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Optimal Growth and Biomass of *Centella asiatica* Using a Twin-Bottle Temporary Immersion Bioreactor

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Abstract: *Centella asiatica* or gotu kola has long been used as a traditional medicinal plant. Here, immersion times and culture systems on growth and biomass production of *C. asiatica* were investigated using a twin-bottle, temporary immersion system. Results indicated that all immersion times gave 100% survival, with a 5 min immersion 12 times/day, providing the highest number of new shoots (3.6 shoots/explant), leaves (10.2 leaves/explant), roots (8.3 roots/explant), and fresh and dry weights of clumps (5.06 g fresh weight and 0.48 g dry weight/clump). The temporary immersion system resulted in more than a three-fold increase in biomass accumulation, with the highest average number of new shoots, leaves, and roots compared to a semi-solid system.

Keywords: *Centella asiatica*; biomass; medicinal plant; plant bioreactor

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1. Introduction

Centella asiatica, commonly referred to as gotu kola or pennywort, is an herbaceous perennial plant that originated from Asia, Australia, and South Africa [1] and has been traditionally used for medicinal purposes for centuries to treat a wide range of health conditions. Ayurvedic and traditional Chinese medicine have utilized the healing properties of this plant for skin diseases, wound healing, and even memory enhancement [1,2]. Bioactive compounds such as triterpenoids, flavonoids, and asiaticoside identified in *C. asiatica* exhibit anti-inflammatory, antioxidant, antibacterial, antiulcer, and anticancer activities [3–7]. The potential skin rejuvenating properties of *C. asiatica* have led to its incorporation as an ingredient in anti-aging products [8] that promote collagen synthesis, increase skin hydration, and improve the appearance of fine lines and wrinkles [9,10]. Conventional methods for propagating *C. asiatica* involve stem cuttings or dividing rhizomes, but these techniques have limitations for cultivation on farms. The plant is difficult to propagate in large quantities, requires specific environmental conditions, and has also shown potential for accumulating heavy metals from the soil [11,12]. To overcome these limitations, alternative propagation methods such as tissue culture and micropropagation for fast mass production of high-quality plant material have recently attracted increased interest.

Plant tissue culture technique is now widely recognized as a successful method for propagating valuable plants and has been used to produce significant quantities of pharmaceutically active compounds without the need for extensive cultivation [13]. The micropropagation of *C. asiatica* has been extensively studied [14–18]. However, the potential for large-scale propagation of plant-derived pharmaceuticals is hindered by high costs, substantial area requirements, and low production efficiency [19]. Cultivation methods for large-scale propagation must be improved. Plant bioreactors have now become essential biotechnological engineering systems for up-scaling the propagation of valuable medicinal plants [20]. The temporary immersion system (TIS) is a plant tissue culture technique that

facilitates automated propagation under sterile conditions by growing plant material in a liquid medium with recurrent immersions the aeration of the bioreactor vessel facilitate growth [21,22]. The twin-bottle TIS is a widely used commercially and it is effective for large-scale propagation of numerous medicinal plants [23,24]. The efficacy of the TIS technique is heavily reliant on the appropriate adjustment of the frequency and duration of immersion in the medium, that determine a precise protocol for plant growth, development, and yield [25–27]. However, limited studies have been conducted to optimize immersion periods and improve the production efficiency of *C. asiatica*. Therefore, this study explored timing and frequency of immersion intervals to maximize large-size production of *C. asiatica* using a twin-bottle TIS.

2. Materials and Methods

2.1. Explant Preparation

Stolon shoot tips of *C. asiatica* were pretreated for 15 min with 1% captan® and 1% carbendazim®, before rinsing with running tap water for 10 min. The explants were sterilized by immersion in 95% ethanol for 30 s, then soaked in 10% sodium hypochlorite solution for 15 min and rinsed trice with sterilized distilled water. After that, the sterilized shoot tips were cultured in Murashige and Skoog (MS) medium [28] supplemented with 15 g L^{-1} sucrose and 0.05 g L^{-1} myo-inositol. The medium pH was changed to 5.8 before sterilization by autoclaving at 121 °C for 20 min. After 2–3 weeks of culture, the new shoots arising that emerged from the buds were utilized as the initial materials for subsequent experiments. In vitro multiplication of the shoots was performed in semi-solid medium composed by the same medium up to the achievement of the sufficient number of shoots to make TIS experiments.

