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Hypocotyl-derived adventitious root in *Boesenbergia rotunda:* Histological insight and medium optimization

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Abstract. *Ghani KA, Azhar SZA, Khalid N, Yusuf NA. 2026. Hypocotyl-derived adventitious root in* Boesenbergia rotunda*: Histological insight and medium optimization. Asian J Agric 10 (1): g100111. https://doi.org/10.13057/asianjagric/g100111. Boesenbergia rotunda*, commonly known as fingerroot, is a medicinal ginger widely valued for its bioactive flavonoids such as panduratin A, cardamonin, and pinostrobin, which exhibit antimicrobial, antioxidant, and anticancer activities. Despite its importance, large-scale pharmaceutical use is constrained by low and inconsistent yields from rhizomes under field conditions. Adventitious Root (AR) cultures represent a promising alternative for scalable biomass and metabolite production, yet the potential of hypocotyl explants remains underexplored. This study investigated the effectiveness of different culture medium types in inducing AR formation from in vitro hypocotyls of *B. rotunda*. Hypocotyl explants were cultured for four weeks on solid and liquid half-strength Murashige and Skoog (½ MS) medium supplemented with 0.5 mg/L Naphthaleneacetic Acid (NAA) (n=6). Induction rate, root number, and root length were quantified. Results showed that liquid medium significantly enhanced AR induction, achieving 100% response compared to 95% in solid medium, with more than twice the number of roots (29.14±2.91 vs. 12.25±2.82) and nearly doubled root length (1.96±0.25 cm vs. 0.94±0.05 cm). Histological analysis confirmed that root apical meristems originated directly from pericycle cells adjacent to phloem tissues, evidencing direct organogenesis. These findings provide the first histological validation of hypocotyl-derived AR in *B. rotunda* and highlight the exciting potential of the liquid culture system for future biotechnological applications and metabolite production.

Keywords: Adventitious root cultures, *Boesenbergia rotunda*, histology, hypocotyl, *temu kunci*

Abbreviations: AR: Adventitious Root, HCl: Hydrochloric acid, MS: Murashige and Skoog, NAA: Naphthaleneacetic Acid, NaOH: Sodium hydroxide, RAM: Root Apical Meristem, TIS: Temporary Immersion Systems

INTRODUCTION

Most plant species belonging to the Zingiberaceae (ginger) family are recognized for their valuable medicinal attributes and have been broadly employed in Ayurveda and ethnomedicines (Kumar et al. 2013). *Boesenbergia rotunda* (L.) Mansf., or finger root, is a ginger species with unique physical features and an aromatic smell. The perennial rhizomatous herb is found in shaded and humid areas across Asian countries, predominantly India, China, and Southeast Asia regions, including Indonesia, Thailand, and Malaysia (Veldkamp 2013).

The underground rhizomes and tubers of *B. rotunda* have previously been used to treat wounds, inflammation, and gastrointestinal-related problems (Morikawa et al. 2008; Tan et al. 2012). Earlier research showed that the rhizomes contain flavonoids, notably flavanones, flavones, and chalcones (Chahyadi et al. 2014). Among the most prominent are panduratin A, cardamonin, and pinostrobin, which appeared to possess antimicrobial, antioxidant, and anticancer properties (Yusuf et al. 2013; Roy and Bharadvaja 2017; Thepthong and Kanjanawattanawong 2025). Recent findings highlight the potential of Adventitious Root (AR) cultures to boost yields of such bioactive compounds through stable, high-productivity culture conditions and elicitation strategies designating *B. rotunda* within broader medicinal plant pipelines (Hussain et al. 2022; Khanam et al. 2022). However, the natural production of these beneficial compounds remains too low and inconsistent to sustain extensive pharmaceutical applications.

