Effects of auxin and cytokinin on biomass and phenolics production in adventitious roots cultures of *Labisia pumila* var. *alata*

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ABSTRACT *Labisia pumila* is a highly valuable medicinal herb in Malaysia which contains many important bioactive compounds. This study described the effects of different concentrations of auxin and auxin-cytokinin combinations on biomass and phenolics production of adventitious roots of *Labisia pumila* var. *alata* (LPA) in liquid shake culture. The roots were cultured in darkness for five weeks and its biomass was determined, followed by extraction and assessment of their production of total phenolics and flavonoids. In single hormone treatment, naphthalene-acetic acid (NAA) (1 mg/L) was the best auxin for biomass production (274.60 ± 11.00 mg FW, 42.65 ± 5.45 mg DW) with highest yield of total phenolics (70.68 ± 8.67 µg) and total flavonoids (31.12 ± 4.58 µg) compared to indole acetic acid (IAA) and indole butyric acid (IBA). However, the combination of 1 mg/L NAA with cytokinins (BAP or KN at 0.1-1 mg/L) failed to enhanced adventitious roots with biomass and bioactives accumulation. The results are beneficial for optimization of LPA adventitious root cultures for phenolics and flavonoids production.

KEYWORDS: Kacip Fatimah, *Labisia pumila*, liquid shake culture, plant growth regulators, secondary metabolites

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INTRODUCTION

Labisia pumila or locally known as Kacip Fatimah in Malaysia, is a small sub-herbaceous plant which belongs to the family Myrsinaceae (Burkill, 1935). This herb is widely used in folk medicine for facilitating childbirth and post-partum recovery (Bodeker, 2009). This plant contains various phytochemicals such as phenolics, flavonoids, carotenoids, ascorbic acids, saponin, alkenyl compounds and benzoquinone derivatives which contribute to its biological activities (Norhanisah *et al.*, 2013). Due to its interesting phytochemical contents, raw materials of commercialized products based on *L. pumila* are harvested directly from the wild environment. This could cause inconsistency in the quality of final products as their phytochemicals content varies due to the inherent complexity of naturally grown medicinal plants, variable nature of cultivated plant and postharvest processing of plant (Kunle *et al.*, 2012). Plant cell, tissue and organ culture is an ideal technology to produce continuous and reliable source of plant-based pharmaceutical products (Rao & Ravishankar, 2002). The production of secondary metabolites in cell culture has a major drawback, which is the occurrence of somaclonal variation. This phenomenon may lead to unstable biochemical behaviour (Flores & Curtis, 1992). In contrast, organ culture, especially adventitious root culture is more favourable due to its fast growth and stable production of secondary metabolites (Murthy *et al.*, 2008). *Eurycoma longifolia* (Hussein *et al.*, 2012), *Hypericum perforatum* (Cui *et al.*, 2010), *Morinda citrifolia* (Baque *et al.*, 2010) and *Rhus javanica* (Taniguchi *et al.*, 2000) are among important medicinal plants, in which adventitious roots have been induced using plant growth regulators for efficient production of high-value secondary metabolites. The induction of adventitious root from leaf and stem explants of *L. pumila* var. *alata* had been reported by Ling *et al.* (2013). However, there is no published work on the growth and secondary metabolites production of adventitious root culture in *L. pumila*. Therefore, the objective of this study was to optimize the biomass and phenolics and flavonoids production by manipulating different concentrations of auxin and cytokinin in adventitious root of *L. pumila* in a liquid shake culture.

METHODOLOGY

Induction of Adventitious Root Culture

Adventitious roots were induced from the leaves of 6 months old LPA derived from previous research by Hartinie & Jualang (2007). The leaf explants were excised approximately 1 cm^2 and cultured on Murashige & Skoog (MS) media containing 3 mg/L IBA, 3% (w/v) sucrose (Sigma) and 0.3% (w/v) Gelrite (Duchefa) which showed the best adventitious root formation (Ling *et al.*, 2013). All the cultures were incubated in a growth chamber (Conviron Adaptis A1000) at 25±2°C with 16 hours light and eight hours dark photoperiod for five weeks. After five weeks, leaf explants with roots were transferred into liquid MS media without hormone and were agitated at 110 rpm on an orbital shaker (Protech SK-71) in darkness at 25±2°C for further root elongation process. The roots were then separated from the explants after four weeks and used in the establishment of LPA root culture.

