

Microbial antagonism against phytopathogenic *Botrytis cinerea* in highbush blueberry (*Vaccinium corymbosum* L.) cultivars. Contribution of leaf-associated microorganisms from native Andean Ericaceae

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Research Article

Keywords: Andean Ericaceous Plants, Biological Control, Blueberry, Microbial Inoculants, Phyllosphere, Phytopathogenic fungi,

Posted Date: September 13th, 2022

DOI: <https://doi.org/10.21203/rs.3.rs-2039166/v1>

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Abstract

Leaf-associated microorganisms are the least studied symbiotic microbes in Ericaceous plants and they represent a novel and interesting source of microbial strains for exploring their biocontrol potential against phytopathogenic fungi. In this study, the diversity of leaf-associated microorganisms of the native Andean ericaceous plants *Gaultheria pumila* and *Empetrum rubrum* were screened to identify and characterize microorganisms with biocontrol capability against the phytopathogenic fungus *Botrytis cinerea* affecting *Vaccinium corymbosum* (highbush blueberry) cultivars. Microbial strains with biocontrol potential against *Botrytis cinerea* were selected, and *in vivo* tests were performed to evaluate the biocontrol activity of the selected strains. Four fungal, four yeast, and five bacterial strains were isolated, including the fungi *Epicoccum nigrum* (strains HFE11 and HFG20), *Epicoccum layuense* (strain HFG13), and *Aspergillus* sp. (strain HFG1), the yeasts *Aureobasidium pullulans* (strains BFG22 and BFG24) *Sporobolomyces roseus* (strains BFE10 and BFE11), and the bacteria *Bacillus mycoides* (strains BFE4 and BFE14), *Bacillus* sp. (strains BFG8), *Pseudomonas fluorescens* (strains BFE6), and *Pseudomonas* sp. (strains BFG21). *In vitro* biocontrol activity of the selected strains (BFE14, BFE6, and HFG13) showed inhibition percentages ranging from 60% to 80. The screening of plant growth-promoting traits detected phosphate solubilization, indole acetic acid production, and ammonium production in most of the isolates. The *in vivo* experiments showed that the inoculation of the isolates BFG22, BFE6, and HFG13 on *V. corymbosum* leaves before infection avoids severe damage to the infected tissues. Additionally, inoculation with BFG22 decreases the lipid peroxidation levels (malondialdehyde 36% lower) when the leaves were infected with *B. cinerea*. Our results provide evidence of beneficial traits of microorganisms inhabiting the phyllosphere of native Andean Ericaceae which can be used as microbial inoculants in agricultural production. These beneficial effects enhance plant growth and avoid damage by *B. cinerea* in *V. corymbosum* cultivars.

1. Introduction

Microorganisms can colonize various environments, including water, soil, air, and plants [1, 2]. The aerial organs of plants are an environment known as the phyllosphere, where several microorganisms such as bacteria, archaea, fungi, and viruses can live in symbiosis [3]. Among them, epiphytic microorganisms can grow on the plant surface, whereas endophytic microorganisms can colonize the inner tissues of the aboveground organs [4, 5]. Bacteria have been described as the most abundant phyllosphere-inhabiting microorganisms (PIMs). However, fungi can also play essential roles in plant health and development [6, 7]. The main biological processes associated with PIMs are related to an improved stress tolerance to environmental changes, production of plant growth regulators, biological nitrogen (N)-fixation, and biological control of phytopathogens that can affect leaves and fruits [8–11]. The identity of the plant species and the environmental conditions influence the diversity and abundance of PIMs, which thrive and survive in this specific environment [12, 13]. The diversity and functionality of microbial taxa in the phyllosphere have been less explored than rhizosphere-inhabiting microorganisms [14, 15]. Still, recent studies have demonstrated a beneficial effect of PIMs on plant growth, stress tolerance, and plant health [16, 17]. Therefore, PIMs represent an essential source of microbial taxa to explore their potential for biotechnological applications such as biological control of phytopathogenic fungi affecting fruit crops.

Among the native flora of Chile, the genera *Gaultheria* and *Empetrum* (Ericaceae) are commonly found growing in pristine Andean ecosystems, including volcanic deposits [18, 19]. Some species, such as *Gaultheria pumila* (L.f.) D.J. Middleton and *Empetrum rubrum* Vahl ex Willd. grow on high altitude slopes in the southern Andes near active volcanoes and under adverse environmental conditions [20–22]. These plants are considered pioneers because they colonize volcanic rock substrates in an environment with strong winds, extreme seasonal temperatures, cold in winter and hot and dry in summer [23]. Several studies on native *Gaultheria* spp. have focused on analyzing antioxidants present in the fruits, whereas the interaction of native ericaceous plants with phyllosphere-inhabiting microorganisms

has been scarcely explored [24–26]. Therefore, the study of microorganisms associated with these plants represents an alternative to exploring direct and indirect plant growth-promoting mechanisms that can be useful in improving plant performance under challenging environmental conditions.

Vaccinium corymbosum L. (Ericaceae), also known as highbush blueberry, is an important fruit crop in southern Chile, Argentina, and the United States [27]. According to the Chilean Blueberry Committee [28], 117 tons were exported in the 2020–2021 season, which represents an 8.4% increase over the previous period with the United States (55%), Europe (34%), and Asia (10%) being the primary consumers. *Botrytis cinerea* can also affect highbush blueberry during the flowering or the post-harvest stage [29–31]. Chemical methods are applied to the crops to avoid economic losses caused by *B. cinerea* [32]. In this sense, symbiotic microorganisms are an interesting tool to be explored and applied in current cultural practices to reduce the use of xenobiotics in the control of phytopathogens.

Nowadays, agriculture is facing several challenges in feeding an increasing global population, especially under the unfavorable environmental conditions caused by climate change, contamination of the food chain with agriculture-derived xenobiotics, decreased yield due to phytopathogen attack, and excessive use of chemicals to ensure high yield but increased contamination of natural resources such as soil and water. Therefore, designing alternatives that exploit the potential of microorganisms for biological control and plant growth promotion is necessary to reduce the use of xenobiotics in current cultural practices [33, 34]. This study aimed to characterize microorganisms inhabiting the leaf surface of ericaceous plants growing in extreme environments in southern Chile and determine their biocontrol capability against *B. cinerea* affecting *V. corymbosum* cultivars.

2. Material And Methods

2.1. Sampling.

Ericaceous plants were localized near the Andes mountains in southern Chile. The selected plants colonize volcanic rock substrates under direct light exposure and no presence of other plants within a 5 m radius. Two species were selected: i) *G. pumila* from Conguillío National Park (38°46'41.7"S 71°38'19.0"W; 1.529 m.a.s.l.) and ii) *E. rubrum* in Malalcahuello National Reserve (38°25'10.6"S 71°32'30.7"W; 1399 m.a.s.l.). Leaf samples were collected from seven individual plants, stored in thermal ice chests, and transported to the laboratory for further processing.

2.2. Isolation of leaf-associated microorganisms.

2.2.1. Epiphytes.

Microbial strains were isolated from the leaf surface following the methodology proposed by Krim et al. [35], with modifications. A total of 10 leaves per plant were imprinted in Petri dishes ($n = 10$) containing modified Luria-Bertani agar (LBA; Difco Laboratories, Detroit, MI, USA) supplemented with cycloheximide at 100 mg L^{-1} to reduce fungal growth, and modified potato dextrose agar (PDA; Difco Laboratories) supplemented with ampicillin at 100 mg L^{-1} to minimize bacterial growth. Half of the Petri dishes were incubated in light, and the other half in darkness at room temperature until no development of new colonies was detected. Growing colonies and strains were selected according to their phenotypic characteristics and growth rate. The isolated leaf-associated microorganisms were stored at 4°C , and in 15% glycerol at -80°C for further analysis.

