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Bioactive compounds in *Laelia speciosa* (Orchidaceae) seedlings grown in temporary immersion bioreactor

Compuestos bioactivos en plántulas de *Laelia speciosa* (Orchidaceae) cultivados en biorreactor de inmersión temporal

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ABSTRACT

Laelia speciosa is an epiphytic orchid endemic to Mexico, enjoying special protected status due to illegal sales and clandestine field extraction. Until now, *in vitro* cultivation of this plant has not been explored using temporary immersion bioreactor (TIB). *L. speciosa* seedlings were grown in a TIB, and the presence of bioactive compounds released into the liquid culture medium was determined. Bioactive compounds such as antioxidants, total polyphenols, and flavonoids were identified. The maximum concentration of total polyphenols was 2.29 ± 0.10 mg GAE/g, and the antioxidant capacity reached 33.7 %. Colorimetric tests revealed the presence of flavanones and flavonols. Gas chromatography-mass spectrometry identified components of the culture medium as 1,3-dihydroxyacetone dimer and 5-(hydroxymethyl)-2-furancarbaldehyde. Fourier transform infrared spectroscopy confirmed the presence of polyphenols in the culture medium. This work provides an alternative method for obtaining bioactive compounds while conserving *L. speciosa* seedlings.

Keywords: Antioxidants, *Laelia speciosa*, propagation, secondary compounds, temporary immersion.

RESUMEN

Laelia speciosa es una orquídea epífita endémica de México, que cuenta con un estatus de protección especial debido a su venta ilegal y extracción clandestina en el campo. Hasta ahora, no se había explorado el cultivo *in vitro* de esta planta utilizando el biorreactor de inmersión temporal (TIB). Se cultivaron plántulas de *L. speciosa* en un TIB y se determinó la presencia de compuestos bioactivos liberados en el medio de cultivo líquido. Se identificaron compuestos bioactivos como los antioxidantes, polifenoles totales y flavonoides. La concentración máxima de polifenoles totales fue de 2,29 ± 0,10 mg GAE/g, y la capacidad antioxidante alcanzó el 33,7 %. Las pruebas colorimétricas revelaron la presencia de flavanonas y flavonoles. La cromatografía de gases-espectrometría de masas identificó componentes como el dímero 1,3 dihidroxiacetona y el 5-(hidroximetil)-2-furancarbaldehido. La espectroscopía infrarroja por transformada de Fourier confirmó la presencia de polifenoles en el medio de cultivo. Este trabajo proporciona un método alternativo para la obtención de compuestos bioactivos conservando las plántulas de *L. speciosa.*

Palabras clave: Antioxidantes, compuestos secundarios, inmersión temporal, *Laelia speciosa*, propagación.

1. INTRODUCTION

Plants have been indispensable to humans due to their medicinal properties and currently represent a promising reservoir of bioactive compounds. Approximately 80 % of the world's population still relies on plants for primary health care, and about 25 % of prescription medicines in Western medicine are derived either directly or indirectly from plants (Fowler, 2006). Furthermore, an observable trend is emerging across various sectors, favoring the utilization of natural products over their synthetic counterparts.

Among the natural substances produced by plants are secondary metabolites that include terpenes, phenolic compounds, glycosides, and alkaloids (Hostettmann *et al.*, 2005). These compounds serve diverse physiological functions, including defense mechanisms, competitive advantages, attraction of pollinators, and protection against various environmental stresses (Bourgaud *et al.*, 2001; Sepúlveda-Jiménez *et al.*, 2003). Moreover, several secondary metabolites have been linked to various bioactive properties, including antioxidant, antimicrobial, cytotoxic, hepatoprotective, antiviral, anti-inflammatory and antiapoptotic capacities (Oskoueian *et al.*, 2011; Chinsamy *et al.*, 2014; Vinaykumar *et al.*, 2020; Dias *et al.*, 2021; Flieger *et al.*, 2021).

