Research article

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Phenolic profile and antioxidant activity from wild and *in vitro* cultivated *Rhynchostele rossii* (Orchidaceae)

Perfil de compuestos fenólicos y actividad antioxidante de *Rhynchostele rossii* (Orchidaceae) silvestre y cultivada *in vitro*

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Abstract:

Background and Aims: *Rhynchostele rossii* is an orchid native to Mexico known as *gallinitas* (little chickens) that is threatened due to overexploitation for ornamental purposes, as a consequence of which it is necessary to realize efforts for its conservation. To date there are no reports of phytochemical studies of this orchid, although it is well known that species of the Orchidaceae family are a good source of bioactive and nutraceutical compounds (e. g. vanillin). Therefore, the main objective of this research was to establish a *R. rossii in vitro* germination protocol for propagation and determination of phenolic compounds that contribute to its phytochemical knowledge.

Methods: A specimen of a wild plant and some seedlings obtained by *in vitro* culture were dried, milled and their components extracted with MeOH; anti-free radical activity (DPPH), total phenols and flavonoids were determined by spectrophotometric methods, and individual phenolic compounds were identified and quantified by liquid chromatography coupled to mass spectrometry (LC-MS).

Key results: The root of the wild plant showed the highest content of total phenols and flavonoids with 121.60 mg GAE g^{-1} and 108.73 mg CE g^{-1}, respectively, and the best anti-free radical activity with an IC_{50} 53.63 µg ml^{-1}. Extracts from seedlings obtained by *in vitro* culture also produced phenolic compounds, showing a total phenolic and flavonoid content of 37.35 mg GAE g^{-1} and 0.16 mg CE g^{-1}, respectively. One coumarin (scopoletin) , three cinnamic acids (4-coumaric acid, ferulic acid and *trans*-cinnamic acid), three benzoic acids (vanillic acid, vanillin and 4-hydroxybenzoic acid) and three flavonoids (quercetin-3-*D*-galactoside, quercetin-3-glucoside and kaempferide) were identified and quantified by LC-MS.

Conclusions: This study demonstrates that *R. rossii* is a potential source of antioxidant metabolites that can be obtained by *in vitro* culture, without harming the wild specimens.

Key words: biotechnology, orchid *in vitro* propagation, secondary metabolites.

Resumen:

Antecedentes y Objetivos: *Rhynchostele rossii* es una orquídea nativa de México conocida como *gallinitas*, que está amenazada debido a su sobreexplotación con fines ornamentales, lo que hace necesario realizar esfuerzos para su conservación. A la fecha, no hay estudios fitoquímicos de esta orquídea, aunque se sabe que las especies de la familia Orchidaceae son una buena fuente de compuestos bioactivos y nutracéuticos (p. ej. vainillina). Por lo anterior, el objetivo principal de esta investigación fue establecer el protocolo de geminación *in vitro* de *R. rossii* para la propagación de la especie y la determinación de compuestos fenólicos que contribuyan al conocimiento fitoquímico de esta planta.

Métodos: Un ejemplar silvestre y algunas plántulas obtenidas por cultivo *in vitro* se secaron, molieron y extrajeron con MeOH; se determinó la actividad de anti-radicales libres (DPPH), fenoles y flavonoides totales por métodos espectrofotométricos y algunos fenoles se identificaron y cuantificaron por cromatografía de líquidos acoplada a espectrometría de masas (LC-MS).

Resultados clave: La raíz de la planta silvestre mostró el mayor contenido de fenoles y flavonoides totales con 121.60 mg GAE g^{-1}, y 108.73 mg CE g^{-1}, respectivamente, y la mejor actividad anti-radicales libres con una IC_{50} de 53.63 µg ml^{-1}. Los extractos de las plántulas obtenidas *in vitro* también produjeron compuestos fenólicos, mostrando un contenido de fenoles y flavonoides totales de 37.35 mg GAE g^{-1} y 0.16 mg CE g^{-1}, respectivamente, mientras que por LC-MS se identificaron y cuantificaron una cumarina (escopoletina), tres ácidos cinámicos (ácido 4-cumárico, ácido ferúlico y ácido *trans*-cinamico), tres ácidos benzoicos (ácido vainillico, vainillina y acido 4-hidroxibenzoico) y tres flavonoides (quercetina-3-*D*-galactósido, quercetina-3-glucósido y kaempferide).

Conclusiones: Este estudio muestra que *R. rossii* es una fuente potencial de metabolitos antioxidantes que pueden obtenerse mediante cultivo *in vitro*, sin perjudicar a los ejemplares silvestres.