2.2.2. Endophytes.

Leaf endophytes were isolated following the methodology proposed by Sturz et al. [36] with modifications. 2 g of fresh leaves per plant were surface sterilized with 5% sodium hypochlorite for 5 min followed by 10% ethanol for 1 min and

10 washes with sterile water. An aliquot of 300 µl from the last wash was plated in LBA and PDA to discard the presence of epiphytic microorganisms. The leaves suspended in sterile phosphate saline buffer (PBS) were ground using a sterile mortar and a pestle. After that, the mixture was serially diluted into 10^{-1} to 10^{-5} in sterile distilled water, and 150 µL were plated in Petri dishes containing 30 ml of modified PDA and LBA for isolating fungal and bacterial endophytes, respectively. The Petri dishes were incubated in light and darkness at 24°C for 15 days. Growing colonies were selected according to their phenotypic characteristics and growth rate and purified. For further analysis, the isolated leaf endophytes were stored at 4°C, and in 15% glycerol at – 80°C.

2.3. Antagonism against *Botrytis cinerea*.

The isolates were confronted with a pathogenic strain previously isolated from infected *V. corymbosum* fruits to determine the biocontrol capability against *B. cinerea*. The biocontrol assay was performed following the methodology proposed by Jamali et al.[37]. Briefly, the isolated strains were grown in Petri dishes containing a mixture of PDA and LBA (1:1). A mycelial disk of the *B. cinerea* was inoculated in the center of the Petri dishes. The tested microorganism was inoculated at both sides (25 mm) from the phytopathogen in the mycelial disk or vertical lines for fungi or bacteria, respectively (n = 5). Control dishes were prepared only with the phytopathogenic fungi and no inoculation of isolates (n = 5). An inhibition percentage was determined using the following equation:

$$\%Inhibition = \left(\frac{C - T}{C} \right) \times 100$$

where C is the diameter of growth in mm in the control and T is the diameter of growth in mm in the treatment.

2.4. Identification of antagonistic leaf-associated microorganisms.

The leaf-associated microorganism that showed biocontrol capability against the phytopathogen *B. cinerea* were selected for further analyses related to their biocontrol capability.

2.4.1. Molecular identification of leaf-associated microorganisms.

The bacterial strains were cultured in 15 ml Falcon tubes containing 5 ml of Luria-Bertani broth in darkness at room temperature and continuous stirring at 120 rpm. After 48 h, the tubes were centrifuged at 5,000 rpm for 5 min, and the bacterial cells were washed with ultrapure water and centrifuged twice at 5,000 rpm for 3 min. According to the manufacturer's recommendations, total DNA was extracted using the E.Z.N.A.® Bacterial DNA kit (Omega Bio-Tek, Norcross, GA, USA). After that, PCR amplifications were performed using the PCR primers 27F and 1492R targeting the 16S rRNA gene [38], following the PCR conditions detailed in Herrera, Novotná, Ortiz, Soto and Arriagada [39].

The fungal strains were cultured in 100 ml Erlenmeyer flasks containing 30 ml of potato dextrose broth (PDB) in darkness at room temperature and continuous stirring at 120 rpm. After 7 days, the culture media was centrifuged at 5,000 rpm for 5 min, and the fungal mycelia were used for DNA extraction using the E.Z.N.A.® Fungal mini kit (Omega Bio-Tek). After that, PCR amplifications were performed using the ITS1 and ITS4 primers targeting the ITS region of the 18S rRNA gene [40], following the PCR conditions detailed in Herrera et al.[41].

The quality of the PCR fragments was verified in 2% agarose gel stained with GelRed® (Biotium Inc., Fremont, CA, USA) and quantified using the Qubit® 2.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). Sequencing was performed by Macrogen (Seoul, Korea), and the sequences were submitted to the GenBank database (accessions OK000985 to OK000992 and OK000903 to OK000908). BLAST searches were conducted to find the closest match, accepting the genus and species classification according to Chen et al. (2011). The obtained sequences were aligned using the ClustalW software [42], and the gaps and deletions were eliminated using the BioEdit software [43]. The

operational taxonomic unit (OTU) estimation was performed based on the similarity matrix obtained in the ClustalW software at 97% sequence similarity.

2.4.2. MALDI–TOF MS identification.

Fungal and bacterial isolates that were not identified by standard PCR-sequencing were analyzed using matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) in an Autoflex Speed™ LT spectrometer (Bruker Daltonics, Bremen, Germany), as described in Egamberdieva et al.[44] with modifications. Briefly, bacterial and fungal samples were prepared following the ethanol/formic acid extraction protocol recommended by Bruker Daltonics. Bacterial and fungal strains were grown in LBA medium 18 and PDA for 24 and 72 h, respectively. A mass of 10–20 mg of bacterial or fungal cells was suspended in 300 µL of ultrapure LC-MS grade water (Merck, Kenilworth, NJ, USA), vortexed to homogenize, and centrifuged at 10,000 rpm for three minutes. Cell pellets were washed three times with ultrapure water and centrifuged. After that, the cell pellets were washed twice with 1 ml of ethanol (99% purity, Merck), followed by centrifugation. The pellet was resuspended in a 1:1 solution of 70% formic acid (LC-MS grade, Merck) and acetonitrile (LC-MS grade, Merck), which were carefully mixed. For fungal strains, an additional sonication step for 10 minutes was performed. After that, the samples were centrifuged, and 1 µL of the supernatant was placed on the spot of a MALDI target and dried at room temperature. The samples were overlaid with 1 µL of a matrix composed of α-ciano-4-hydroxycinnamic acid in 50% aqueous acetonitrile containing 2.5% trifluoroacetic acid. Mass spectra were acquired in a mass range of 2–20 kDa. The raw spectra were imported into the MALDI Biotyper™ software and then analyzed by comparing the obtained spectra against the reference spectra in the MALDI Biotyper Compass 4.1 reference database (Bruker Daltonics), and a matching score was calculated.

2.5. Antagonism through volatile substances.

A double plate-chamber method was used to evaluate the effect of volatile compounds produced by the isolates on the growth of *B. cinerea*, following Boukaew et al. [45] with modifications. The leaf-associated microorganisms with biocontrol potential were inoculated in Petri dishes containing PDA or LB for fungi and bacteria, respectively, and incubated in darkness at room temperature. After 3 d for bacteria and 7 d for fungi, the Petri dishes containing the leaf-associated microorganism were covered with another Petri dish containing PDA inoculated with a 5 mm mycelial plug of *B. cinerea*. Then, the two plates were sealed with parafilm to obtain the double-plate chamber. The double plates were incubated for an additional 7 d period at room temperature and in darkness, and the inhibition percentages were periodically estimated.

2.6. Production of hydrolytic enzymes.

A spot plate methodology was performed to detect the production of hydrolytic enzymes (amylase, protease, cellulase, and lipase) by the isolated leaf-associated microorganisms. The results of these enzymatic activities were estimated using the following equation:

$$EI = \frac{Dh}{Dc}$$

Where EI is the enzymatic index, Dh is the diameter of the hydrolysis halo in mm, and Dc is the diameter of the colony in mm [46], where an index higher than 2.0 was considered efficient for enzyme production [47].