Boesenbergia rotunda is highly susceptible to soil-borne pathogens such as soft rot and leaf spot, which spread easily through traditional rhizome planting (Chan and Thong 2004; Paret et al. 2010). Conventional propagation by rhizome segments is slow, harvest requires extended periods, and monocotyledonous gingers generally limit the range of explants that can be used (Lincy and Sasikumar 2010). These constraints reduce the efficiency of biomass production and metabolite yield, underscoring the need for in vitro culture technologies as a sustainable alternative (Yusuf et al. 2011).

Plant tissue culture enables the controlled regeneration and multiplication of cells, tissues, and organs under defined nutrient conditions (Meskaoui 2013). Among these systems, AR cultures are increasingly favored for metabolite production because they are simple to establish, genetically stable, and suitable for long-term biomass accumulation. Beyond flask systems, modern AR platforms employ various bioreactor, and Temporary Immersion Systems (TIS), to improve oxygen transfer and medium homogeneity, thereby accelerating biomass growth and metabolite titers (Wang et al. 2024; Krol et al. 2025). These scalable configurations position AR cultures for industrial translation (Ahmad et al. 2015; Noviyanti et al. 2017; Khanam et al. 2022).

Adventitious roots are roots that arise from non-root tissues, such as stems, leaves, or hypocotyls, and their initiation pathways vary among plant species (Verstraeten et al. 2013; Bellini et al. 2014; Hussain et al. 2022; Khanam et al. 2022). In *B. rotunda,* adventitious roots derived from shoot buds have been reported previously (Azhar et al. 2018; Yusuf et al. 2018), yet hypocotyls, a faster-organizing explant, remain underexplored and lack histological validation (Ng et al. 2016; Thepthong and Kanjanawattanawong 2025). Recent studies emphasize that identifying the precise cellular origin of root apical meristems is essential because AR primordia may arise from distinct vascular or cambial tissues depending on species identity (Bellini et al. 2014; Lakehal and Bellini 2019). Clarifying this developmental pathway through histological analysis is essential for identifying the specific tissues involved in RAM initiation and provides critical insights into plant morphogenesis therefore strengthening the application of hypocotyl explants in propagation and metabolite production pipelines (Azhar et al. 2019; Wang et al. 2024).

The physical consistency of culture media plays a decisive role in organogenesis. Solid media provide only surface contact between explants and nutrients, whereas liquid media under agitation allow full immersion, enhanced aeration, and more uniform uptake of plant growth regulators (Hung et al. 2006; Akdemir et al. 2014; Arya et al. 2014). These advantages have been shown to accelerate induction, elongation, and secondary metabolite accumulation in diverse species cultured under shake-flask or bioreactor conditions (Mohamed et al. 2006; Zhang et al. 2013; Hussein et al. 2014; Bayanati et al. 2015). Despite these reports, the effect of liquid medium on adventitious root induction in *B. rotunda* hypocotyls has not yet been evaluated. Recent advances in bioreactor configurations further highlight the need to test liquid systems for this species, due to improve oxygen transfer and nutrient homogeneity (Wang et al. 2024; Krol et al. 2025; Maskani et al. 2025). Therefore, this study was designed to compare the efficiency of solid and liquid media for root induction in *B. rotunda* hypocotyls and to determine the cellular origin of root apical meristem initiation. We hypothesized that (i) liquid medium would significantly enhance the efficiency and rate of adventitious root induction compared with solid medium, and (ii) root apical meristems in *B. rotunda* hypocotyls would originate from pericycle cells adjoining phloem tissues.

MATERIALS AND METHODS

Plant materials

The in vitro hypocotyls used in this research were obtained from *B. rotunda* plantlets maintained at the Plant Biotechnology laboratory, Faculty of Plantation and Agrotechnology, University Teknologi MARA (UiTM), Puncak Alam, Malaysia. The plantlets were originally induced from shoot buds' explants grown on the solid half-strength Murashige and Skoog (MS) medium (Murashige and Skoog 1962) (Duchefa Biochemie, The Netherlands) supplemented with 0.5 mg/L 1-Naphthaleneacetic Acid (NAA) (Azhar et al. 2018; Ghani et al. 2020).

