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Cultural Characterization and Antagonistic Activity of *Cladobotryum virescens* against Some Phytopathogenic Fungi and Oomycetes

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Abstract: In this study, the characteristic growth of *Cladobotryum virescens* on nine culture media was analyzed. The growing behavior of this fungus was dependent on the culture medium. In vitro analysis showed that oat agar was better than other media tested with the highest conidia production. The antifungal activity against *Fusarium chlamydosporum* and *Alternaria brassicicola* was evaluated by the Dual Culture method. *C. virescens* displayed high activity against both pathogens acting through antibiosis and mycoparasitism. This effect was increased by a higher competitiveness of the strain for the substrate. Furthermore, the crude ethyl acetate extract of the culture broth was tested in vitro against *Botrytis cinerea* and *Septoria tritici*, as well as the hemibiotrophic oomycete *Phytophthora infestans* using a microtiter plate assay at different concentrations. The extract showed excellent inhibition even below 5 ppm. According to these results, we concluded that *C. virescens* can be considered as a potential biological control agent in agriculture. To the best of our knowledge, this is the first study to investigate *C. virescens* as a biocontrol agent for different diseases caused by five relevant pathogens that affect cereals and vegetables.

Keywords: phytopathogens; antifungal; *Fusarium*; *Alternaria*; *Botrytis*; *Septoria*; *Phytophthora*

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1. Introduction

Traditionally, natural products have been widely employed for the development of new control strategies aiming to improve agricultural production. The scientific community focuses on plant and fungal extracts and secondary metabolites isolated from these sources as natural, environmentally friendly fungicides. Food production is affected, among other factors, by the presence of phytopathogenic fungi and oomycetes which have a negative impact on the development, yield, and quality of crops. Pathogens such as *Fusarium chlamydosporum* Wollenw and Reinking (fusarium wilt pathogen on several crops) [1,2], *Alternaria brassicicola* Schwein (black spot disease on *Brassica* spp. leaves) [3,4], *Botrytis cinerea* Pers. (grey mold pathogen on many crops, including *Fragaria* × *ananassa (Weston)* Duchesne ex Rozier and *Vitis vinifera* L.) [5,6], *Septoria tritici* Rob. ex Desm. (causing agent septoria leaf blotch of *Triticum* species) [7,8], and *Phytophthora infestans* (Mont.) de Bary (causal agent of the late blight disease on *Solanum tuberosum* L. and *Solanum lycopersicum* L.) [9,10] are among the most aggressive. Their control is very difficult due to their genotypic mutability and adaptive capacity, amongst other factors, which allow them to develop fungicide resistances [11,12]. Among the biggest challenges in agricultural production is the risks reduction and negative

impacts of chemical products and fertilizers on the environment and human health. Therefore, the search for mycopathogenic fungi to be used as tools for fungal disease control has been a growing area of research throughout the last century. In addition, it is very important to know the types of substrates in which the fungi grow and develop and the habitat conditions in which they show different phenotypic expressions. Different substrates and habitats modify the characteristics of the fungus, and as such it is important to analyze how it behaves in the different environments and establish the most suitable conditions for its study [12]. The production of biologically active secondary metabolites (antifungal activity) has been attributed to various microorganisms; however, these substances are produced only under specific physiological conditions, and there is also great variability in the metabolic production of each particular strain. Surface growth in solid media is one of the basic methods to study the physiology of microorganisms, since the diameter of the colonies and the radial growth rate are parameters widely used in bioassays and physiological investigations. It is of great interest in the study of the physiology of a microorganism to test the use of different culture media, since it has been observed that the complexity of the medium has a significant influence on the growth of the mycelium and, therefore, on the speed of biomass production [11,12]. To achieve a higher production of these antifungal or bioactive metabolites, optimal conditions must be ensured during the trophophase for good development of the microorganism [13,14].

