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Low Nitrogen Concentration and Acidic pH Enhance the Antifungal Activity Against *Botrytis cinerea* of an Endophytic *Alternaria* sp. Isolated from *Opuntia ficus-indica*

Leonora Mendoza [1,*], Camilo Aguirre [1], Rodolfo Parada [1], Hristo Parada [1], Paulo Castro [1], Freddy Navarro [1], Araceli Vidal [2] and Milena Cotoras [1,*]

[1] Laboratorio de Micología, Facultad de Química y Biología, Universidad de Santiago de Chile, Avenida Bernardo O'Higgins 3363, Estación Central, Santiago 9170022, Chile; camilo16aguirre@gmail.com (C.A.); rodolfo.paradaf@usach.cl (R.P.); hristo.parada@usach.cl (H.P.); paulo.castro@usach.cl (P.C.); freddy.navarro@usach.cl (F.N.)

[2] Laboratorio de Microbiología Aplicada, Departamento de Ciencias Básicas, Facultad de Ciencias, Universidad Santo Tomás, Ejército Libertador 146, Santiago 8370003, Chile; aracelividalag@santotomas.cl

* Correspondence: leonora.mendoza@usach.cl (L.M.); milena.cotoras@usach.cl (M.C.)

Abstract

Endophytic fungi from the Cactaceae family are an underexplored source of bioactive secondary metabolites with potential applications in sustainable agriculture. This study investigated an endophytic fungus obtained from healthy cladodes of *Opuntia ficus-indica* growing in the Chilean Andean Precordillera. The influence of culture conditions, specifically pH and nitrogen concentration, on the production of diffusible and volatile antifungal compounds against the phytopathogen *Botrytis cinerea* was evaluated using dual-culture (confrontation) and sandwiched Petri dish assays. Morphological characteristics and molecular analyses confirmed that the isolate belongs to the genus *Alternaria*. Antifungal activity increased significantly under acidic conditions and limited nitrogen availability. The strongest inhibition by volatile compounds occurred at pH 4.5 and the lowest concentration of ammonium tartrate. Furthermore, ethyl acetate extracts at 40 mg/L obtained from *Alternaria* sp. cultures grown at pH 4.5 with 2.3 g/L ammonium tartrate inhibited *B. cinerea* mycelial growth by 60%. The study provides a framework for improving the yield of antifungal metabolites produced by *Alternaria*, contributing to the development of biofungicides for gray mold control.

Keywords: *Botrytis cinerea*; cactus endophytic fungus; biofungicide

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1. Introduction

In recent decades, the interest in endophytic fungi has increased due to their potential as producers of bioactive secondary metabolites [1]. It has been reported that endophytic fungi produce secondary metabolites with antibacterial, antifungal, immunosuppressants, antiviral, antiparasitic, antioxidant, anti-inflammatory, and anticancer properties [2–4]. Bioactive compounds produced by endophytes isolated from Cactaceae have been less studied [5–12]. Cacti are found in a wide variety of ecosystems, including arid, semi-arid environments and tropical forests [12]. It has been proposed that endophytic fungi isolated from plants growing in dry or semi-dry environments can produce new molecules with biotechnological interest [13]. For instance, Silva-Hughes et al. [9] isolated a hundred-eight endophytes from native cactus *Opuntia humifusa* in the United States. Six extracts of these

endophytes showed antifungal activity. On the other hand, Castro et al. [14] isolated six endophytic fungi from the roots of *Echinopsis chiloensis*, an endemic columnar cactus species growing in the central Andean Precordillera of Chile. This region has a Mediterranean climate, with a prolonged dry season lasting approximately 4 to 6 months [15]. Among the isolates obtained from *E. chiloensis*, one exhibited inhibition of mycelial growth of the phytopathogenic fungus *Botrytis cinerea* through antibiosis mediated by the secretion of secondary metabolites [14].

Endophytic fungi represent a promising reservoir of novel antifungal compounds for sustainable management of *B. cinerea* [16]. However, one of the challenges associated with the production of secondary metabolites by endophytic fungi is that subculturing on defined media often results in a marked reduction in metabolite yield [17]. The composition of culture medium (carbon and nitrogen sources) and pH significantly influence the production of fungal metabolites [18–20]. Production of mycotoxins by *Alternaria* was influenced by pH and C:N ratio of the growth medium [21]. The effect of pH has been particularly noted in studies on the genus *Alternaria*, where the production of secondary metabolites such as alternariol and tenuazonic acid is favored under acidic conditions, especially between pH 4.0 and 4.5. Notably, conidial germination did not occur when the pH dropped below 3.5 [21]. Based on evidence that pH and the C:N ratio of the growth medium affect mycotoxin production by *Alternaria* sp. [22], it is hypothesized that the antifungal activity against *B. cinerea* produced by the endophytic fungus isolated from *O. ficus-indica* is modulated by pH and nitrogen concentration.

