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Isolation and Establishment of Cambial Meristematic Cells (CMCs) from *Panax vietnamensis* var. *fuscidiscus*

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Key words: P. vietnamensis var. *fuscidiscus,* Cambial meristematic cells (CMC), Callus

Abstract

Panax vietnamensis var. *fuscidiscus* is an endemic medicinal plant of Vietnam, renowned for its high content of ocotillol-type saponins. While plant cell culture presents a promising strategy for producing these valuable compounds, large-scale application remains limited by the instability and heterogeneity of dedifferentiated cells (DDCs). To overcome these limitations, inherently undifferentiated cambial meristematic cells (CMCs) from *P. vietnamensis* var. *fuscidiscus* were successfully isolated and cultured. Optimal induction of callus was achieved using WPM (Woody plant medium) supplemented with 1.0 mg/l picloram and 2.0 mg/l BAP, resulting in a CMC formation frequency of 28.67% and a mean fresh weight of 568 ± 32 mg per explant after one month induction. CMCs were characterized by distinct morphological and cytological traits: small, isodiametric cells with multiple small vacuoles and a non-aggregative, slow-growing proliferation pattern. These features were confirmed through neutral red staining and long-term culture. Notably, the established CMC lines remained morphologically stable and proliferative over a six-month subculture period. This is the first report on the successful isolation and maintenance of CMCs in *P. vietnamensis* var. *fuscidiscus*, providing a robust cellular system for downstream applications such as somatic embryogenesis, metabolic engineering, and the production of secondary metabolites. The CMC-based platform offers new opportunities for the conservation and sustainable utilization of this pharmacologically important species.

Introduction

Panax vietnamensis var. *fuscidiscus*, commonly known as Lai Chau ginseng, is a rare and valuable medicinal plant endemic to the mountainous regions of northwestern Vietnam. It belongings to the family Araliaceae. This variety is a closely related taxon of *Panax vietnamensis* Ha et Grushv. (Vietnamese ginseng), and is taxonomically distinguished by its morphological characteristics and phytochemical composition. It is especially notable for its high levels of ocotillol-type saponins, including majonoside-R2 and vina-

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ginsenoside-R2, which have demonstrated significant pharmacological properties such as anti-fatigue, immunomodulatory, hepatoprotective, and adaptogenic effects (Nguyen et al. 2017). Due to the increasing demand for natural medicinal products and the limited distribution of *P. vietnamensis* var. *fuscidiscus*, the species is under significant pressure from overharvesting and habitat destruction. This has led to the depletion of wild populations and raised concerns over the long-term conservation and sustainable use of this valuable resource (Phan et al. 2021). Conventional methods of propagation such as seed germination or vegetative propagation are not only slow but often yield low efficiency due to dormancy and poor rooting. Therefore, the development of effective *in vitro* propagation and metabolite production systems is essential both for conservation purposes and for meeting pharmaceutical demands.

