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Assessing the Growth Performance of *In-Vitro* Tissue Culture Plantlets of *Labisia pumila* in Mixed Soil Media over Extended Cultivation Periods

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ABSTRACT

The medicinal properties and economic value of Labisia pumila (Kacip Fatimah) make it a significant cultivation crop. Despite the promising use of the tissue culture propagation technique in enhancing the growth of L. pumila, current research findings are limited to the nursery stage. Hence, this study was performed to assess the growth performance of tissue culture plantlets of L. pumila over 30 months of cultivation period in a mixture of soil media. In-vitro plantlets were subjected to the acclimatisation stage in sphagnum peat and coir fibre media for 24 weeks (6 months). This was followed by the growth phase in a cultivation bed containing a mixture of topsoil and organic soil for 24 months. The growth parameters, including plant height, leaf area, and root length, were evaluated, while soil samples were taken for soil analysis and heavy metal detection. Results indicate distinct growth patterns during the cultivation periods and soil properties. After the 6-month acclimatisation phase, L. pumila exhibited early growth with varying degrees of vigour in the sphagnum peat and coir fibre media. By the end of the 30-month growth phase, substantial variations in growth performance were recorded, demonstrating the favourable conditions for sustained growth in soil mixture with improved soil properties. The growth performance also increased significantly, with 5.61 times taller plants, 22.14 times larger leaf areas, and 100% root growth. Remarkably, rutin was the only flavonoid compound detected in the plant extract, which is known to possess robust pharmacological properties. Overall, this study underscores the significant influence of soil properties on the growth trajectory of tissue culture plantlets of L. pumila over extended cultivation periods.

Keywords: *Labisia pumila*, tissue culture, topsoil, organic soil, growth pattern

INTRODUCTION

Labisia pumila is a herbaceous plant native to Southeast Asia and is prevalent in Malaysia. Locally known as *Kacip Fatimah*, this herbal plant has garnered significant attention in recent years owing to its renowned medicinal properties and potential health benefits, ranging from improving women's reproductive health to protecting from harmful free radicals in the body (Chua *et al*., 2012; Sanusi *et al*., 2013).

The economic significance of *L. pumila* as a potential cash crop has driven research and development in optimising cultivation methods to maximise its yield and quality (Jamaluddin *et al*., 2015). Tissue culture propagation offers a promising approach for the mass production of *L. pumila*, with genetically identical plantlets possessing desirable traits. Implementing measures to aid in product authentication would be beneficial for *L. pumila*, mainly through an integrated strategy utilising deoxyribonucleic acid (DNA) barcoding, such as internal transcribed spacer 2 (*ITS2*) and ribulose 1,5-biphosphate carboxylase (*rbc*L) (Tarmizi *et al*., 2021). Nevertheless, detailed attention to environmental factors, particularly soil properties, is essential for the successful cultivation of tissue culture plantlets, given their crucial role in determining growth and development.

Previous studies have explored soil amendments for enhancing the growth of *L. pumila* and its total phenolic compound, albeit limited to the nursery stage (Vijayanathan *et al*., 2019; Saffie *et al*., 2017). Considering the advantages of tissue culture propagation for cultivating *L.*

pumila, this study was conducted to investigate the growth performance of tissue culture plantlets of *L. pumila* over a 30-month cultivation period. The impact of soil media composition on the growth performance was also assessed by evaluating the growth parameters, such as plant height, leaf area, and root length. The evaluation of the growth patterns and examination of soil properties conducive to optimal growth in this research will contribute to the understanding of crucial factors that affect the growth performance and productivity of *L. pumila* cultivation.

MATERIALS AND METHODS

Initiation and propagation of *In-Vitro* culture

Sterilised cut leaves of *L. pumila* (leaf size = 2 cm × 2 cm) were cultured *in-vitro* on Murashige and Skoog (MS) semi-solid media supplemented with 1 mg/L of naphthaleneacetic acid (NAA). After being cultured for 36 weeks, the explants were shortened by trimming off shoot tips taller than 3 cm. The explants were subcultured in MS liquid media supplemented with 1 mg/L of NAA to allow further propagation for another 4 weeks. The culture environment for both the 36-week culture period and the 4 additional weeks included a 16-hour light and 8-hour dark cycle, with a constant room temperature of 23–25°C. Light-emitting Diode (LED) lamps (intensity level = 20 lux) were used as the source of light.

