https://doi.org/10.30799/jnpr.114.25100101 J. Nat. Prod. Resour. - Volume 10 Issue 1 (2025) 299–301

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ISSN: 2455-0299

Production of Phenolic Compounds in *Colobanthus quitensis* Kunth (Bartl.) through Cultivation in a Temporary Immersion Bioreactor

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ARTICLE DETAILS

Article history:
Received 26 January 2025
Accepted 23 February 2025
Available online 16 March 2024

Keywords:
Colobanthus quitensis
Phenolic Compounds
Temporary Immersion Bioreactor
Flavonoids
Bioactive Metabolites

ABSTRACT

The Antarctic vascular plant *Colobanthus quitensis* has developed unique mechanisms to survive in extreme environmental conditions, such as high UV-B radiation and nutrient scarcity. This study evaluated the effects of immersion frequency in a temporary immersion bioreactor (TIB) on biomass growth, phenolic compound accumulation, and flavonoid synthesis. Plants subjected to 24-hour immersion pulses exhibited the highest biomass yield (192.7%), phenylalanine ammonia-lyase (PAL) activity (52 µkats/kg protein), and total phenolic content (TPC; 40 mg GAE/g dry weight). Flavonoids such as neoschaftoside, saponarin, and schaftoside significantly accumulated under these conditions, demonstrating the metabolic shift induced by controlled nutrient deprivation and improved aeration. The findings highlight that intermittent stress optimises secondary metabolite production, providing a sustainable pathway for bioactive compound extraction. These phenolic metabolites have potential applications in agriculture as antifungal agents, in cosmetics for UV-B protection, and functional foods as antioxidants. This study underscores the relevance of TIB systems as scalable and efficient tools for enhancing plant-derived bioactive compounds, contributing to sustainability across various industries.

1. Introduction

Antarctica, a unique and extreme continent, presents challenging environmental conditions such as extremely low temperatures, strong winds, and elevated levels of UV-B radiation. These adversities limit life development, pushing plants to the edge of their physiological capabilities [1].

Among the species that have successfully colonised this hostile environment is the Antarctic pearlwort (*Colobanthus quitensis*), one of only two native vascular plants in the region. During the Holocene, this species established itself in maritime Antarctica, inhabiting the Antarctic Peninsula and surrounding islands [2]. Previous studies have shown that *C. quitensis* positively response to rising temperatures and UV-B radiation, particularly harmful during spring. In response, the plant has developed strategies, including the synthesis of metabolites such as phenolic compounds [3].

Phenolic compounds, widely distributed in the plant kingdom, represent one of the most diverse and functional groups of secondary metabolites. This group includes lignin, flavonoids, and phenolic acids, which are essential for plant development and defence [4]. Flavonoids, in particular, are notable for their benzo-γ-pyran structure and their widespread occurrence as glycosides in vascular plants. Their synthesis involves metabolic pathways derived from shikimate and acetate-malonate, followed by modifications such as hydroxylation, methylation, and glycosylation, resulting in significant functional diversity [5].

In this context, the enzyme phenylalanine ammonia-lyase (PAL) plays a central role in catalysing the phenylpropanoid pathway, which gives rise to a wide range of secondary metabolites, including flavonoids, stilbenes, anthocyanins, and lignin [5]. These pathways, which emerged approximately 500 million years ago, reflect the early adaptations of terrestrial plants to a hostile environment marked by high UV radiation levels and microbial stress [6].

Interest in these compounds has driven the development of strategies for their study and production. However, logistical difficulties associated with access and fieldwork in Antarctica limit the biotechnological potential of *C. quitensis*. Considering these constraints, controlled cultivation emerges as a relevant tool to explore the capabilities of this plant. Micropropagation, an advanced biotechnological system, has proven effective for clonal plant production and the study of plant physiology under specific conditions [7].

The success of in vitro cultures largely depends on the type of medium used. Semi-solid media, which employ gelling agents such as agar-agar, are widely used during the initial cultivation phase. However, temporary immersion systems, such as bioreactors, offer significant advantages by optimising nutrient and gas availability for explants. These systems enable more uniform contact with the culture medium, promoting growth and metabolite accumulation [8].