Influence of Plant Growth Regulators on Root Growth and Secondary Metabolites Production

Adventitious roots (50mg/flask, 2 cm in length) were inoculated in 100 mL shake flask culture containing 25 mL MS medium with 3% (w/v) sucrose and different concentrations (1, 3 and 5 mg/L) of IAA, IBA and NAA. In combined treatment, the roots were cultured in medium containing 1 mg/L NAA and 0.1, 0.5 and 1.0 mg/L cytokinins (6-benzylaminopurine (BAP) or kinetin (KN). All flasks were agitated at 110 rpm on an orbital shaker (Protech SK-71) in darkness at 25±2°C. After five weeks, the roots biomass was determined, followed by extraction and then assessed for their production of total phenolics and flavonoids.

Extraction Method

Extracts were prepared using method by Harborne (1998) with some modification. The dried samples were first grounded into fine powder. Then, the samples were soaked with methanol (99% (v/v), Fisher Scientific) using a ratio of 1:10 (w/v) and left to stand for overnight. The extracts were filtered using Whatman® paper No.1. The collected filtrates were dried using rotary evaporator (Heidolph Laborota 4000) at 37°C and then stored in universal bottle at 4°C until used.

Determination of Root Biomass

The harvested roots were separated from the medium by filtration through a 1 mm stainless steel sieve, and the fresh weight was measured after rinsing once with distilled water and blotting away the surface water with towel tissue papers (Cui *et al.*, 2010). The roots were oven-dried (Protech FAC-50) to constant weight at 37°C for three days and the root dry weight was recorded.

Determination of Total Phenolics Content

The total phenolics content in the extracts were determined using Folin-Ciocalteu method as described by Ainsworth & Gillespie (2007). In brief, 200 µL of 10% (v/v) Folin-Ciocalteu's reagent (Sigma) were added to 100 µL of extracts (1 mg/mL) or standard solution of gallic acid (Merck) (20, 40, 60, 80, 100 µg/mL) in methanol. Methanol (99% (v/v), Fisher Scientific) was used as blank. After three minutes, 800 µL of 700 mM sodium carbonate (Sigma) solution was added and the contents were mixed thoroughly. Reaction mixtures were incubated for two hours at room temperature and protected from light. An aliquot (200 µL) of the sample, standard or blank were transferred into a 96-well microplate and the absorbance was measured at 765 nm using a microplate reader (Thermo Scientific™ Multiskan™ GO 51119200). The calibration curve was plotted using gallic acid ($y=0.0061x + 0.0126$, $R^2=0.9904$). Results of total phenolic contents were expressed as mg of gallic acid equivalent (GAE) per gram dry weight. Phenolics yield was calculated by multiplying dry weight of roots with total phenolics content.

Determination of Total Flavonoids Content

Total flavonoids content was measured by the aluminium chloride colorimetric assay based on Chang *et al.* (2002) with some modifications. Briefly, the method consisted of mixing 100 µL of extracts, standard solution of quercetin (Sigma) (20, 40, 60, 80, 100 µg/mL) or methanol (99% (v/v), Fisher Scientific) (blank) with 270 µL methanol, 18 µL of 10% (w/v) aluminium chloride (Sigma), 18 µL of 1 M potassium acetate (Sigma) and 170 µL of distilled water in 96-well microplate. Reaction mixtures were incubated for 30 minutes at room temperature and protected from light. The absorbance was measured at 415 nm using a microplate reader (Thermo Scientific™ Multiskan™ GO 51119200). The calibration curve was plotted using quercetin ($y=0.0043x + 0.0017$, $R^2=0.9978$). Total flavonoid compound of extracts were expressed as mg quercetin equivalent (QUE) per gram dry weight. Flavonoids yield was calculated by multiplying dry weight of roots with total flavonoids content.