Culture conditions and medium preparation

A half-strength concentration of MS medium (Murashige and Skoog 1962) supplemented with 0.5 mg/L of NAA was employed in this study. The MS medium consisted of 30.0 g/L sucrose was solidified with 2.0 g/L gelrite (Duchefa Biochemie, The Netherlands) to procure a solid medium, while the liquid medium did not contain gelrite. The pH of the media was adjusted to 5.8 with 1.0 M Sodium hydroxide (NaOH) or 1.0 M Hydrochloric acid (HCl) before being autoclaved. Finally, the cultures were maintained in a growth room at a controlled temperature of 25±1°C under total darkness. For liquid treatments, cultures were placed on an orbital shaker at 100 rpm to ensure continuous agitation.

Experimental design and statistical analysis

Each treatment comprised 6 explants per flask (liquid medium) and petri dish (solid medium). The experiment followed a completely randomized design. Data on induction percentage, root number, and length were expressed as mean ± Standard Deviation (SD). Comparisons between solid and liquid media were performed using two-tailed Student's T-tests ($p \leq 0.05$). Statistical analyses were conducted in SPSS version 21.0 (IBM Corp., Armonk, NY, USA).

Histology

Non-cultured in vitro hypocotyls were the control samples in the current study. The cultured hypocotyls with attached adventitious roots were harvested from the solid and liquid media. The samples were then incised into smaller pieces approximately 0.5 cm long, procuring transverse and longitudinal sections. The samples were fixed according to the protocol outlined by Jalil et al. (2008).

The samples in this study were fixed in Glutaraldehyde-Paraformaldehyde-Caffeine (GPC) (Sigma Chemical Co. brand, USA) at room temperature for 24 h to 48 h. The 100 mL GPC consisted of 50 mL 0.2 M phosphate buffer, pH 7.2, 20 mL of 10% (v/v) paraformaldehyde, 4 mL of 25% (v/v) glutaraldehyde, 1 g of caffeine, and distilled water. All in vitro hypocotyl samples were then dehydrated in 30%, 50%, 70%, 80%, 90%, and 95% ethanol (EtOH) for 30 mins, 45 mins, 60 mins, 60 mins, and 60 mins, respectively, before being dehydrated twice in pure (100%) EtOH for 1 h.

Technovit 7100 resin was employed during tissue culture breaching in this study. The samples were left to solidify at 4°C for 24 h. The solidified blocks were sliced with a microtome into 3.5 µm semi-thin segments and soaked in 100% EtOH. The samples were placed in distilled water-filled glass containers on black paper to ease the picking up of the sections.

Following placement on clean, dry slides, the segments were stained with 0.5% (w/v) toluidine blue stain [Sigma, United States of America (USA)] to assess their conditions. Selected sections were then double stained with 1% (w/v) periodic acid for 5 min and rinsed four times in distilled water at pH 4.5. Subsequently, the sections were soaked in Schiff's reaction for 20 min in total darkness before being rinsed four times in distilled water (pH 4.5). Naphthol blue black (Sigma, USA) was then employed to stain the slides for 5 min at 60°C. Finally, the slides were mounted with Surgi Path mounting medium and left to dry for 24 h after they were thoroughly rinsed with distilled water.

The photomicrographs in this study were obtained with a Leica camera (LEITZ DMRB) attached to a light microscope (×20/0.5, ×40/0.7, and ×100/1.3). Longitudinal and transverse sections of the samples were observed at 200× to 800× magnification to determine the presence of Root Apical Meristem (RAM). The tissues involved in RAM development during adventitious root induction were also observed.

RESULTS AND DISCUSSION

The effects of medium type on *B. rotunda* adventitious root induction

After four weeks of culture, all hypocotyl explants responded positively to adventitious root induction. Explants cultured in the liquid half-strength MS medium supplemented with 0.5 mg/L NAA achieved 100% adventitious root induction, whereas those cultured on the solid counterpart showed a 95% response. As shown in Table 1, the in vitro hypocotyls agitated in the liquid culture induced adventitious roots on the 7th day, while the adventitious roots from the in vitro hypocotyls grown in the solid medium emerged on the 14th day.