Palabras clave: biotecnología, metabolitos secundarios, propagación *in vitro* de orquídeas.

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Introduction

The Orchidaceae family is constituted by 736 genera and ca. 28,000 species, being one of the largest and diverse plant groups worldwide (Chase et al., 2015; Fay, 2018). There are 1260 species in Mexico, of which 433 grow in the state of Veracruz (Castañeda-Zárate et al., 2012; Baltazar-Bernal et al., 2014). Some of these species have traditionally been used in ethnomedicine for the treatment of illnesses (Cano Asseleih et al., 2015) and consequently, some of them have already been phytochemically studied or biotested for finding a biological potential. Bioactive orchid secondary metabolites include terpenoids, polyphenols and flavonoids, which have shown antioxidant activity and proven application as antibacterial agents (Bhattacharyya et al., 2014; Chand et al., 2016; Bhatnagar et al., 2017).

Rhynchostele rossii (Lindl.) Soto Arenas & Salazar is an epiphytic orchid native from Mexico that grows in the cloud forest region of the state of Veracruz, where it is given the common name of *gallinitas* (little chickens). Its distribution extends from Mexico to Guatemala, El Salvador, Honduras and Nicaragua (Menchaca-García and Moreno-Martínez, 2010). Due to its recognized use as ornament, which has resulted in illegal overexploitation, this orchid belongs to the category of threatened according to the Mexican environmental protection norm NOM-059-SEMARNAT-2010 (SEMARNAT, 2010). Plants in this category are considered in danger of disappearing in the short or medium term, if factors that negatively affect their viability continue decreasing the size of populations, such as the looting of specimens for selling, destruction of their habitat and low germination percentage (Rasmussen et al., 2015; Hinsley et al., 2018). Although *R. rossii* is not traditionally used for medicinal purposes, on the basis of the ecological and chemotaxonomic criteria, it is necessary to look for alternatives for their rescue, conservation and rational use as source of bioactive metabolites with applications in sectors such as pharmaceutical, food, cosmetology, among others.

In vitro plant tissue culture is an alternative to satisfy the commercial demand of *R. rossii* and to contribute to the conservation of wild specimens. This technique offers an effective method for the multiplication of threatened plants and to obtain secondary metabolites with biological activities (Pant, 2013; Utami et al., 2017). An example of important biological metabolites for health, food and biotechnological sectors are phenolic compounds. Some beneficial effects of these compounds have been ascribed to their antimicrobial and antioxidant activity, which helps to prevent infectious and degenerative diseases (Pandey and Rizvi, 2009).

Phenols are one of the most abundant groups of molecules, with at least 10,000 different compounds with a diverse range of beneficial bioactivities (Rasouli et al., 2017). In plants, polyphenols protect against UV radiation, pathogens and oxidative stress, among other biotic and abiotic factors (Di Ferdinando et al., 2014). In the human body, polyphenols act as antioxidants, and have diverse biological properties such as antidiabetic, anticancer, anti-inflammatory, neuroprotective, hepatoprotective, antifungal, antibacterial and antiaging (Ganesan and Xu, 2017). In this sense, *in vitro* culture has enabled the accumulation of phenolic compounds in cells grown under controlled conditions (Bhattacharyya et al., 2014), which makes this technique an alternative not only for the conservation of threatened species, but also for obtaining secondary metabolites without collecting wild plants. The main objectives of this investigation were to establish the protocol of *R. rossii in vitro* germination for its propagation and to determine the phenolic compounds content, as well as its antioxidant activity in wild plants compared to *in vitro* plants.

Materials and Methods

Collection of biological material

The collection of the orchid *R. rossii* was carried out in the municipality of Chiconquiaco, in the state of Veracruz, Mexico. A capsule was collected for *in vitro* culture, as well as a small plant for phytochemical analysis and a flowering specimen for its taxonomic identification by the staff of the herbarium XAL of the Instituto de Ecología, A.C., where it has been deposited as the voucher *Gutiérrez 1* (XAL).

In vitro germination

For the *in vitro* germination, a previously reported and standardized protocol with a good percentage of seed germination was used (Ješvnik and Luthar, 2015). For this, the capsule obtained from the wild plant was disinfected by soaking in detergent for 10 min, and immersed in 0.5% Bravo 500®(aqueous solution; Phthalonitrile 0.36%, Syngenta) for

10 min. Next, it was immersed in 70% EtOH (aqueous solution) for 10 seconds and finally placed for 20 min in 20% of commercial bleach (NaClO; aqueous solution) with 0.1 ml l^{-1} of Tween 20. Between each of the steps indicated and at the end of the process, it was rinsed with sterile distilled water.