Statistical Analysis

Data collected were analysed using SPSS (Statistical Package for Social Science) version 20 and subjected to One-Way analysis of variance (ANOVA). The mean values were compared by Duncan's multiple range test at $p<0.05$.

RESULT AND DISCUSSION

Effect of Auxin Types and Concentrations

Adventitious root explants were cultured on liquid MS media supplemented with various concentrations of auxins (IAA, IBA or NAA; 1, 3 or 5 mg/L; Figure 1). The greatest response in terms of biomass production (274.60±11.00 mg FW and 42.65 mg DW) was observed in medium containing 1 mg/L NAA. However, the fresh and dry weight of the roots decreases with the increment of NAA concentrations (Table 1). In addition, treatment of 1 mg/L NAA significantly produced LPA roots with the highest phenolics (70.68±8.67 µg) and flavonoids (31.12±4.58 µg) yield compared to the other treatments. Hence, 1 mg/L NAA was selected as the best auxin for biomass production in LPA adventitious roots which was favourable in achieving high yield of phenolics and flavonoids. LPA adventitious roots cultured in NAA generally showed favourable response to biomass production despite producing low total phenolics and flavonoids content. In contrast, LPA adventitious roots supplemented with IBA which showed poor biomass accumulation contained higher total phenolics and flavonoids content. Hence, it can be postulated that there is an inverse relationship between the growth of LPA adventitious roots and its production of secondary metabolites. The possible reason causing this phenomenon is that the dedifferentiation process might be inhibiting the accumulation of secondary metabolites (Lindsey & Yeoman, 1985; Khani *et al.*, 2012). Auxin plays a critical role in adventitious roots formation (Arroo *et al.*, 1995; Aloni *et al.*, 2006; Hou *et al.*, 2010; Guan *et al.*, 2015). The initiation of adventitious roots starts with asymmetric divisions of founder cells triggered by the signal from auxin. Exogenous auxin must go through a series of processes of absorption, transportation and metabolization before the action of inducing adventitious roots formation takes place. Auxin affects both growth and secondary product formation. The low secondary metabolites formation in auxin-treated culture is probably due to the improper cell specialization, hence causing inefficient biosynthesis (Arroo *et al.*, 1995). Previous researches have suggested that the effect of auxin on adventitious root growth varies from species to species. The maximum biomass accumulation in *Prunella vulgaris* (Fazal *et al.*, 2014) and *Silybum marianum* (Khan *et al.*, 2015) were also achieved in liquid MS medium supplemented with 1 mg/L NAA. Root-promoting effects of NAA was also reported in *Dendrobium lowii* (Jualang *et al.*, 2016). In contrast, IBA was more effective than NAA in stimulating lateral root formation in *Panax ginseng* (Kim *et al.*, 2003). Kollárová *et al.*

(2004) reported that IBA was the best auxin compared to IAA and NAA in adventitious root elongation and lateral root induction on *Karwinskia humboldtiana* root cultures. Meanwhile, Mohd Geoffrey & Sani (2017) stated that IBA did not affect the formation of adventitious roots in *Synsepalum dulcificum*.

Table 1. Effect of auxin type and concentration on biomass and secondary metabolite production in LPA adventitious root after five weeks of culture