The adventitious roots in the solid and liquid culture media continued to develop and elongate until the fourth week of culture. The *B. rotunda* adventitious root induction rates from the in vitro hypocotyls in both media in the current study were considerably more rapid than those previously reported for shoot bud explants, which required almost 40 to 80 days to emerge (Azhar et al. 2018; Yusuf et al. 2018). Morphological observations indicated that the adventitious roots induced from in vitro hypocotyls cultured in the liquid medium shake flask system were physically larger and longer than the adventitious roots induced from in vitro hypocotyls cultured on the solid medium, which were thinner and shorter (Figure 1). Liquid-cultured hypocotyls produced denser root clusters per explant. Similar morphological enhancement under liquid conditions has been reported in *Lessertia frutescens* (L.) Goldblatt & J.C.Manning and *Salvia apiana* Jeps., where liquid shake-flask or bioreactor systems increased biomass and metabolite content (Wang et al. 2024; Krol et al. 2025).

The average number of adventitious roots observed in the liquid culture was also significantly higher (29.14±2.91) than in the solid medium (12.25±2.82) ($p \leq 0.05$). The adventitious roots in the liquid medium were significantly longer, averaging 1.96±0.25 cm, than their counterparts grown in the solid culture, averaging 0.94±0.05 cm (Table 1). Azhar et al. (2018) reported that shoot bud explants induced an average of 7.667±2.08 adventitious roots and 1.733±0.42 cm root length when cultured in a solid ½ MS medium supplemented with 0.5 mg/L NAA.

This study confirmed that the liquid medium is more effective than the solid medium for AR induction of *B. rotunda* from hypocotyl explants. Constant agitation in the shake-flask system produced direct and consistent contact between explant surfaces, nutrients, and plant growth regulators, promoting induction and escalating root biomass. AR in liquid culture produces larger numbers, longer and higher frequency compared with those in solid culture, revealing the potential of liquid systems for scalable biomass production. These findings are particularly relevant for B. rotunda, where bioactive flavonoids such as panduratin A, cardamonin, pinocembrin, and pinostrobin have been reported in previous phytochemical studies (Ng et al. 2016). Although the present work did not evaluate flavonoid content, the enhanced adventitious root induction observed under liquid culture provides a promising foundation for future studies that aim to link developmental responses with secondary metabolite production. Thepthong and Kanjanawattanawong (2025) further established advances in *B. rotunda* micropropagation, supporting the importance of in vitro systems that can sustain propagation fidelity and metabolite stability. By integrating hypocotyl-derived AR cultures with the strategies, it is possible to demonstrate a rapid and genetically stable system that supports both micropropagation and secondary metabolite production.

These results agree with earlier research reporting the benefit of a liquid culture system for in vitro propagation and superior plantlet quality (Hussein et al. 2014; Bayanati et al. 2015). These findings are also in parallel with Zhang et al. (2013) showed that liquid culture escalated both adventitious root biomass and secondary metabolite production in *Psammosilene tunicoides* W.C.Wu & C.Y.Wu (Ng et al. 2016; Thepthong and Kanjanawattanawong 2025). Similarly, other findings have attributed such responses to the constant agitation of explants in liquid systems, which ensures maximum contact with plant growth hormones and nutrients and enhances aeration (Akdemir et al. 2014; Arya et al. 2014). Mohamed et al. (2006) also agreed that liquid systems enhance nutrient and hormone uptake, promoting induction and organogenesis. Therefore, the combination of morphological robustness, histological authentication, and current proliferation improvements emphasizes the exclusive value of hypocotyl explants. Unlike shoot bud-derived ARs, hypocotyl-derived roots provide a faster and more uniform response, potentially shortening culture cycles while ensuring stable metabolic outputs.