To detect the presence of amylase activity, the isolated microorganisms were spot-inoculated in trypticase soy agar containing 1% soluble starch. After 7 days of incubation in darkness at room temperature, 5 ml of a 1% iodine solution was added to each plate. A clear halo around the strain was considered a positive result. The protease activity was determined according to Zilda et al.[48]. Briefly, the isolates were spot inoculated in minimal synthetic medium (1 g L⁻¹

K₂HPO₄, 1 g L⁻¹ NaCl, 0.1 g L⁻¹ MgSO₄ * 7 H₂O, 0.5 g L⁻¹ yeast extract and 15 g L⁻¹ agar) supplemented with 10 ml l⁻¹ skim milk. The dishes were incubated in darkness for 7 days at room temperature, and the presence of a clear halo around the strain was considered a positive result. Cellulase activity was determined according to Kasana et al.[49] with modifications. The isolated leaf-associated microorganisms were spot-inoculated in carboxymethylcellulose agar containing 0.2% NaNO₃, 0.1% K₂HPO₄, 0.05% MgSO₄, 0.05% KCl, 0.2% carboxymethylcellulose sodium salt, 0.02% peptone, and 1.7% agar). The Petri dishes were incubated for 7 d in darkness at room temperature. After that, the plates were flooded with Lugol solution for 30 min to reveal the hydrolyzation zones. Lipase activity was determined according to Kumar et al.[50], with modifications. The leaf-associated strains were incubated in both Tween 80 agar and tributyrin agar plates in darkness at room temperature for 7 days. A clear halo near colonies

indicated a positive result for lipase activity.

2.7. Detection of exopolysaccharides.

The production of exopolysaccharides was evaluated according to Freeman et al.[51] with modifications. The leaf-associated microorganisms were grown in LB or PDA agar for bacteria and fungi, respectively, supplemented with saccharose 30 g L⁻¹ and 0,4 g L⁻¹ Congo red reagent. The Petri dishes were incubated for 7 days in darkness and at room temperature. The change of the colony to a darker tone was considered a positive result.

2.8. Detection of siderophores.

The isolates were cultured in chrome azurol S (CAS) agar for 5 days to determine siderophore production. The CAS reaction was determined by a color change in the blue CAS agar, as detailed in Soto et al.[52].

2.9. Screening of plant growth-promoting traits.

The production of indole acetic acid (IAA) was estimated according to the methodology proposed by Bric et al.[53] with modifications. The isolated leaf-associated microorganisms were grown in LB (50%) for bacteria and PDB (50%) for fungi, supplemented with 100 µg mL⁻¹ of L-tryptophan. The strains were incubated for 48–72 h at 100 rpm in darkness and at room temperature. The isolates were centrifuged for 15 min at 6,000 rpm. An aliquot of 1 ml of supernatant was mixed with 2 ml of the Salkowski reagent and incubated for 30 min at room temperature. The production of IAA was measured in a Biobase BK-UV 1800 spectrometer (Biobase, Jinan, China) at an absorbance of 535 nm. Similarly, siderophore production was estimated as described in Herrera et al.[54], evaluating the color change in a blue CAS agar for 5 days after 7 to 10 days incubation in darkness at room temperature. Solubilization of phosphate in the form of Ca₃(PO₄)₂, AlO₄P, FeO₄P * 2 H₂O and phytic acid were evaluated using the National Botanical Research Institute's phosphate growth medium (NBRIP) [55]. The isolated leaf-associated microorganisms were incubated in NBRIP agar (glucose 10 g l⁻¹, MgCl₂ 2,5 g l⁻¹, MgSO₄ 0,25 g l⁻¹, KCl 0,2 g l⁻¹, (NH₄)₂SO₄ 0,1 g L⁻¹ y agar 15%, pH 7.0) supplemented with 5 g L⁻¹ of each of the tested phosphate sources. The leaf-associated microorganisms were grown in 4% peptone broth and incubated for 7 days in darkness under stirring at 100 rpm and room temperature to detect ammonia production. After the incubation period, Nessler's reagent was added to the cell suspension and measured at 450 nm in a BK-UV1800 spectrophotometer (Biobase) [56].

2.10. Greenhouse experiments.

A greenhouse experiment was designed to know the effect of inoculating the isolated leaf-associated microorganisms in *V. corymbosum* plantlets. One-year-old *V. corymbosum* variety legacy plantlets were inoculated with the selected antagonistic strains. The experimental design included: i) control plantlet (without microorganisms); ii) control plantlet inoculated with the isolated yeast BFG22; iii) control plantlet inoculated with the bacteria BFE6; iv) control plantlet inoculated with the fungus HFG13; v) plant infected with *B. cinerea*; vi) plant infected with *B. cinerea* and the yeast

BFG22; vii) plant infected with *B. cinerea* and inoculated with the bacteria BFE6; and viii) plant infected with *B. cinerea* and inoculated with the fungus HFG13. The selected leaf-associated microorganisms were inoculated in healthy *V. corymbosum* plantlets by aspersions of a 10 ml solution containing 3×10^7 cells counting in a Neubauer chamber for fungi and to an optical density of 0.35 measured in a spectrometer at 600 nm (approximately 10^8 colony forming-units per ml) [57] for bacteria and yeast. After 5 days of incubation, *B. cinerea* was inoculated in the leaves as described for fungi and re-inoculated on days 7 and 14 after the first inoculation. The inoculated leaves were monitored periodically for 30 days. Then the infected leaves were screened to evaluate the presence of fungal spores and hyphae in the leaves using a SU 3500 scanning electron microscope (SEM) (Hitachi, Tokyo, Japan) at a work distance of 5–7.2 mm. The inoculated/infected leaves were harvested for further analysis.

2.10.1. Determination of chlorophyll content in leaves.

The estimation of chlorophyll A and B was performed according to Hiscox and Israelstam [58] with modifications. A total of 50 mg of leaves were deposited in 15 ml test tubes containing 3 ml of dimethyl sulfoxide (DMSO), and the tubes were incubated at 65°C for 30 min. After that, the supernatants were transferred to new test tubes and filled to 5 ml using DMSO, and an aliquot of 1 ml was measured in a BK-UV1800 spectrophotometer (Biobase) at an absorbance of 645 and 663 nm. The chlorophyll content was estimated using Arnon's equation [59].

2.10.2. Determination of lipid oxidation in leaves.

The malondialdehyde (MDA) content in leaves was estimated according to the methodology proposed by Du and Bramlage [60] with modifications. A total of 150 mg of frozen leaves were macerated using a mortar and a pestle and 2.5 ml of 01% trichloroacetic acid (TCA). The samples were centrifuged for 5 min at 10,000 rpm and 4°C, and an aliquot of 0.5 ml of the supernatant was mixed with 2 ml of a solution containing 20% TCA and 0.5% thiobarbituric acid. The samples were incubated at 95°C for 30 min and then were set on ice to stop the reaction. Finally, the samples were centrifuged at 10,000 rpm for 10 min and 4°C. The samples were measured in a BK-UV1800 spectrophotometer (Biobase) at 440, 532, and 600 nm.

2.10.3. Re-isolation of microorganisms from leaves.

A segment of the inoculated leaves was submitted to the isolation of epiphytic microorganisms after four months of incubation as described in Activity 2.2.1 to detect the presence of the previously inoculated microorganisms. The isolates that match the phenotypic characteristic of the inoculated leaf-associated microorganisms were submitted to proteomics-based identification as described in Activity 2.4.2.