PGR	Conc. (mg/L)	Fresh weight (mg)	Dry weight (mg)	TPC	Phenolics yield (µg)	TFC	Flavonoids yield (µg)
MSO	0	86.25±1.35^{d}	13.85±1.25^{de}	1.32±0.02^{f}	18.35±1.83^{f}	0.18±0.07^{e}	2.41±0.75^{f}
IAA	1	81.90±7.20^{d}	13.20±0.90^{de}	2.25±0.05^{c}	29.71±2.47^{de}	0.41±0.03^{d}	5.41±0.18^{ef}
	3	87.40±3.60^{d}	18.85±2.05^{c}	2.35±0.02^{bc}	44.25±4.63^{c}	0.70±0.06^{c}	13.21±2.49^{cd}
	5	90.60±1.00^{d}	17.20±0.80^{cd}	1.99±0.03^{d}	34.28±2.04^{d}	0.96±0.11^{b}	16.48±1.37^{cd}
IBA	1	63.90±2.00^{e}	10.45±0.55^{e}	2.37±0.14^{b}	24.73±1.67^{ef}	0.73±0.26^{c}	7.64±2.83^{e}
	3	108.70±7.10^{c}	20.55±1.85^{c}	2.60±0.09^{a}	53.43±5.79^{b}	1.09±0.08^{b}	22.26±2.02^{b}
	5	64.90±0.80^{e}	12.50±1.50^{de}	2.61±0.08^{a}	32.50±2.91^{de}	1.39±0.08^{a}	17.31±1.93^{c}
NAA	1	274.60±11.00^{a}	42.65±5.45^{a}	1.66±0.01^{e}	70.68±8.67^{a}	0.73±0.04^{c}	31.12±4.58^{a}
	3	136.60±1.60^{b}	27.95±1.75^{b}	1.61±0.02^{e}	44.96±2.85^{c}	0.57±0.09^{cd}	15.71±1.69^{cd}
	5	113.40±1.60^{c}	27.50±5.50^{b}	1.37±0.01^{f}	37.67±7.73^{cd}	0.47±0.06^{d}	12.92±2.37^{d}

Values are expressed as mean ± standard deviation. Mean followed by the same letter in the same column were not significantly different at $p<0.05$ according to Duncan's Multiple Range Test. IAA = Indole acetic acid; IBA = Indole butyric acid; NAA = Naphthalene-acetic acid; MSO=MS medium without PGR; TPC = Total phenolics content (mg GAE/g DW); TFC = Total flavonoids content (mg QUE/g DW).