Histological observation of root initiation

Histological examinations confirmed the presence of Root Apical Meristems (RAM) and associated cell types responsible for adventitious root formation. Adventitious roots clearly originated from the pericycle cells adjacent to phloem tissues, and both solid and liquid treatments exhibited RAM development, indicating successful root initiation. The RAM appeared as dome-shaped primordia with organized root caps, cortex, and vascular initials (Figures 2.A-B). These developmental events are consistent with reports that in monocotyledonous species, AR primordia frequently arise from the phloem-pole pericycle (Bellini et al. 2014; Lakehal and Bellini 2019). Such an organization reflects a direct organogenic pathway, which is advantageous for maintaining the genetic stability of regenerated tissues and has also been highlighted in recent studies on AR induction in cereals such as *Oryza sativa* L. and *Zea mays* L. (Wang et al. 2024). Notably, transverse and longitudinal sections showed that explants cultured in liquid medium displayed more prominent and numerous RAM clusters, particularly in longitudinal views (Figure 3.B), compared with those cultured on solid medium (Figures 2.A-B). Similar enhancements of RAM vigor under liquid conditions were also reported in other medicinal species (Maskani et al. 2025), suggesting that liquid culture not only accelerates root induction but also promotes stronger RAM development in *B. rotunda.*

The direct origin of adventitious roots from the pericycle without an intervening callus phase was evident in both treatments. This pathway reflects direct organogenesis, which is generally more desirable for clonal propagation because it minimizes the risk of somaclonal variation and ensures greater genetic stability (Maskani et al. 2025). Such constancy is particularly important for medicinal plants like *B. rotunda,* where maintaining metabolite reliability is critical for therapeutic applications. Previous reports have shown that shoot bud explants of *B. rotunda* were also capable of direct AR formation under solid medium (Azhar et al. 2018; Yusuf et al. 2018). However, the present study demonstrates that hypocotyl explants not only follow the same direct histological pathway but also respond faster and more uniformly, especially under liquid culture conditions. This efficiency highlights the potential of hypocotyl-derived AR cultures to complement or even surpass shoot bud–derived systems in propagation and metabolite production pipelines.

The Root Apical Meristem (RAM) is a critical growing tip that continuously generates new cells, forming the basis for diverse tissues and organs in plants (Pop et al. 2011; Rost 2011). Sustained mitotic activity of meristematic cells ensures root elongation and organogenesis, making RAM the foundation of adventitious root development. RAM clusters typically exhibit a dome-shaped structure enclosed by root caps, which protect the meristem, secrete mucilage to facilitate root penetration, and function as gravity-sensing tissues (Aragón-Raygoza et al. 2020). In the present study, such dome-shaped RAM structures were clearly observed in *B. rotunda* hypocotyl explants under both solid and liquid conditions (Figures 2.A-B and 3.A-B).

Table 1. The effects of medium types on the induction of *B. rotunda* adventitious roots via in vitro hypocotyls

Type of ½ MS media	Percentage of adventitious roots induced from explants	Day of adventitious roots emergence	The average number of induced adventitious roots	The average length of adventitious roots (cm)
Solid	95	14	12.25±2.82b	0.94±0.05b
Liquid	100	7	29.14±2.91a	1.96±0.25a