Orchids constitute a diverse group encompassing numerous species that have been used for cultural, economic, ceremonial, medicinal, culinary, and ornamental purposes (Emeterio-Lara *et al.*, 2016). Among orchids, *L. speciosa* stands out as an epiphytic

species endemic to Mexico, capable of producing bioactive molecules. The contemporary distribution of *L. speciosa* is 52,892 km^2, representing 4 % of Mexican territory, with only 0.6 % of the distribution falling within protected areas (Flores-Tolentino *et al.*, 2020). *L. speciosa* holds a designation under NOM-059-2010 (SEMARNAT, 2010), classifying it in the special protection category due to issues of illegal trade and clandestine field extraction. Although *L. speciosa* has been propagated *in vitro* (Díaz & Salgado-Garciglia, 2006; Ávila-Díaz *et al.*, 2009; Sarabia-Ochoa *et al.*, 2010), its potential to produce antioxidants, total polyphenols, and other bioactive molecules has not been evaluated so far.

Some molecules, such as flavonoids, alkaloids, tannins, and enzymes with anti-collagenase, anti-cyclooxygenase, or anti-elastase properties, have been reported in orchids such as *Epidendrum secundum*, *Dendrobium nobile*, *D. moschatum*, *A. graminifolia*, *Coelogyne nitida*, *Dendrobium* spp., *Eulophia hereroensis*, *E. petersii*, *Ansellia africana*, *Bulbophyllum scaberulum*, *Cyrtorchis arcuata*, and *Malaxis acuminata* (Chinsamy *et al.*, 2014; Bose *et al.*, 2017; Athipornchai & Jullapo, 2018; Natta *et al.*, 2022; Cazar *et al.*, 2023). Additionally, vasorelaxant effects have been observed in *L. speciosa* and *L. anceps* (Vergara-Galicia *et al.*, 2013). It is noteworthy that these biomolecules were either directly extracted from the plants (flowers, leaves, roots, etc.) or the plant has been obtained from local markets, which could affect conservation efforts given the large volumes of plants used.

An alternative approach for obtaining orchid biomass involves *in vitro* plant tissue culture. These techniques allow the aseptic propagation of plant material, including protoplasts, cells, tissues, and organs, and under appropriate nutritional and environmental conditions, can induce various morphological and physiological responses, including the production of secondary compounds. Traditionally, solid media have been employed in these techniques, which can escalate costs. Temporary immersion bioreactor (TIB) represent a more efficient alternative. TIB has been successfully applied in the cultivation of various plant species such as *Dianthus caryophyllus* (Ahmadian *et al.*, 2017), *Gynura procumbens* (Pramita *et al.*, 2018), *Elaeis guineensis* (Marbun *et al.*, 2015), among others, but its potential has not yet been explored in the context of *L. speciosa*. Furthermore, the composition of the culture medium in which the plants are immersed and which may potentially contain valuable secondary metabolites has not been reported. In this study, *L. speciosa* was cultivated in TIB, and the presence of bioactive molecules in the culture medium was investigated.

2. MATERIALS AND METHODS

2.1. Obtaining *L. speciosa* seedlings and reagents

L. speciosa seeds were obtained from Metztitlán, Hidalgo, Mexico. The seedlings were germinated *in vitro* in half-strength Murashige & Skoog medium (1962) and 1 g/L of activated charcoal, gelled (0.8 % agar, w/v) as detailed in Aguilar-Morales & López-Escamilla (2013).

The following reagents were used: Folin-Ciocalteu reagent (Hycel), 2,2-diphenyl-1-picrylhydrazyl, zinc metal, gallic acid, sodium carbonate (Sigma Aldrich), hydrochloric acid, and sodium hydroxide (Meyer).

2.2. Cultivation of *L. speciosa* in temporary immersion bioreactor

Eight pairs of temporary immersion bioreactor were used. Each pair contained two glass flasks (1 L). The first flask contained 250 mL of Murashige & Skoog medium supplemented with 1 mg/L benzylaminopurine and 2 mg/L naphthaleneacetic acid. The second flask held 2 g of *L. speciosa* seedlings. Silicone tubing was inserted through the lid of each container to establish a connection between the flasks, facilitating the displacement of culture medium to the plant flask. Hydrophobic filters (0.2 µm) were used to ensure air sterility. Seedling immersion frequency in the liquid medium was one minute every eight hours. The cultures were then maintained for a duration of eight weeks at a controlled temperature of 26 °C ± 2, under a photoperiod of 16/8 h, and with a light intensity of 30 µmol $m^{-2}s^{-1}$.