2.11. Statistical analyses.

Quantitative data was analyzed by ANOVA. If the p-value indicated significant differences between treatments ($p < 0.05$), post hoc pair-wise comparisons were performed, using the standard deviation of means and Tukey's multiple range test. Statistical significance was set at $p < 0.05$. All statistical tests were conducted using the R software (R Core Team 2018; <https://www.R-project.org>).

3. Results

3.1. Isolation of leaf-associated microorganisms.

The isolation of microorganisms from *E. rubrum* resulted in 97 bacterial colonies (91 epiphytic and 1 endophytic) which were grouped in 13 epiphytic and 1 endophytic strain based on phenotypic characteristics. For fungal isolates,

83 fungal colonies were accounted (67 epiphytic and 16 endophytic), which were reduced to 11 epiphytic and 3 endophytic strains based on the phenotypic characteristics (Table S1). In the case of *G. pumila*, 127 bacterial colonies were accounted for (99 epiphytic and 28 endophytic) and grouped in 17 epiphytic and 1 endophytic strain based on phenotypic characteristics. For fungal isolates, 113 fungal colonies (89 epiphytic and 24 endophytic) were reduced to 19 epiphytic and 8 endophytic strains based on the phenotypic characteristic (Table S1). In total, 30 epiphytic and two endophytic strains were considered for further analysis.

3.2. Screening of antagonisms against *B. cinerea*.

Of the selected strains, 13 showed growth inhibition against *B. cinerea* ranging from 34–81% (Table 1). The isolates BFE14 and BFE6 showed the highest inhibition percentages of 81% and 68% respectively (Table 1). 13 out of 60 epiphytic strains showed inhibition potential. By contrast, no endophytic strains showed biocontrol potential (Table 1).

Table 1

Molecular identification of leaf-associated microorganisms isolated from *Gaultheria pumila* and *Empetrum rubrum* phyllosphere showing biocontrol capability against *Botrytis cinerea*.

Isolate	Source plant	Inhibition (%)	Molecular identification	Identity (%)	GenBank accession	MALDI-TOF identification (score)
FUNGI						
HFE11	<i>Empetrum rubrum</i>	59,6 ± 0,61	<i>Epicoccum nigrum</i>	100	OK000990	-
HFG1	<i>Gaultheria pumila</i>	48,6 ± 0,87	<i>Aspergillus</i> sp.	100	OK000985	-
HFG13	<i>G. pumila</i>	61,4 ± 0,50	<i>Epicoccum layuense</i>	100	OK000988	-
HFG20	<i>G. pumila</i>	61,4 ± 0,66	<i>E. nigrum</i>	100	OK000989	-
YEAST						
BFE10	<i>E. rubrum</i>	35,4 ± 0,51	-	-	-	<i>Sporobolomyces roseus</i> (2.440)
BFE11	<i>E. rubrum</i>	33,7 ± 0,57	-	-	-	<i>S. roseus</i> (2.000)
BFG22	<i>G. pumila</i>	48,2 ± 0,25	-	-	-	<i>A. pullulans</i> (2.020)
BFG24	<i>G. pumila</i>	54,6 ± 0,68	<i>Aureobasidium pullulans</i>	100	OK000992	-
BACTERIA						
BFE6	<i>E. rubrum</i>	67,6 ± 0,61	<i>Pseudomonas fluorescens</i>	100	OK000903	-
BFE4	<i>E. rubrum</i>	56,6 ± 0,61	<i>Bacillus mycoides</i>	100	OK000908	-
BFE14	<i>E. rubrum</i>	80,5 ± 0,70	<i>B. mycoides</i>	100	OK000904	-
BFG21	<i>G. pumila</i>	43,6 ± 0,46	<i>Pseudomonas</i> sp.	100	OK000907	-
BFG8	<i>G. pumila</i>	54,5 ± 0,52	<i>Bacillus</i> sp.	100	OK000905	-

3.3. Identification of leaf-associated microorganisms with biocontrol potential

Molecular identification of the bacterial isolates with biocontrol potential revealed the presence of four OTUs mainly related to Proteobacteria and Firmicutes: OTU1 *Pseudomonas fluorescens* (isolate BFE6); OTU2 *Pseudomonas* sp. (isolate BFG21); OTU3 *Bacillus* sp. (isolate BFG8); and OTU4 *Bacillus mycoides* (isolates BFE4 and BFE14) (Table 1). Specifically, the isolates BFE6 and BFG21 were assigned to bacteria included in the phylum Proteobacteria. The isolates BFE4, BFE14, and BFG8 were assigned to taxa included in the phylum Firmicutes.

Regarding fungal isolates with biocontrol potential, the analyses also revealed the presence of 4 OTUs related to Ascomycota: OTU1 *Aspergillus* sp. (isolate HFG1); OTU2 *Epicoccum layuense* (isolate HFG13); OTU3 *Aureobasidium pullulans* (isolate HFG24); and OTU4 *Epicoccum nigrum* (isolates HFG20 and HFE11).

The MALDI-TOF-based identification of the isolates that did not show a significant match in the GenBank database, or failed in the PCR amplification, showed the presence of the species *A. pullulans* (isolate BFG22) and *Sporobolomyces roseus* (isolates HFE10 and HFE11) (Table 1).

3.4. Screening for biocontrol mechanisms.

The screening of biocontrol activity showed a variable capability in the tested isolates (Table 1, Table 2 and Table 3). The leaf-associated microorganisms showed the presence of a set of hydrolytic enzymes, with the isolates BFG24, BFE11, and BFE14 showing the highest enzymatic indexes for amylase (4.3 cm), protease (5.1 cm), cellulase (5.7 cm), and lipase (3.1 cm) (Table 2). Similarly, in the test of antagonisms through the emission of volatile substances, the isolates HFG1 and HFG13 showed the highest inhibition percentages ranging from 64 to 59%, respectively (Table 3).

Table 2
Hydrolytic enzymatic index of leaf-associated microorganisms isolated from the phyllosphere of *Gaultheria pumila* and *Empetrum rubrum* showing biocontrol capability. Values are means $\pm$ standard deviation for n = 10

Isolate	Protease	Lipase	Amylase	Cellulase
HFE11	-	2 $\pm$ 0.01 ^a	1 $\pm$ 0.00 ^b	1 $\pm$ 0.00 ^c
HFG1	-	2.1 $\pm$ 0.25 ^a	1.5 $\pm$ 0.03 ^{cd}	2.1 $\pm$ 0.06 ^d
HFG13	-	1.7 $\pm$ 0.03 ^{ab}	1.1 $\pm$ 0.01 ^{ab}	1 $\pm$ 0.00 ^c
HFG20	-	1.3 $\pm$ 0.00 ^b	1.1 $\pm$ 0.01 ^{ab}	1.1 $\pm$ 0.02 ^c
BFE10	-	2.1 $\pm$ 0.11 ^{ad}	1.4 $\pm$ 0.08 ^{ac}	2.6 $\pm$ 0.02 ^{ad}
BFE11	-	3.1 $\pm$ 0.03 ^c	1.9 $\pm$ 0.03 ^{de}	3.1 $\pm$ 0.03 ^{ab}
BFG22	1.9 $\pm$ 0.02 ^a	2.6 $\pm$ 0.08 ^{cd}	1.3 $\pm$ 0.01 ^{abc}	3.5 $\pm$ 0.03 ^b
BFG24	5.1 $\pm$ 0.11 ^d	2.7 $\pm$ 0.11 ^c	2.1 $\pm$ 0.19 ^e	3.2 $\pm$ 0.08 ^{ab}
BFE6	2.9 $\pm$ 0.03 ^b	2 $\pm$ 0.04 ^a	-	2.9 $\pm$ 0.91 ^{ab}
BFE4	3.6 $\pm$ 0.07 ^c	1.7 $\pm$ 0.02 ^{ab}	1.4 $\pm$ 0.02 ^{ac}	5.2 $\pm$ 0.08 ^e
BFE14	-	-	4.3 $\pm$ 0.79 ^f	5.7 $\pm$ 0.79 ^e
BFG21	1.9 $\pm$ 0.13 ^a	-	-	-
BFG8	2.7 $\pm$ 0.02 ^b	1.8 $\pm$ 0.01 ^{ab}	-	-