B. cinerea is a necrotrophic fungus that produces gray mold disease, causing losses in fruit production by around 15 to 35% depending on climate conditions [23,24]. The control of this phytopathogenic fungus mainly involves synthetic fungicides [25]. The indiscriminate use of fungicides can cause problems in human health and soil and water contamination. Furthermore, *B. cinerea* isolates resistant to all families of fungicides, including multi-resistant isolates, have been reported [25]. Due to this, alternative control methods have been extensively investigated [26,27]. The two most investigated alternative control methods include biological control agents and secondary metabolites obtained from plants or endophytic fungi [22,26,28].

The objective of this work was to analyze the effect of the pH and the nitrogen concentration on the antifungal activity against *B. cinerea* of an endophytic fungus isolated from the cactus *Opuntia ficus-indica* growing in the central Andean Precordillera of Chile.

2. Materials and Methods

2.1. Isolation of Fungal Endophytes

Cladodes of *O. ficus-indica* without visible disease symptoms were collected from plants growing in El Ingenio, Cajón del Maipo, in the central Andean Precordillera of Chile (Latitude: −33°46′8.76″; Longitude: −70°16′35.03″ and Altitude: 1200 m). The collected samples were placed in polythene bags and then stored at 4 °C. The surface of cladode fragments was sterilized by sequential washing with 2% sodium hypochlorite, 0.1% Tween® 20, and 75% ethanol (all chemicals and reagents were purchased from Merck Millipore, Santiago, Chile, unless otherwise stated). After each washing, the cladodes were submerged in sterile distilled water for 2 min. The efficient disinfection of the plant surface was confirmed by inoculation of 0.5 mL of water from the last rinse onto malt-yeast agar (2% malt extract, 0.2% yeast extract, and 1.5% agar; Becton Dickinson and Company, Sparks, MD, USA). Then, four fragments of 1 cm × 1 cm were inoculated in Petri dishes. Ten Petri dishes containing malt-yeast agar supplemented with 50 µg/mL kanamycin and 34 µg/mL chloramphenicol were inoculated with four pieces of the fragments of 1 cm × 1 cm of cladodes. Therefore, a total of 40 cladode fragments were used. Cultures were incubated

at 22 °C. The mycelia growing from the cladode fragments were transferred to new Petri dishes containing fresh culture media.

2.2. B. cinerea Isolate and Culture Conditions

In this study, the G29 isolate of *B. cinerea* was used. This isolate was originally obtained from a naturally infected grapevine (*Vitis vinifera*) [29] and was maintained on malt-yeast agar (2% (*w*/*v*) malt extract, 0.2% (*w*/*v*) yeast extract, and 1.5% (*w*/*v*) agar) at 4 °C. The fungus was grown in a rich medium: malt-yeast agar or potato dextrose agar (PDA; Becton Dickinson and Company, Sparks, MD, USA).

2.3. Confrontation Assay

The antifungal activity of the endophytic fungi was evaluated using a dual culture confrontation assay. Mycelial disks (1 cm diameter) of both the endophytic isolate and *B. cinerea* were co-inoculated on 9 cm Petri dishes containing culture medium. The disks were placed at the periphery of the Petri dish, on opposite sides, with a distance of 6 cm between them. In the control treatment, *B. cinerea* mycelium was inoculated on both sides of the plates. After 10 days of incubation, the inhibition percentage of the radial mycelium growth of *B. cinerea* was evaluated according to Gothandapani et al. [30]. For this, the following formula was used:

$$Inhibition\ (\%) = \left(\frac{R_t - R_c}{R_t}\right) \times 100 \quad (1)$$

where R_c is the radial growth of *B. cinerea* in the presence of the endophytic fungus and R_t is the radial growth of *B. cinerea* in the absence of the endophytic fungus. The assays were performed four times, with three replicates per assay.

2.4. Identification of the Endophytic Fungus

Morphological identification of the endophytic fungus was performed using the microculture technique. Briefly, sterile pieces of 2 cm^2 of malt-yeast extract agar were placed onto sterile microscope slides. These pieces of solid medium were inoculated at the center with the endophytic fungus and then covered with a coverslip. Then, the slides containing the inoculated solid culture medium were incubated in a humid chamber, as previously described [31]. After incubation, mycelium, conidia, and conidiophores were examined microscopically following staining with lactophenol blue.

Additionally, molecular methods were used for genus-level identification of the fungus. For this, the mycelium was inoculated in malt-yeast broth (2% (*w*/*v*) malt extract, 0.2% (*w*/*v*) yeast extract) and incubated at 22 °C for seven days at 150 rpm. After this period, mycelia were frozen and ground in liquid nitrogen. DNA was extracted using the methodology previously described [32,33]. Then, the internal transcribed spacer (*ITS*) region was amplified from genomic DNA using the primers *ITS1* (5′-TCCGTAGGTGAACCTGCGG-3′) and *ITS4* (5′-TCCTCCGCTTATTGATATGC-3′) as described [34]. Once amplified, the PCR product was purified and sequenced (both strands) using the automatic sequencing service from Macrogen, Seoul, Republic of Korea. Sequences obtained were assembled and edited using ChromasPro version 1.5.