In a laminar flow hood, a transversal cut was made in the capsule and the seeds were spread on MS medium (Murashige and Skoog, 1962) with 1 g l^{-1} of activated charcoal and 30 g l^{-1} of sucrose at pH 5.8±0.5. The seeds were left to germinate initially in the dark at room temperature (20-25 °C). After germination (~90 days), once the seed volume began to increase due to water absorption, these were replanted and incubated at 22±2 °C with a 16 h photoperiod and a 1180 Lux white light lamp (Prendo INO650V-9, Puebla, Mexico). Six months later, 2.25 g of seedlings were taken for a subsequent extraction of metabolites and the remaining material was kept in *in vitro* conditions.

Preparation of extracts

Fresh material of leaves (15 g), pseudobulbs (695 g) and roots (61 g) from wild plant and *in vitro* germinated seedlings (2.25 g) were dried in an oven (Labtron Scientific VDO-6050D, Hampshire, UK) at 45±2 °C for 3 to 4 days. The dried material was ground and placed in Erlenmeyer flasks. MeOH was added to completely cover the tissues, and these were placed on an orbital shaker (Dragon lab, SK-033-PRO, Beijing, China) at 150 rpm for 24 hours. Finally, the extracts were filtered and concentrated to dryness in a rotary evaporator under reduced pressure (BÜCHI R210, Flawil, Switzerland).

Quantification of total phenols and flavonoids

For total phenols quantification, the Folin-Ciocalteu method was used as previously reported by Singleton and Rossi (1965) and Bhattacharyya et al. (2014). For this, a calibration curve was made from 0.004 to 0.4 mg ml^{-1} of gallic acid in H_2O/MeOH (1:1). 250 µl of each calibration point, plant extracts and blank (H_2O) were taken and placed in tubes protected from light. Five hundred µl of Folin-Ciocalteu reagent (Sigma-Aldrich, F9252) (1:10 in H_2O) was added to each tube, and allowed to react for 5 min. Then, 500 µl of 7% Na_2CO_3 (aqueous solution) was added, homogenized and, after incubation for 90 min at room temperature in darkness, the absorbance was measured in a UV/Vis spectrophotometer (Thermo Scientific Genesys 10, Waltham, Massachusetts, USA) at λ=750 nm. Total phenolic content was expressed as mean of three replicates ± standard deviation in milligrams of gallic acid equivalents per gram of dry weight (mg GAE g^{-1}±SD).

The quantification of flavonoids was performed according to the $AlCl_3$ test (Zhishen et al., 1999; Bhattacharyya et al., 2014). A catechin calibration curve was prepared in H_2O-MeOH (1:1) from 0.004 to 0.4 mg ml^{-1}. From the dilutions of the standard, extract and blank, 150 µl was taken and placed in test tubes protected from light. After this, 40 µl of 5% $NaNO_2$ (aqueous solution) was added and tubes were incubated for 5 min. Then, 40 µl of 10% $AlCl_3$ (aqueous solution) was added and left one more minute. Finally, 250 µl of 1 M NaOH (aqueous solution) and 750 µl of distilled water were added. This solution was homogenized, and the absorbance was measured at λ=510 nm in a UV/Vis spectrophotometer (Thermo Scientific Genesys 10, Waltham, Massachusetts, USA). The total flavonoid content in the samples was expressed as the mean of three replicates ± standard deviation in milligrams of catechin equivalents per gram of dry weight (mg CE g^{-1}±SD).

DPPH free radical scavenging

Free-radical scavenging activity was determined by the method of 2,2-diphenyl-1-picrylhydrazine (DPPH; Sigma-Aldrich, D9132) according to Brand-Williams et al. (1995). As a standard, a vitamin C calibration curve from 0 to 20 µg ml^{-1} was performed, and for each extract evaluation a curve from 10 to 100 µg ml^{-1} was prepared. From each dilution (vitamin C or extract), 250 µl was added to 750 µl of 0.1 mM DPPH MeOH solution. The tubes were protected from light and incubated at room temperature for 30 min. Finally, the absorbance was measured with a spectrophotometer at λ=517 nm in a UV/Vis spectrophotometer (Thermo Scientific Genesys 10, Waltham, Massachusetts, USA). MeOH was used as a blank and the concentration capable of neutralizing 50% of the free radicals DPPH (IC_{50}) was determined.