Table 3
Biocontrol capability by volatile production index of leaf-associated microorganisms isolated from the phyllosphere of *Gaultheria pumila* and *Empetrum rubrum*. Values are means $\pm$ standard deviation for n=10

Isolate	% Inhibition <i>B. cinerea</i>
HFE11	58,4 $\pm$ 0,50
HFG1	64,1 $\pm$ 0,36
HFG13	59,7 $\pm$ 0,51
HFG20	57,7 $\pm$ 0,49
BFE10	52,5 $\pm$ 0,40
BFE11	50,2 $\pm$ 0,49
BFG22	56,3 $\pm$ 0,46
BFG24	49,6 $\pm$ 0,57
BFE6	34,3 $\pm$ 0,55
BFE4	46,1 $\pm$ 0,20
BFE14	24,3 $\pm$ 0,53
BFG21	54,3 $\pm$ 0,45
BFG8	32,3 $\pm$ 0,57

3.5. Plant growth-promoting traits.

The screening of plant growth-promoting traits in Table 4 showed that exopolysaccharide production was detected in two bacterial and three fungal isolates. Similarly, positive activity for siderophore production was detected in three bacterial and six fungal isolates. IAA production was detected in eight isolates, with the strains BFG22 and HFG20 having the highest values (31 and 13 $\mu\text{mol}/\text{ml}^{-1}$, respectively). Phosphate solubilization was also detected in seven isolates, and the isolates BFG24 and BFE6 had the highest phosphate solubilization capability. Ammonia production was detected in ten isolates, with the isolates BFE14 and HFG1 demonstrating the highest ammonia production (47 and 85 $\mu\text{mol}/\text{ml}^{-1}$, respectively).

Table 4

Plant growth-promoting traits of leaf-associated microorganisms isolated from the phyllosphere of *Gaultheria pumila* and *Empetrum rubrum* showing biocontrol capability. Values are means $\pm$ standard deviation for n=10

Isolate	Phosphate solubilization				Indole acetic acid ($\mu\text{mol}/\text{ml}^{-1}$)	Ammonium Production ($\mu\text{mol}/\text{ml}^{-1}$)	Siderophores	Exopolysaccharides
	<i>AlO₄P</i>	<i>Phytic acid</i>	<i>FePO₄ 2H₂O</i>	<i>Ca₃(PO₄)</i>				
HFE11	5.1 $\pm$ 0.17 ^{ab}	1.7 $\pm$ 0.02 ^c	4.5 $\pm$ 0.5 ^d	0.4 $\pm$ 0.02 ^{ab}	2.5 $\pm$ 0.62 ^a	77 $\pm$ 6.6 ^d	+	-
HFG1	5.2 $\pm$ 0.17 ^{ab}	0.6 $\pm$ 0.09 ^d	2.2 $\pm$ 0.21 ^{ab}	0.6 $\pm$ 0.02 ^{bcd}	-	85 $\pm$ 2.6 ^d	+	-
HFG13	4.8 $\pm$ 0.17 ^a	1.1 $\pm$ 0.02 ^e	3.1 $\pm$ 0.02 ^c	0.2 $\pm$ 0.02 ^a	1.2 $\pm$ 0.18 ^a	39 $\pm$ 7.5 ^c	+	-
HFG20	4.8 $\pm$ 0.17 ^a	2.2 $\pm$ 0.21 ^{ac}	4.7 $\pm$ 0.33 ^d	0.5 $\pm$ 0.02 ^{abc}	13 $\pm$ 2.3 ^b	62 $\pm$ 2.1 ^e	+	-
BFE10	-	-	-	-	1.5 $\pm$ 0.2 ^a	16 $\pm$ 1.5 ^{ab}	-	-
BFE11	-	-	-	-	-	-	-	-
BFG22	5.7 $\pm$ 0.17 ^{bc}	2.2 $\pm$ 0.05 ^{ab}	2.3 $\pm$ 0.17 ^{abc}	1.3 $\pm$ 0.02 ^e	31 $\pm$ 2.6 ^d	19 $\pm$ 1.6 ^a	+	+
BFG24	6.3 $\pm$ 0.14 ^c	2.3 $\pm$ 0.02 ^{ab}	2.8 $\pm$ 0.05 ^{bc}	0.8 $\pm$ 0.01 ^d	12 $\pm$ 2.9 ^b	15 $\pm$ 0.7 ^a	+	+
BFE6	6.3 $\pm$ 0.14 ^c	2.7 $\pm$ 0.05 ^b	1.5 $\pm$ 0.03 ^a	0.7 $\pm$ 0.01 ^{cd}	2.5 $\pm$ 1 ^a	42 $\pm$ 3.0 ^c	+	+
BFE4	-	-	-	-	-	-	+	+
BFE14	-	-	-	-	-	47 $\pm$ 3.8 ^c	-	-
BFG21	-	-	-	-	6.9 $\pm$ 0.9 ^c	11 $\pm$ 0.6 ^b	-	-
BFG8	-	-	-	-	-	-	+	+

3.6. Greenhouse experiments

The greenhouse experiments showed a negative effect of the inoculation of *B. cinerea* on the infected leaves compared to the control or leaves previously inoculated with the isolated leaf-associated microorganisms (Fig. 1). The inoculated leaves showed a difference in the development of microbial structures inhabiting the leaf surface, as revealed by scanning electron microscopy (Fig. 2). Specifically, the presence of *B. cinerea* hyphae was considerably reduced. Even modifications to the phenotypic characteristics of the hyphae were detected in the treatment inoculated with the isolate HFG13 (*E. layuense*). The chlorophyll content was affected by the inoculation of the microorganisms, showing that the

chlorophyll content was directly increased by inoculation of the strains BFG22 (*A. pullulans*), being 13.8% higher than control (Fig. 3). Similarly, the MDA content in leaves was affected by the inoculation of the selected strains. Specifically, the MDA content was lower in the treatments, with the isolate BFG22 (*A. pullulans*) being 36.8% lower than the control (Fig. 4). After four months of culture under greenhouse conditions, the inoculated strains were isolated from the selected leaf surface, making it possible to purify and identify, confirming its viability even after four months of incubation even under *in vivo* conditions (Table 5).

Table 5: Re-identification by MALDI-TOF of leaf-associated microorganisms inoculated in plants *Vaccinium corymbosum* in greenhouse conditions.