Plant cell and tissue culture technologies have long been employed to produce plant biomass and valuable secondary metabolites in a controlled environment. Among these techniques, callus cultures derived from dedifferentiated plant cells (DDCs) have been widely used in numerous medicinal plant species. DDC-based cultures have successfully produced high-value secondary metabolites in various medicinal plants. For instance, cell suspensions of *Taxus media* produced paclitaxel at levels up to ~2.0 mg/l following elicitation with methyl jasmonate and precursor feeding (Kim et al. 2004, Facchini et al. 2005). Callus cultures of *Panax ginseng* and *P. notoginseng* were shown to accumulate ginsenosides, with elicitation or suspension culture enhancing total saponin content up to 2-3% of dry weight, compared to <1% in non-elicited controls (Zheng et al. 1989, Langhansova et al. 2005). Similarly, callus cultures of *Ocimum basilicum* accumulated phenolic compounds, particularly rosmarinic acid, reaching 40-50 mg/g dry weight, demonstrating the effectiveness of DDC systems in producing phenolic secondary metabolites (Nazir et al. 2019, Nazir et al. 2020). However, DDCs often show significant limitations including genetic instability, low reproducibility, somaclonal variation, and a decline in metabolite productivity over time (Sanchez-Munoz et al. 2019, Duta-Cornescu et al. 2023). These factors have posed considerable obstacles to the industrial-scale application of DDC-based cultures for secondary metabolite production. To overcome these limitations, researchers have recently turned their attention to a more promising and stable cell type known as cambial meristematic cells (CMCs). Cambial meristematic cells (CMCs) are inherently undifferentiated cells derived from the vascular cambium, the tissue responsible for secondary growth in plants. Unlike dedifferentiated cell (DDC) cultures, CMCs retain meristematic, stem-cell–like properties, exhibit sustained proliferation capacity, and maintain characteristic small size with abundant small vacuoles under appropriate culture conditions (Lee et al. 2010b, Mehring et al. 2020). Several studies have demonstrated that CMC cultures show higher genetic stability and more consistent accumulation of secondary metabolites compared with conventional DDC-based cultures (Ochoa-Villarreal et al. 2015, Mehring et al. 2020). These features make CMCs a promising platform for scalable and reliable production of high-value plant secondary metabolites. Moreover, CMCs typically exhibit unique cytological characteristics, including small cell size, dense cytoplasm, high nucleus-to-cytoplasm ratio, and multiple small vacuoles. These features distinguish them from callus-derived cells and make them highly suitable for consistent and efficient production of bioactive compounds.

The superiority of CMCs over DDCs has been well documented in several plant species, particularly within the genus *Panax*. In a landmark study, Lee et al. (2010b) successfully isolated and characterized CMC lines from *Panax ginseng*, demonstrating not only higher proliferation rates but also enhanced ginsenoside accumulation compared to callus cultures. Similarly, studies on *Panax notoginseng* have shown that CMCs exhibit more stable growth and greater biosynthetic potential for notoginsenosides, important pharmacologically active components in traditional Chinese medicine (Kim et al. 2014c). These findings suggest that CMC culture systems offer significant advantages for both basic research and industrial-scale applications in medicinal plants. Despite the increasing interest in CMCs, no prior studies have reported the successful isolation or establishment of CMCs from *Panax vietnamensis* var. *fuscidiscus*. Research on this species has largely focused on micropropagation via nodal segments (Nguyen et al. 2015), somatic embryogenesis (Do et al. 2020), or the identification of chemical constituents (Nguyen et al. 2017), with limited exploration into cellular-level approaches for metabolite production. The lack of CMC-based studies represents a significant knowledge gap, especially considering the species high pharmacological value and conservation priority.

Isolating and maintaining CMCs from *P. vietnamensis* var. *fuscidiscus* presents a novel and potentially transformative approach. To achieve successful CMC establishment, several factors must be carefully considered, including explant source, basal media composition, and plant growth regulator (PGR) combinations. Therefore, the present study was conducted with the following objectives: (i) to isolate CMCs from *P. vietnamensis* var. *fuscidiscus* using different explant types, (ii) to evaluate the effects of various combinations of basal media and plant growth regulators on the induction and development of CMCs, and (iii) to characterize the morphological and cytological features of the obtained CMC lines to confirm their identity. This study represents the first effort to develop a CMC-based culture system for *P. vietnamensis* var. *fuscidiscus* and lays the groundwork for future research in mass propagation, metabolite production, and conservation of this highly valuable medicinal species.

Materials and Methods

The mature individual (one year old) of *Panax vietnamensis* var. *fuscidiscus* was collected from a natural population in Lai Chau Province, northern Vietnam. The exact geographic coordinates of the collection site were recorded using a GPS device (latitude: 22°21′19.4″ N, longitude: 103°14′54.4″ E). Photographs were taken to document the morphology of the plants and the surrounding habitat (Fig. 1A, 1B).

Fig. 1. Plant materials of *Panax vietnamensis* var. *fuscidiscus* used in this study: (A) a one-year-old individual maintained in a nursery bag, (B) multiple seedlings cultivated at the collection site.

