of shoot and leaf per segment. Data were collected every 2 weeks up to 12 weeks of incubation.

Individual plantlets with fully developed shoots and expanded leaves were transferred to rooting medium consisted of semi-solid MS

basal medium, 3 % (w/v) sucrose, 0.8% (w/v) agar and 0.05, 0.5 or 2.5 μM NAA. Medium without NAA served as control. Growth conditions were similar as for regeneration study. Percentage of rooting plantlet, number of root and root length were collected every 3 weeks up to 16 weeks of culture. The 4-months-old plantlets (2-3 leaves) were then selected for plant acclimatization. Plantlets were removed from the culture flasks and washed thoroughly in running tap water to remove traces of medium. They were then transplanted in plastic pots containing peat moss or soil obtained from commercial nursery. The pots were covered with transparent plastic bags to maintain high humidity. Plants were grown in the nursery and irrigated at 3 days intervals with tap water. Plant growth was monitored weekly and the plantlet height, size and number of leaves and plantlet survival were observed after 6 months.

Experiments were conducted in a completely randomized design (CRD). Each treatment consisted of 3 explants per flask and conducted in triplicates, representing a total of 9 explants per treatment. The data were analyzed by SPSS (Statistical Package for Social Science) version 17.0 and subjected to one way ANOVA. The mean differences were tested using Duncan Multiple Range Test at $p<0.05$.

III. RESULTS AND DISCUSSIONS

A. Shoot Regeneration and Multiplication

Table 1 shows the effect of PGRs on shoot regeneration from stem node of *L. pumila* var. *pumila* observed after 12 weeks of culture. Shoot induction started after 4 weeks of culture and continue to multiply until week 12 (Figure 1 b-c). Cytokinins treatment showed ability to induce shoot regeneration and recorded the highest response (up to 88.90 ± 19.20% of responsive explant). The number of shoots per responsive explant was significantly increased with increasing concentrations of ZEA. Off all treatments, addition of 20 μM ZEA promotes the highest number of shoots multiplication (6.32 ± 0.81).ZEA at 1-7mg/l was also reported to induced up to 100% shoot regeneration in *L.pumila* var. *alata* (Ling *et al.,* 2013) but with lesser number of shoot (1.4±0.3) compared to our finding. In accordance with our result, ZEA has also been superior compared to other cytokinin during shoot regeneration of *Thermopsis turcica* (Tekdal & Cetiner, 2014). The presence of single ZEA in the medium also found efficient for shoot regeneration in *Solanum nigrum* (Li *et al.,* 2017) and induction of adventitiousbuds from leaf explants of *Vaccinium corymbosum* (Chen *et al*. 2018). Zeatin known as a major cytokinins in plant (Murai, 2014) and cZ (*cis-zeatin*) have been reported to be in high concentration in many in vitro-cultured plants (Aremu *et al.,* 2017).The stimulatory effect of ZEA during shoot regeneration might be due to the lower affinity of cytokinin degrading enzyme for ZEA, thus allowing ZEA to be in a relatively higher concentration, therefore having a higher probability of effecting organogenesis in plants than any of the other cytokinin at the same

concentration(Nikolic et al. 2006; Masekesa et al. 2016). On the other hand, all auxin treatments (NAA and 2,4–D) except for the 5 μM NAA failed to induced shoot regeneration. It was observed that NAA treatments promote root organogenesis from the explant after 4 weeks of culture. Roots are mostly induced in the presence of an auxin (Chae, 2016) and NAA was found the most effective auxin in root induction from stem explant of *L. pumila* (Hasan & Hussein, 2013).

The effect of NAA and ZEA combinations on shoot regeneration from stem segment is presented in Table 2. Initiation of shoot bud was observed after 4 weeks of culture on control medium followed by medium supplemented with ZEA as well as in combinations of 20 μM NAA and 5 μM ZEA after 6 weeks of culture. It was suggested that the application of ZEA alone was sufficient for direct shoot regeneration and shoot multiplication. Addition of ZEA and NAA combination in media induced formation of undesirable hard callus within 6 weeks of culture initiation, resulted to indirect organogenesis and delay on shoot regeneration (up to 10 weeks of culture). An average of 1.3-2.5 shoots per explants was observed on these media. This result may be explained by the fact that the balance and interaction between two plant hormones, auxin and cytokinin at certain concentrations determine the cellular differentiation and organogenesis in tissue and organ cultures. The application of exogenous auxin and cytokinin of an intermediate ratio can promote callus induction(Ikeuchi et al. 2013). Generation of shoot from growing callus can be elicited by culture in media in different ratio of the same plant hormones (Gautheret, 2003). The production of regenerable organogenic callus on medium fortified with NAA in combination with ZEA or other cytokinin were also reported in *Thermopsis turcica* (Tekdal & Cetiner, 2014) and *Cerasus humilis (Wang et al., 2016).*