Identification MALDI-TOF	Score
<i>Botrytis cinerea</i>	2.110
<i>Epicoccum sp</i>	2.210
<i>Pseudomonas sp</i>	2.020
<i>Aureobasidium pullulans</i>	2.120

4. Discussion

This study isolated and characterized leaf-associated microorganisms from the native Andean ericaceous plants *G. pumila* and *E. rubrum* colonizing volcanic deposits in a segment of the Andes mountain range in south-central Chile. The information about the diversity of leaf-associated microorganisms and beneficial traits is crucial to exploring their biotechnological potential for current agronomic practices, especially strains with biocontrol capability against phytopathogens.

One of the principal beneficial attributes of leaf-associated microorganisms is biocontrol capability [61]. This study isolated 13 leaf-associated microorganisms that showed biocontrol capability against a pathogenic *B. cinerea* strain isolated from infected blueberry fruits (Table 1, Fig. 1). Several of the identified microorganisms have already been reported as colonizing the plant phyllosphere, including *Epicoccum* sp. and *Aureobasidium* sp. strains in *Vitis vinifera* [62, 63], *Bacillus* sp. in *Zea mays* [64], *Pseudomonas* sp. in *Prunus domestica* [65], *Sporobolomyces* sp. in *Lactuca sativa* [66], and *Aspergillus* sp. in *Oryza sativa* [67]. These studies agree with our results and confirm the widespread presence of these microbial taxa in fruit crops different from Ericaceae. Therefore, this information can be essential for designing bioinoculants for fruit crops such as blueberry.

Several mechanisms have been described as crucial for the biocontrol of phytopathogens on the leaf surface, including niche competition, biosynthesis of antimicrobial compounds, production of volatile organic compounds, reduced adhesion, production of hydrolytic enzymes, and siderophore production [68, 69]. The screening of these biocontrol mechanisms in the isolates showed positive results for production of volatile compounds, hydrolytic enzymes and siderophores (Table 2, Table 3 and Table 4) and suggests that multiple biocontrol activity in several strains can be beneficial to limiting fungal diseases in nature, especially under the severe conditions in which the selected plants grow.

Hydrolytic enzymes have been described as the primary mechanisms for colonization of the leaves and the biocontrol of phytopathogenic fungi that can affect the tissue, thus directly affecting fungal membrane stability [70]. In this study,

the selected isolates demonstrated the production of cellulase, protease, lipase, and amylase activities (Table 2). Strains of *Bacillus* spp. and *Pseudomonas* spp. isolated from *Fragaria ananassa* and *Solanum lycopersicum*, respectively, have demonstrated the production of hydrolytic enzymes such as proteases and lipases during the biocontrol of *B. cinerea* [71]. Similarly, proteases produced by *A. pullulans* have been described as a component of post-harvest biological control in several fruits [72]. Lipase activity was the primary mechanism that we detected in our analyses, as almost all the isolates showed positive results for this enzyme (Table 2). Additionally, this mechanism can also support the primary colonization of the leaf by yeast, as this enzyme can metabolize the fatty acids produced by the plant host [73]. In our study, the isolates BFG22, BFG24, BFE6, BFE4 and BFG8 were positive for the production of biofilms by exopolysaccharide (Table 4). It is the main mechanism involved in leaf colonization by microorganisms and also has essential roles in abiotic stress tolerance and defense of the host plant [74, 75]. Similarly, competition for nutrients and space on the leaf surface has been described as a mechanism that enables colonization of the leaf surface [76, 77]. Therefore, the presence of these mechanisms in the isolates can be crucial to avoid colonization of the leaf by pathogenic strains.

Plant growth-promoting traits are another mechanism present in microbial strains isolated from the plant phyllosphere [78]. This study detected plant growth-promoting traits in the selected leaf-associated microorganisms, including IAA, ammonia production, and phosphate solubilization (Table 4). The ammonia production capability has been linked to beneficial traits for the host, such as a usable source of nitrogen, improving growth in the leaves and roots of *Zea mays* [79]. Similarly, the phosphate solubilization capability of fungal taxa included in the genus *Aspergillus* has been widely reported [80, 81]. However, this capability has been scarcely reported in the genus *Epicoccum*, with the strains belonging to this genus being the most effective for phosphate solubilization (Table 4). Similarly, yeasts from the genus *Aureobasidium* also showed phosphate solubilization capability, which is consistent with recent studies analyzing the beneficial effects of *Aureobasidium* strains for plant growth promotion [82, 83]. By contrast, only one bacterium belonging to the genus *Pseudomonas* showed positive results for this trait, a previously reported characteristic [84].

Our results demonstrated that the selected strains can be inoculated in *V. corymbosum* leaves without causing damage to the leaf and preventing infection by a pathogenic *B. cinerea* strain. This can be directly observed in the MDA content, which reflects the oxidative destruction of unsaturated fatty acids in the cellular membrane [85]. The results demonstrated that the inoculation of *B. cinerea* in the blueberry leaves significantly increased the MDA content compared to the control group (Table 4). However, the application of the isolate BFG22 (*A. pullulans*) reduced the MDA content (Table 4), which agrees with Lidon et al. [86], who showed a similar trend in the MDA levels of *A. pullulans* in infected *Pyrus communis* fruits. Similarly, another negative effect of *B. cinerea* infections is the decrease in total chlorophyll content by rapid degradation at the infection sites [87, 88]. In this study, the treatment with BFG22 demonstrated a significant increase in chlorophyll concentration in treatment without *B. cinerea* (Fig. 3), showing a positive effect on leaves since numerous studies positively correlate the chlorophyll concentration with increasing plant growth [89, 90]. The result demonstrated that the isolate BFG22 was the most effective at avoiding a reduction in chlorophyll content in the presence of *B. cinerea* (Fig. 3).

The yeast *A. pullulans* isolate BFG22 was an effective strain for biocontrol of *B. cinerea* (Fig. 1), as it positively reduced the leaf colonization in *V. corymbosum* (Fig. 2). Similarly, the other analyzed strains, BFE6 (*P. fluorescens*) and HFG13 (*E. layuense*), also performed well in both *in vitro* and *in vivo* tests. Overall, the study results have demonstrated the viability of isolating and applying leaf-associated microorganisms from native plants, which showed biocontrol capabilities and plant growth-promoting traits. However, the molecular mechanisms underlying the biocontrol capability of yeasts are mainly unknown, and further studies must address this research topic.

5. Conclusion

This study demonstrated that native ericaceous plants interact with a set of beneficial microorganisms colonizing the leaf environment, which showed biocontrol capability and plant growth-promoting traits. Additionally, the study results demonstrated the ability of the isolates to be applied as a leaf inoculant effective at inhibiting the pathogenicity of *B. cinerea* in a *V. corymbosum* plantlet. Further studies must evaluate the efficiency of the obtained strains against other phytopathogens that affect fruit crops (Omics approaches).

Declarations

Acknowledgments This research was funded by the “Fondo Nacional de Desarrollo Científico y Tecnológico” of Chile, grant number 1211857 to C.A. This study is dedicated for the memory of Prof. Domingo Contreras Fernandez (1952–2021), Chilean botanist, unforgettable friend and from who the authors learned much that could not have been learned elsewhere

Author Contribution T. S. and H. H. designed the experiments. T. S. analyzed data. H. H. drafted the manuscript. H. H. and C. A reviewed the first manuscript draft. All authors reviewed, improved, and approved the final version of the manuscript.