2.3. Biomass quantification

The fresh biomass of *L. speciosa* was quantified after 8 weeks of incubation. The mass of seedlings from each bioreactor was extracted under sterile conditions, and its weight was calculated using an analytical balance. Subsequently, the extracted biomass was returned to its respective bioreactor. Results were reported in grams.

2.4. Obtaining the TIB culture extract

The liquid medium in the TIB, in where the seedlings were submerged, was collected on a weekly basis until week 8 (extracts M1 to M8) and subsequently replaced with fresh medium. The presence of antioxidants, total polyphenols and bioactive compounds was determined directly in the collected culture medium. The liquid medium without any contact with the plants served as a control.

2.5. Determination of biomolecules from the liquid culture medium

2.5.1. Total polyphenols

The Folin-Ciocalteu technique was employed to determine total polyphenols, following the procedure outlined by Choi *et al.* (2006). The reaction mixture comprised 200 µL of culture extract, 2.0 mL of 2 % sodium carbonate, and 100 µL of 50 % Folin-Ciocalteu reagent. After incubating for 30 minutes at room temperature in the dark, the absorbance was measured at 750 nm using a UV-VIS spectrophotometer (Genesys 10S, Thermo Scientific). A calibration curve ranging from 0 to 100 mg/mL of gallic acid (GAE) was constructed, and the results were reported as mg GAE equivalents/mL.

2.5.2. Antioxidant capacity

The antioxidant capacity was assessed using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging technique, following the procedure outlined by Choi *et al.* (2006).

The reaction mixture comprised of 800 µL of 0.2 mM DPPH in methanol and 200 µL of culture extract. After thorough mixing for 10 minutes in the absence of light, the absorbance was measured at 520 nm using a UV-VIS spectrophotometer (Genesys 10S, Thermo Scientific). The results were expressed as the percentage of DPPH radical scavenging and calculated according to equation 1.

$$\text{Ec. 1. Percentage of DPPH radical scavening } = 1 - \frac{\text{Sample absorbance}}{\text{Absorbance of the control}} x\ 100$$

2.5.3. Flavonoids

Flavonoids present in the culture extract were quantified using a colorimetric test based on the Zn/HCl reduction and alkaline reagent method, following the procedure outlined by Sajjalaguddam & Paladugu (2015) with slight modifications. For the Zn/HCl reduction, 2.0 mL of culture extract was mixed with 0.10 g of metallic Zn°, and concentrated HCl drops were added to the mixture. The development of a reddish color indicated the presence of flavonoids. In the alkaline test, 2.0 mL of culture extract was combined with 500 µL of NaOH 1N. The appearance of a yellow color signified the presence of flavonoids.

2.6. Determination of other biomolecules by gas chromatography-mass spectrometry (GC/MS)

The M8 culture extract was lyophilized and selected for GC/MS analysis using an Agilent 7810A with a 5975C mass detector. A capillary column (Agilent DB5) was utilized for analysis. A 2 µL sample was loaded and run for 32 minutes with a 15 °C/min temperature ramp to 300 °C and a 1.5 mL/min carrier gas flow. Data were acquired in scan mode, analyzed by Chemstation software and identified using the Nist 2.0 library.

2.7. Analysis by Fourier transform infrared spectroscopy (FTIR)

The lyophilized M8 extract was analized using Fourier transform infrared spectroscopy. A Nicolet iS10 FTIR instrument (Thermo Scientific) was employed in the range of 4000 - 600 cm^{-1} with a resolution of 4 cm^{-1}.

2.8. Statistical analysis

The results were analyzed by ANOVA using the Fisher test at $\alpha < 0.05$, employing Minitab software, version 13 (USA), to determine the least significant difference.

3. RESULTS

3.1. Biomass obtained from the TIB culture

L. speciosa plants cultivated in TIB remained green and free from contamination. Furthermore, during the incubation period of the plants, the culture medium (initially colorless) gradually transitioned from a pale salmon-red hue to an intense salmon-red color

(Fig. 1). The biomass obtained after 8 weeks of cultivation was 30.54 ± 1.49 g, representing a notable increase of 1527 % over the initial 2 g of seedlings.
This underscores the effectiveness of TIB as a valuable alternative for propagating *L. speciosa*.