Identification of phenolic compounds by LC-MS

The identification and quantification of individual phenolic compounds in the wild and *in vitro* plants was performed by liquid chromatography (LC) (Agilent Technologies 1290 In-

finity series, Santa Clara, California, USA) coupled to a triple quadrupole mass spectrometer (MS-QqQ) (Agilent Technologies 6460, Santa Clara, California, USA), following the dynamic multiple reactions monitoring method reported by Juárez-Trujillo et al. (2018). All the solvents used were MS grade and purchased from Sigma-Aldrich. The chromatographic analysis was carried out on a ZORBAX SB-C18 column (1.8 µm, 2.1 × 50 mm) of Agilent Technologies (Santa Clara, California, USA). The column oven at 40 °C and the mobile phase consisted of water (A) and acetonitrile (B), both with 0.1% of formic acid. The gradient conditions were: 0 min, 1% of B; 0.1-40 min, linear gradient 1-40% of B; 40.1-42 min, linear gradient 40-90% of B; 42.1-44 min, isocratic 90% of B; 44.1-46 min, linear gradient 90-1% of B; 46.1-47 min, isocratic 1% of B. Total run time was 47 min. The flow rate was 0.1 ml min^{-1}. For injection, 20 mg of each sample extract was diluted in 1 ml of 0.1% formic acid in MeOH and then filtered with a 0.2 µm membrane, with 5 µl as the injection volume. The electrospray ionization source was operated in positive and negative ionization modes. The desolvation temperature was 300 °C, the cone gas (N_2) flow rate was 5 l min^{-1}, the nebulizer pressure was 45 psi, the sheath gas flow was 11 l min^{-1} and the capillary voltage (positive and negative) was 500 V. The fragmentor voltage was 100 V and the cell accelerator voltage was 7 V for all compounds. The phenolic compounds identity was confirmed by coelution with authentic standards analyzed under the same conditions. For quantitation, a calibration curve in a concentration range from 1 to 9 µM was prepared for each standard. r^2 values ≥0.97 were considered for the quantification range. The data were processed with the MassHunter workstation software, version B.06.00 of Agilent Technologies (Santa Clara, California, USA). The results were expressed as mean of three replicates ± standard deviation of µg g^{-1} of dry weight.

Results

In vitro germination

The successful germination of seeds of *R. rossii* was achieved, starting about two weeks after sowing, when the seeds increased in size due to water absorption and changed from a white to intense green color. The first leaf development was observed 14 weeks (approximately 95-100 days; Fig. 1A) after sowing and finally one year after its maintenance *in vitro,* the seedlings reached an average height of 5 cm (Fig. 1B).

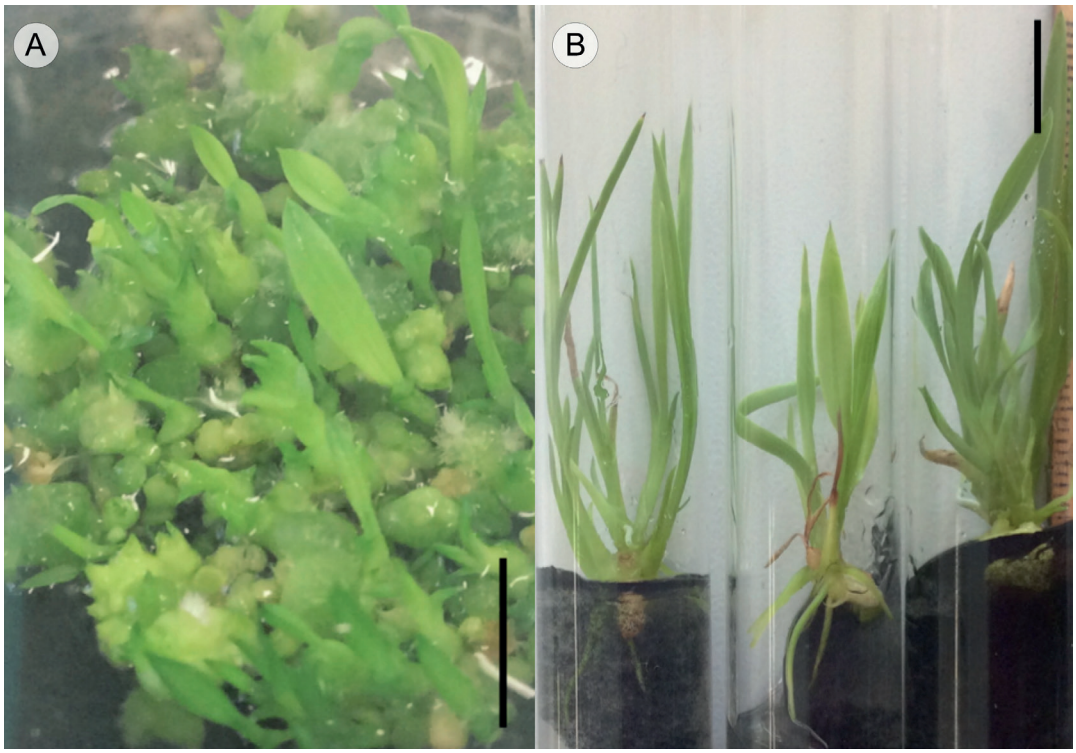

Figure 1: *In vitro* germination of *Rhynchostele rossii* (Lindl.) Soto Arenas & Salazar. A. tissues 195 days after germination (Bar: 1 cm); B. *R. rossii* seedlings after a year of *in vitro* development (Bar: 1 cm).