Data Availability All sequences are in GenBank and will made public after publication OK000985 - K000992 and OK000903 - OK000908

Ethics approval Not Applicable

Consent to participate Not Applicable

Consent for publication All authors agree with the final version of the manuscript as well as the submission to Microbial Ecology Journal

Conflict of interest The authors declare no conflict of interest.

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Figures

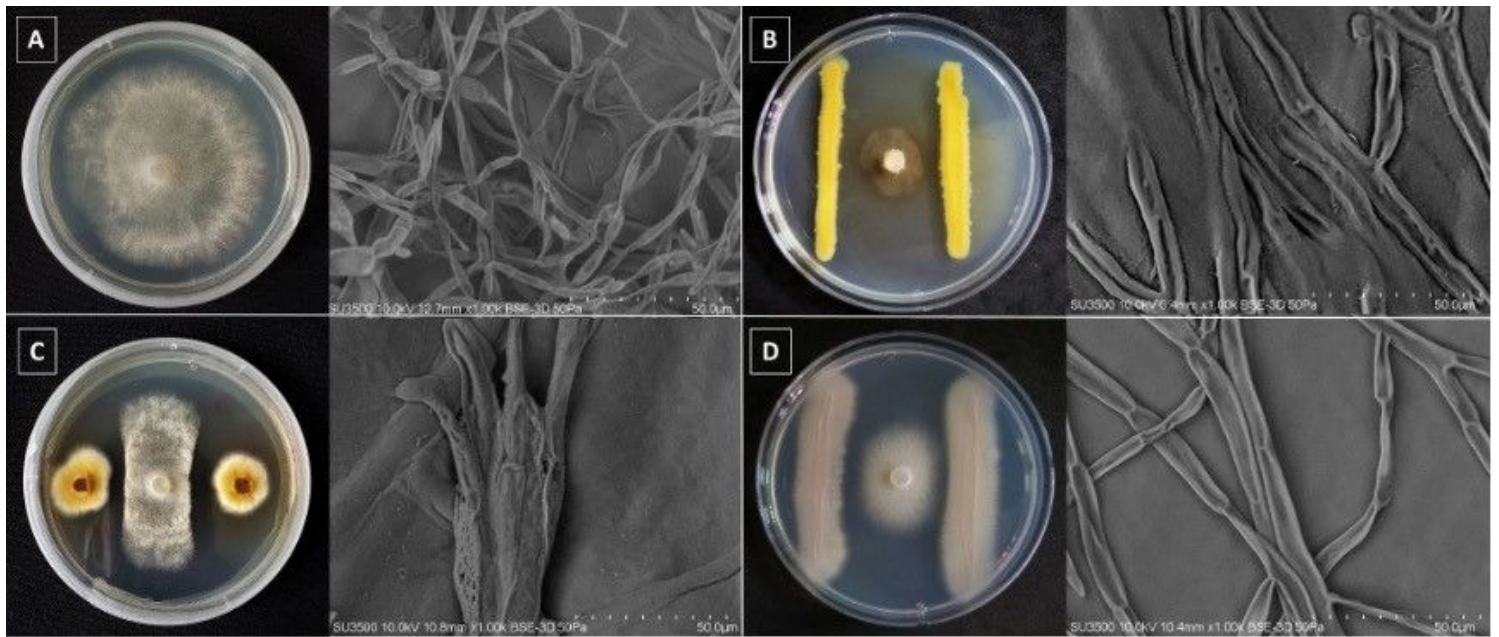


Figure 1

Morphological alterations of *Botrytis cinerea* hyphae in the presence of leaf-associated isolate microorganisms of *Gaultheria pumila* and *Empetrum rubrum*. A: hyphae of *B. cinerea* in control conditions; B: hyphae of *B. cinerea* inhibited by the presence of the isolate BFE6; C: hyphae of *B. cinerea* with alterations by the presence of the isolate HFG13; D: hyphae of *B. cinerea* inhibited by the presence of the isolate BFG22.

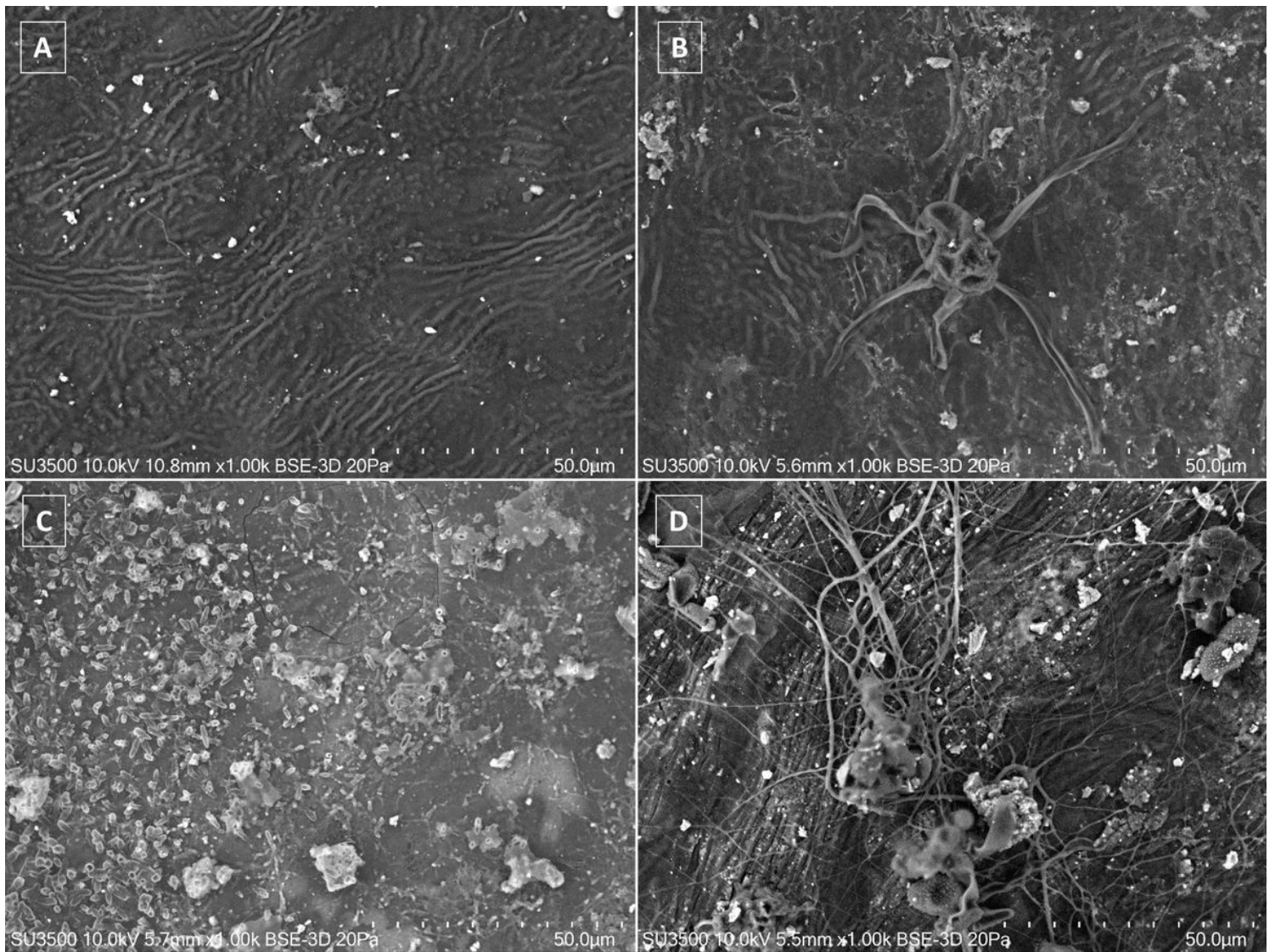


Figure 2

Colonization of the leaf surface of *V. corymbosum* by hyphae *B. cinerea* in the presence of leaf-associated isolates with the phyllosphere of *Gaultheria pumila* and *Empetrum rubrum*. A: leaf surface with treatment control (without *B. cinerea*); B: leaf surface treated with HFG13-*B. cinerea*, where the colonization by spores of the isolate HFG13 is observed; C: leaf surface treated with BFG22- *B. cinerea*, where the great colonization by the isolate BFG22 is observed; D: leaf surface with treatment control- *B. cinerea*, where the great colonization by hyphae of *B. cinerea* is observed.