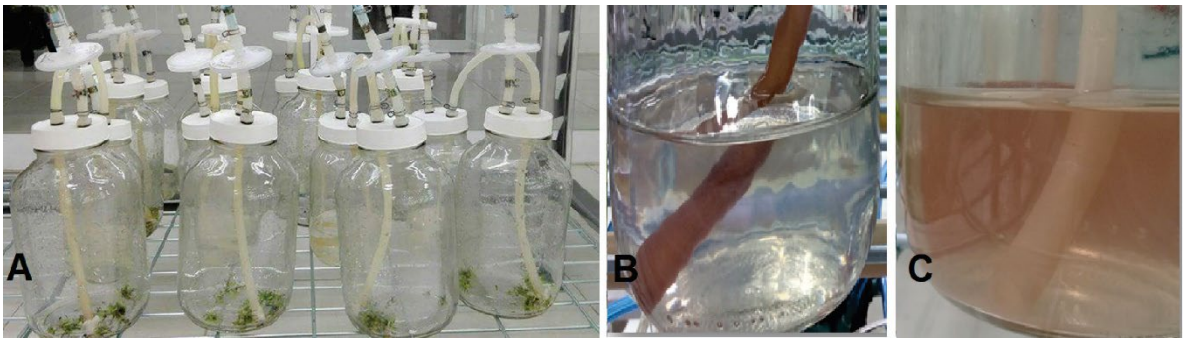

Fig. 1. Assembly of the temporary immersion bioreactor. Twin flask system with *L. speciosa* seedlings (A), culture medium at the beginning of the experiment (B), and pink-shaded culture medium after 8 weeks of incubation (C).

3.2. Total polyphenols, antioxidant activity and flavonoids

Total polyphenols were present in all samples and the concentration increased as the weeks of incubation passed (Fig. 2). The M8 extract had significantly higher levels than other weeks, reaching a maximum of 2.29 ± 0.10 mg GAE/mL. These results demonstrate that *L. speciosa* produces polyphenols that can be extracted into the culture medium using TIB.

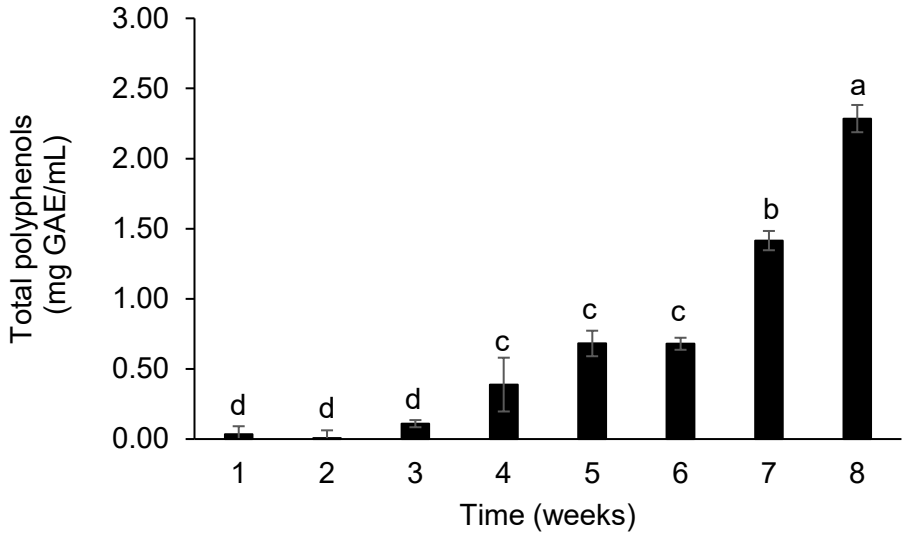

Fig. 2. Total polyphenols from *in vitro* culture of *L. speciosa* in a temporary immersion system. Different letters indicate a statistically significant difference ($p < 0.05$).

The DPPH assay revealed a free radical scavenging percentage ranging from 19.02 to 33.7 % in extracts M1 to M8. Statistically significant differences were observed at week 7 compared to weeks 2-3 (Fig. 3).