Antioxidant activity, phenolic and flavonoid content

Total phenols and flavonoids were determined in plants of wild origin and *in vitro* germinated seedlings (Table 1). In the wild plants, the highest content of phenols and flavonoids was present in the root with 121.60±2.99 mg GAE g^{-1} and 108.73±1.13 mg CE g^{-1}, respectively. Seedlings obtained *in vitro* presented a total phenolic and flavonoids content of 37.34±3.45 mg GAE g^{-1} and 0.16±0.08 mg CE g^{-1}, respectively. These concentrations are lower than those in the roots, but higher than those in the pseudobulbs of the wild plants.

Finally, the highest antioxidant activity was found in the extract of wild plant roots with an IC_{50} of 53.64±0.82 µg ml^{-1}. This results correlates with the high content of phenols and flavonoids present in this tissue. The second highest antioxidant activity was present in the MeOH extract of *in vitro* seedlings with an IC_{50} of 126.68±0.98 µg ml^{-1}, followed by wild plant pseudobulbs and leaves with the lowest antioxidant activity, since it requires up to 423.11±1.34 µg ml^{-1} to inhibit 50% of DPPH free radicals.

Identification and quantification of phenolic compounds

A total of 10 phenolic compounds were identified for the first time in MeOH extracts of plants of wild origin of *R. rossii*. From the identified compounds, three were cinnamic acids, three flavonoids, three benzoic acids and one coumarin (Table 2). Representative chromatograms of authentic standards and analyzed samples are shown in Fig 2. The compounds identified are 4-hydroxybenzoic acid (1), vanillic acid (2), vanillin (3), 4-coumaric acid (4), scopoletin (5), ferulic acid (6), quercetin-3-*D*-galactoside (7), quercetin-3-glucoside (8), *trans*-cinnamic acid (9) and kaempferide (10) (Fig. 2). In wild plants, five phenolic compounds were shared by pseudobulb, leaf and root tissues (4-coumaric, ferulic, *trans*-cinnamic, vanillic and 4-hydroxybenzoic acids). One flavonoid was only found in the pseudobulb (kaempferide), while the quercetin derivatives did only occur in leaf tissues. The wild and *in vitro* plants shared seven phenolic compounds (4-coumaric, ferulic, vanillic, 4-hydroxybenzoic acids, vanillin, quercetin-3-*D*-galactoside and kaempferide). Interestingly, scopoletin, *trans*-cinnamic acid and quercetin-3-glucoside were only found in wild tissues, while no phenolic compounds were detected exclusively in *in vitro* plants (Table 2).

The major cinnamic acids found in wild plants were ferulic acid (99.37±3.58 µg g^{-1}) and 4-coumaric acid (20.68±0.27 µg g^{-1}) in roots, while *trans*-cinnamic acid presented the maximum concentration in leaves (10.11±0.29 µg g^{-1}). In contrast, *in vitro* plants showed higher concentration of 4-coumaric acid (34.37±1.86 µg g^{-1}) and ferulic acid (20.66±1.97 µg g^{-1}), while *trans*-cinnamic acid was not found. Scopoletin is a coumarin that was only observed in roots of wild plants (0.65±0.07 µg g^{-1}). Regarding flavonoids in wild plants, quercetin-3-*D*-galactoside and quercetin-3-glucoside were detected in leaf tissues (22.94±3.30 and 35.12±2.58 µg g^{-1}, respectively), while kaempferide did only occur in the pseudobulbs (2.62±0.00 µg g^{-1}). In contrast to what was observed in wild plants, only quercetin-3-*D*-ga-

Table 1: Phenols and flavonoids total content and free antiradical activity in MeOH extracts of wild *Rhynchostele rossii* (Lindl.) Soto Arenas & Salazar tissues and *in vitro* seedlings. Results are expressed as the mean ±SD (n=3). Ascorbic acid with an experimental IC_{50} of 14.55±0.16 µg ml^{-1}, similar to Espinosa-Leal et al. (2015) who report an IC_{50} of 14.13 µg ml^{-1}. Different letters indicate significant differences between samples ($p<0.05$). GAE: Gallic acid equivalents; CE: Catechin equivalents; IC: Inhibitory concentration.