Acclimatisation phase

At the end of the 4-week subculture period, the plants were deflasked and transferred into a potting medium containing sphagnum peat and coir fibre media. The plants were carefully positioned within a seedling tray for the next 24 weeks (6 months) of the acclimatisation phase. The top cover of the seedling tray was temporarily removed twice each week for 4 weeks, allowing the plants to adapt to the new environment. Adequate watering was also ensured for proper hydration. Then, the top cover of the seedling tray was permanently removed over the remaining 20 weeks to allow new shoot establishment and root development. The average humidity during the acclimatisation phase was set at 50%, and an average temperature of 30–35°C. The plants were watered twice a day. The growth parameters of the cultivated plants, including plant height and leaf area, were recorded. The experiments were conducted in triplicates.

Growth phase on the cultivation bed

Following the 24-week (6-month) acclimatisation stage, the plantlets were transplanted into a cultivation medium containing a mixture of topsoil and organic soil (mixing ratio of 1:1) for the growth phase. The temperature and humidity were maintained around 35–38°C and 30%, respectively. The cultivation bed was constructed in a shade house covered with 70% sun-shade netting to protect the plants from direct sunlight, and at the same time relied on rainwater for adequate watering. The growth parameters of the plants, including plant height, leaf area, and root length, were meticulously monitored and measured throughout the 24-month growth phase.

Soil analysis

Approximately 200 g of soil sample was collected during the growth phase after the harvesting process and sent to a soil analysis laboratory to assess the influence of the soil properties on plant growth. Each parameter was determined according to the respective method: pH level and soil texture (pipette method), organic carbon (Wakley and Black method), available phosphorus (Bray and Kurtz method and UV-visible spectrophotometer), total nitrogen (Kjeldahl and distillation method), exchangeable cations (Inductively Coupled Plasma; ICP),

and Cation Exchange Capacity; CEC (flow analyser) in topsoil, organic soil, and mixed soil (topsoil+organic soil).

Microbial and heavy metal analysis

The leaves, stems, and roots of 30-month-old plant samples were first harvested. Then, they were washed and dried in a vacuum oven at 50°C for 3–4 hours. The dried samples (mixed leaves, stems, and roots) were ground into smaller pieces and outsourced to a commercial laboratory to conduct several microbial contamination tests, including Total Aerobic Microbial Count (TAMC), Total combined Yeasts/Moulds Count (TYMC), and *Escherichia coli* and *Salmonella* spp. detection assays. The testing methods followed the protocols in Appendix XVI B and F (British Pharmacopoeia, 2022). Meanwhile, heavy metal analysis was conducted to determine the presence of lead (Pb), cadmium (Cd), mercury (Hg), and arsenic (As) via an in-house method with reference testing based on BS EN 14084:2003 and Perkin Elmer Recommended Analytical Conditions.

High-Performance Liquid Chromatography (HPLC) analysis

Prior to HPLC analysis, ground leaf samples from 30-month-old plants were subjected to methanolic extraction. Approximately 20 mg of the dried sample was dissolved in 1 mL of methanol. Subsequently, the methanolic extract was transferred into an HPLC vial before being analysed using a WATERS 2535 quaternary gradient pump, WATERS 2707 autosampler, and WATERS 2998 PDA equipped with an HPLC Phenomenex Luna C18 column (5 µM, 250 mm × 4.6 mm). The gradient system was employed, which comprised solvent A (0.1% formic acid in water) and solvent B (acetonitrile) at a flow rate of 1 mL/min. The HPLC chromatograms were viewed at wavelengths of 254 nm. In this study, four reference standards, including gallic acid, caffeic acid, kaempferol and rutin were applied in the HPLC analysis to compare and detect the presence of respective compounds in the sample. The retention time for each standard was recorded and used as a reference, as follows: gallic acid (7.414 min), caffeic acid (13.452 min), kaempferol (25.770 min) and rutin (13.794 min).