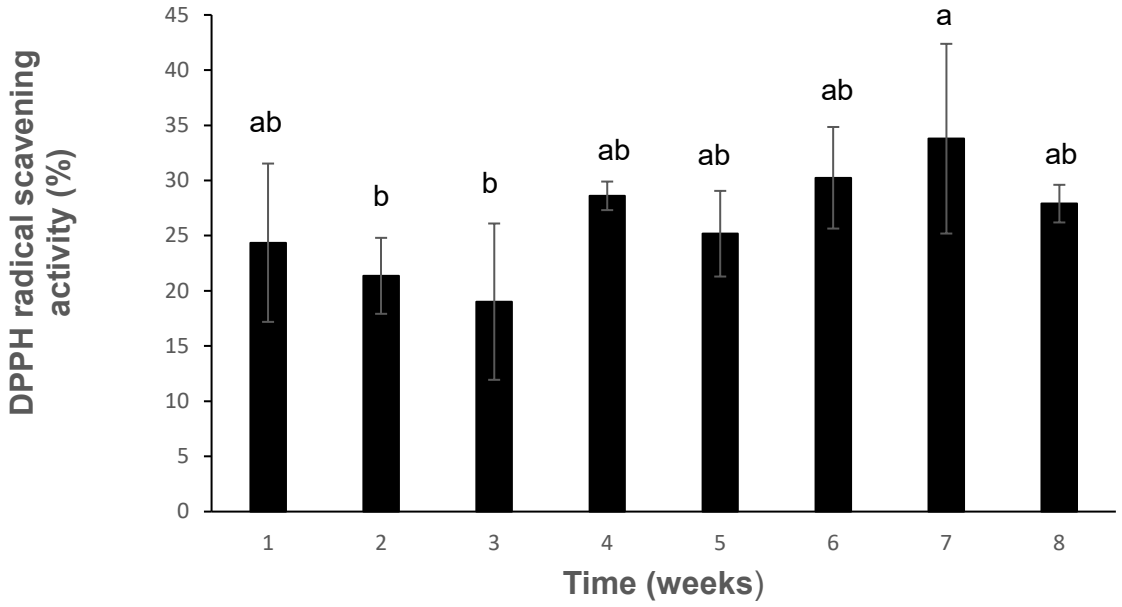

Fig. 3. Antioxidant capacity of *in vitro* culture of *L. speciosa* in a temporary immersion system. Different letters indicate a statistically significant difference ($p < 0.05$).

Furthermore, the Zn/HCl reduction test was positive for all extracts. A weak pink coloration was observed, indicating the presence of flavanones and flavonols. Additionally, the alkaline reagent test produced a yellow coloration in all extracts, confirming the presence of flavones and flavonols (Fig. 4).

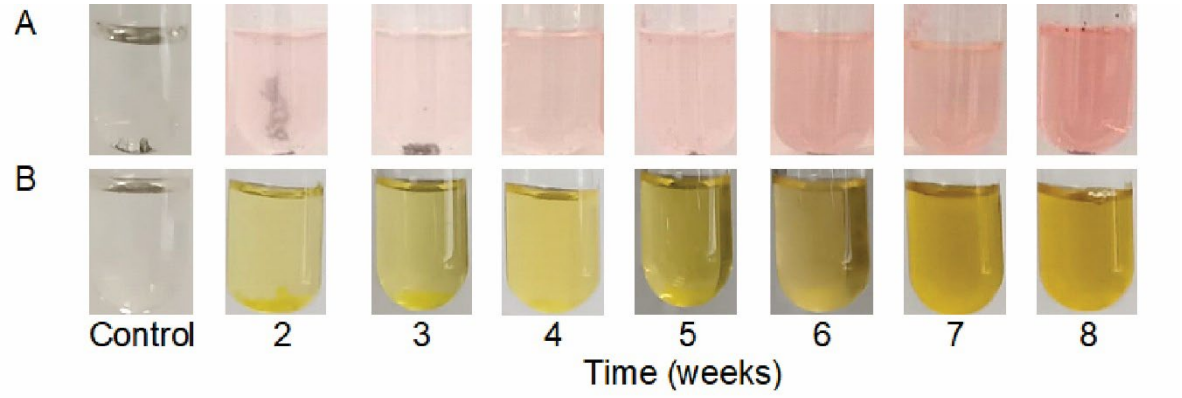

Fig. 4. Flavonoids from *in vitro* culture of *L. speciosa* in a temporary immersion system. Zn/HCl reduction test (A) and alkaline reagent test (B).