To confirm the taxonomic identity of the collected samples, total genomic DNA was extracted from fresh leaf tissue using the cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle 1990). Three commonly used DNA barcode regions, namely nuclear ITS and chloroplast rpoB and trnH-psbA were amplified and sequenced using standard primers (Kress and Erickson 2007, Chen et al. 2010, Dong et al. 2012). The obtained sequences were aligned with reference sequences from GenBank using MAFFT. A Maximum Likelihood phylogenetic tree was then constructed using PhyML via the NGPhylogeny.fr platform (Lemoine et al. 2018), with 1,000 bootstrap replicates to assess branch support. The resulting tree was visualized using the Interactive Tree of Life (iTOL) v6. The analyzed sample, designated as AGI-FUS2025, was deposited as a voucher specimen at the Agricultural Genetics Institute (Hanoi, Vietnam).

One-year-old *Panax vietnamensis* var. *fuscidiscus* plants were used as experimental materials. Samples were transported from nurseries and acclimatized under laboratory conditions with weekly fertilizer application and fungicide spraying to reduce environmental microbial contamination and improve sterilization efficiency. Stem segments were washed under running water, cut into 5-7 cm pieces, and stored in 0.56 M ascorbic acid solution at 4°C for up to 5 days (Lee et al. 2010b). Samples were pretreated with 3 g/l Metalaxyl for 10 min, rinsed with clean water, and sterilized in a laminar flow cabinet. Surface sterilization involved 70% ethanol (2 min), followed by rinsing with sterile distilled water. Samples were then treated with 2 and 1% NaClO for 15 and 20 min, respectively, with 1-2 drops of Tween 20. Residual disinfectants were removed with 5-7 rinses using sterile distilled water.

Stem segments were trimmed to 0.5-1.0 cm in length and longitudinally bisected. Stem cross-sections were stained for lignin detection using phloroglucinol-HCl solution (0.5% [w/v] phloroglucinol in 6 N HCl) for 5 min, followed by microscopic observation to identify cambial meristematic tissue. Under a stereomicroscope (Meiji techno co., LID., Model: MT5300H)), the central pith and xylem were carefully removed using a scalpel. The remaining tissue, including cambial meristem, phloem, cortex, and epidermis was placed horizontally on the culture medium, with the cambial side facing upward and the outer cortex in contact with the medium surface (Yun et al. 2012).

Explants were cultured on WPM medium supplemented with 1.0 mg/l picloram (auxin), 2.0 mg/l BAP (cytokinin), 30 g/l sucrose, and 3 g/l phytagel, adjusted to pH 5.6, maintained in darkness at 25 ± 1°C. Callus induction and morphology were recorded after 4 weeks. Callus derived from the stem cambium was selected for further subculture and characterization.

To distinguish cambial meristematic cells (CMCs) from dedifferentiated callus cells (DDCs), vacuolar morphology was examined using neutral red staining, following a modified method of Lee et al. (2010b). Cells were isolated from friable callus tissue, stained with 0.01% (w/v) neutral red (3-amino-7-dimethylamino-2-methylphenazine hydrochloride; GHTech, China) for 10 min, and rinsed with 0.1 M phosphate buffer (pH 7.2). Samples were prepared using the squash smear technique and observed under a light microscope (Meiji Techno) equipped with an Infinity3 Lumenera digital camera. CMCs were identified based on the following characteristics (Lee et al. 2009, Kim et al. 2014c): (i) Small, isodiametric cell shape, (ii) Presence of multiple small vacuoles, (iii) Non-aggregative, slow-growing proliferation pattern.

Selected CMC lines were maintained and subcultured every three weeks over a period of six months. Each treatment included 30 explants per replication, with three biological replicates. Data were collected for: (i) Callus induction rate (%), (ii) Fresh weight of callus (mg), (iii) Time to initial callus formation (days).

