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Research article

Impact of culture systems on growth and plumbagin yield of *in vitro Drosera binata* Labill.

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Abstract

Importance of the work: Guidelines are required for the conservation of the carnivorous plant *Drosera binata* Labill. in the wild, to ensure sufficient supply for pharmaceutical use.

Objectives: To compare the effects of different cultivation systems on the *in vitro* growth, development and plumbagin production of *D. binata*.

Materials and Methods: Young shoots of *D. binata* were cultured using three different cultivation systems: semi-solid (SSS), continuous immersion (CIS) and temporary immersion (TIS). Half-strength Murashige and Skoog medium was utilized in all three culture systems. The growth and plumbagin production were examined at the 8th week of culture.

Results: CIS promoted superior growth and development compared to SSS and TIS, as evidenced by higher fresh and dry weights per clump and an increased shoot number. However, the leaf length was greatest using TIS. Considering growth performance per replication at the 8th week of culture, explants cultured in CIS had significantly higher numbers of shoots, as well as greater fresh and dry weights, than those cultured in SSS or TIS. In addition, the maximal biomass using CIS was more than twice that from TIS or SSS. The highest plumbagin production was obtained from TIS, followed by CIS and SSS.

Main finding: The findings should serve as a useful foundation for the tissue culture of *Drosera* species, ensuring sufficient supply for future conservation in nature and the production of pharmaceutical compounds.

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Introduction

Drosera binata Labill., a carnivorous plant belonging to the Droseraceae family, is native to New Zealand, occurring on the North, South, Stewart and Chatham Islands, as well as being found in Australia (Thorsen et al., 2009). Its striking appearance makes it a popular ornamental plant (Junipers et al., 1989). Notably, this species contains medicinally valuable compounds, particularly naphthoquinones such as plumbagin, that exhibit anticancer, inflammation and antioxidant activities, leading to their widespread use in traditional medicine (Tilak et al., 2004; Checker et al., 2010; Xu and Lu 2010). *D. binata* is known to accumulate high levels of plumbagin (Marczak et al., 2005). However, natural populations of *D. binata* face major challenges. The plant has low seed production and poor survival rates due to its restricted growth period during the rainy season and a general decline in populations (Baranyai and Joosten, 2016). Furthermore, human activity, natural disasters and environmental changes threaten this genus, exacerbating the population decline and contributing to a shortage of raw materials for plumbagin production and hinder consistent, year-round availability (Kavi Kishor et al., 2025). *D. binata* propagates naturally through sexual reproduction via seeds and asexually via division; however, these methods yield limited quantities and are slow (Fretwell., 2005). Consequently, propagation approaches, such as plant tissue culture, offer a promising alternative for large-scale propagation. Plant tissue culture enables the production of larger quantities of plants in a shorter timeframe, effectively meeting demand. While plant tissue culture has been applied successfully to propagate various plant species, including endangered commercial plants and herbs, and initial successes have been achieved with *Drosera* (Hussain et al., 2012), current methods have not yet produced sufficient additional *Drosera* plant numbers or plumbagin production to satisfy existing needs and the present production levels remain inadequate to compensate for the rapid decline of natural populations and support effective conservation efforts (Kunakhonnuruk et al. (2019). Therefore, further research is crucial to ensure the sustainable production of *D. binata* and plumbagin through the optimization of culture systems to underpin the availability of sufficient plants for both plumbagin production and long-term utilization.

There are three widely used primary culture systems: semi-solid (SSS), continuous immersion (CIS) and temporary immersion (TIS). Numerous studies have compared these systems across various plant species. For example, Martínez-Estrada et al. (2019) found that TIS was the most effective for shoot proliferation in *Anthurium andreanum* Linden ex André. surpassing CIS and SSS. In tulips, An et al. (2016) reported high shoot regeneration rates with both TIS and CIS (80.86% and 95.21%, respectively), but TIS yielded greater plant height. Similarly, Ramos-Castellá et al. (2014) demonstrated that TIS promoted the highest number of shoots, shoot size and leaf count in *Vanilla planifolia* Andrews. Furthermore, research has explored the impact of these systems on the production of valuable compounds. Grzegorczyk-Karolak et al. (2017) observed that TIS resulted in the highest baicalin yield in *Scutellaria alpina*. Likewise, Kunakhonnuruk et al. (2019) documented that TIS maximized plumbagin production in *Drosera communis*, achieving 17.31 µg per replication. Collectively, these studies underscore the major impact of culture systems on plant growth and development across various species. Consequently, the current study aimed to assess the impact of various culture systems on the *in vitro* growth, morphological development and plumbagin production of *D. binata*. The findings should provide valuable insights into the optimal cultivation of *Drosera* species under *in vitro* conditions. In addition, these results should inform practical guidelines for the efficient and rapid production of this plant in sufficient quantities to meet demand, thereby facilitating the replacement of wild populations and supporting pharmaceutical applications.