Cladobotryum spp. cause cobweb disease and are described as harmful in different mushroom-producing countries. Cobweb is characterized by the growth of coarse mycelium over the affected mushrooms [15]. On the other hand, several *Cladobotryum* spp. produced inhibitory effects on some phytopathogenic oomycetes [16]. Ortiz and coworkers evaluated three species of *Cladobotryum* and their crude ethyl acetate extracts in the control of *Corynespora cassiicola* (Berk and M.A. Curtis) C. T. Wei by dual culture and bioautographic TLC methods [17]. The obtained results showed that the strains produced compounds with antagonistic effects on the pathogen. Similar results were reported by Ramos-García [18] when studying the in vitro and in vivo activity of these isolates against phytopathogenic fungi. To the best of our knowledge, these are the only reports regarding the use of the anamorphic *Cladobotryum virescens* G.R.W. Arnold (conidial state of teleomorphic *Hypomyces virescens* G.R.W. Arnold and K. Põldmaa) [19,20] (Supplementary Materials, Figures S1 and S2) as a biocontrol agent of diseases caused by phytopathogenic fungi.

The aim of this study was to analyze the eco-morphological characterization of *Cladobotryum virescens* growth on nine culture media, the in vitro antagonistic activity of the strain and ethyl acetate crude extract from the culture broth against five pathogens that affect agricultural production.

2. Materials and Methods

2.1. Fungal Strain, Growth Media and Conditions

The registration numbers of strains are shown in Table 1.

Table 1. Registration number of strains used in this research and preserved at INIFAT and IPB culture collection.

Species	Strain Number
Alternaria brassicicola Schwein	INIFAT 4145
Botrytis cinerea Pers.	IPB KSH 892
Cladobotryum virescens G.R.W. Arnold	INIFAT C10/110
Fusarium chlamydosporum Wollenw and Reinking	INIFAT 4147
Phytophthora infestans (Mont.) de Bary	IPB KSH 894
Septoria tritici Rob. ex Desm.	IPB KSH 895

INIFAT: Institute of Fundamental Research in Tropical Agriculture; IPB: Institute of Plant Biochemistry; TFC: Tartu Fungal Culture Collection.

Cladobotryum virescens G.R.W. Arnold, was collected and identified by R. F. Castañeda and preserved at the international recognized World Federation Culture Collections (WFCC 853) at INIFAT. The isolates were transferred into Petri dishes (9 cm diameter) and were

grown on oat agar medium (OA) [21] for 10 days at 25 ± 2 °C. The phytopathogenic fungi, *Fusarium chlamydosporum* and *Alternaria brassicicola,* from the same culture collection, were grown under identical conditions. In addition, *Botrytis cinerea*, *Phytophthora infestans* and *Septoria tritici*, deposited in the culture collection at the Leibniz Institute of Plant Biochemistry, Halle (Saale), Germany, were grown according to the protocol established by Stammler et al. [22–24] (Table 1).

2.2. Culture Characterization of Cladobotryum virescens INIFAT C10/110

A total of 20 mL of each of nine culture media used in this experiment (potato dextrose agar (PDA), carrot agar, malt extract agar (MEA), rice agar, oat agar (OA), agar poor in synthetic nutrients (SNA), yeast agar (YA), Czapeck agar (CZA), and Sabouraud agar (SABG)) were poured into Petri dishes (9 cm). All of them were prepared according to the protocol established at the Fungal Biodiversity Centre (CBS) [25]. *C. virescens* INIFAT C10/110 cultures were grown for 10 days and then the abovementioned different culture media were inoculated with a mycelial plug of the fungal cultures with a diameter of 0.7 cm, from culture on OA media, and incubated at 25–27 °C, HR = 66–75% and under a 12/12 h darkness and light cycle. The growth of colonies was measured every day, and culture characterization was evaluated after 10 days of inoculation. The following qualitative characteristics were analyzed: the color of mycelium, superficial texture, shape, edge, height, and internal structure, according to Frey et al. [26] and Bernal et al. [27]. The experiments were performed with two repetitions by quintuplicate. Furthermore, conidia production on the nine culture media was analyzed after 10 days of inoculation under an Axioscop 40 microscope, and their concentration was determined by a Thomas BRAND "Blaubrand" counting chamber. Each experiment was carried out in triplicate.

2.3. Ethyl Acetate Crude Extract

C. virescens was grown in twelve Erlenmeyer flasks (size 500 mL), each containing 200 mL of liquid oat extract, where oats were boiled and then filtered, and the broth adjusted to pH 6.5. The culture was incubated under stirring conditions at 150 rpm for 15 days (T = 25 °C ± 2 °C, y HR = 63–65%). All cultures were homogenized and filtered using glass wool to separate the mycelium from the culture medium. The culture filtrate was extracted with ethyl acetate by liquid–liquid partition (1:1, *v*/*v*). The organic solvent layer (upper layer) was separated using a separating funnel and concentrated under vacuum, yielding a dark red crude extract (460 mg) [17].