Results and Discussion

To verify the taxonomic identity of the collected specimen AGI-FUS2025, a DNA barcoding approach was employed using three commonly accepted molecular markers: the nuclear internal transcribed spacer (ITS) and two chloroplast regions, rpoB and trnH-psbA. These loci are widely used in plant molecular taxonomy due to their relatively high interspecific variation and universal primer availability (Kress and Erickson 2007, Chen et al. 2010, Dong et al. 2012). The concatenated sequences were aligned and subjected to phylogenetic analysis using the Maximum Likelihood (ML) method. As shown in Fig. 2, the AGI-FUS2025 sample clustered tightly with reference sequences of *Panax vietnamensis* var. *fuscidiscus*, forming a distinct clade supported by a bootstrap value of 847. Although slightly below the conventional 900-1000 threshold for "very strong" support, a value of 847 still indicates high confidence in the inferred relationship (Hillis and Bull 1993). This grouping clearly separates AGI-FUS2025 from other closely related taxa such as *P. notoginseng*, *P. japonicus*, and *P. ginseng*, which formed separate clades with robust bootstrap support (ranging from 484 to 1000). These results are consistent with previous molecular studies on the genus *Panax*. For example, Zuo et al. (2011) successfully distinguished *P. vietnamensis* from *P. ginseng* and *P. notoginseng* using ITS and trnH-psbA sequences. Similarly, Pham et al. (2013) demonstrated that DNA barcoding could effectively differentiate *Panax* species native to Vietnam, reinforcing the applicability of this approach in both wild population authentication and conservation genetics.

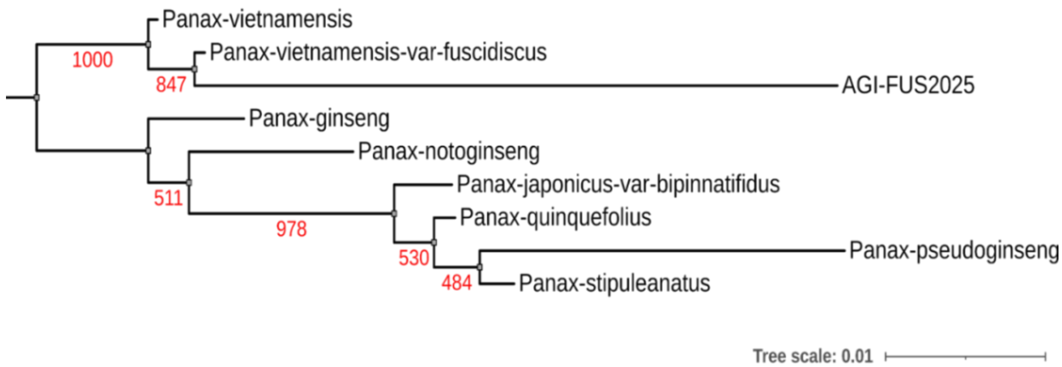

Fig. 2. Maximum Likelihood phylogenetic tree based on concatenated ITS, rpoB, and trnH-psbA sequences of various *Panax* species. Bootstrap values (1,000 replicates) are shown in red.

To ensure precise excision of the cambial region, transverse and longitudinal sections of fresh stems were observed under a stereomicroscope. The vascular cambium was clearly identified as a continuous ring of small, densely arranged cells located between the xylem and phloem tissues (Fig. 3). The cross-sectional view revealed a distinct cambial layer surrounding the central xylem region, with phloem distributed peripherally (Fig. 3A). Longitudinal sections confirmed the alignment and density of the cambial initials along the stem axis (Fig. 3B). To further validate the presence of lignified xylem cells, phloroglucinol-HCl staining was applied. The resulting red coloration highlighted secondary cell wall lignification in xylem elements, thus demarcating the cambium as the unstained, meristematic band bordering the lignified region (Fig. 3C).