Materials and Methods

Plant material preparation

The *in vitro* young shoots of *D. binata* explants at age 4 wk, with 2–3 cm height and 5–6 leaves, were transferred to containers containing semi-solid, half-strength Murashige and Skoog (1962) medium supplemented with 10 g/L of sucrose and 2 g/L of gelrite. They were cultured for 5–7 d to check for contamination and to select uniform explants.

Semi-solid, continuous immersion and temporary immersion of D. binata

A half-strength Murashige and Skoog (1962) medium supplemented with 30 g/L sucrose and 0.1 g/L myo-inositol was used for the *in vitro* culture. The medium was adjusted to pH 5.8 prior to sterilization in an autoclave at 121°C for 15 min. In the SSS, one explant was cultured in a four-ounce glass bottle containing 20 mL of medium solidified with 2 g/L Gelrite (Gelzan™G3251). For the CIS, one explant was cultured in a 125 mL conical flask containing 20 mL of liquid medium

and placed on a rotary shaker at 110 rpm. For TIS, a twin-bottle type was used. Then, 20 explants produced for each of the three systems were cultured in the growth chamber and 400 mL of culture medium (equivalent to 20 mL per explant) were added to the medium reservoir. The medium was pumped into the growth vessel every 4 hr., with the leaves being immersed for 5 min before the medium was drained out. All culture systems were maintained at 25 ± 2°C under a warm-white light-emitting diode lamp, with a light intensity of 40 µmol/m²s for 12 hr. daily. Each culture system consisted of three replications, with 20 pre-induction leaves used for each replication.

Examination of growth and biomass

At the 8th week of culture, the numbers of shoots and roots per explant, leaf length and root length of each shoot were recorded. Fresh weight was measured after drying the clumps with tissue paper and dry weight was measured after drying the clumps at 50°C in a hot-air oven for 2 d.

Plumbagin content analysis

Plumbagin extraction and analysis were carried out following the protocol described by Wongsa et al. (2018). A 0.1 g portion of fine, powdered sample was mixed with 1 mL of methanol based on vortexing for 1 min and then sonicating in an ultrasonic water bath (Sonicor® DSC-120TH; USA) at 35 kHz for 30 min at room temperature. After that, the mixture was centrifuged at 10,000 revolutions per minute (rpm) for 3 min before collecting the supernatants in a new tube. Then, the residue was extracted twice. The supernatants were pooled and then dried in a vacuum oven (LabTech LVO-2030; Republic of Korea) at 40°C. The crude extracts were reconstituted in 1 mL of methanol and subsequently passed through a 0.45 µm nylon membrane filter (ANPEL Laboratory Technologies; China). The plumbagin content in the filtered extract was determined using high-performance liquid chromatography (HPLC) with an Agilent 1260 Infinity II LC System (Agilent Technologies; Germany) equipped with an Agilent G7116A photodiode array detector (Agilent Technologies; Germany). A 10 µL aliquot of each sample was injected automatically into the HPLC system. Separation was performed using a Purosphe® STAR RP-18 end-capped column (250 mm × 4 mm internal diameter, 5 µm particle size; Waters; USA). The mobile phase consisted of methanol and 0.1 M aqueous acetic acid in a ratio of 45:55 (volume per volume) and was delivered at a flow rate of 1.0 mL/min. The isocratic mobile phase was delivered at a flow rate of 0.75 mL/min at room temperature and plumbagin was detected at 270 nm using an ultraviolet detector. The plumbagin content in each injection was quantified using the regression equation derived from the standard curve, which was generated by plotting peak areas against the concentration of genuine plumbagin (CAS No: 481-42-5; Sigma-Aldrich; USA). Each sample was analyzed in triplicate.