This present work presenting the ability of *C. quitensis* to produce phenolic compounds and its sensitivity to cultivation conditions, the use of advanced systems such as bioreactors could generate new opportunities to maximise both its growth and bioactive potential, laying the groundwork for future biotechnological applications.

2. Experimental Methods

2.1. Plant Material and Treatments

In vitro pre-established explants were obtained from samples collected near the Henryk Arctowski base during the summer. These were micropropagated monthly at a ratio of four explants per plant until sufficient biomass was achieved for subsequent studies. The culture medium used was Murashige-Skoog (4.46 g/L) supplemented with 3% w/v sucrose, 0.1 g/L myo-inositol, benzyl amino purine (BAP, 1.33 µM), indole acetic acid (IAA, 0.33 µM), and 0.2% activated carbon at pH 4.7 ± 0.1, with 2.0 g/L phytagel. Plants were grown at 14 ± 2 °C with a 16/8-hour light/dark photoperiod [3,7].

One-month-old plants were collected, weighed under sterile conditions, and transferred to a flask connected to a temporary immersion bioreactor (TIB). The basal culture medium was the same as above but without the gelling agent or activated carbon. The bioreactor was programmed with the following immersion conditions: 5-minute pulse every 6 hours; 5-minute pulse every 12 hours and 5-minute pulse daily. These conditions were maintained for 15 days under the same temperature and photoperiod.

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https://doi.org/10.30799/jnpr.114.25100101

Cite this Article as: Rodrigo A. Contreras, Karla Sepúlveda, Gustavo E. Zúñiga, Production of phenolic compounds in *Colobanthus quitensis* Kunth (Bartl.) through cultivation in a temporary immersion bioreactor, J. Nat. Prod. Resour. 10(1) (2025) 299-301.

Biomass yield was determined using Eq.(1), with initial and final biomass measured on an analytical balance. Water content was calculated by drying a tissue fraction at 65 °C for 72 hours and using Eq.(2):

$$\left(\frac{bm_f - bm_0}{bm_0}\right) \times 100 = yield\ (\%) \quad (1)$$

where, bm is biomass, f is final, and 0 is initial.

$$\left(\frac{bm_{fresh} - bm_{dry}}{bm_{dry}}\right) \times 100 = water\ content\ (\%) \quad (2)$$

where, bm_{fresh} is fresh biomass, and bm_{dry} is dry biomass.

2.2 Metabolite Extraction

Samples from two-week-old cultures, including regular in vitro conditions and the three TIB treatments, were collected, and growth rates were determined by comparing their initial and final masses. Ten grams of fresh tissue were ground in liquid nitrogen, followed by the addition of 5 mL methanol, and sonicated in an ice bath at 60 Hz for one hour. The extract was centrifuged at 10,000 × g, and the supernatant was washed five times with hexane to remove non-polar components. The methanolic phase was dried under nitrogen flow, weighed to calculate yield, and re-dissolved in ethanol at a standard concentration of 1 mg/mL [9].

2.3 Total Phenolic Content

The total phenolic content was determined using the Folin-Ciocalteu method. For this, 8 µL of the standardised extract were dissolved in 112 µL ultrapure water, followed by the addition of 20 µL Folin-Ciocalteu reagent. The reaction was kept in the dark for 15 minutes, and 60 µL of 7% w/v sodium carbonate was added. After one hour in the dark, absorbance was measured at 740 nm using a microplate spectrophotometer (Tecan Infinite M200Pro). Results were expressed as gallic acid equivalents (GAE), using a previously described calibration curve [10].

2.4 PAL Enzyme Activity

Samples of 100 mg tissue from each treatment were ground in liquid nitrogen and extracted in 1 mL Tris-HCl buffer (50 mM, pH 8.5) supplemented with polyvinylpyrrolidone (PVP, 5%) and β-mercaptoethanol (14.4 mM). The suspension was centrifuged at 10,000 × g for 15 minutes at 4 °C, and the supernatant was collected for analysis.