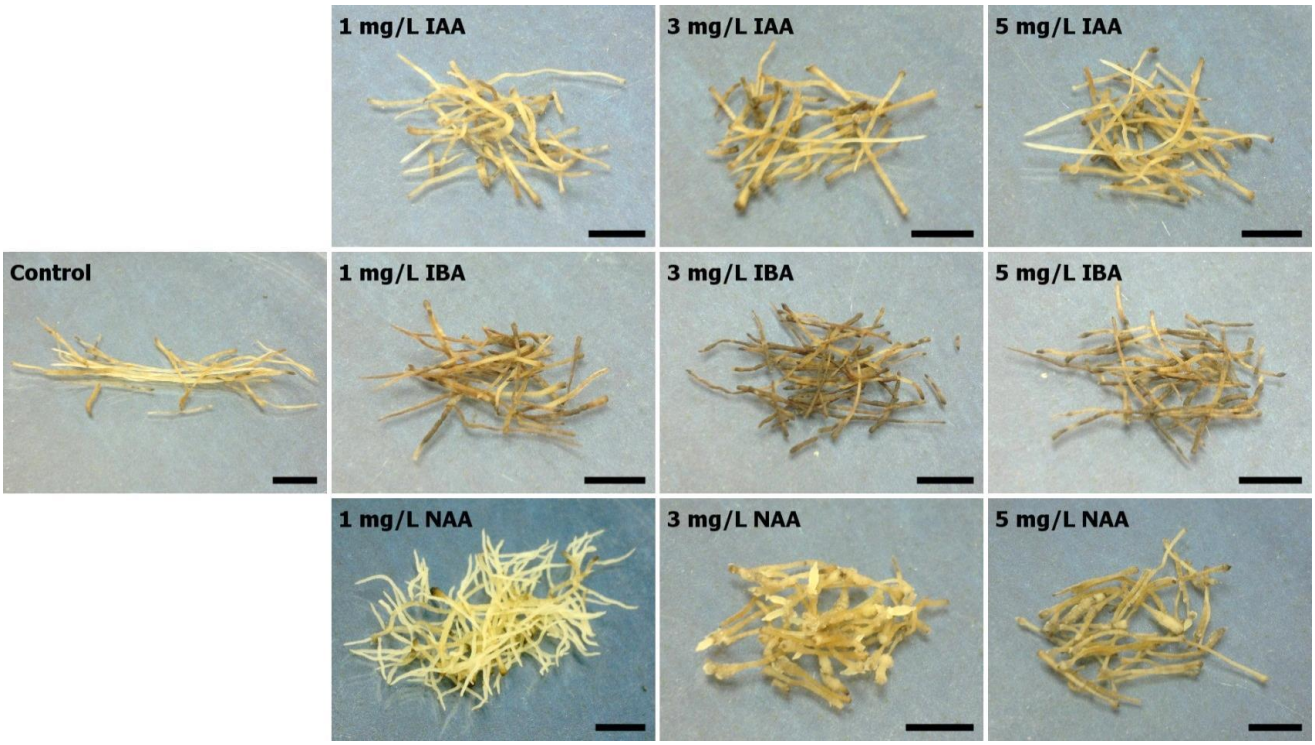

Figure 1. Proliferation of LPA adventitious root in liquid MS medium supplemented with auxin after five weeks of culture (Bar = 5 mm; IAA = Indole acetic acid; IBA = Indole butyric acid; NAA = Naphthalene-acetic acid)

Effect of Auxin and Cytokinin Combinations

For the subsequent experiment, NAA 1 mg/L was selected as the auxin as it showed the best proliferation rate among other auxins tested (Table 2, Figure 2). When both auxin and cytokinin were added into the culture medium, root growth and secondary metabolites production could be improved compared to that when using auxin alone (van Staden *et al*., 2008). In this context, the type and concentration of auxin or cytokinin, and the auxin to cytokinin ratio are the major determinants in controlling root growth and secondary compounds accumulation (Baque *et al*., 2010). In this study, the combination of NAA (1 mg/L) with either BAP or KN did not significantly influence biomass production as compared to the control (NAA 1 mg/L). This might be caused by the cytokinin itself, as it can prevent promotive effect of auxin in root initiation (Slav *et al*., 1967; Wightman & Thimann, 1980) and root growth especially in dark conditions (Baque *et al*., 2010). Hence, it can be pointed out that the combination of auxin-cytokinin decreases the biomass production in LPA adventitious root cultures. When increasing level of BAP and KN were combined with 1 mg/L NAA, the contents of phenolics and flavonoids also gradually increased. However all the auxin and cytokinins combination tested did not manage to produce higher phenolic and flavonoid contents compared to the cytokinin-free medium (TPC=1.72±0.05 mg gallic acid/g DW, TFC=0.75±0.03 mg quercetin/g DW). Both phenolics (74.71±5.06 µg) and flavonoids (32.53±0.83 µg) yield were also significantly higher in the control medium. Based on the data obtained, it is apparent that the combination of 1 mg/L NAA with KN or BAP respectively was not favourable for enhanced LPA30 adventitious roots biomass and secondary metabolite accumulation.

Table 2. Effect of auxin and cytokinin combination on biomass and secondary metabolite production in LPA adventitious root after five weeks of culture

PGR (mg/L)	Fresh weight (mg)	Dry weight (mg)	TPC	Phenolics yield (µg)	TFC	Flavonoids yield (µg)
Control (NAA 1)	268.27±9.50^{a}	43.32±2.52^{a}	1.72±0.05^{a}	74.71±5.06^{a}	0.75±0.03^{a}	32.53±0.83^{a}
NAA 1 + BAP 0.1	190.03±38.08^{bc}	33.73±8.24^{b}	0.78±0.01^{f}	26.49±6.70^{bc}	0.18±0.05^{c}	5.75±0.04^{c}
NAA 1 + BAP 0.5	114.90±17.00^{e}	20.20±1.55^{c}	1.07±0.01^{d}	21.56±1.63^{c}	0.20±0.05^{c}	4.02±0.89^{c}
NAA 1 + BAP 1.0	129.27±9.28^{de}	21.00±2.40^{c}	1.42±0.03^{c}	29.67±2.80^{bc}	0.26±0.06^{c}	5.36±1.45^{c}
NAA 1 + KN 0.1	196.50±1.47^{b}	35.93±1.35^{ab}	0.61±0.01^{g}	21.86±1.21^{c}	0.16±0.06^{c}	5.81±2.01^{c}
NAA 1 + KN 0.5	156.40±15.24^{cd}	33.97±1.47^{b}	0.97±0.02^{e}	33.11±2.07^{b}	0.18±0.05^{c}	6.06±1.71^{c}
NAA 1 + KN 1.0	111.23±31.23^{e}	21.93±7.21^{c}	1.56±0.03^{b}	34.34±11.61^{b}	0.63±0.06^{b}	13.56±3.76^{b}