Properties	Tissue			
	Pseudobulb	Leaf	Root	*In vitro*
Total phenols (mg GAE g^{-1})	35.18±1.61^{a}	45.36±2.90^{b,c}	121.60±2.99^{c}	37.34±3.45^{a,b}
Total flavonoids (mg CE g^{-1})	13.58±1.07^{a,c}	25.20±2.29^{a,b}	108.73±1.13^{b}	0.16±0.08^{c}
Antiradical activity IC_{50} (µg ml^{-1})	190.40±4.97^{a,b}	423.11±1.34^{a}	53.64±0.82^{c}	126.68±0.98^{b,c}

Table 2: Phenolic compounds identified in MeOH extracts from *Rhynchostele rossii* (Lindl.) Soto Arenas & Salazar. Concentrations are expressed in µg g^{-1} of dry weight. Results are expressed as the mean±SD (n=3). RT: Retention time. ND=Not detected. NQ: Detected but not quantified due to is low value. *Concentration below the limit of quantification.

Chemical group	Metabolites	Precursor ion (m/z)	Product ion (m/z)	RT (min)	Tissue			
					Pseudobulb	Leaf	Root	*In vitro*
Coumarins	Scopoletin	355.10	193.00	18.6	ND	ND	0.65±0.07	ND
	4-Coumaric acid	163.05	119.00	16.4	2.31±0.01	3.44±0.02	20.68±0.27	34.37±1.86
Cinnamic acids	Ferulic acid	193.10	133.90	18.8	1.63±0.01	8.90±0.28	99.37±3.58	20.66±1.97
	trans-Cinnamic acid	147.01	103.05	28.7	5.93±0.08	10.11±0.29	2.95±0.04	ND
	Vanillic acid	169.04	151.04	12.0	5.05±0.14	57.51±0.82	7.46±0.09	26.04±3.92
Benzoic acids	Vanillin	153.00	93.00	15.3	NQ	NQ	4.77±0.02	16.89±0.04
	4-Hydroxybenzoic acid	137.02	93.03	9.4	2.19±0.01	16.96±1.18	1.00±0.00	9.46±0.64
	Quercetin-3-*D*-galactoside	465.10	303.04	20.6	NQ	22.94±3.30	ND	11.70±0.8*
Flavonoids	Quercetin-3-glucoside	465.20	303.40	20.9	NQ	35.12±2.58	ND	ND
	Kaempferide	301.07	258.05	43.3	2.62±0.00	NQ	ND	2.10±0.08*

lactoside (11.70±0.80 µg g^{-1}) and kaempferide (2.10±0.08 µg g^{-1}) were detected in *in vitro* plants. Moreover, some benzoic acids were found in wild and *in vitro* plants. Vanillic (57.51±0.82 µg g^{-1}) and 4-hydroxybenzoic (16.96±1.18 µg g^{-1}) acids did mainly occur in leaf tissues, while vanillin (4.77±0.02 µg g^{-1}) was found only in roots of wild plants. In *in vitro* plants, vanillic acid (26.04±3.92 µg g^{-1}), vanillin (16.89±0.04 µg g^{-1}) and 4-hydroxybenzoic acid (9.46±0.64 µg g^{-1}) were detected.

Discussion

In vitro germination

To date, only two studies have been conducted on species of *Rhynchostele* Rchb. f., first to analyze its macronutrients content (Jiménez-Peña et al., 2018) and the second focused on the isolation and study of associated mycorrhizal fungi (Cruz-Higareda et al., 2015). However, none of them has focused on their *in vitro* culture, although they are highly valuable resources for ornamental purposes (Castillo-Pérez et al., 2019). In this study, the asymbiotic *in vitro* germination of the wild orchid *R. rossii* was achieved. The disinfection method applied to the capsule was effective to obtain an aseptic *in vitro* culture. Only full-strength MS medium was tested with good results for germination and development of tissues; nevertheless, for other orchids, half or quarter strength MS medium is commonly used with optimal results (Chen et al., 2015). Hence, it would be appropriate to continue testing different media and concentrations to evaluate their effect on growth and optimize production. Seed germination started about two weeks after sowing, like Paul et al. (2012) reported for *Dendrobium hookerianum* Lindl., when the seeds increased in size due to water absorption and changed from a white to intense green color.