B. Rooting and Acclimatization

For rooting experiment, all newly regenerated shoots produce roots after 8 weeks of culture regardless of NAA concentrations as well as control medium (Table 3). Our finding also indicated that 2.5 μM of NAA was capable to enhance rooting efficiency. This showed by the significant increased on number of root from 2.9 (in control medium) to 7.1 (Figure 1e). Even though 2.5 μM of NAA promotes the highest number of roots, the control medium however has the highest root length (12.1 ± 2.9 cm). Addition of exogenous auxin is important for *in vitro* rooting because auxin increases the availability of carbohydrate at the site of root development (Middleton *et al.,* 1980). Promoting effect of NAA on rooting was also reported in *Laburnum anagyroide* (Timofeeva, 2014), *Cerasus humilis* (Wang *et al.,* 2016)and *Tulbhagia simmleri* (Kumari *et al.,* 2018)

The result of plantlets acclimatization after 6 months of transplantation is shown in Table 4. In this preliminary study, 90% and 70% of acclimatized plantlets has survived on cocoa

peat medium and on soil medium, respectively. Plantlets acclimatized on peat moss media produced healthy plant that is morphologically similar to the donor plant with a mean of 4.2 ± 0.8 cm height plantlet accompanied with 6.9±1.5 leaves per plantlet (Figure 1f). In contrast, plantlets transplanted on potting soil achieved a mean of 7.7 ± 1.3 cm height and appear with longer internode segments as well as smaller and less number of leaf. This might be due to the different soil composition used in the experiment while in nature, *L. pumila* favours to grow on humus-rich soils (Sunarno, 2005). It was reported that a mixture of top soil: leaf compost: sand (2:3:1) gave higher performance for *L.pumila* var *alata* (Syafiqah Nabilah *et al.,* 2017).This preliminary result indicates that the rooting and acclimatization stage were not the critical aspect for *L. pumila* var. *pumila*.

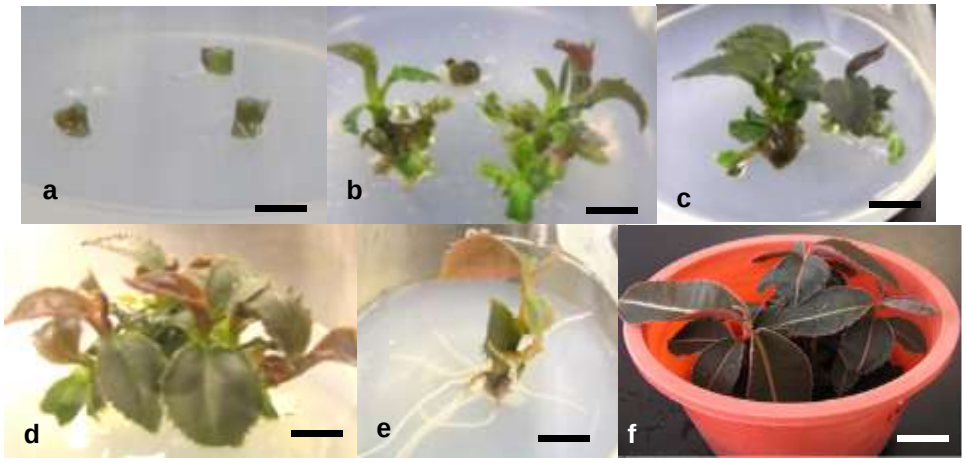

Figure 1. Establishment of *L. pumila* var. *pumila* plantlet through multiple shoot regeneration from stem explant cultured on MS medium supplemented with 20 µM ZEA. a) Initial culture b) Shoot initiation (4-6 weeks) c) Shoot multiplication (12 weeks) d) Shoot elongation e) Rooting stage f) Acclimatization (6 months). Bars: 10mm

Table 1. Effect of different PGRs on regeneration from stem node of *L. pumila* var. *pumila* after 12 weeks of culture

PGR	Concentration (µM)	Responsive explant (%) (Mean± SD)	Number of shoot per responsive explant (Mean ± SD)	Number of root per explant (Mean ± SD)	Time to initiate shoot/root (weeks)
Control	-	100±0^{a}	1.00±0^{e}	-	4
2,4-D	5	5.56±9.62^{g}	-	0.22±0.39^{d}	4
	10	Nr	-	-	-
	15	Nr	-	-	-
	20	Nr	-	-	-
NAA	5	5.56±9.62^{g}	0.89±0.42^{c}	1.50±0.88^{ab}	4
	10	Nr	-	1.06±0.42^{bc}	4