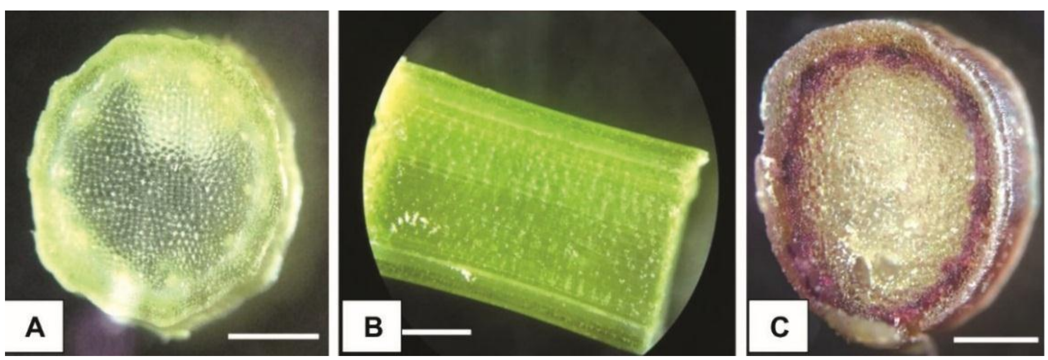

Fig. 3. Morphological observation of the stem segments for cambial tissue identification: (A) cross section of a young green stem, (B) longitudinal section of the same stem and, (C) cross section of the stem stained with phloroglucinol-HCl. Scale bar = 1 mm.

This anatomical identification provided a reliable basis for accurate sampling of cambial tissues for subsequent callus induction and CMC isolation. Similar histological methods have been widely used to define cambial regions in woody and herbaceous species (Lee et al. 2010a).

After 4 weeks of culture on WPM medium supplemented with 1.0 mg/l picloram and 2.0 mg/l BAP, stem explants of *Panax vietnamensis* var. *fuscidiscus* initiated callus formation primarily from the cambial region. The first signs of callus emergence were typically observed between 10 and 14 days post-inoculation. Morphologically, CMCs appeared as a translucent, friable region within the callus mass (Fig. 4A), clearly distinguishable from the surrounding yellow, compact DDC regions. CMC-rich sectors showed a non-aggregative, slow-growing proliferation pattern even after repeated subculturing, further supporting their identity. The resulting calli were initially creamy-white and compact (Fig. 4A). Callus induction was observed in approximately 28.67% of the explants, with a mean fresh weight of 568 ± 32 mg per explant. Notably, calli arising specifically from the cambial zone displayed slow-growing, non-aggregative proliferation pattern morphological traits suggestive of cambial meristematic cell (CMC) identity. No callus formation was detected from the epidermal or xylem tissues, reinforcing the histological evidence that the cambial zone is the primary responsive region under the tested culture conditions. These results support the effective use of picloram in combination with BAP for selective induction of callus from cambial tissues, aligning with previous studies in *Panax* and other medicinal plants (Lee et al. 2010a,d; Kim et al. 2014c).

To distinguish cambial meristematic cells (CMCs) from dedifferentiated callus cells (DDCs), neutral red staining was employed to examine vacuolar morphology. After staining, DDCs typically exhibited large central vacuoles and irregular shapes, often forming aggregated clusters (Fig. 4B). In contrast, CMCs were characterized by small, isodiametric cells containing multiple small vacuoles (Figs 4C, D). These features are consistent with the established criteria proposed by Lee et al. (2010b) and Kim et al. (2014c).

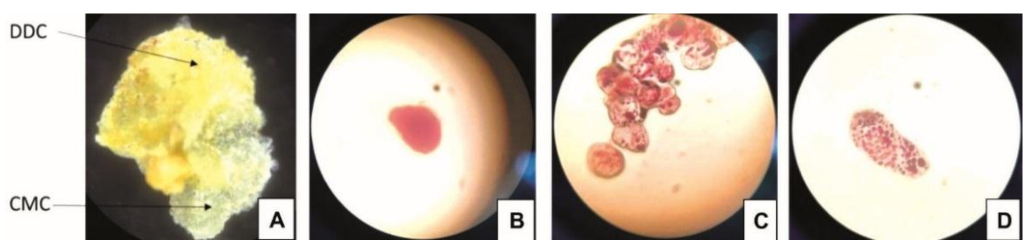

Fig. 4. Morphological and cytological distinction between dedifferentiated callus cells (DDCs) and cambial meristematic cells (CMCs) derived from stem explants of Panax vietnamensis var. fuscidiscus: (A) callus tissue after 4 weeks of induction showing distinct regions: yellow, compact DDCs (upper region) and translucent, friable CMCs (lower region), (B) DDCs with large central vacuoles and irregular shapes, forming aggregated clusters (neutral red staining), (C) cluster of CMCs displaying isodiametric shape and dense cytoplasm with numerous small vacuoles, and (D) two CMCs showing small size and multiple small vacuoles. Scale bars: 1 mm (A); 50 μm (B–D).