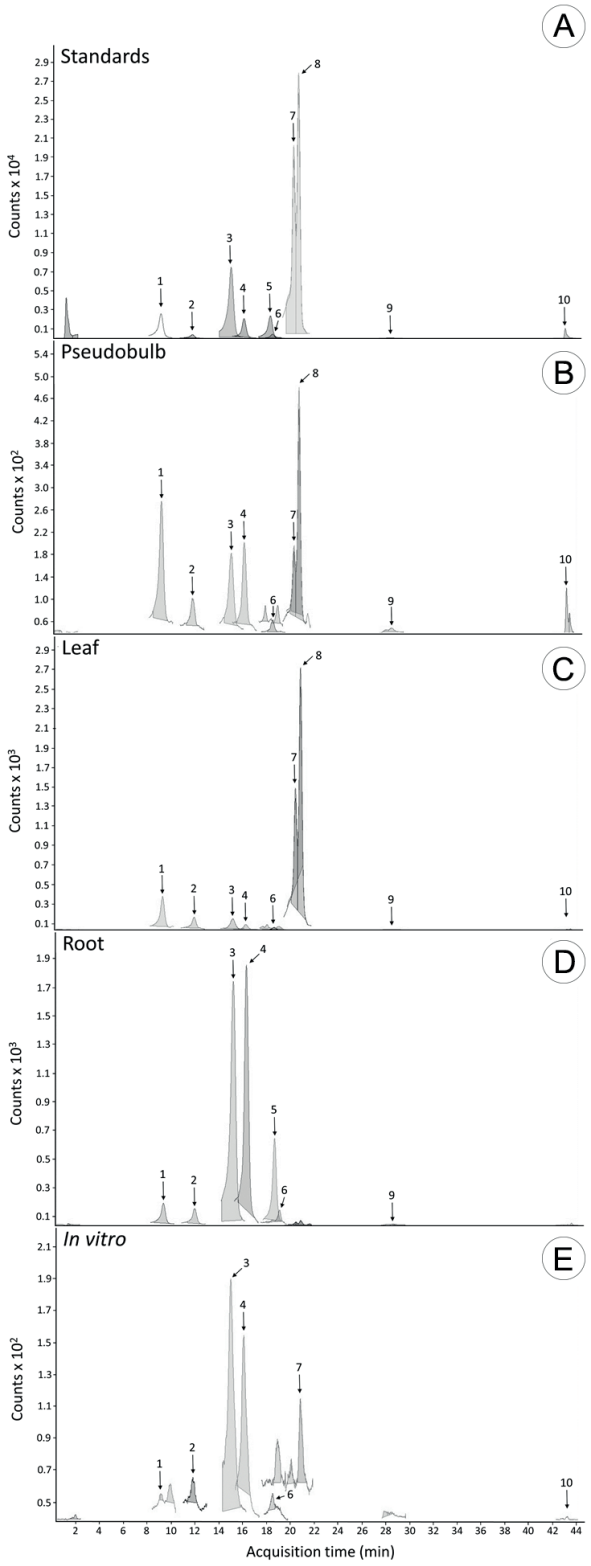

Figure 2: Representative chromatograms of: A. standards; B. pseudobulb; C. leaf; D. root; E. *in vitro* plants of *Rhynchostele rossii* (Lindl.) Soto Arenas & Salazar. The compounds identified are: 4-hydroxybenzoic acid (1), vanillic acid (2), vanillin (3), 4-coumaric acid (4), scopoletin (5), ferulic acid (6), quercetin-3-*D*-galactoside (7), quercetin-3-glucoside (8), *trans*-cinnamic acid (9) and kaempferide (10).

In vitro culture of *R. rossii* is a viable alternative for its propagation, and this biotechnological strategy can be applied for commercial aims, but most importantly for rescue, conservation and even long-term reintroduction purposes (Khamchatra et al., 2016). This technique can also be applied for the phytochemical study of this species, without extracting or damaging wild plants and to obtain metabolites of interest for food or pharmaceutical industries in a more efficient and controlled manner (Giri et al., 2012). Phenols are one of the main natural compounds that already have been reported as compounds in orchids, in different tissues such as leaves, roots or stems (Minh et al., 2017), and it is also known that they are naturally produced under *in vitro* conditions (Chugh et al., 2009).

Antioxidant activity, phenolic and flavonoid content

Phenolic compounds produced by plants can act as antioxidant, UV protection, signal and defence compounds (Petruk et al., 2018). In human health, they are very important for their anti-inflammatory, antioxidant or antiproliferative activity (Lin et al., 2016). For example, flavonoids are a kind of polyphenol metabolites with various bioactive effects such as antiviral, cardioprotective or antidiabetic (Wang et al., 2018).