Note: n=6, p≤0.05

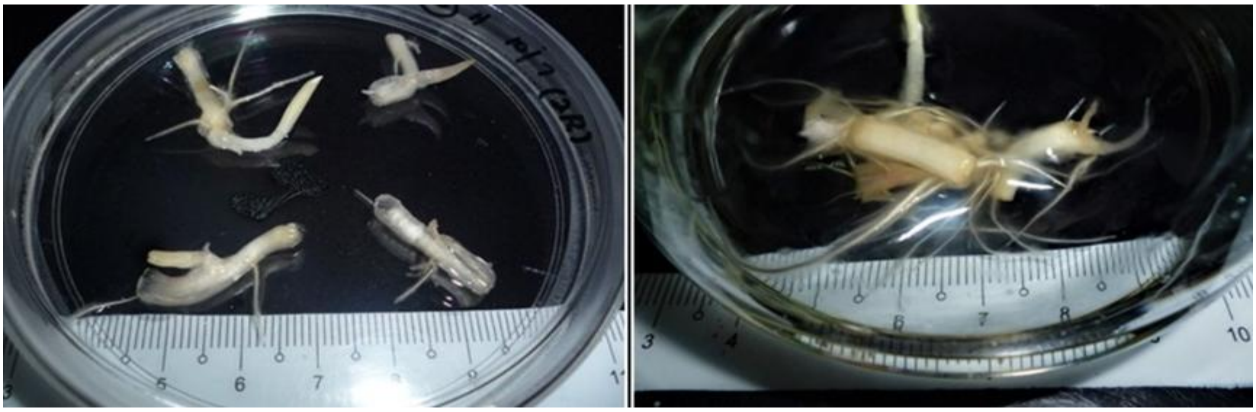

Figure 1. The adventitious roots induced from in vitro hypocotyl cultured on the A. Solid medium and B. Liquid medium of half-strength MS medium +0.5 mg/L NAA after 14 days of culture

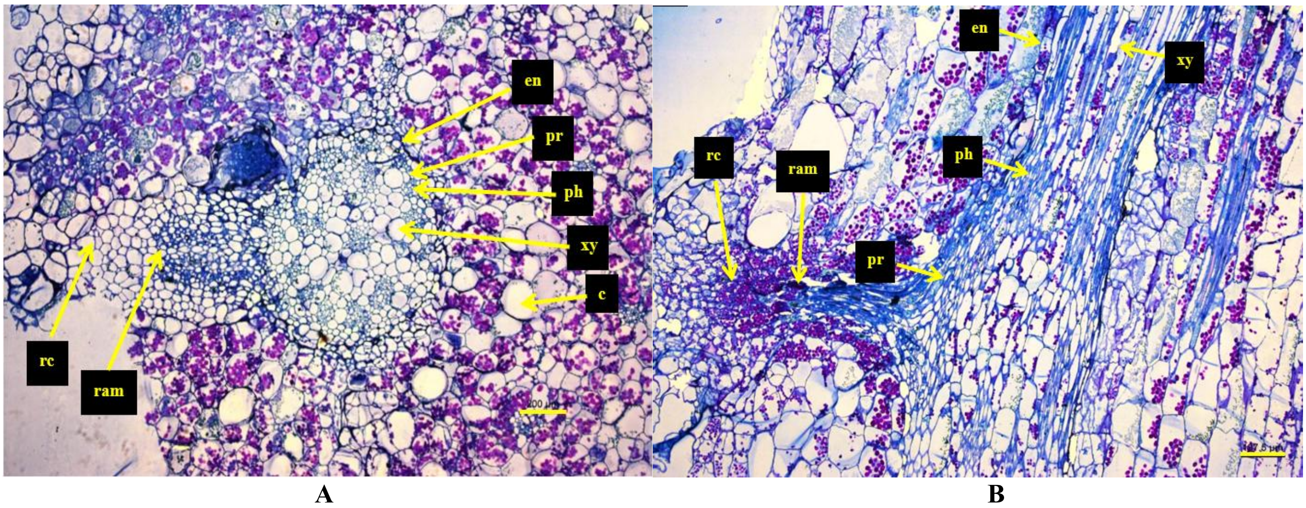

Figure 2. The A. Cross and B. Longitudinal sections of the adventitious roots induced from in vitro hypocotyl on the solid ½ MS medium supplemented with 0.5 mg/L NAA after four weeks of culture at 200× magnification. Note: Bar = 200 um, c: Cortex, en: Endodermis, ep: Epidermis, pr: Pericycle, ph: Phloem, xy: Xylem, ram: RAM, rc: Root cap