2.4. Antagonistic Assays

In order to evaluate the in vitro antagonistic activity of *C. virescens* INIFAT C10/110, a dual culture method was performed in Petri dishes (9 cm diameter) with OA [28]. In this experiment, 0.7 cm mycelial plug of the culture of *C virescens* was placed on the Petri dish, while a plug with the pathogens (*F. chlamydosporum* and *A. brassicicola*, independently) was placed on the opposite side, with a distance of 4 cm between both fungal plugs. A plug of OA medium was used as the control treatment, while the pathogen plug was placed on the other side. All plates were incubated at 25 ± 2 °C under a 12/12 h darkness and light cycle for ten days, after which the distance between the mycelia fronts of the pathogen and the fungal test strain was measured. The results were transformed into the percentage of radial growth inhibition (PRGI) of the pathogen. All tests were performed in quintuplicate. Three different modes of action were evaluated:

1. Competition for substrate: Evaluations were made every 24 h by analysis of the diameter of the colonies in cm. All tests were carried out in quintuplicates, and results were analyzed with a bifactorial variance analysis using the Statgraphics 5 program. Differences between treatments were determined by a Tukey test at a 5% significance level;

2. Antibiosis: The percentage of radial growth inhibition (PRGI) was evaluated at three and five days after inoculation (before the colonies had contact) and was calculated as PRGI = (R1 − R2)/R1 * 100, where R1: radial growth of pathogen in control plates; R2: radial growth of pathogen in dual culture plates before the points of interaction [29];
3. Mycoparasitism: Different types of mycoparasitism were evaluated: penetration (P), vacuolization (V), lysis (L), and coiling (C) [30]. From the area of interaction between both fungi (*C. virescens* and pathogen), three samples were taken. All the tests were carried out in triplicate. A drop of Lactofenol was added to the sample, which was analyzed with an optical microscope (Axioscop 40) at 100× and 400×.

2.5. Antifungal Screening of Ethyl Acetate Crude Extract

The ethyl acetate crude extract was tested in a 96-well microtiter plate assay against *B. cinerea, P. infestans*, and *S. tritici* according to the protocol established by the Fungicide Resistance Action Committee (FRAC) with slight modifications [22–24]. The ethyl acetate crude extract was redissolved in DMSO and evaluated in different final concentrations (125, 42, 14, 5, and 1.5 ppm). The solvent DMSO was used as a negative control, and the commercially used fungicides Terbinafine and Epoxiconazole (Merck) served as reference compounds. Eight days after inoculation, the pathogen growth was evaluated by measuring the optical density (OD) at λ = 405 nm with a Tecan GENios Pro microplate reader (5 measurements per well using multiple reads in a 3 × 3 square). Each experiment was carried out in triplicate.

2.6. Statistical Analysis

Data were analyzed in Excel 2016. Statistical analyses were processed using analysis of variance (ANOVA) Turkey test $p \leq 0.05$ with Statgraphics 5 program. Differences between treatments were determined by a Tukey test at a 5% significance level.

3. Results

3.1. Culture Characterization

Figure 1 shows the changes in the diameter growth of colonies and conidia production of *C. virescens* INIFAT C10/110 in the different culture media evaluated (Table 2). The results showed that the culture medium significantly influenced the growth of the colonies, since there were differences between all nine tested culture media (Figure 1). The strain showed greater growth in the oat agar, agar malt, potato dextrose agar (PDA) and Czapek media, while the reduced growth was observed in the synthetic nutrient-poor agar (SNA), rice agar and yeast agar media.