	15	Nr	-	2.07±1.02[a]	4
	20	Nr	-	0.50±0..50[cd]	4
KN	5	88.89±9.62[a]	3.00±0.29[b]	-	4
	10	72.22±25.46[ab]	3.78±0.92[b]	-	4
	15	50.00±16.67[cd]	0.89±0.42[c]	-	4
	20	33.33±16.67[de]	0.61±0.42[cd]	-	4
BAP	5	55.56±9.62[bc]	0.78±0.48[cd]	-	4
	10	27.78±9.62[ef]	0.28±0.10[de]	-	4
	15	38.89±9.62[cde]	0.55±0.25[cde]	-	4
	20	11.11±19.24[fg]	0.11±0.19[e]	-	4
ZEA	5	33.31 ±0[d]	1.00±0.00[e]	-	6
	10	44.40±19.2[cd]	3.83±0.62[b]	-	6
	15	53.76±9.63[bc]	3.99±0.92[b]	-	6
	20	88.90±19.2[a]	6.32±0.81[a]	-	6

Each value is mean of three replicates. Values followed by the same letter are not significantly different at P< 0.05 according to Duncan Multiple Range Test.Nr-Not responsive

Table 2: Effect of various concentrations of NAA and ZEA on regeneration from stem node of *L. pumila* var. *pumila* after 12 weeks of culture

PGRs (µM) NAA	PGRs (µM) ZEA	Responsive explant (%) (Mean± SD)	Number of shoot per responsive explant (Mean± SD)	Number of leaves per shoot (Mean± SD)	Number of root per explant (Mean±SD)	Time to initiate shoot/root (weeks)
0	0	100 ± 0[a]	1.0±0[e]	3.8±0.2[a]	-	4
	5	33.3 ±0[d]	1.0±0[e]	3.2±0.4[a]	-	6
	10	44.4±19.2[cd]	3.8±0.6[b]	1.8±0.3[bc]	-	6
	20	88.9±19.2[ab]	6.3±0.8[a]	1.4±0.3[c]	-	6
10	0	44.4±19.2[cd]	1.3±0.6[de]	2.3±0.6[b]	2.2±0.3[de]	10
	5	44.4±19.2[cd]	1.3±0.3[de]	2.2±0.4[b]	2.7±0.6[d]	10
	10	66.7±0[bc]	2.5±0.9[c]	2.0±0.3[bc]	2.3±0.6[d]	10
	20	44.4±19.2[cd]	1.3±0.3[de]	2.1±0.9[bc]	-	10
20	0	-	-	-	-	-
	5	88.9±19.2[ab]	1.3±0.3[de]	3.2±0.7[a]	3.8±0.9[c]	6
	10	44.4±19.2[cd]	1.3±0.6[de]	1.9±0.2[bc]	6.0±1.0[b]	10
	20	66.7±0[bc]	2.2±0.8[cd]	1.4±0.4[c]	2.7±0.8[d]	10
30	0	-	-	-	-	-
	5	66.7±0[bc]	-	-	6.5±0.5[ab]	10
	10	66.7±0[bc]	2.5±0.5[c]	3.9±0.4[a]	1.4±0.5[e]	10
	20	100±0[a]	-	-	6.9±0.5[a]	10

Each value is mean of three replicates. Values followed by the same letter are not significantly different at P< 0.05 according to Duncan Multiple Range Test

Table 3: Effect of NAA concentration on root formation of *L. pumila* var. *pumila* plantlet after 16 weeks of culture

NAAConcentration(μM)	Percentage of rooting plantlet (%)	Number of roots (Mean ± S.D)	Root length(cm) (Mean ± S.D)
Control	100	2.9 ± 0.6[b]	12.1 ± 2.9[a]
0.05	100	3.0 ± 1.0[b]	5.7 ± 0.5[b]
0.5	100	3.0 ± 1.0[b]	6.9 ± 0.9[b]
2.5	100	6.7 ± 1.2[a]	6.4 ± 1.0[b]

Each value is mean of three replicates. Values followed by the same letter are not significantly different at P< 0.05 according to Duncan Multiple Range Test

Table 4: *Ex-vitro* performance of *L. pumila* var. *pumila* plantlets after 6 months of transplantation

Media	Survival (%)	Plantlet height(cm) (Mean ±SD)	Leaf (Mean ±SD)			Appearance
			Number	Length (cm)	Width (cm)	
Peat moss	90	4.2 ± 0.8	6.9±1.5	4.3 ± 1.3	2.3 ± 0.3	Normal
Soil	70	7.7 ± 1.3	4.9 ±1.5	4.0 ± 0.9	2.3 ± 0.3	Longer internode segments

IV. SUMMARY

The present study offers an improved protocol for shoot multiplication of *L. pumila* var. *pumila*.The type and concentration of PGRs significantly influence shoot multiplication from stem explant. Our findings also suggest possible method for rooting and acclimatization of the regenerated shoots. Further research on the effect of other factors such as explant orientation, carbon sources and others could provide an optimization, efficient and rapid clonal propagation of this industrial important species.

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