2.2. Effect of Immersion Time on Growth and Biomass Production of Centella asiatica

Combinations of immersion frequencies and durations were investigated for propagation efficiency and biomass production of *C. asiatica* using a twin-bottle TIS, which was developed by the Plant Tissue Culture Research Unit at Naresuan University. Medium feeding times as 3, 6, or 12 immersions/day for 1, 5, or 10 min regulated by a digital timer were evaluated. Fifteen explants of *C. asiatica* were placed into each TIS vessels (1 L glass bottle; Ø 80 cm wide mouth; DURAN®, Wertheim, Germany) and 400 mL of MS medium supplemented with 30 g L^{-1} sucrose, 0.1 mg L^{-1} myo-inositol, 1.0 mg L^{-1} 6-Benzylaminopurine (BA), 1.0 mg L^{-1} 1-Naphthaleneacetic acid (NAA), and 0.25 mg L^{-1} Gibberellic acid (GA) was added in each medium reservoir. The medium pH was changed to 5.8 before autoclaving at the same previous condition. Each TIS set was connected to air tube lines attached with solenoid valves. The liquid medium dislocated by air pressure was transported from medium reservoir to plant vessel. The air pressure from the air pump (oil-free air compressor, 1000 W, 30 L) was sterilized by a membrane air filter (0.2 µm pore; PTFE; Acro®50, New York, NY, USA), connected with the silicone tube on top of each container. Small glass beads (3 mm diameter) were added in the plant chamber as a supportive material for the trig explants. All treatments were cultured under warm-white LED lamps (T8, 18 W, Philips, Amsterdam, The Netherlands) at 40 µmol m^{-2} s^{-1} light intensity for a 12 h photoperiod. All TIS vessels remained in a growth room where the temperature was maintained at 25 ± 2 °C for a duration of 5 weeks. All experiments were accomplished with three replicates. Biomass accumulation of *C. asiatica* was monitored after 5 weeks of culture.

2.3. Comparison of Different Culture Systems on Growth and Biomass Production of Centella asiatica

Semi-solid system (SSS) and temporary immersion system (TIS) were investigated to compare the propagation efficiency of *C. asiatica*. MS medium supplemented with 30 g L^{-1} sucrose, 0.1 mg L^{-1} myo-inositol, 1.0 mg L^{-1} BA, 1.0 mg L^{-1} NAA, and 0.25 mg L^{-1} GA was used as propagation medium. pH of the medium was changed to 5.8 by 1 N NaOH or

HCl and the medium was sterilized by autoclaving at the same conditions mentioned above. For the SSS treatment, two explants were placed in 4 oz glass bottles containing 20 mL of semi-solid medium, solidified with 2.0 g L^{-1} of gelrite. For the TIS treatment, fifteen explants were cultured into vessels containing 400 mL of liquid medium in the medium reservoir. TIS treatment was set to feed 12 times/day for 5 min/time. Each experiment was undertaken in three replicates, one set of TIS and thirty bottles of SSS/replicate. The culture systems were maintained in a growth room at 25 ± 2 °C and a 12 h photoperiod using warm-white LED lamps with 40 µmol m^{-2} s^{-1} of light intensity. After 5 weeks of incubation, the growth and biomass production of *C. asiatica* were evaluated and compared.

2.4. Experimental Design and Data Analysis

All experiments were performed using a completely randomized design. To evaluate significant differences between each parameter, one-way ANOVA was employed, followed by Duncan's new multiple range test (DMRT) with a significance level of $p \leq 0.05$.