Comparisons with GenBank sequences were performed using Geneious Prime® 2020.0.4. The sequences were compared using the *ITS* region of the rDNA of *Alternaria* species, which showed a high percentage of identity. Then 14 sequences (Table S1) were aligned using Clustal Omega 1.22 algorithm with 100 refinement iterations. The alignment obtained was used to generate a phylogenetic tree that was constructed using the Tamura-Nei genetic distance model and Neighbor-Joining build method with 1000 bootstraps and using *Pleospora herbarum* as the outgroup. The quality of the tree was assayed by bootstrap, resampling the data sets with 1000 replicas.

2.5. Effect of pH and Nitrogen Concentration in the Culture Media on the Antifungal Activity of Endophytic Fungus Against B. cinerea

The effect of pH and nitrogen concentration in the culture media on the antifungal activity produced by diffusible compounds was evaluated using confrontation assays. The following culture media were used: malt-yeast agar (as a positive control) and minimum solid media (1.5% *w*/*v* agar, 1 g/L KH_2PO_4, 0.5 g/L K_2HPO_4, 0.5 g/L $MgSO_4 \times 7H_2O$, 0.5 g/L KCl, 0.001 g/L $FeSO_4 \times 7H_2O$) supplemented with ammonium tartrate at 2.3, 4.6, or 9.2 g/L and 1% (*w*/*v*) glucose. The pH in these culture media was adjusted to 4.5, 6.1, or 7.0. The cultures were incubated at 22 °C for 10 days.

The effect of culture media on the antifungal activity against *B. cinerea* produced by volatile compounds from the endophytic fungus was also evaluated using the sandwiched Petri dishes assay as described Li et al. [35]. These experiments used minimum solid media (pH 4.5 or 7.0) supplemented with ammonium tartrate at 2.3 or 9.2 g/L and 1% (*w*/*v*) glucose. A mycelium disk of the endophytic fungus was inoculated in the center of a Petri dish base (90 mm) containing culture medium, and a second base was inoculated with a mycelial disk of *B. cinerea*. Plates containing *B. cinerea* were always placed on top of endophytic fungus plate, allowing the headspace containing volatile compounds to be shared. The paired plates were hermetically sealed together using Parafilm®. Control sets were prepared similarly, but both Petri dish bottoms were inoculated with *B. cinerea*. The cultures were incubated at 22 °C for 10 days. The inhibition percentage was determined as mentioned above in Formula (1).

2.6. Extraction of Secondary Metabolites from Culture Media of the Endophytic Fungus

The antifungal diffusible compounds produced by the endophytic fungus were analyzed. For this, the endophytic fungus was grown for 10 days at 22 °C on the surface of the following culture media: malt-yeast agar or minimum solid media (pH 4.5) supplemented with 2.3 g/L ammonium tartrate. After the incubation, the mycelium was removed, and the compounds present in the culture media were extracted with ethyl acetate. Pieces of solid media were extracted three times with ethyl acetate for 4 h at room temperature. To obtain crude extracts, organic fractions were combined and concentrated to dryness under a reduced vacuum. As a negative control, the extraction of solid culture media without inoculating with the fungus was used.

2.7. Effect of the Extract on the Mycelium Growth of B. cinerea

The effect of the extract obtained from malt-yeast agar and minimum medium at pH 4.5 and 2.3 g/L ammonium tartrate on the mycelium growth of *B. cinerea* was evaluated *in vitro*. Extracts dissolved in acetone were added to the culture media at different concentrations. The final acetone concentration was identical in the negative control and treatment assay. After acetone evaporation in a laminar-flow cabinet (ESCO Lifesciences, Singapore), the culture media were inoculated with 0.5 cm agar disks from an actively growing culture of *B. cinerea*. Cultures were incubated in the dark at 22 °C for three days. Mycelium diameter was measured daily in two perpendicular directions. Inhibition percentages were calculated after 72 h of incubation. These experiments were performed in triplicate.

To identify fractions with antifungal activity of the extract, bioautography was performed using thin-layer chromatography (TLC) [14,36]. After solvent evaporation, the TLC plate was placed on a malt-yeast agar medium supplemented with 50 µg/mL kanamycin. Then, 10 mL of malt-yeast agar inoculated with a conidial suspension of *B. cinerea* (1×10^6 conidia/mL) was poured on the chromatogram. Cultures were incubated at 22 °C for 10 days. The active fraction, identified by bioautography, was separated from the extract by preparative thin-layer chromatography (PTLC) on pre-coated silica gel 60 F_{254}

using a chloroform:methanol (95:5) mobile phase. The bioactive zone was scraped from the chromatographic plate, and compounds were extracted from the silica gel using acetone.

This new fraction was used to perform another TLC under the described conditions and visualized under UV light at 254 and 365 nm.

In addition, a preliminary characterization of the bioactive fraction was carried out using different stain solutions. The used stain solutions were (i) sulfuric acid (25% v/v) for organic compounds, (ii) Dragendorff's reagent was used for alkaloids, and (iii) a solution of vanillin–sulfuric acid (3.5% w/v vanillin in methanol and 0.625% v/v of sulfuric acid) for terpenoids, phenols, and flavonoids [37].