Protein concentration was determined using the Bradford method. PAL activity was measured by combining 200 µL supernatant with 800 µL substrate (0.2% w/v L-phenylalanine), with 0.2% w/v D-phenylalanine as a negative control. Reactions were incubated at 38 °C for 2 hours, and the formation of trans-cinnamic acid was quantified by measuring absorbance at 290 nm in a spectrophotometer [3]. The calculation followed Eq.(3):

$$PAL\ activity = \frac{\left(A_{290}^{L-phe} - A_{290}^{D-phe}\right) \times V \times 10^6}{\varepsilon \times l \times t \times m \times C_P} \quad (3)$$

where A290 is absorbance at 290 nm for L-phe and D-phe, V is volume (1 mL), ε is the molar extinction coefficient (9,630 M^{-1} cm^{-1}), l is path length (1 cm), t is incubation time (2 hours), and m is mass, and C_p is protein concentration determined by Bradford method. Results were expressed in µkats/kg protein.

2.5 Phenolic Compound Analysis by LC-MS/MS

The standardised extract was filtered through a 0.45 µm PVDF membrane, and 20 µL were injected into a triple quadrupole LC-MS system with a Zorbax Eclipse C18 column (4.6 × 150 mm, 5 µm) at 25 °C. The mobile phase was binary: solvent A (10 mM ammonium acetate, pH 4.0) and solvent B (10 mM ammonium acetate). The gradient elution was: 0–5 min, 0–50% B; 5–10 min, 50–52% B; 10–20 min, 52–100% B; 20–35 min, 100–65% B; 35–40 min, 65–0% B.

MS/MS analysis was conducted in negative electrospray ionisation (ESI) mode using an Agilent 6410 detector. Key parameters included a capillary voltage of 4,000 V, nebulisation pressure of 2.8 bar, nitrogen flow rate of 60 L/h, and a temperature of 330 °C. Flavonoids were identified using a custom database and expressed as relative concentrations compared to the control.

2.6 Statistical Analysis

All assays were conducted in triplicate. Significant differences between treatments were evaluated using analysis of variance (ANOVA) with GraphPad Prism 10 software, considering a significance level of $p < 0.05$.

3. Results and Discussion

This study evaluated the effect of different immersion frequencies in a temporary immersion bioreactor (TIB) system specifically designed for in vitro cultures (Fig.1). The system consists of a main container holding the culture medium and an upper compartment where the plant tissues were placed. The medium is transferred to the upper compartment using negative pressure generated by a vacuum pump, enabling controlled immersions while ensuring proper aeration between pulses.

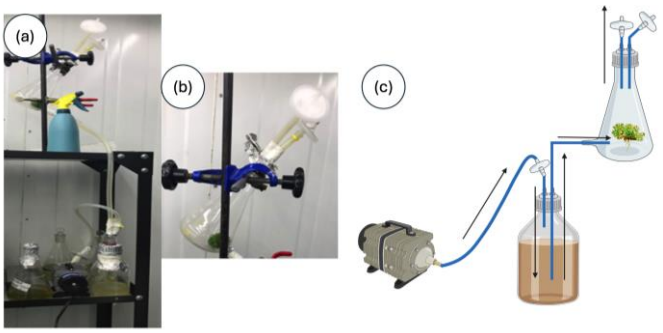

Fig. 1 Schematic and experimental configuration of the TIB. (a) Overview of the TIB system, showing the setup of the upper chamber containing the plant tissue, the connection to the vacuum pump, and the lower chamber containing the culture medium. (b) Detail of the upper chamber with the plant tissue and the air inlet and outlet connections. (c) Schematic representation of TIB operation: the culture medium is transferred to the upper chamber through negative pressure generated by a vacuum pump, allowing controlled immersion of the plant tissue at specific intervals. Aeration occurs during resting periods, ensuring gas exchange and preventing hypoxia