3. Results

Different growth rates of *C. asiatica* were observed in TIS after 5 weeks of culture (Figure 1). All TIS treatments showed 100% survival rate (Table 1). Results indicated that the propagation and elongation of *C. asiatica* were influenced by the flooding frequency and duration, with a 5 min immersion 12 times/day the highest number of new shoots (3.6 shoots/explant), leaves (10.2 leaves/explant), and roots (8.3 roots/explant) were recorded (Table 1). Decreased frequency of immersion (3–6 times/day) led to reduced growth (Table 1 and Figure 1), while increase in immersion duration generally resulted in a high rate of shoot proliferation, except when using the highest immersion frequency (12 times/day) (Table 1). Using 12 immersions/day resulted in the most significant leaf elongation, exceeding 10 cm/leaf (Table 1 and Figure 1), while decrease in immersion frequency and duration resulted in decline of these growth parameters. About the parameters related to biomass production, the fresh and dry weights demonstrated similar trends to plantlet leaf elongation. The highest fresh and dry weights were achieved using 12 immersions/day for 5 min each (5.06 g fresh weight and 0.48 g dry weight/clump) (Table 1). For the total amount of dry weight obtained from each culture container, using 12 immersions/day for 5 min each time provided the highest achievable dry weight/container at 7.27 g (Table 1). The total dry weight obtained/container was highest when using 5 min of immersion compared to the other immersion times (Table 1 and Figure 1).

To determine the optimal cultivation system for biomass production of *C. asiatica*, semi-solid and temporary immersion systems were compared. After 5 weeks of culture, results indicated that *C. asiatica* seedlings responded differently to diverse culture systems (Figure 2). Survival rates were 100% in both culture systems (Table 1). The TIS treatment demonstrated the highest average number of *C. asiatica* seedling new shoots, leaves, and roots with 3.6 shoots/explant, 10.2 leaves/explant, and 8.3 roots/explant, which was three-fold higher than in the semi-solid medium (Table 1). In the TIS treatment, leaf elongation was over 10.2 cm and significantly higher than the semi-solid medium (3.3 cm/leaf) (Table 1). Biomass accumulation was also statistically significantly different, with the highest values recorded in the TIS treatment for clump fresh and dry weights at 5.06 g and 0.48 g, respectively. Total dry weight of 7.27 g/container was also obtained from the TIS treatment (Table 1).