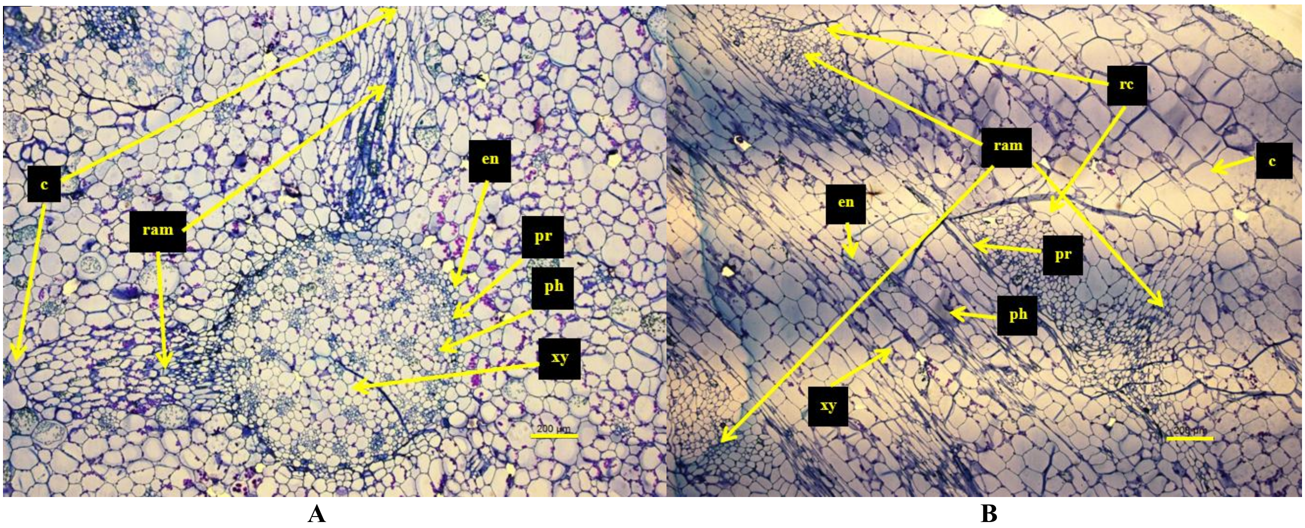

Figure 3. The A. Cross and B. Longitudinal sections of the adventitious roots induced from in vitro hypocotyl on the liquid ½ MS medium supplemented with 0.5 mg/L NAA after four weeks of culture at 200× magnification. Note: Bar = 200 um, c: Cortex, en: Endodermis, ep: Epidermis, pr: Pericycle, ph: Phloem, xy: Xylem, ram: RAM, rc: Root cap

However, liquid culture promoted more abundant and larger RAM clusters, with denser meristematic activity (Figure 3.B), suggesting that continuous immersion and improved nutrient uptake enhanced the proliferative capacity of RAM. These histological findings reinforce the functional role of RAM described in earlier developmental studies and demonstrate how culture conditions can modulate the vigor and frequency of meristem initiation in a medicinal monocot species.

Histological observations in this study confirmed that adventitious root meristems in *B. rotunda* hypocotyls originated from pericycle cells adjoining phloem tissues. These cells are divided periclinal and anticlinal to generate complete root primordia. This condition is consistent with earlier reports that vascular-associated pericycle cells are the primary source of adventitious roots in many species (Lakehal and Bellini 2019). Our results agree with Bellini et al. (2014), who found that the developmental origin of AR resembles lateral roots, even though the exact pattern depends on plant taxonomy. In dicotyledonous species like *Arabidopsis thaliana* (L.) Heynh*, Raphanus sativus* L.*,* and *Helianthus annuus* L., AR typically occurs from pericycle cells neighboring xylem poles; however, in monocot species, for example, rice and maize, AR generally initiates at pericycle cells adjacent to phloem poles. The finding that *B. rotunda* AR initiates from phloem-associated pericycle cells is consistent with the monocotyledonous character.