Values are expressed as mean ± standard deviation. Mean followed by the same letter in the same column were not significantly different at $p<0.05$ according to Duncan's Multiple Range Test. NAA = Naphthalene-acetic acid; BAP = 6-benzylaminopurine; KN = Kinetin; TPC = Total phenolics content (mg GAE/g DW); TFC = Total flavonoids content (mg QUE/g DW).

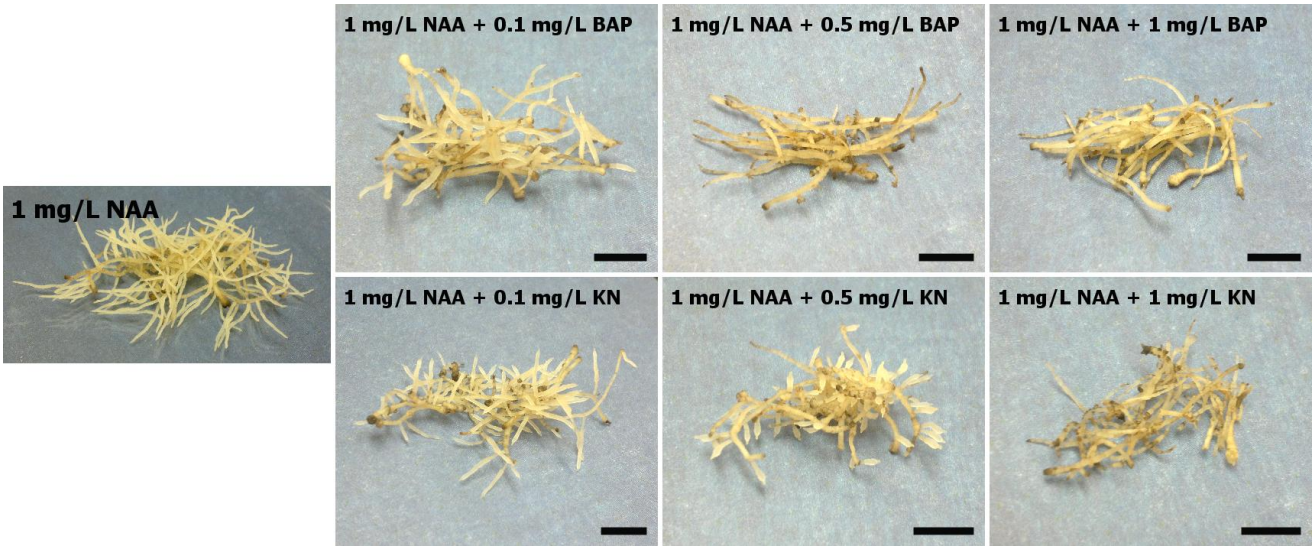

Figure 2. Proliferation of LPA adventitious root in liquid MS medium supplemented with 1 mg/L NAA in combination with BAP or KN after five weeks of culture (Bar = 5 mm, NAA = Naphthalene-acetic acid; BAP = 6-benzylaminopurine; KN = Kinetin)

CONCLUSION

This is the first report on the establishment of adventitious roots culture of *L. pumila* in liquid shake culture system for the production of biomass and secondary metabolites. Among the hormones tested, 1 mg/L NAA successfully enhanced the biomass production which leads to high metabolite yield owing to the high dry weights of the LPA adventitious roots. The combination of auxin and cytokinin negatively affected LPA adventitious roots biomass and secondary metabolite contents. Further optimizations need to be carried out in order to maximize the phenolic and flavonoid production in adventitious roots culture of *L. pumila.*

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