2.8. Statistical Analysis

Data from four independent experiments (performed in triplicate) were analyzed using a one-way ANOVA. Means were separated using Fisher's LSD post hoc test with a significance level of $p < 0.05$, using InnerSoft STATS v1.1 software.

3. Results

3.1. Isolation and Identification of an Endophytic Fungus with Antifungal Activity Against B. cinerea Isolated from O. ficus-indica

After two weeks of incubation of cladodes of *O. ficus-indica*, three fungi emerged from the plant tissue. These fungi were purified, and the antifungal activity was evaluated by confrontation assays. Only one fungus, the isolate OF1, exhibited antifungal activity against *B. cinerea*. The isolate was deposited in the Chilean Collection of Microbial Genetic Resources (CChRGM) with the number RGM 3384.

Microscopic examination (Figure 1) revealed septate hyphae and production of catenate, obclavate to ovoid muriform conidia (dictyospores). The conidia exhibited a short conical beak and possessed transverse and longitudinal septa. They were pale to dark brown, measuring 20–37 × 9–18 µm. Based on the catenate fructification pattern and conidial morphology, the isolate is suggested to belong to the genus *Alternaria* [38].

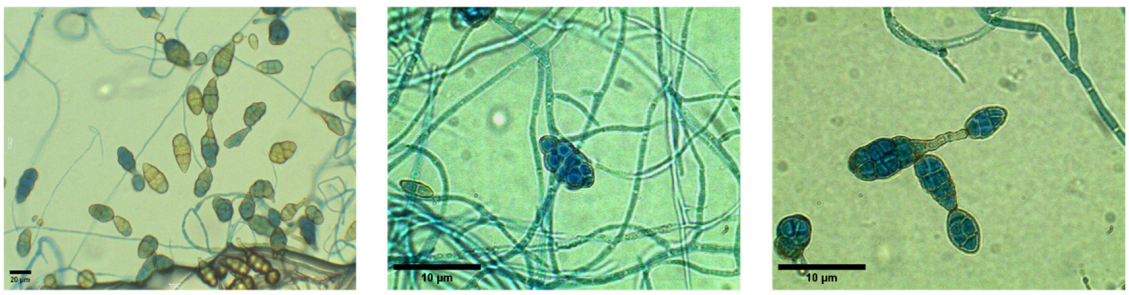

Figure 1. Conidia and mycelia of isolate OF1 cultured using the microculture technique on malt-yeast agar. Fungal structures stained with lactophenol blue were observed under a microscope at 40× (**a**) and 100× (**b**,**c**) magnification.

To confirm the genus of the isolated endophytic fungus (OF1), the region flanked by the primers *ITS1–ITS4* was amplified. The resulting fragment (~600 bp) was purified, sequenced, and deposited in NCBI GenBank (accession number OP106422).

After sequencing, it was determined that the length of the fragment was 521 bp. A megaBLAST (version 2.10.1) similarity search in the NCBI database using the sequence *ITS* region identified this fungus as belonging to the genus *Alternaria*. The highest identity was with *A. tenuissima* ATCC 16423 and *A. tenuissima* AT3 (both with a 96.9% of identity).

Additionally, a phylogenetic tree was constructed using the neighbor-joining method using the *ITS* sequence of the endophytic fungus and 14 *ITS* sequences of *Alternaria*

(Table S1). The endophytic fungus was grouped in the same clade as *A. alternata* and *A. tenuissima* (Figure 2).

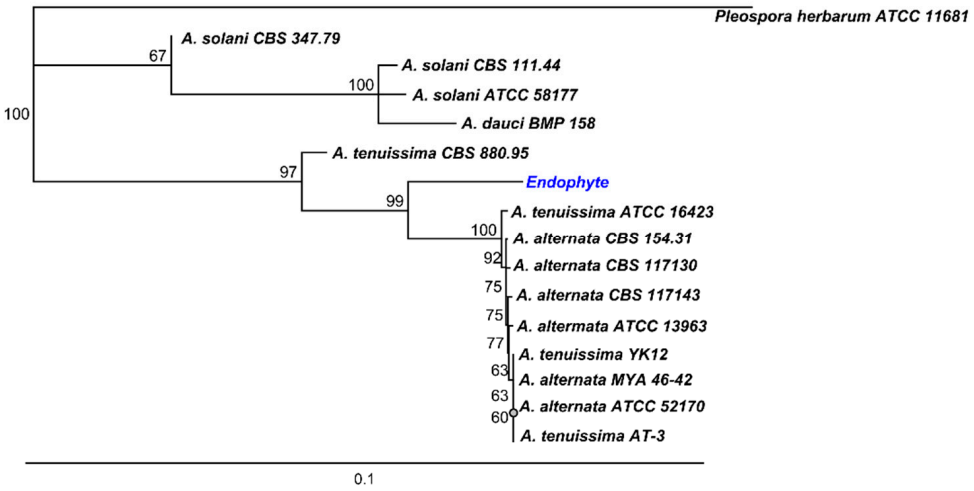

Figure 2. Neighbor-joining tree based on fungal *ITS* sequences of OFI isolate, 14 *Alternaria* spp., and *Pleospora herbarum* as outgroup. Numbers labeled at each node indicate the bootstrap value (%) from 1000 replicas. The term "Endophyte" is highlighted in blue to denote the isolate (OF1) obtained in this study.