Current histological research in other monocots additionally supports this pattern. For example, *O. sativa* and *Z. mays* demonstrated phloem pole pericycle initiation that leads to speedy root development and vigorous vascular connection (Wang et al. 2024). Moreover, Maskani et al. (2025) emphasized that pericycle-derived AR in monocot species tends to bypass callus phases, producing direct organogenesis and plummeting the risk of somaclonal variation. This is a significant advantage for *B. rotunda*, where metabolic reliability is crucial to ensure reliable yields of panduratin A and pinostrobin. By proving the cellular origin and division pattern of AR, our studies supported the developmental framework of *B. rotunda*. They reinforced its suitability for both clonal propagation and secondary metabolite production.

Previous research reveals that AR can arise via two pathways: direct induction through pericycle cell reactivation, or indirect induction (rhizogenesis) facilitated by callus proliferation (Falasca and Altamura 2003). In the present study, the direct origin of AR from pericycle cells suggests a more effective pathway, improved by the presence of exogenous auxin (NAA) promoted cell division and RAM differentiation. This is also agreed by previous findings that auxin-driven rhizogenesis involves endodermal and cortical exfoliation, exposing pericycle tissues to new developmental contexts that favor root initiation (Bellini et al. 2014).

The documentation of hypocotyl-derived AR from pericycle cells has significant implications for progressive biology and applied biotechnology. Confirming the cellular origin reinforces the reliability of hypocotyls as an efficient explant source, supplementing former reports on shoot buds' explants and plantlets of *B. rotunda* (Azhar et al. 2018, 2019). The superiority of the liquid system for AR induction specifies promising potential for scaling up hypocotyl-derived AR cultures in advanced systems such as bioreactors. Various bioreactor systems, such as air lift, balloon, and column, could provide continuous agitation support, uniform nutrient absorption, and high biomass production (Zhang et al. 2013; Wang et al. 2024). This outcome supports that AR cultures provide scalable routes for bioactive metabolite production (Hussain et al. 2022). Moreover, recent advances in *B. rotunda* clonal propagation confirm the viability of maintaining clonal fidelity and metabolic stability under in vitro conditions (Thepthong and Kanjanawattanawong 2025).

Further studies should relate these developmental findings to metabolite profiling to determine whether enhanced AR induction correlates with increased flavonoid production, such as panduratin A and pinostrobin. Furthermore, testing other auxins or auxins and cytokinin combinations will possibly further optimize induction proficiency and genetic stability in long-term cultures. Integrating elicitation strategies and scaling up research in temporary immersion or air lift bioreactors will also be critical to bridge the gap between laboratory cultures and industrial applications.

In conclusion, this study concluded that in vitro hypocotyl-derived explants of *B. rotunda* are very promising for the induction of AR cultures. Histological analyses confirmed that RAM originated directly from pericycle cells adjacent to phloem tissues, providing the first cellular-level evidence for hypocotyl-derived AR in this species. The use of liquid systems significantly improved root induction, numbers, and elongation compared with solid systems, highlighting their superiority for biomass accumulation. These findings establish hypocotyls as a reliable and efficient explant source and validate liquid shake-flask systems as promising platforms for initiating and propagating root culture. The results also highlight the potential of scaling up liquid-based AR cultures of *B. rotunda* in bioreactors to produce consistent and metabolite-rich biomass. Assimilation of developmental insights with metabolite profiling, optimizing hormone types and combinations, and assessing genetic fidelity during long-term culture will be critical steps forward. Moreover, integrating elicitation strategies in airlift, column, balloon, or TIS bioreactors could further enhance the production of importance bioactive compounds such as panduratin A and pinostrobin. These directions will strengthen the application of hypocotyl-derived AR for both the conservation of Zingiberaceae species and the sustainable production of high-value pharmaceutical compounds.

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