Selected CMC-rich callus lines, identified based on morphological and cytological criteria, were subcultured onto fresh WPM medium supplemented with 1.0 mg/l picloram and 2.0 mg/l BAP every three weeks. Over a six-month period, these lines maintained their characteristic slow-growing, non-aggregative proliferation pattern. Unlike DDC regions, which tended to form compact masses and displayed signs of early browning or necrosis upon prolonged culture, CMCs retained a friable texture and translucent appearance (Fig. 5A, B).

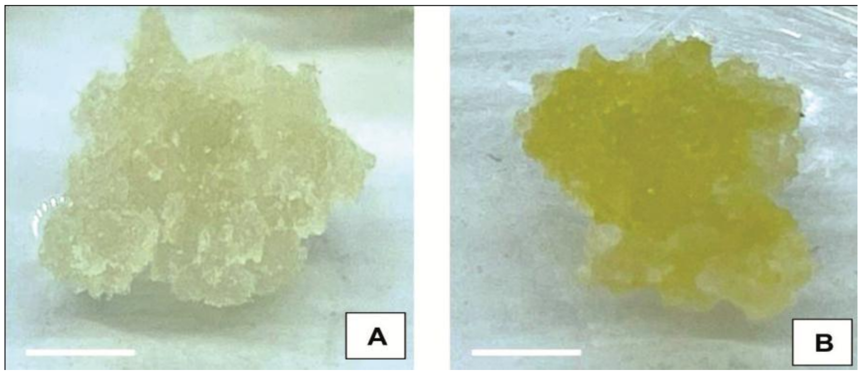

Fig. 5. Morphological characteristics of CMC (A) and DDC (B) after 6 months of continuous subculture on WPM medium supplemented with 1.0 mg/l picloram and 2.0 mg/l BAP. (A) CMC appeared as translucent, friable, non-aggregative tissues; (B) DDC exhibited dense, compact morphology with a yellowish color and tendency to aggregate. Scale bars = 1 mm.

Histological observations confirmed that CMCs preserved meristematic features such as dense cytoplasm and small, evenly distributed vacuoles across multiple subculture cycles. These properties are indicative of sustained cellular totipotency and low differentiation status. Notably, the CMC cultures did not give rise to organized shoot or root structures under the tested hormonal conditions.

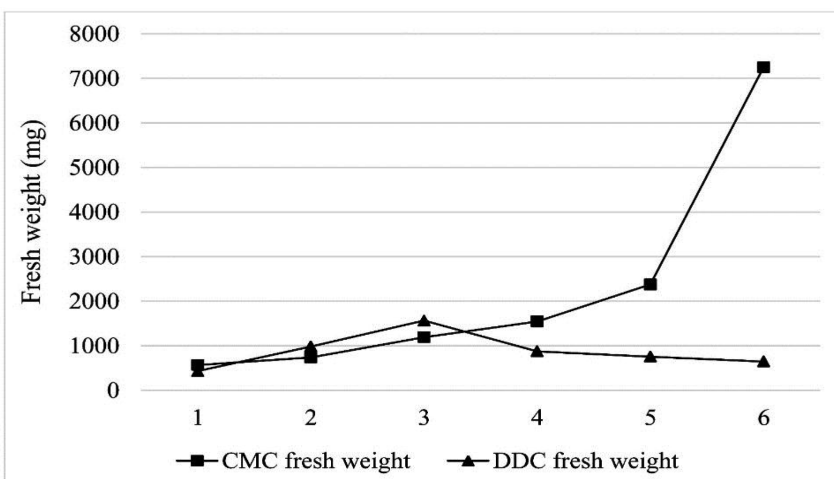

Fig. 6. Fresh weight (mg) of CMC and DDC calli over 6 months of subculture.

To evaluate the long-term proliferative capacity of CMCs, both CMC and DDC calli were subcultured monthly on the same induction medium and their fresh weights were recorded for six consecutive months. As shown in Fig. 6, CMC calli exhibited a consistent increase in fresh weight throughout the culture period, particularly accelerating after the fourth month and reaching approximately 7251 mg by month six. In contrast, DDC calli showed a modest increase during the initial three months, peaking at around 1564 mg, followed by a gradual decline in biomass.