Experimental design and data analysis

The experiment was set up using a completely randomized design. Differences in each parameter were statistically analyzed using one-way analysis of variance, followed by Duncan's new multiple range test for mean comparison, with significant differences tested at $p < 0.05$.

Results

Growth and biomass performance in different culture systems

At the 8th week of culture, all young shoots survived and successfully developed into shoot clumps across the various culture systems, although the rates of growth and proliferation differed between the systems. CIS promoted better growth and development of single shoots separated from individual clumps than TIS and SSS (Fig. 1). The significantly highest fresh weight of a *D. binata* individual clump was obtained from CIS at 0.92 g fresh weight (FW) per clump (Fig. 2A). However, there were no significant differences in dry weight per clump among the culture systems used. (Fig. 2B). Furthermore, the highest number of shoots per clump was obtained from CIS (12 single shoots per clump) followed by SSS (7.26 single shoot per clump) and TIS (6.88 single shoots per clump) (Fig. 2C). There was an increase in root numbers, with the CIS treatment inducing the highest new root production per clump (6.9 roots per clump), approximately 2–3 times greater than SSS and TIS; however, there were no significant differences in root length among the three systems (Figs. 2D–2E). The TIS treatment produced a significantly greater leaf length (7.26 cm per leaf) than the SSS and CIS treatments (Fig. 2F). Regarding growth performance per replication (Table 1), Of the three systems, CIS produced a significantly higher number of shoots (240.3 shoots per replication) and fresh weight (21.5 g per replication), approximately two times greater than SSS (144.7 shoots per replication, 10.7 g FW per replication) and TIS (137.7 shoots per replication, 11.2 g FW per replication). Nonetheless, there were no significant differences in dry weight per replication among the culture systems.

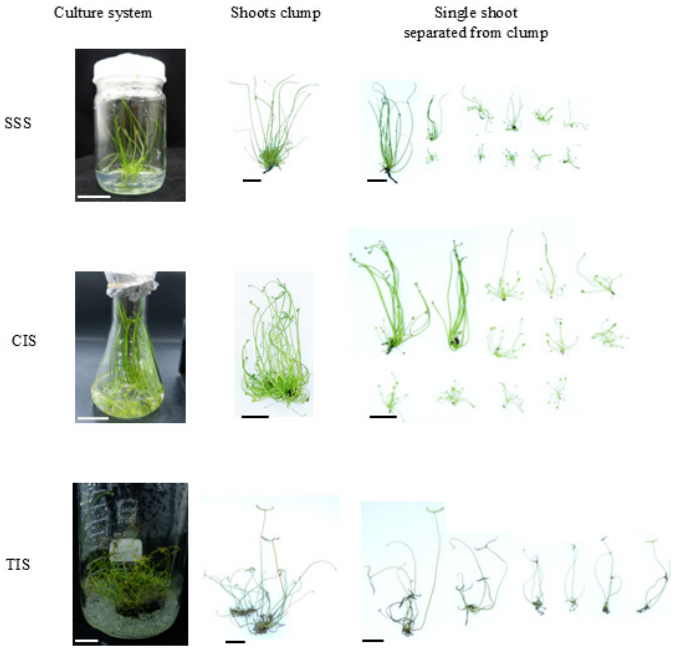

Fig. 1 Growth and shoot proliferation (shoot clumps and single shoots) from young shoots of *Drosera binata* after 8 weeks of culture using SSS (semi-solid system), CIS (continuous immersion system), or TIS (temporary immersion system). The basal medium was half-strength Murashige and Skoog (1962). For TIS, the medium was supplied every 4 hr with a 5-min immersion period. Scale bar = 2 cm.