3.3. Other biomolecules identified by GC/MS

The main compounds identified in the M8 extract were 1,3-dihydroxyacetone dimer (retention time 6.719 min, 10.06 % area) and 5-(hydroxymethyl)-2-furancarbaldehyde (retention time 9.679 min, 29.08 % area) (Fig. 5).

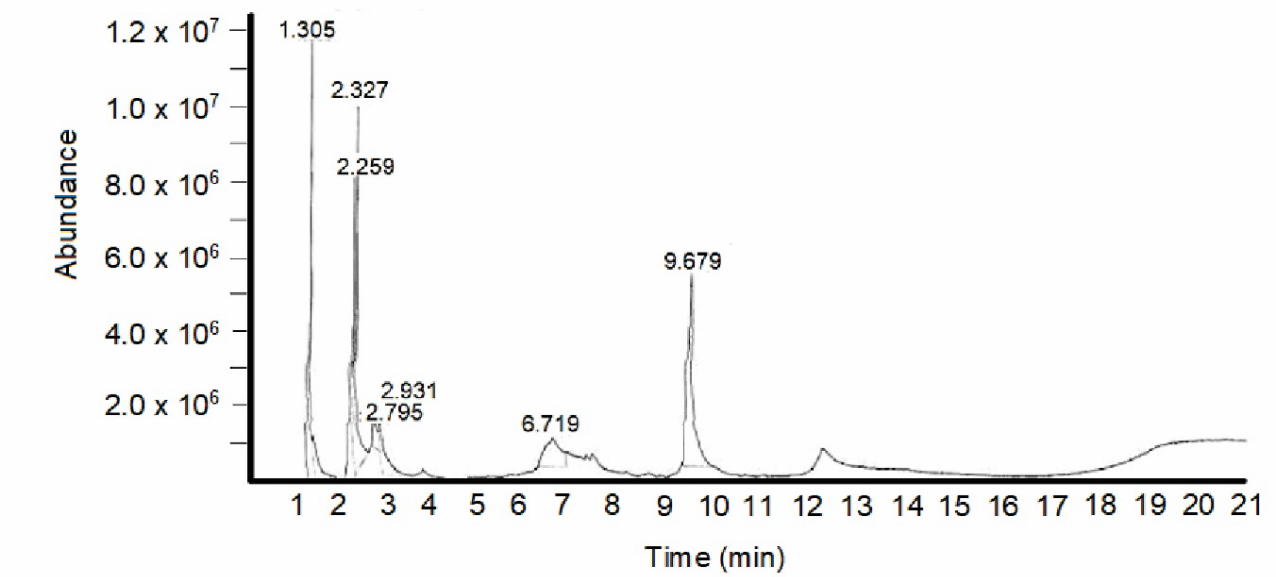

Fig. 5. GC/MS spectra of *in vitro* culture of *L. speciosa* in a temporary immersion system.

3.4. FTIR

The FTIR spectrum of the M8 culture extract showed the presence of polyphenols. The υ(OH) absorption at 3297 cm^{-1} and υ(C-O) stretching bands at 1100 cm^{-1} confirmed this result (Fig. 6).