3.2. Effect of Culture Conditions on the Antifungal Activity Against B. cinerea of the Isolated Endophytic Fungus (OF1)

The effect of pH in the culture media on the antifungal activity produced by diffusible compounds was evaluated using confrontation assays. In these experiments, 4.6 g/L tartrate ammonium was used, and the cultures were incubated at 22 °C (Figure 3). The antifungal activity at different pH values was compared the malt-yeast agar medium. The highest antifungal activity, about 26% of mycelial growth inhibition, was reached when the confrontation assays were carried out in minimum media at pH 4.5, this antifungal activity was similar to that obtained in malt-yeast extract medium.

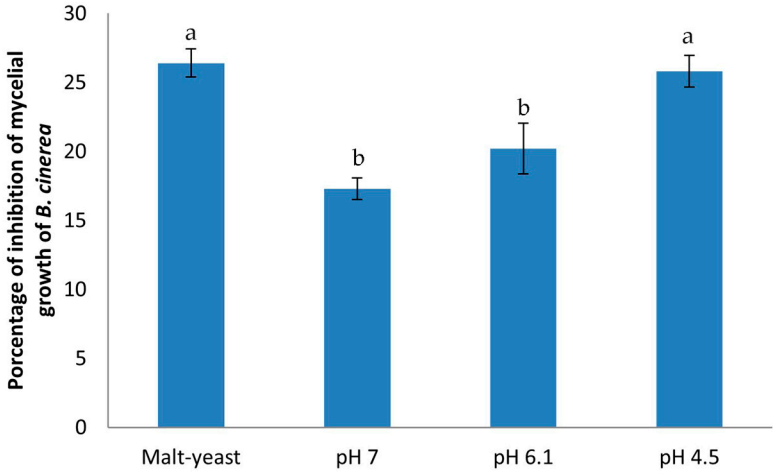

Figure 3. Effect of malt-yeast medium and minimum media at different pH on the antifungal activity against *B. cinerea* produced by the endophytic fungus. After 10 days of incubation at 22 °C, the inhibition percentage of mycelium growth of *B. cinerea* related to the control was evaluated. Each bar corresponds to the mean of four independent experiments ± standard deviation. Different letters correspond to a significant difference ($p < 0.05$) according to Fisher's LSD test.

Additionally, the effect of ammonium concentration in the culture media on antifungal activity against *B. cinerea* was analyzed (Figure 4). It can be observed that the antifungal effect was lower at higher ammonium tartrate concentration.

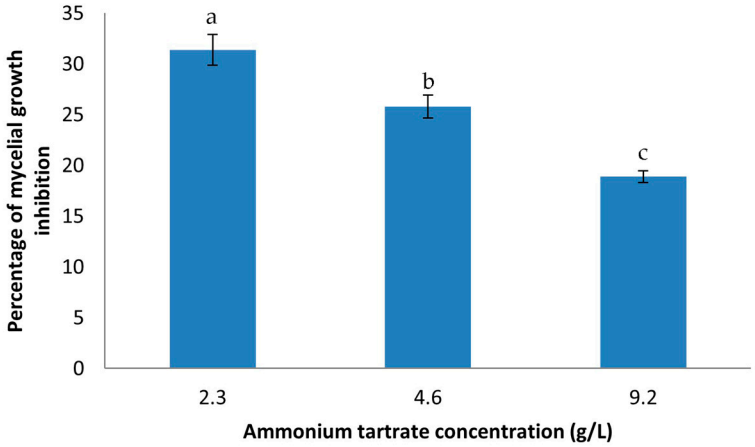

Figure 4. Effect of ammonium tartrate concentration of the culture medium on the antifungal activity against *B. cinerea* produced by the endophytic fungus. After 10 days of incubation at 22 °C, the inhibition percentage of mycelium growth of *B. cinerea* related to the control was evaluated. Each bar corresponds to the mean of four independent experiments ± standard deviation. Different letters correspond to a significant difference. ($p < 0.05$) according to Fisher's LSD test.

Extracts obtained from the minimum medium (pH 4.5 and 2.3 g/L tartrate ammonium) at 40 mg/mL or from the rich medium between 50 and 200 mg/L inhibited the mycelial growth of *B. cinerea* in 60% with statistically indistinguishable differences (Figure 5).