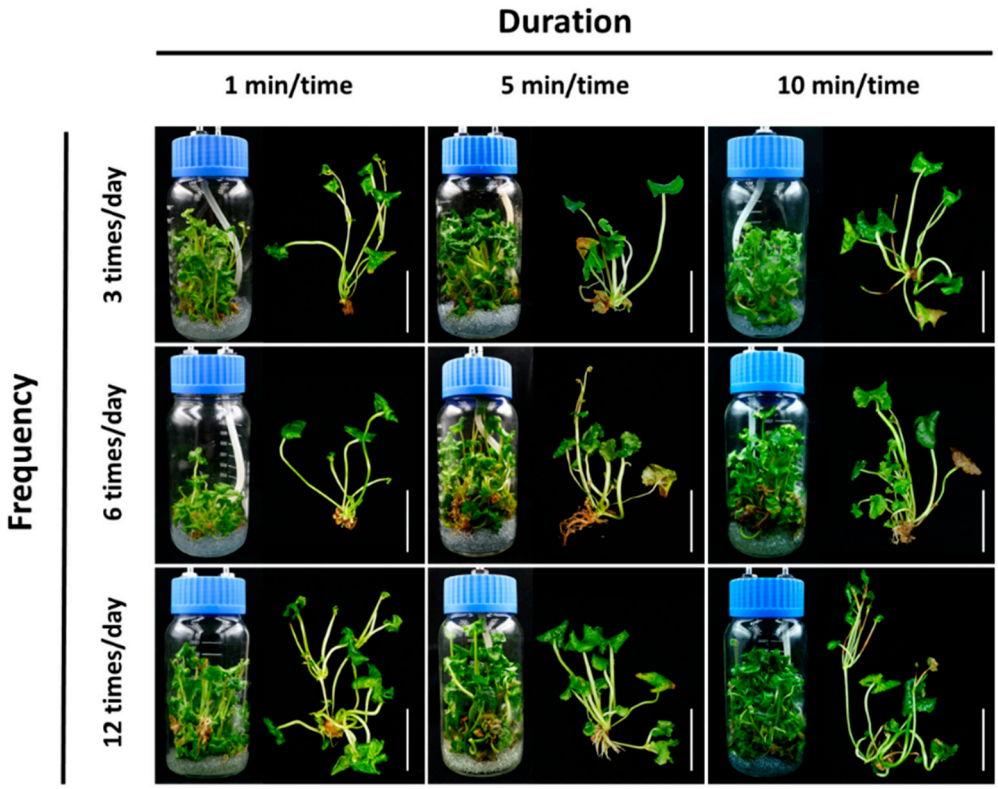

Figure 1. Growth and development of *Centella asiatica* shoots after 5 weeks of cultivation under different immersion frequencies and durations. One bottle contained 15 explants; scale bar = 5 cm for comparison of individual plantlet.

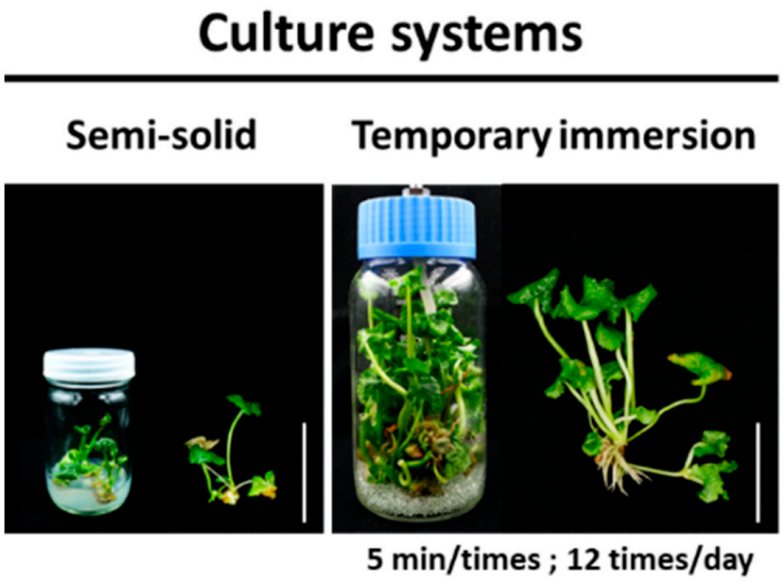

Figure 2. Growth and development of *Centella asiatica* after 5 weeks of cultivation using semi-solid or temporary immersion systems supporting material with small glass beads in the bottom. Scale bar = 5 cm.

Table 1. Survival and growth parameters of *Centella asiatica* after 5 weeks of cultivation.