RESULTS AND DISCUSSION

The growth analysis revealed distinct patterns across the two cultivation periods (acclimatisation and growth phase). After the initial 24-week/6 months phase in Spaghnum peat and coir fibre media, *L. pumila* exhibited vigorous growth and establishment, albeit varying vigour among individual plantlets. Subsequent transplantation into the mixed topsoil and organic soil media sustained growth over the next 30 months. These findings emphasise the crucial role of soil properties in influencing the growth and development of *L. pumila* tissue culture plantlets during the prolonged growth phase following the 6-month acclimatisation phase. Topsoil, comprising a mixture of sand (coarse and fine sand), silt, and predominantly clay, contains high organic material content. It serves as the most fertile layer and provides essential nutrients for plant root uptake. In contrast, organic soil utilised in this study comprised organic fertiliser, seedling soil, coconut fibre, roasted husk, and effective microbes. Thus, the observed growth performance underscores the rich soil composition of the mixed soil in supplying adequate nutrients and structural support conducive to plant growth.

Plant height and leaf area were measured and recorded over the 24 weeks/6 months of the acclimatisation phase, while the plant height, leaf area, and root length of the 30-month-old plantlets were harvested and measured before proceeding with downstream analysis. Based on the results, at 24 weeks/6 months of age, the average leaf area and plant height were 7.24 cm and 5.13 cm, respectively. At this stage, the roots have yet to be developed. On the contrary, at 30 months of age, the average leaf area and plant height increased significantly to 160.32 cm^2 and 28.78 cm. Additionally, the root length was 13.91 cm. As summarised in **Table 1**, the

growth performance improved substantially, with the plant height recording 5.61 times taller, the leaf area 22.14 times wider, and the root length showing a 100% increase.

Noticeable improvements in growth performance were observed after 30 months of cultivation, suggesting conducive conditions for sustained plant growth under enhanced mixed soil comprising topsoil and organic soil. Interestingly, the mixed soil exhibited considerably improved characteristics, including a pH of 5.7, carbon content of 2.92%, available phosphorus (Av. P) of 1.05 ppm, nitrogen content of 0.29%, exchangeable potassium (Exc. K) of 0.13 cmol/kg, exchangeable calcium (Exc. Ca) of 4.87 cmol/kg, exchangeable magnesium (Exc. Mg) of 1.72 cmol/kg, and CEC of 11.30 cmol/kg, as presented in Table 2. Studies have shown that N and K levels in soils impact the total phenolic compound (TPC), suggesting that the mixed soil containing adequate N and K concentration may likely contribute to the increased TPC in *L. pumila* (Ibrahim *et al*., 2013; Sharafzadeh, 2011). Past research has also revealed that the utilisation of organic composts derived from organic soils can boost the antioxidant activities of specific plants, consequently elevating their TPC levels (Fricke *et al*., 1994). It is also worth noting that the mixed soil maintained an acceptable pH level (5.70). While most tropical plants, including *L. pumila,* thrive in acidic soil conditions, excessively acidic soils can still retard the growth of certain tropical species (Pinho *et al*., 2019). Furthermore, soil pH influences soil nutrient availability, particularly micronutrients, which can affect the content of total phenolic compounds. Hence, the pH level of the mixed soil is unlikely to be a limiting factor for *L. pumila* cultivation (Norhaiza *et al*., 2009; Ibrahim *et al*., 2013).

Additionally, the microbial contamination test revealed acceptably low levels of microbial growth: 1.2×10^5 CFU/g for TAMC, 5.0×10^4 CFU/g for TYMC, and less than 10 PN/g for *E. coli* presence. In fact, *Salmonella* spp. was undetected in the dried sample. Likewise, the heavy metal analysis recorded acceptable concentrations of Pb (1.12 mg/kg), Cd (0.11 mg/kg), and As (0.27 mg/kg), Hg was undetected. The minimal microbial infection and heavy metal concentration indicate that the proposed two-phase cultivation of *L. pumila* in mixed soil yielded healthy tissue culture plantlets.