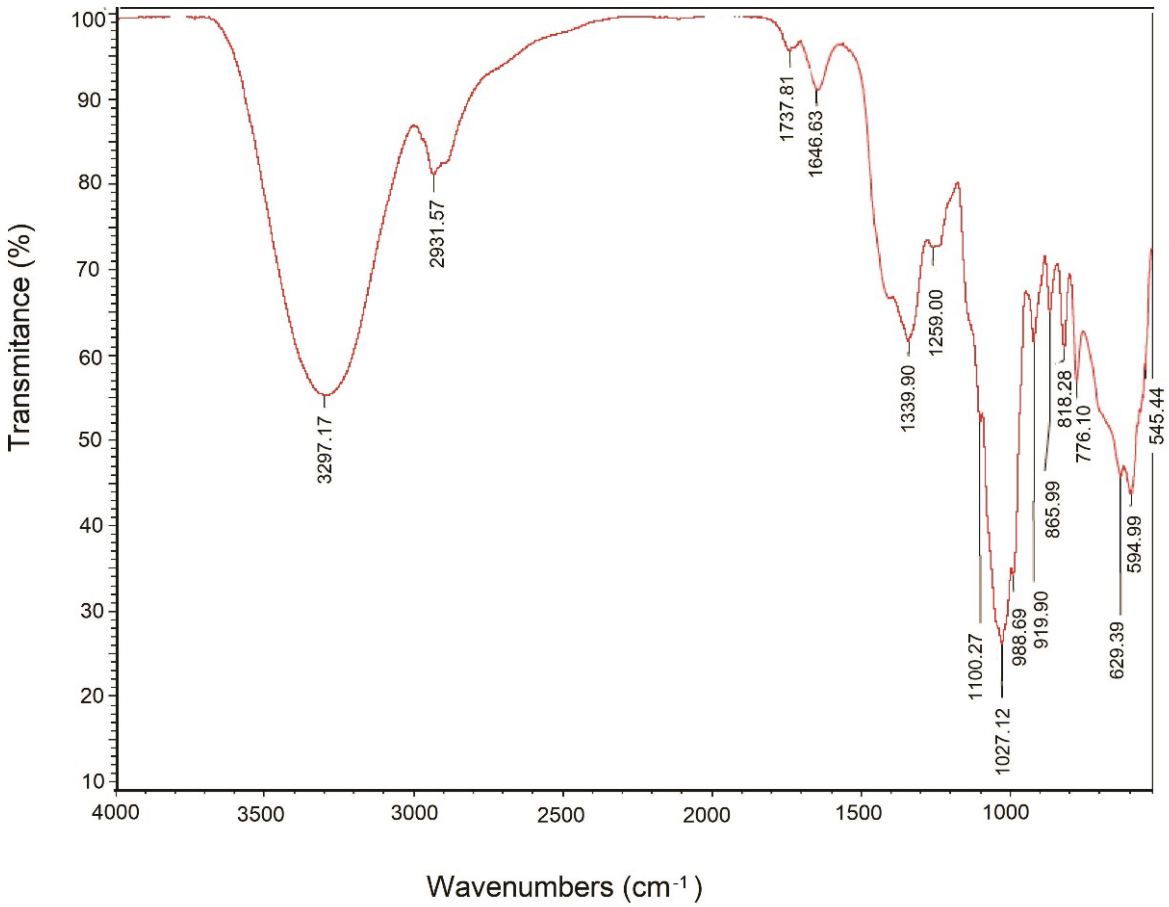

Fig. 6. FTIR spectra of *in vitro* culture of *L. speciosa* in a temporary immersion system.

4. DISCUSSION

The TIB utilized in this study facilitated both seedling growth and the extraction of bioactive compounds into the liquid medium. This is the first work where the cultivation of *L. speciosa* in a TIB has been documented. Until now, temporary immersion systems have been used for the cultivation of other orchids, such as *Phalaenopsis* sp., *Vanilla planifolia*, *Cattleya walkeriana*, *Dendrobium candidum* and *Epidendrum fulgens* (Nongdam *et al.*, 2023), but not for *L. speciosa*. Among the advantages of using the TIB are cost reduction and greater simplicity when scaling the process. Furthermore, automation could be used for large-scale production of plants. In contrast to the traditional cultivation of orchids *in vitro* on solid or semi-solid systems, which has limitations in terms of multiplication rates and incurs high production costs due to frequent medium changes. The liquid medium employed in this study supported culture for 8 weeks. The successful cultivation of *L. speciosa* biomass in the TIB can likely be attributed to favorable operating parameters, as factors such as immersion time, medium volume, and photoperiod are known to influence tissue growth in temporary immersion systems (Nongdam *et al.*, 2023).