Factors	Survival	Number/Explant [a]			Leaf Length [a]	Clump (g/Clump) [a]		Total Dry Weight [b]
	(%)	Shoots	Roots	Leaves	(cm)	Fresh Weight	Dry Weight	(g/Replication)
Temporary immersion								
3 times/day for 1 min	100 ± 0.0 ^{ns}	2.6 ± 0.0 ^{abc}	3.7 ± 0.0 ^{c}	6.2 ± 0.1 ^{bc}	7.2 ± 0.1 ^{cd}	2.05 ± 0.02 ^{d}	0.18 ± 0.02 ^{c}	2.75 ± 0.87 ^{c}
5 min	100 ± 0.0	2.2 ± 0.0 ^{bc}	3.4 ± 0.1 ^{c}	7.0 ± 0.1 ^{b}	8.6 ± 0.2 ^{bc}	3.22 ± 0.10 ^{bc}	0.31 ± 0.02 ^{b}	4.72 ± 1.03 ^{b}
10 min	100 ± 0.0	2.6 ± 0.1 ^{abc}	3.8 ± 0.6 ^{c}	6.6 ± 0.2 ^{b}	7.1 ± 0.3 ^{cd}	2.05 ± 0.15 ^{d}	0.17 ± 0.01 ^{c}	2.60 ± 0.70 ^{c}
6 times/day for 1 min	100 ± 0.0	1.5 ± 0.1 ^{c}	4.6 ± 0.5 ^{bc}	4.2 ± 0.1 ^{c}	5.8 ± 0.1 ^{d}	0.81 ± 0.09 ^{e}	0.07 ± 0.00 ^{d}	1.02 ± 0.14 ^{d}
5 min	100 ± 0.0	2.5 ± 0.1 ^{abc}	10.9 ± 0.3 ^{a}	8.4 ± 0.1 ^{ab}	9.4 ± 0.2 ^{ab}	4.20 ± 0.11 ^{ab}	0.44 ± 0.01 ^{a}	6.54 ± 0.43 ^{a}
10 min	100 ± 0.0	3.1 ± 0.2 ^{ab}	3.7 ± 0.2 ^{c}	8.1 ± 0.3 ^{ab}	9.2 ± 0.1 ^{ab}	3.12 ± 0.05 ^{bcd}	0.32 ± 0.00 ^{b}	4.74 ± 0.24 ^{b}
12 times/day for 1 min	100 ± 0.0	3.6 ± 0.3 ^{a}	4.8 ± 0.4 ^{bc}	8.4 ± 0.6 ^{ab}	7.5 ± 0.2 ^{c}	2.48 ± 0.29 ^{cd}	0.23 ± 0.02 ^{bc}	3.39 ± 1.29 ^{bc}
5 min	100 ± 0.0	3.6 ± 0.2 ^{a}	8.3 ± 1.2 ^{ab}	10.2 ± 0.2 ^{a}	10.7 ± 0.2 ^{a}	5.06 ± 0.13 ^{a}	0.48 ± 0.01 ^{a}	7.27 ± 0.60 ^{a}
10 min	100 ± 0.0	3.2 ± 0.1 ^{ab}	4.6 ± 0.5 ^{bc}	7.8 ± 0.6 ^{b}	9.3 ± 0.4 ^{ab}	4.60 ± 0.27 ^{a}	0.43 ± 0.02 ^{a}	6.44 ± 1.17 ^{a}
Culture systems								
Temporary immersion	100 ± 0.0 ^{ns}	3.6 ± 0.2 *	8.3 ± 1.2 *	10.2 ± 0.2 *	10.7 ± 0.2 *	5.06 ± 0.13 *	0.48 ± 0.01 *	7.27 ± 0.60 *
Semi-solid	100 ± 0.0	1.3 ± 0.0	0.2 ± 0.0	3.6 ± 0.0	3.3 ± 0.0	0.46 ± 0.02	0.05 ± 0.00	0.68 ± 0.11

The same letters within a row are not significantly different at $p \leq 0.05$ according to DMRT. ns; not significantly different. [a] Values are mean ± SE of three replications (15 explants/replication). [b] Values are mean ± SD of three replications (15 explants/replication). * Significant differences between culture systems of each parameter according to the independent *t* test at $p \leq 0.05$.

4. Discussion

Temporary immersion system (TIS) has now become a successful alternative plant propagation method to traditional plant bioreactors for large-scale production of horticultural crops using plant tissue culture techniques [29–31]. The plant material is periodically immersed in a liquid medium, and the optimal immersion time promotes nutrient uptake with increased plant multiplication rates. The critical parameters for the efficiency of TIS in terms of multiplication rate and yield are the frequency and duration of immersion [21,23,32]. Many researchers have attempted to establish the optimal procedures for the mass propagation and biosynthesis of secondary metabolites in medicinal plants such as *Eurycoma longifolia* [33], *Rhododendron tomentosum* [34], *Lucas aspera* [35], *Stevia rebaudiana* [36], and *Anthurium andreanum* [37].