The HPLC analysis of the extracted samples only identified rutin at 254 nm with a retention time of 13.725 min (Figure 1). Rutin, also known as quercetin-3-O-rutinoside, is a glycosylated flavonoid commonly found in plants and known for its significant pharmacological properties (Chua, 2013; Chua *et al*., 2017). The demand for natural rutin has steadily increased over the years, driven by growing scientific evidence supporting its health benefits (Erlund *et al*., 2000). On the contrary, the absence of gallic acid raised concerns as it is among the highest compounds produced and easily detected in *L. pumila*. For example, a previous study demonstrated the highest yield of gallic acid, protocatechuic acid, epigallocatechin, and rutin of 0.0293, 0.0081, 0.0057, and 0.0038 mg/g DW, respectively, from *L. pumila* extract obtained through the Ultrasonic Assisted Extraction (UAE) method (Yeop *et al*., 2019). It was assumed that the extraction technique and soil nutrient content (in the mixed topsoil+organic soil) in this study could have influenced the presence of gallic acid in the extracted sample. When compared with the methanolic extraction employed in this study, the results indicate that UAE is a more effective extraction method in terms of extraction time, overall harvest yield, and extraction efficiency. Thus, future studies should consider employing UAE or other effective extraction methods to maximise the yield of the targeted bioactive compounds from *L. pumila*.

Based on the characterised soil properties and compound analysis associated with improved growth performance, this study provides valuable insights for farmers seeking to optimise the yield and quality in *L. pumila* cultivation.

Table 1. Growth performance of *L. pumila* at 6 months and 30-month-old

Plant age	Leaf area (cm^2)	Plant height (cm)	Root length (cm)
6 months	7.24	5.13	0.00
30 months	160.32	28.78	13.91

Table 2. Soil properties of topsoil, organic soil, and mixed soil

Type of soil	Wet pH	Organic carbon (%)	N (%)	Av. P (ppm)	Exc. K (cmol/kg)	Exc. Ca (cmol/kg)	Exc. Mg (cmol/kg)	CEC (cmol/kg)
Mixed soil (topsoil+organic soil)	5.7 (acidic)	2.92	0.29	1.05	0.13	4.87	1.72	11.30
Topsoil	4.6 (acidic)	0.46	0.08	0.29	0.05	0.49	0.10	14.72
Organic soil	6.7 (acidic)	2.63	0.13	1.65	1.17	4.52	2.65	14.86

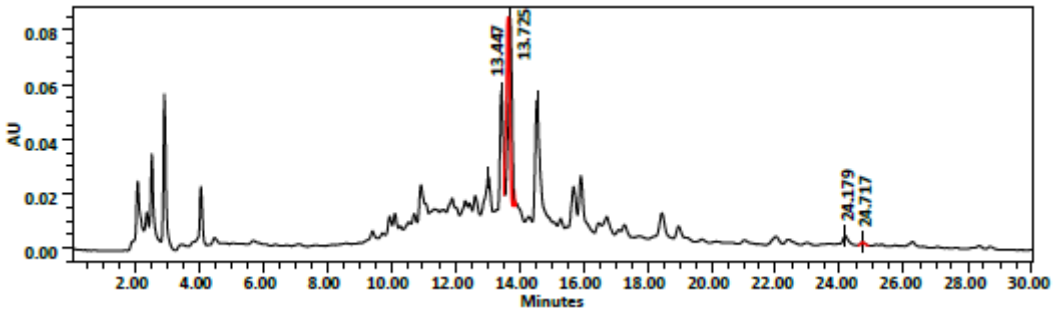

Figure 1. HPLC chromatogram detected the presence of rutin

CONCLUSION

In conclusion, this study demonstrated the growth performance of *L. pumila* in mixed soil media via *in-vitro* tissue culture propagation method over 30 months of cultivation period. This study also revealed the significant correlations between soil properties and growth parameters, with the soil pH, organic carbon content, and nutrient levels essentially influencing the growth trajectories of *L. pumila*. The plant height, leaf area, and root length recorded substantial increments under favourable mixed soil conditions characterised by optimal nutrient availability and soil texture. Furthermore, selecting appropriate potting media during the acclimatisation phase is crucial, as it facilitates faster root development by stimulating the growth of fibrous roots within the Sphagnum peat and coir fibre media through air pruning.

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