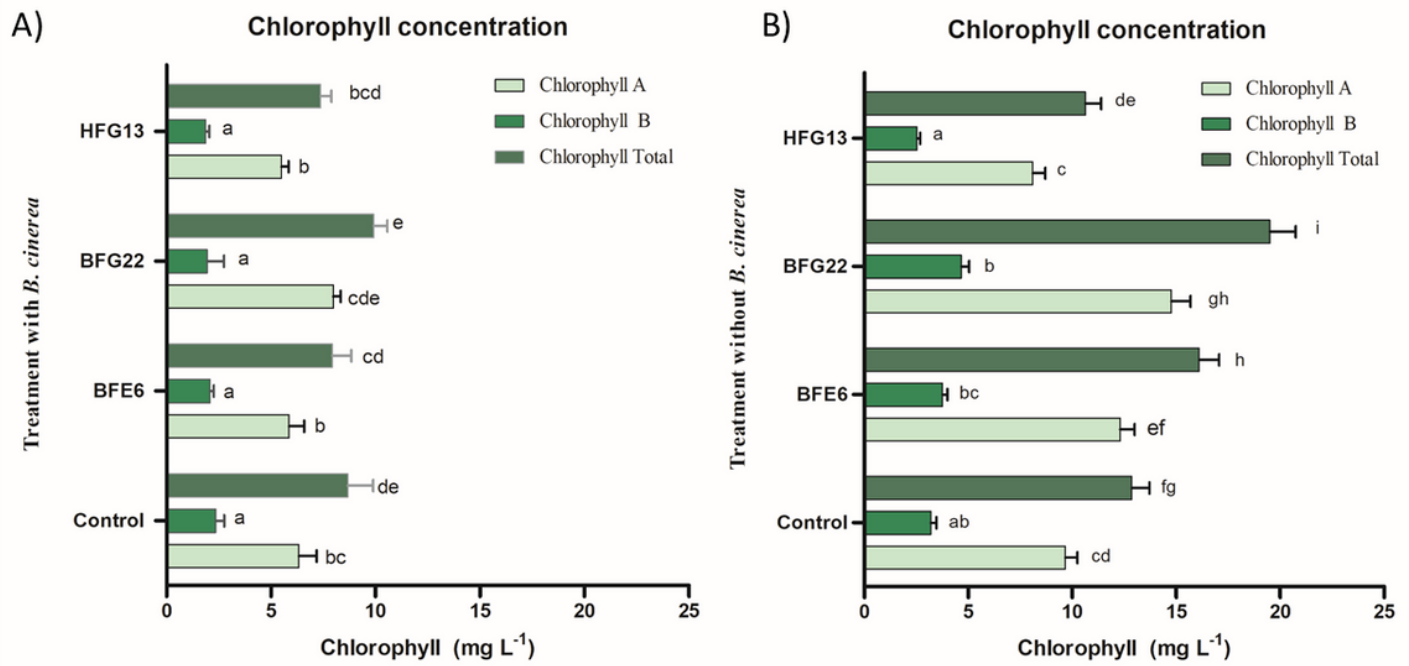


Figure 3

Chlorophyll concentration in leaf of *V. corymbosum* in treatments with leaf isolates of phyllosphere of native Ericaceae *Empetrum rubrum* and *Gaultheria pumila*. Values are means $\pm$ standard deviation for n=10.

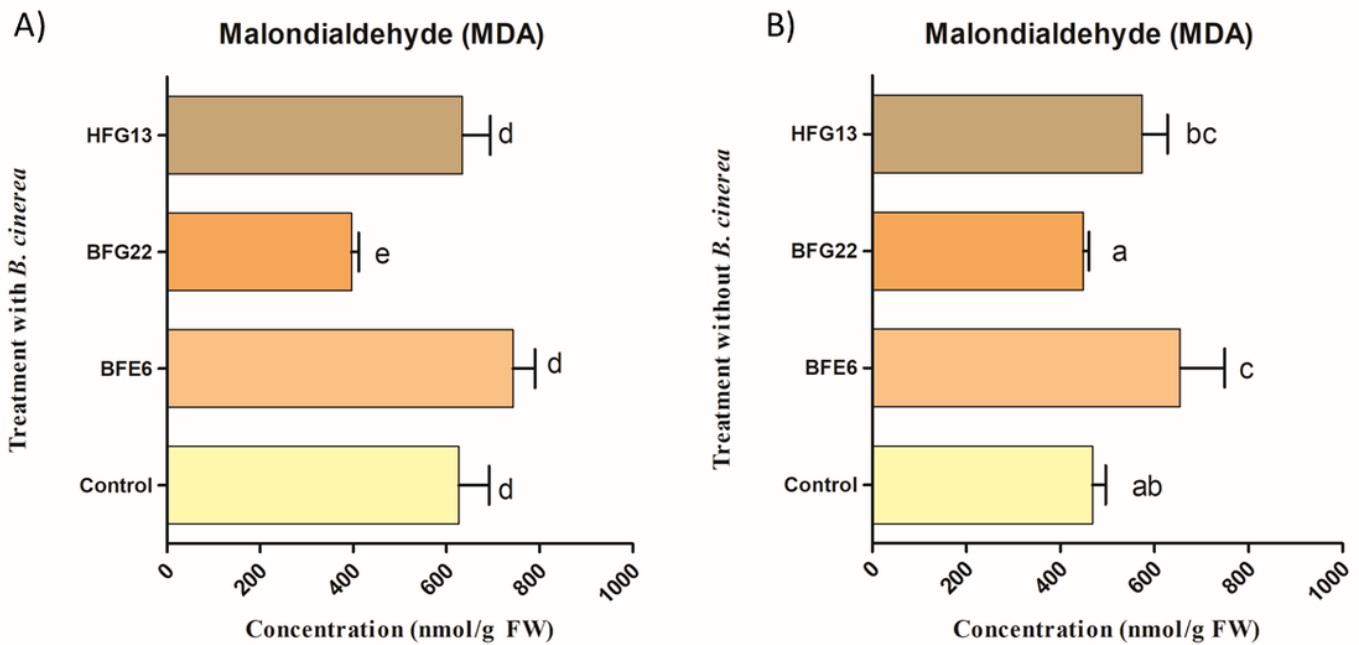


Figure 4

Lipid peroxidation of leaf of *V. corymbosum* in treatments with leaf isolates of phyllosphere of native Ericaceae *Empetrum rubrum* and *Gaultheria pumila*. Values are means $\pm$ standard deviation for n=10

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