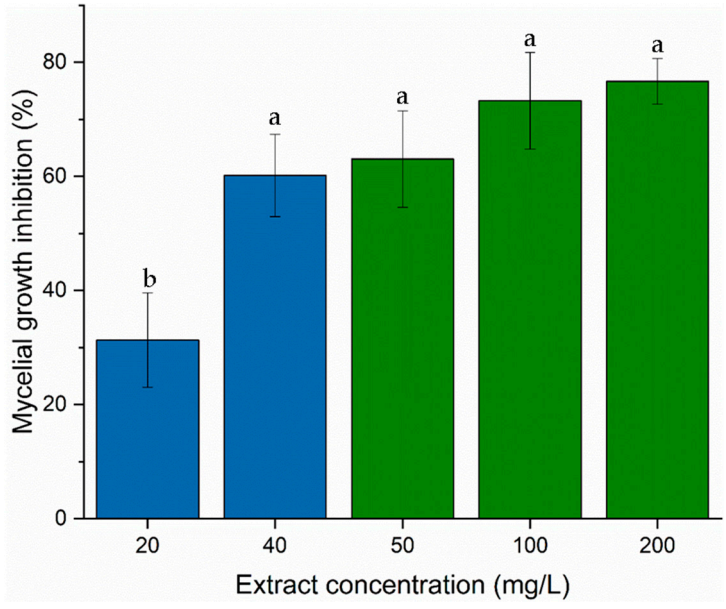

Figure 5. Comparative antifungal activity against *B. cinerea* using extracts derived from different culture conditions. The minimal medium (blue bars) extract, obtained from cultivation at pH 4.5 with 2.3 g/L tartrate ammonium, was applied at 20 and 40 mg/L. The rich medium (green bars) extract was applied between 50–200 mg/L. Each bar corresponds to the mean of four independent experiments ± standard deviation. Different letters correspond to a significant difference ($p < 0.05$) according to Fisher's LSD test.

Additionally, the rich medium extract was subjected to thin-layer chromatography. In the chromatogram, various compounds produced by the endophyte can be observed

(Figure 6a, lane 2). These compounds were absent in the extract obtained from the culture medium without inoculating with the endophytic fungus (Figure 6a, lane 1). A bioautography was performed to identify the fraction in the extract with antifungal activity (Figure 6b). In this technique, the antifungal activity of the separated compounds can be visualized as an absence of growth over the compound in the chromatogram (clear zone). One fraction at a high retention factor (R_f 0.75–0.88) exhibited antifungal activity against *B. cinerea*. This fraction was scraped from the chromatographic plate and was subjected to other TLC and visualized at UV 254 and 365 nm light (Figure 6c). Various spots can be observed at 254, and one of these fluoresces blue under ultraviolet light at 365 nm. The compounds present in these spots inhibited fungal growth (Figure 6d). The bioactive fraction was preliminarily characterized by using different stain solutions. This fraction was positive for vanillin-sulfuric acid reagent but was negative for Dragendorff reagent (Figure S2).

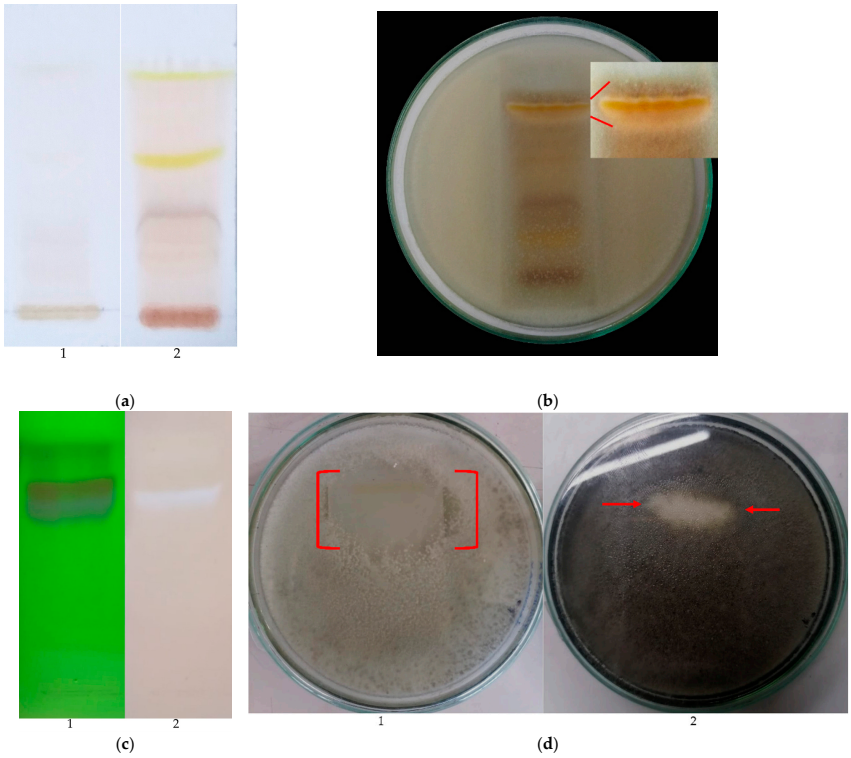

Figure 6. (**a**) TLC of compounds extracted from culture media in the absence (line 1) or presence (line 2) of endophytic fungus after 10 days of incubation at 22 °C. (**b**) Bioautography of the chromatogram shown in (**a**), lane 2, *B. cinerea* was grown on the chromatogram for 10 days, and the inhibition zone is shown in the amplified image to the right, indicated with red arrows. (**c**) TLC of the active fraction at 254 nm (1) or 365 nm (2). (**d**) Bioautography of the chromatogram of active fraction *B. cinerea* was grown on the chromatogram for (1) three or (2) seven days of incubation at 22 °C; the red brackets and lines highlight the areas of fungal growth inhibition.