These findings suggest that CMCs possess a sustained proliferative ability, which was not only superior to DDCs but also continues to increase over time, indicative of stable cell division activity and meristematic characteristics. Meanwhile, the observed reduction in DDC biomass after prolonged subculture implies cellular senescence or loss of mitotic activity, consistent with previous reports on the limited propagation potential of dedifferentiated calli (Lee et al. 2010d, Kim et al. 2014a).

This sustained proliferation of CMCs over extended culture periods is consistent with reports in other *Panax* species and woody medicinal plants, where cambial-derived cells have shown potential for long-term maintenance without loss of regenerative capacity (Kim et al. 2014b, Xu et al. 2017). The ability to isolate and maintain CMCs *in vitro* provides a promising cellular platform for subsequent studies on somatic embryogenesis, genetic transformation, and secondary metabolite production.

This study demonstrated the successful induction of callus from stem explants of *Panax vietnamensis* var. *fuscidiscus* using WPM medium supplemented with 1.0 mg/l picloram and 2.0 mg/l BAP. Callus formation occurred primarily from the cambial region, with approximately 28.67% induction efficiency. Histological and cytological analyses confirmed the presence of cambial meristematic cell (CMC)-like structures, distinguishable from dedifferentiated callus cells (DDCs) based on morphology and vacuolar features. CMC-rich calli exhibited long-term stability and uniform proliferation over six months of subculture. These findings suggest that CMCs represent a promising cell population for further development of plant regeneration and metabolic production systems in *P. vietnamensis*.

In this study, induced callus formation from stem explants of *Panax vietnamensis* var. *fuscidiscus* was successfully achieved using WPM medium supplemented with picloram and BAP. Notably, callus was predominantly initiated from the cambial region, while other tissues such as epidermis and xylem showed no responsiveness under the tested conditions. This observation is consistent with previous findings in other Panax species, where the cambial zone was identified as the most responsive tissue for callus induction due to its inherent meristematic activity (Lee et al. 2010b, Kim et al. 2014c).

The callus induction rate in our experiment was approximately 28.67%, which, although moderate, is within the expected range for woody medicinal plants. The relatively low induction frequency may reflect the physiological constraints of *P. vietnamensis* var. *fuscidiscus*, a slow-growing and highly recalcitrant species. Nonetheless, the quality of the induced calli particularly those arising from the cambial zone was morphologically consistent with cambial meristematic cells (CMCs), characterized by translucent, friable texture and non-aggregative proliferation. These traits align well with previously established criteria for identifying CMCs in Panax spp. and other plant systems (Lee et al. 2009).

Further distinction between CMCs and dedifferentiated callus cells (DDCs) was confirmed by vacuolar staining using neutral red. As reported by Kim et al. (2014c), DDCs typically exhibit a large central vacuole and irregular morphology, while CMCs are small, isodiametric cells containing multiple small vacuoles—hallmarks of cells retaining meristematic potential. Our microscopic observations reaffirmed these cellular differences, reinforcing the identification of CMC-like sectors within the callus mass.

Importantly, the CMC-rich calli maintained their unique morphological characteristics over six months of subculture, exhibiting slow, uniform proliferation without tissue browning or somaclonal variation. This long-term stability highlights the potential of these cells for downstream applications such as somatic embryogenesis, transformation, and secondary metabolite production. Previous studies have demonstrated the utility of CMCs as a uniform and genetically stable platform for plant regeneration and metabolic engineering in *Panax ginseng* (Lee et al. 2010b).

The present findings therefore not only confirm the suitability of picloram-BAP combination for CMC induction from *P. vietnamensis* var. *fuscidiscus*, but also provide a foundation for future work on optimizing CMC proliferation and differentiation. Further molecular characterization, including transcriptomic or epigenetic profiling, may help delineate the regulatory mechanisms underlying CMC maintenance and dedifferentiation in this valuable medicinal species.

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