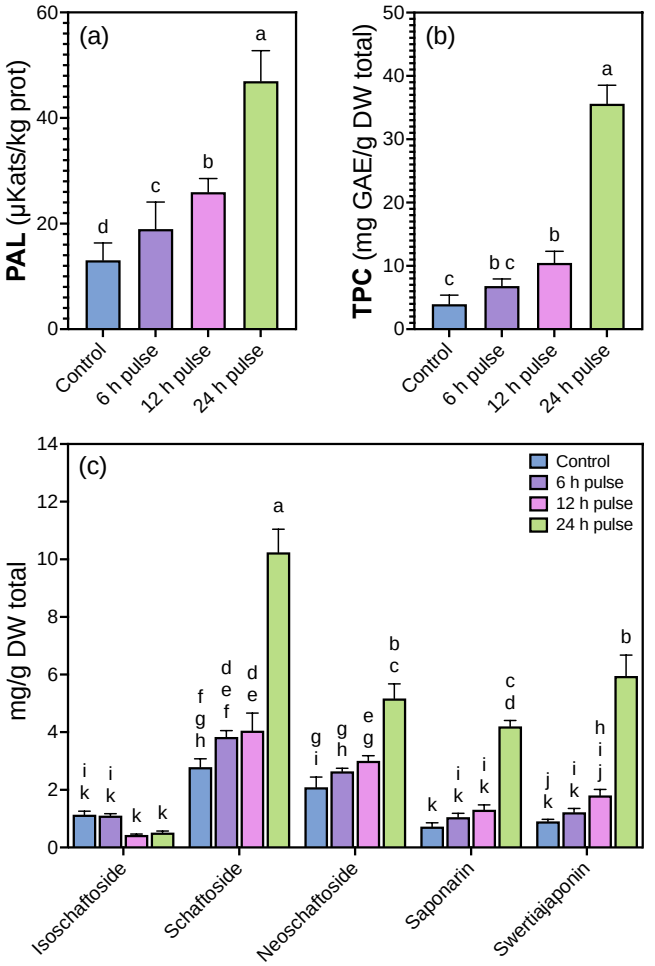

Fig. 2 Effect of temporary immersion pulse frequency on phenolic compound metabolism. (a) PAL activity expressed in µkat/kg protein. (b) Total phenolic content (TPC) expressed in mg gallic acid equivalents (GAE) per gram of dry weight (DW). (c) Concentrations of individual flavonoids, including isoschaftoside, schaftoside, neoschaftoside, saponarin, and swertiajaponin, expressed in mg/g DW. Treatments correspond to the control (traditional cultivation) and immersion pulse frequencies of 6, 12, and 24 hours in the culture medium. According to Tukey letters above bars indicate statistically significant differences ($p < 0.05$). Data are presented as mean ± standard deviation ($n = 9$)

https://doi.org/10.30799/jnpr.114.25100101

3.1 Biomass Yield

Biomass yields, calculated as the ratio of final to initial biomass, were significantly higher in treatments with 24-hour pulses (192.7 ± 14.6%) compared to the control (78.3 ± 8.4%), 6-hour pulses (121.5 ± 9.7%), and 12-hour pulses (131.3 ± 11.2%). This suggests that lower immersion frequency favours plant tissue growth in the TIB system, likely due to an optimal balance between aeration and nutrient availability.

3.2 PAL Activity and TPC

The activity of the enzyme phenylalanine ammonia-lyase (PAL), a key component in flavonoid biosynthesis, showed a significant increase with 24-hour pulses (Fig. 2a). The highest values reached 52 μkats/kg protein in this treatment, followed by 12-hour pulses (36 μkats/kg). The control exhibited the lowest activity (17 μkats/kg). Similarly, total phenolic content (TPC) levels showed an upward trend, reaching 40 mg GAE/g dry weight under 24-hour pulses, which were significantly higher compared to the 12-hour and 6-hour treatments and the control (Fig. 2b). This suggests that longer immersion pulses create favourable conditions for the accumulation of phenolic compounds.