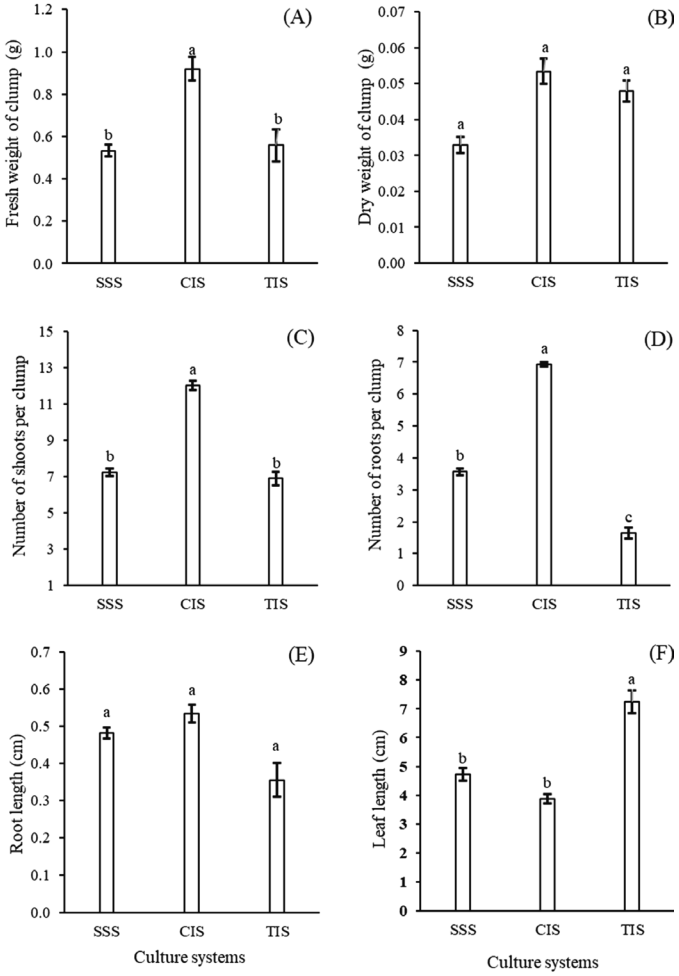

Fig. 2 Bar charts of *Drosera binata* at the 8th week of culture in different culture systems: (A) fresh weight per clump; (B) dry weight per clump; (C) shoot number per clump; (D) root number per clump; (E) root length; (F) leaf length. SSS = semi-solid system; CIS = continuous immersion system; and TIS = temporary immersion system. Results are mean ± SD values of three replicates, with each replicate consisting of up to 20 plantlets depending on survival in each treatment. Different lowercase letters above the bars indicate significant differences ($p < 0.05$), according to DMRT.

Table 1 Number of shoots, biomass per replicate and plumbagin production of *Drosera binata* at the 8th week of culture in three different systems: SSS (semi-solid system), CIS (continuous immersion system) and TIS (temporary immersion system).

Growth parameter (per replication)	Culture system			P-values
	SSS	CIS	TIS	
Number of shoots	144.7 ± 9.7^{b}	240.3 ± 13.3^{a}	137.7 ± 20.0^{b}	14.62
Fresh weight (g)	10.7 ± 1.4^{b}	21.5 ± 2.8^{a}	11.2 ± 1.3^{b}	4.46
Dry weight (g)	0.7 ± 0.1^{a}	1.1 ± 0.2^{a}	1.0 ± 0.2^{a}	1.96
Plumbagin production	SSS	CIS	TIS	
Content (µg/g DW)	5.42 ± 1.4^{b}	85.50 ± 80.5^{ab}	254.97 ± 44.7^{a}	5.746
Yield (µg/clump)	0.29 ± 3.6^{b}	3.76 ± 0.1^{b}	11.54 ± 0.2^{a}	7.546
Yield (µg/replication)	5.85 ± 1.6^{b}	75.19 ± 72.5^{b}	230.76 ± 4.0^{a}	7.541

(3 replicates, 20 explants per replicate) within the same row with different lowercase superscript are significantly different ($p < 0.05$), according to DMRT. DW = dry weight.

Plumbagin production of D. binata in different culture systems

Plumbagin was clearly separated from the other sample components using HPLC, showing sharp, symmetrical peaks with a retention time of around 26.2 min (Fig. 3A). The standard curves for plumbagin were linear over the range 0.625–2.5 mg/mL, which could be described by the regression equation: Y = 1,609.08909X + 21.950 (coefficient of determination = 0.99983), where Y is the peak area of the analyte and X is the concentration in milligrams per milliliter.

The plumbagin content and yield were evaluated at the 8th week of culture. The TIS culture system prodcued the highest plumbagin content (254.97 µg/g dry weight, DW), significantly exceeding that of CIS (85.50 µg/g DW) and SSS (5.42 µg/g DW) (Table 1). Similarly, the plumbagin yield per clump was significantly higher for TIS (11.54 µg per clump) than for CIS (3.76 µg per clump) and SSS (0.29 µg per clump). A system comparison of yield productivity showed that TIS produced approximately twice as much as CIS and 10 times that of SSS. Furthermore, the plumbagin yield per replication was significantly elevated in TIS (230.76 µg per replication) compared to CIS (75.19 µg per replication) and SSS (5.85 µg per replication).