Different TIS immersion frequencies and durations had no effect on the survival rate of *C. asiatica*. Results indicated that *C. asiatica* showed optimal growth and biomass at high immersion frequency of 12 times/day for 5 min each compared with the other feeding conditions. Frequent immersion of the culture vessel creates a microenvironment with specific conditions such as humidity and liquid pressure that promote growth and development of the shoot. By contrast, previous research indicated that the incidence of physiological disorders was observed to rise when explants were frequently immersed in the liquid medium [26]. Both the frequency and duration of the immersion process had an impact on the absorption of nutrients and the renewal of gases within the internal culture vessel [21]. Perez-Alonso et al. [38] improved growth and secondary metabolite production in *Digitalis purpurea* using TIS. Their results confirmed that fresh and dry weight varied during culture under different immersion frequencies. Likewise, high immersion frequency (every 3 h) led to a higher multiplication growth rate, whereas a shorter frequency of immersion (every 7 h) promoted the height of the shoot in Musa AAB plantlets [39]. Kunakhonnuruk et al. [24] and Vendrame et al. [40] indicated that the biomass of plantlets cultivated with frequent and prolonged immersion times demonstrated significantly higher growth compared to other feeding times. It is possible that this is because the plantlets have more time to contact with the culture medium, allowing them to accumulate and absorb nutrients, sugars, and water.

To evaluate the effect of the culture system on the in vitro mass propagation efficiency of *C. asiatica*, a comparison was made between the semi-solid and temporary immersion systems. The use of microcontainers for large-scale production of medicinal plants is not a cost-effective option, whereas bioreactors are expensive for mass production [29,41]. The experiments showed that TIS was an optimized culture system for large-scale production of *C. asiatica* due to its ability to increase growth and biomass accumulation. Shoots obtained from TIS showed improved, leaf, and root numbers, with increased biomass compared to conventional culture systems. The TIS micropropagation technique has been shown to be advantageous for growing various plant species. The intermittent contact between the liquid medium and explants provides essential nutrients and oxygen, reduces the accumulation of toxic gases and hydrodynamic forces, and enhances the overall quality of the plants [25,42,43]. Bayrakter [36] improved growth of *Stevia rebaudiana* shoot using TIS more than in a small culture container. TIS micropropagation of *Lessertia frutescens* L. provided a suitable multiplication rate of shoot compared with a continuous immersion system (CIS) and SSS [44], while SSS growth in microcontainers obstructed shoot proliferation and elongation of *C. asiatica*. Escalona et al. [45] found that the periodic exchange of gases within the culture vessel and intermittent contact between the explants and liquid medium in TIS improved photosynthesis in the plants. Improved aeration and CO_2 concentration within culture containers were also found in TIS than in SSS systems [29,41]. Zhao et al. [46] informed that the utilization of TIS with forced ventilation was found to be effective in reducing morphological disorders, enhancing shoot quality and increasing the multiplication rate of medicinal plants, *Rhodiola cremulata*, while Aragon et al. [47] and Jova et al. [48] found that TIS resulted in a higher photosynthetic rate and optimal growth of the plantlets, as compared to continuous immersion. The TIS technique combined the

benefits of both gelling and liquid media, leading to an overall improvement in the growth of the plantlets [21,24,49].

5. Conclusions

This is the first report providing important information on *C. asiatica* cultivation using a temporary immersion bioreactor. The temporary immersion system generated a large biomass in a small space, with optimal immersion times, stimulating growth and multiplication, giving improved biomass production of *C. asiatica* in an in vitro culture. Growth and biomass *C. asiatica* using the temporary immersion system method was higher than the conventional propagation technique in a microcontainer. Moreover, this study reported that the TIS could be a promising approach for large-scale commercial production of *C. asiatica* to meet the increasing demand in the pharmaceutical industry.

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