Roots are the tissue where the largest accumulation of phenolic compounds occurs, which could be due to the structure called velamen that covers the roots and that is responsible for the absorption of water and nutrients from the medium (Zotz and Winkler, 2013). It is also important to mention that in nature, this type of orchids establish symbiotic relationships with mycorrhizal fungi (Zhang et al., 2018), so this is an important biotic factor to consider in the biosynthesis and storage of secondary metabolites in *R. rossii* roots.

On the other hand, the phenolic content of an *in vitro* plant is low when comparing with leaf and root tissues from the wild plant and with *in vitro* cultivated *Dendrobium nobile* Lindl., whose MeOH extract presented a total phenolic content of 41.39±0.1 mg GAE g^{-1} dry weight (Bhattacharyya et al., 2014). Hence, it will be necessary to evaluate other culture conditions such as pH, light, carbon source or minerals, to fine-tune and evaluate how these factors influence

the production and accumulation of phenolic compounds (Ramakrishna and Ravishankar, 2011; Murthy et al., 2014). Even the use of plant growth regulators for generation of callus and cell suspension or *in vitro* culture of roots can be tested (Tokuhara and Mii, 2001; Hussain et al., 2012).

Identification and quantification of phenolic compounds

All phenolic compounds have a building block that is a carbon skeleton C_6-C_3, which, due to multiple biochemical reactions results in a variety of molecules such as coumarins (C_6-C_3), flavonoids (C_6-C_3-C_6), cinnamic acids (C_6-C_3) or benzoic acids (C_6-C_1) (Pereira et al., 2009). The phenolic compounds identified in this study are biosynthesized by the phenylpropanoids pathway which starts from amino acid phenylalanine to produce the cinnamic acids *trans*-cinnamic, 4-coumaric and ferulic acids. The cinnamic acids are the precursors of benzoic acids, flavonoids and coumarins. Interestingly, *in vitro* seedlings present contrasting phenolic content of cinnamic acids compared to wild plants and this results in changes in the levels of downstream phenylpropanoid pathway metabolites. Undoubtedly, factors as age, nutriments, and light exposure, among others may be influencing the metabolomic fluxes (Naik and Al-Khayri, 2016).

The great structural diversity observed in plant phenolic compounds may result in several biological activities that can be displayed by products containing these molecules. For example, scopoletin is a coumarin with anti-inflammatory activity associated with the inhibition of eicosanoid biosynthesis (Ding et al., 2008), 4-coumaric acid is a phenol with anti-inflammatory capacity suppressing the activity of cyclooxygenase 2 (Luceri et al., 2004), vanillic acid has the capacity to prevent liver diseases by reducing bilirubin (Atefipour et al., 2016) and ferulic acid, a major phenolic compound present in all tissue extracts of *R. rossii*, is an important compound in the prevention of oxidative stress and has a nephroprotective effect (Bami et al., 2017).

Finally, one of the most relevant phenolic compounds identified in *R. rossii*, which can be used either for medicinal or biotechnological purposes, are those also found in the orchid *Vanilla planifolia* Andrews, one of the primary sources for vanilla flavouring in food industry due to its high vanillin and vanillic acid content, among others (Divakaran et al., 2016). Although vanilla flavouring can now be produced by synthetic approaches, natural sources of these compounds are preferred by consumers around the world due to the flavour contribution of other compounds different from vanillin and vanillic acid. This has been one of the main reasons why research groups are interested in the development of culturing strategies for *V. planifolia* either in *in* or *ex vitro* conditions (Divakaran and Babu, 2009; Ramírez-Mosqueda and Iglesias-Andreu, 2017). A similar phenolic profile found in *in vitro* cultivated plants represents the capacity and opportunity that *in vitro* culture techniques offer to produce secondary metabolites of interest like wild plants do (Guerriero et al., 2018). Hence, the identification of novel sources of phenolic compounds with known applications from additional natural sources still is an opportunity area for scientists and very attractive for food, pharmaceutical and biotechnological industries.

Conclusions

This is the first report of phenolic compounds in wild plants and *in vitro* tissues of *R. rossii*, being the roots those that presented the highest concentration of phenolic compounds. In this study it is shown that through biotechnological method of plant tissues *in vitro* culture it is possible to support the rescue, care and study of endangered species, as well as to propose strategies to obtain bioactive compounds than can be exploited for several areas of industry without disturbing the environment.

Author contributions

AGS, NSMC, YCR conceived and designed the study. JAGA and JLMV contributed to the acquisition and interpretation of data by LC-MS. AGS wrote the manuscript with the help of NSMC, YCR, JLMV and JAGA. All authors contributed to the discussion, revision and approved the final version of the manuscript.

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