Discussion

Studies comparing culture systems across various plant species have consistently shown that species exhibit unique responses to different systems (Jones et al., 2007; Lulu et al., 2015; Yancheva et al., 2019; Hwang et al., 2022). In the current study, CIS proved to be the most effective system for *in vitro* micropropagation of *D. binata*, outperforming both TIS and SSS methods. Specifically, CIS yielded higher numbers of shoots and roots, as well as greater fresh and dry weights than the other two systems. These findings aligned with *D. binata*'s natural habitat of continually wet bogs and swamps (Gilbert, 1984). Liquid culture systems, such as CIS, offer several advantages over solid cultures, including accelerated growth rates, efficient nutrient and growth regulator uptake and the dilution of inhibitory exudates, such as phenolic compounds (Martínez-Estrada et al., 2019).

Despite the observed differences in shoot and root development, CIS promoted a fresh weight higher than the other two systems. However, there were no significant variations in dry weight biomass among the three cultivation systems. Notably, the temporary immersion system (TIS) promoted the greatest leaf length and the fastest overall shoot growth in *D. binata*. A key advantage of TIS lies in its enhanced ventilation within the culture vessel that allows for the removal of volatile compounds, such as ethylene (Roels et al., 2006), while simultaneously facilitating the recirculation of the carbon dioxide necessary for photosynthesis (Aragón et al., 2014). The duration and frequency of immersions in TIS significantly influence nutrient availability, the internal atmosphere of the culture vessel and the occurrence of hyperhydricity (Teisson et al., 1996). Fundamentally, TIS's effectiveness stems from its ability to provide optimal ventilation and intermittent contact between the plant tissues and the liquid medium (Berthouly and Etienne, 2005).

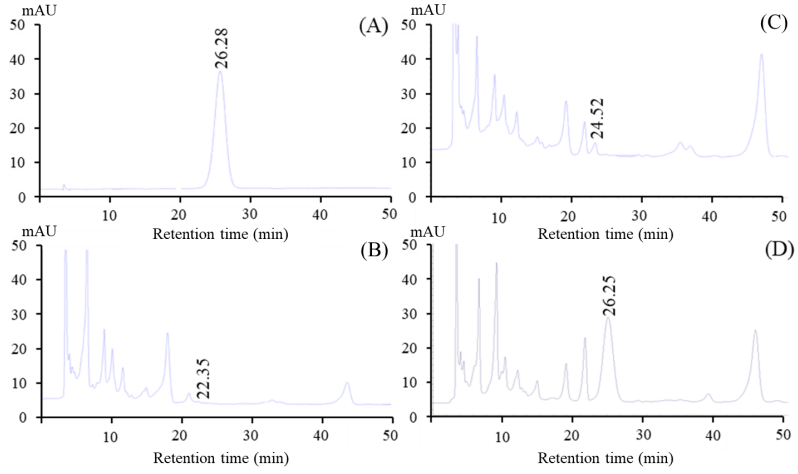

Fig. 3 High-performance liquid chromatography (HPLC) chromatograms of (A) the plumbagin standard; (B) *Drosera binata* sample extracts at the 8th week of culture in the semi-solid system; (C) the continuous immersion system; and (D) the temporary immersion system. The plumbagin peak (retention time 22.35–26.28 min) was detected at a wavelength of 270 nm.

While the three culture systems yielded comparable amounts of biomass, analysis of plumbagin content revealed that TIS produced significantly higher levels per gram dry weight compared with the SSS and CIS methods (Table 1). This likely was due to the greater maturity of the *D. binata* plants cultured in TIS (Fig. 1). Mature leaves, known to be the primary site of plumbagin accumulation in *D. binata*, are capable of producing and storing higher concentrations of secondary metabolites (Kunakhonnuruk et al., 2019). Similarly, TIS yielded the highest amount of plumbagin per shoot clump and per container. Furthermore, TIS required less operational time and labor, particularly for subculturing in small containers, leading to considerable cost reductions compared to SSS and CIS. Based on these results, TIS is a suitable method for the large-scale production of *D. binata* as a raw material for plumbagin production. Therefore, TIS represents a suitable system for potential future applications in the micropropagation of various other medicinal plant species.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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