On the other hand, the effect of pH and nitrogen concentration in the culture media on the antifungal activity of the endophytic fungus through the production of volatile compounds was evaluated. The sandwiched Petri dishes assay was used to study the production of volatile compounds. Figure 7 shows that the endophytic fungus produced volatile compounds that inhibited *B. cinerea* growth. The inhibitory effect was affected by the culture conditions. The best inhibitory effect was produced when the endophytic fungus was grown at pH 4.5 and low ammonium tartrate concentration.

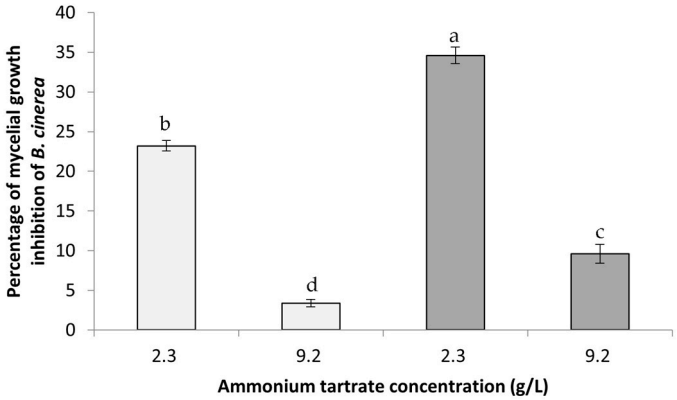

Figure 7. Effect of ammonium tartrate concentration and pH on the volatile compound production with antifungal activity by the endophytic fungus. These experiments used minimum solid media at pH 4.5 (gray bars) or 7.0 (white bars) supplemented with ammonium tartrate at 2.3 or 9.2 g/L. After 10 days of incubation at 22 °C, the inhibition percentage of mycelium growth of *B. cinerea* compared to the control was evaluated. Each bar corresponds to the mean of four independent experiments ± standard deviation. Different letters correspond to a significant difference ($p < 0.05$) according to Fisher's LSD test.

4. Discussion

In this work, an endophytic fungus of the genus *Alternaria* with antifungal activity against *B. cinerea* was isolated from the cactus *O. ficus-indica*. Fungi of the genus *Alternaria* have not been reported as endophytes in the cladode of *O. ficus-indica*. *Alternaria* spp. were isolated as endophytes from other species of cacti in the United States [6,9]. In Chile, fungi of the genus *Alternaria* have been isolated as endophytes from *Chenopodium quinoa* growing near the salt lakes of the Atacama Desert and from *Embothrium coccineum*, a small tree endemic to South American temperate forests [39,40]. On the other hand, species of *Alternaria* have also been reported as endophytes of numerous plants worldwide [41].

In a previous work, forty-four endophytic fungi were isolated from *O. ficus-indica* from a semi-arid Brazilian northeast region. Among the most abundant were *Cladosporium cladosporioides* and *C. sphaerospermum* [7]. *Aspergillus niger* was also isolated as an endophyte from *O. ficus-indica* fruit peels, and this isolate produced antimicrobial compounds against multidrug-resistant bacteria [42].

The endophyte *Alternaria* isolated in this study inhibited the mycelial growth of *B. cinerea* through the production of diffusible and volatile antifungal compounds. Extracts obtained from the culture media displayed activities comparable to those of grape pomace extracts, pure compounds, such as the terpenoids salvic acid and acetylsalvic acid, and the commercial fungicide iprodione. Nevertheless, their activity was lower than that of the purified extract from *Solanum tuberosum* and extracts enriched with anthocyanins obtained from various varieties of grape pomace (Table 1).

Table 1. Antifungal activity of extracts, pure compounds and the fungicide iprodione against *B. cinerea* isolate G29.

Compound or Extract	ED_{50} (mg/L)	Reference
Extracts enriched with anthocyanins obtained from various varieties of grape pomace	1.45 ± 0.18 to 5.50 ± 0.09	[43]
Purified extract from *S. tuberosum*	4.3 ± 0.9	[44]
Extracts from grape pomace	35.7 ± 5.2 to 2832.0 ± 105.3	[26]
Terpenoids (salvic acid and acetylsalvic acid)	53.1 ± 4.6 to 60.2 ± 8.4	[45]
Phenylpropanoids (*p*-coumaric acid and kaempferol, among others)	95.8 ± 3.6 to 390.0 ± 19.8	[26]
Terpenoids (sclareol and 13-epi-sclareol)	237.6 ± 12.2 and 268.8 ± 9.8	[46]
Iprodione	35 ± 1.5	[45]

Extracts obtained from culture media of the *Alternaria* genus have shown antifungal activity [41]. Some of these fungi have been isolated as endophytes [9,47,48]. There are a few reports of *Alternaria* endophytes exhibiting antifungal activity against *B. cinerea* [33,49,50]. Numerous metabolites produced by fungi of the genus *Alternaria* have been reported. Among them, terpenoids, polyketides, aromatic polyketides, alkaloids, pyranones, quinones, and phenolic compounds isolated from endophytic *Alternaria* have shown antifungal activity [17,50–57]. For example, polyketides, such as benzopyrones and a meroterpenoid derivative, showed antifungal activity against *B. cinerea* with MIC values ranging from 32 to 64 μg/mL [58,59]. The active fraction obtained in this work would contain terpenoids, phenylpropanoids, phenols, and flavonoids or polyketides, due to the presence of a blue/violet band at UV 365 light.