3.3 Flavonoid Accumulation

The accumulation of specific flavonoids (Fig. 2c) varied significantly among treatments. Notably, neoschaftoside showed the highest concentration (10.8 mg/g dry weight) under 24-hour pulses, while 6-hour and 12-hour treatments exhibited intermediate concentrations, and the control had the lowest levels. A similar pattern was observed for schaftoside, saponarin, and swertiajaponin, which reached their maximum values with 24-hour pulses. In contrast, isoschaftoside did not show significant differences across treatments.

3.4 Discussion

The results of this study demonstrate that the frequency of immersion in TIB has a significant impact on biomass growth and the accumulation of secondary metabolites, such as phenolic compounds and flavonoids [11]. Prolonged immersion frequencies (every 24 hours) enhanced both biomass yield and PAL activity, which in turn promoted the synthesis of TPC and specific flavonoids, such as neoschaftoside, saponarin, and schaftoside. This optimal balance between aeration and periodic nutrient supply offer an environment that stimulates secondary metabolic pathways, leading to the accumulation of high-value bioactive metabolites.

Temporal nutrient deprivation not only reduces competition between primary and secondary metabolic pathways but also acts as a controlled metabolic stress trigger, activating defence pathways and secondary metabolite synthesis through hormonal signalling and reactive oxygen species (ROS) [12]. This process is reflected in the increased PAL activity and potentially the accumulation of metabolic precursors, such as phenylalanine, that are channelled into flavonoid biosynthesis [3]. Furthermore, active aeration periods prevent hypoxic conditions, promoting energy homeostasis and supporting both growth and bioactive compound production.

From an applied perspective, these phenolic compounds and flavonoids hold significant potential in various industrial sectors [13]. In agriculture, flavonoids such as swertiajaponin, schaftoside, and saponarin have demonstrated efficacy in inhibiting key virulence enzymes in pathogens like *Botrytis cinerea* [14]. These compounds represent sustainable alternatives to synthetic fungicides, whose efficacy is declining due to pathogen resistance and their negative environmental impact. Additionally, flavonoid-rich extracts have proven effective in postharvest fruit preservation, such as table grapes, protecting quality and extending shelf life [9].

In the cosmetics sector, the photoprotective capacity of flavonoids, especially in species adapted to extreme conditions like *C. quitensis*, offers opportunities for developing products that protect against UV-B radiation. These compounds form protective films on the external layers of plant tissues, reducing UV transmission and minimising oxidative damage. In the food industry, phenolic compounds can be utilised as functional ingredients, not only for their antioxidant and anti-inflammatory properties but also to improve the oxidative stability of fat-rich foods, such as oils and emulsions [3].

4. Conclusion

In conclusion, TIB systems with prolonged immersion pulses present an effective approach to optimising both growth and the production of high-value secondary metabolites, addressing challenges in agriculture, cosmetics, and functional foods. This approach not only provides a sustainable solution for bioactive compound production but also opens new opportunities for designing innovative products with functional and environmental benefits. Future research should focus on integrating these strategies with advanced metabolomics and transcriptomics tools to maximise the production of specific compounds and explore new industrial applications. Overall, this study underscores the importance of controlled biotechnological systems in generating sustainable and high-impact solutions.

Author contributions

Rodrigo A. Contreras: Conceptualization, Writing – Original Draft, Project Administration, Investigation, Methodology, Experiments;
Karla Sepúlveda: Methodology, Investigation, Experiments, Data Analysis;
Gustavo E. Zúñiga: Supervision, Data Curation, Formal Analysis, Project Administration.

Funding

This work was funded by project Fondecyt 3160274 to RAC.

Competing interests

The authors declare that they have no competing interests.

Acknowledgement

The authors would like to thank Marisol Pizarro for her collaboration in the development of this work. They also extend their gratitude to Elizabeth Barría for her support in micropropagation and the preparation of sterile materials.

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https://doi.org/10.30799/jnpr.114.25100101

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