This study identified the presence of both total polyphenols and flavonoids with antioxidant capacity. This fact is not unusual given that orchids have been recognized as synthesizers

and accumulators of various bioactive compounds, including alkaloids, saponins, carotenoids, flavonoids, and polyphenols, within their cellular and tissue structures. The presence of these compounds suggests potential medicinal properties (Natta *et al.*, 2022). These compounds play a vital role in scavenging reactive oxygen species, including hydroxyl, peroxyl, hypochlorous acid, superoxide anion, and peroxynitrite radicals, which are associated with cell damage and diseases (Giri *et al.*, 2012). Notably, this study represents the first report evaluating total polyphenol levels and antioxidant capacity in the culture medium of *L. speciosa*. There are also no studies that determine the concentration of antioxidants directly in flowers, leaves, stems and roots of *L. speciosa*. The concentration of total polyphenols has been reported in other orchids, such as in *Epidendrum secundum* (leaves) with 4.03 mg GAE/g (Cazar *et al.*, 2023), in extracts of *Dendrobium* sp., *A. graminifolia* and *Coelogyne* with 5-40 mg GAE/g (Natta *et al.*, 2022), in *Dendrobium* flowers with 5.34 mg GAE/g (Athipornchai & Jullapo, 2018), in *Habenaria edgeworthii* with 14.3 mg GAE/100 g (Giri *et al.*, 2012) and in *Malaxis acuminata* with 7.82-32.59 mg GAE/g (Bose *et al.*, 2017). It is worth noting that the antioxidant capacity observed in this study exceeded the 9 % reported for *Eulophia macrobulbon* roots (Schuster *et al.*, 2017).

Although the concentration of total polyphenols increased as the weeks of cultivation progressed, this same profile was not observed with the antioxidant capacity. It has been reported that, in addition to total polyphenols, there are other molecules that contribute to antioxidant effects. Notable contributors include enzymes such as catalase, superoxide dismutase, glutathione peroxidase, and glucose-6-phosphate dehydrogenase, as well as vitamins like ascorbic acid, retinol, and tocopherols. Additionally, low molecular weight compounds such as uric acid and various amino acids, along with protein-bound metals like ferritin and trace elements such as selenium, zinc, and manganese, are implicated in these antioxidant effects (Flieger *et al.*, 2021). Furthermore, it is worth noting that seedlings may generate oxidants as part of an oxidative stress response, which can counteract the actions of antioxidants.

On the other hand, the compound 5-(hydroxymethyl)-2-furancarbaldehyde, identified by GC/MS in this work, has been reported in several sources, including the fruit of *Gardenia gummifera* (Vinaykumar *et al.*, 2020), extracts of *Callistemon lanceolatus* with anticancer properties (Ahmad *et al.*, 2018) and in honey (Montaser *et al.*, 2023). It has also been found to exhibit potent antioxidant effects in aged garlic (Wang *et al.*, 2016). Additionally, the presence of 1,3-dihydroxyacetone dimer was detected, a compound previously identified in *Moringa oleifera* leaves (Bhalla *et al.*, 2021) and floral nectar from *Ericomyrtus serpyllifolia* and *Verticordia chrysantha* (Obeng-Darko *et al.*, 2023). This compound has also found utility in biomaterials applications (Zelikin & Putnam, 2005).

The choice of solvent for extracting antioxidants can significantly influence the types and concentrations of compounds recovered. Various extraction solvents have been reported in the literature, including chloroform, acetone, methanol, and ethanol (Cazar *et al.*, 2023; Natta *et al.*, 2022; Bose *et al.*, 2017), and the yields can vary depending on the plant species and the plant part used for extraction. In this study, we employed water from the *L. speciosa* culture medium as the solvent for extraction. This approach circumvented the need for direct plant utilization and contributed to the conservation of resources.

Furthermore, it is worth noting that the use of water as a solvent carries a lower environmental impact when compared to chloroform or methanol. Our findings suggest a practical approach for the extraction of bioactive compounds from TIB. Specifically, we recommend removing the culture medium from TIB after the eighth week to extract valuable metabolites while simultaneously replenishing the medium to facilitate ongoing seedling growth. This dual-purpose strategy allows the culture system to efficiently generate biomass for subculture while obtaining essential metabolites from the culture medium.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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