Production of fungal secondary metabolites is a multifactorial process influenced by numerous variables (e.g., carbon sources, micronutrients, and epigenetic modifiers) [60]. The present study focused on essential factors, namely pH and nitrogen concentration, because both factors are recognized as important regulators of fungal metabolism [60].

Modifications of culture conditions have been employed to optimize the production of active fungal metabolites [19,61], and specifically, it has been reported that nitrogen concentration in the culture media affects the biosynthesis of many fungal secondary metabolites [62]. In *Aureobasidium pullulans*, it was reported that a low nitrogen concentration increased the biosynthesis of polymalic acid, and the TOR signaling pathway could mediate this response [63]. In *F. fujikuroi*, gibberellic acid and bikaverin production were induced in low nitrogen concentration [64]. This response is consistent with the mechanism of Nitrogen Catabolite Repression (NCR), where low nitrogen availability leads to the activation of global transcription factors, primarily AreA. This factor is known not only to induce biosynthetic gene clusters for secondary metabolites but also to mediate chromatin remodeling, thereby increasing gene accessibility in filamentous fungi. Furthermore, recent models suggest that NCR involves a complex interplay between AreA and the GATA factor AreB to fine-tune this response [62].

On the other hand, the pH of the culture media was also important in the antifungal effect against *B. cinerea*. This could be due to the higher production of antifungal compounds, volatiles, and diffusibles by the endophyte at lower pH. In *Fusarium verticillioides*, *Aspergillus ochraceus*, and *Fusarium graminearum*, the secondary metabolites are mainly produced at low pH [65–68]. Acidic conditions induced the production of harzianic acid and related polyketide-derived compounds in *Trichoderma harzianum* [69]. Adaptation of fungi to environmental pH fluctuations is primarily regulated by the conserved Pal/Rim signaling pathway, in which the zinc-finger transcription factor PacC (or its homolog Rim101) serves as the principal effector [70]. According to Zong et al. [71], biomass formation in *Penicillium expansum* and the production of the mycotoxin patulin are favored at pH values between 3 and 5, which correlates with the upregulation of numerous genes putatively involved in patulin biosynthesis. Furthermore, the same group demonstrated that the transcription factor PacC is essential for patulin production [72].

Finally, this work demonstrates that culture conditions, particularly pH and nitrogen concentration, enhance the production of volatile and diffusible antifungal metabolites by the endophytic fungus *Alternaria* sp. Extracts obtained from the culture media contain bioactive compounds with potential applications in the development of biofungicides against gray mold. The use of endophytic fungi as a source of natural antifungal agents facilitates more sustainable alternatives to conventional chemical fungicides. In future work, it would be interesting to determine the chemical identity of the antifungal compounds produced by *Alternaria* sp.

5. Conclusions

In conclusion, an endophytic fungus identified as *Alternaria* sp. was isolated from the cactus *Opuntia ficus-indica*. This fungus produced both volatile and diffusible compounds with antifungal activity against *B. cinerea*. Moreover, low ammonium tartrate concentrations and an acidic pH in the culture medium enhanced the antifungal effect against *B. cinerea* of the endophytic fungus. This study highlights the importance of the fungal culture medium in the production of secondary metabolites with antifungal activity, and, as a projection, provides alternative biological resources for the production of secondary metabolites to control phytopathogenic fungi.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/microorganisms14020376/s1, Table S1: *ITS* sequences used in this study and their GenBank accession numbers; Figure S1: Confrontation assay between (A) endophytic fungus (right) and *B. cinerea* (left) and (B) between *B. cinerea* (control) in PDA medium after 10 days of incubation; Figure S2: TLC profiling of the bioactive fraction on Silica Gel 60 F_{254} plates. (a) Visualization under visible light; (b) visualization under UV light (365 nm); (c) derivatization with vanillin-sulfuric acid.

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Abbreviations

The following abbreviations are used in this manuscript:

CChRGM	Chilean Collection of Microbial Genetic Resources
DNA	Deoxyribonucleic acid
ITS	Internal transcribed spacer
NCBI	National Center for Biotechnology Information
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PTLC	Preparative thin-layer chromatography
R_c	Radial growth of *B. cinerea* in the presence of the endophytic fungus
rDNA	Ribosomal deoxyribonucleic acid
R_t	Radial growth of *B. cinerea* in the absence of the endophytic fungus
TLC	Thin-layer chromatography

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