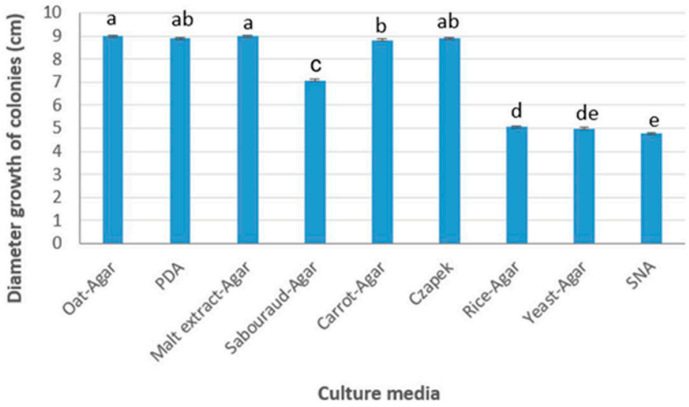

Figure 1. Diameter growth of colonies (cm) on nine different culture media after 10 days. Mean and standard deviation is expressed in the error bar (n = 3). Different letters indicate a significant difference (Tukey test, $p \leq 0.05$).

Table 2. Conidia production of *C. virescens* INIFAT C10/110 on different culture media after 10 days.

Culture Media	Conidia Production Spores/mL
Oat agar	28.2×10^6
Potato dextrose agar	25.0×10^6
Malt extract agar	23.7×10^6
Sabouraud agar	18.4×10^6
Carrot agar	27.8×10^6
Czapek agar	24.0×10^6
Yeast agar	-
SNA agar	-
Rice agar	8.2×10^6

C. virescens INIFAT C10/110 was studied in terms of the measured qualitative parameters, depending on the variants used. Regarding the coloration of the faster growing colonies, they were pinkish-purple with variable intensity; the internal structure, in general, consisted of coarse and filamentous grains. However, in the media with less growth, the internal structure consisted of very fine and translucent grains, with scarce mycelium. The height of the colonies of the strain was rather similar on all nine tested culture media, and the edges only varied in some of them, where there were slightly undulating; in the rest, they were regular edges.

The production of conidia is an important element of the biological cycle in fungi. In the studied case, the best results were obtained in oat agar, potato dextrose agar (PDA), malt extract agar, carrot agar, and Czapek agar, which had the most abundant conidia production and the greatest diameter of the colonies (approximately 9 cm).

According to these results, it was decided to study the liquid cultivation of *C. virescens* in potato dextrose and oat liquid media in order to analyze the differences in coloration, pellet formation, and mycelium production after 5, 10, and 15 days of incubation, respectively. In this experiment, the best results were observed in oat medium (Table 3) [31].

Table 3. Characteristics of *C. virescens* colonies in liquid medium of potato dextrose and oats respectively after 15 days.

Strain	Culture Media	Coloration			Pellet Formation			Mycelium		
		Day 5	Day 10	Day 15	Day 5	Day 10	Day 15	Day 5	Day 10	Day 15
C. virescens INIFAT C10/110	PDA	Light yellow	White	White	-	Low	Low	Low	Low	Low
	Oat agar	Red	Red	Red	High	High	High	Abundant	Abundant	Abundant

3.2. Antagonistic Assays

3.2.1. Competition for Substrate

In Table 4 and Figure 2 it is presented the competition for nutrients in the interaction between *F. chlamydosporum* and *C. virescens***.** The analysis of the *C. virescens* INIFAT C10/110 control exhibited a lower growth rate compared with the *F. chlamydosporum* control; however, when both strains were confronted, the results showed in vitro antagonistic activity of *C. virescens* INIFAT C10/110 against the pathogen. This effect is statistically significant from day four to the end of the experiment.

Results of the inhibition of the radial mycelial growth of *A. brassicicola* are shown in Table 5 and Figure 3. The analysis of the proportion of media between *Cladobotryum* and pathogen controls showed a similar growth rate for both fungi. However, *C. virescens* had an inhibitory effect on the mycelial growth of the pathogen according to the media ratio values when they were confronted. This effect is significant from the second day of inoculation to day 10.

Table 4. Competition for nutrients. In vitro inhibition activity of *C. virescens* on mycelial growth (cm) of *F. chlamydosporum* from day 1 to 10 on OA medium. Media proportion was calculated as: mycelial growth diameter of *C. virescens*/*F. chlamydosporum* CT and Cc refer to tested and control *C. virescens*, respectively. Pc and PT refer to tested and control pathogen *F. chlamydosporum*, respectively.

Days	*C. virescens* Control (Cc)	Pathogen Control (Pc)	*Cladobotryum virescens* Tested (CT)	Pathogen Tested (PT)	Media Proportion (Cc/Pc)	Media Proportion (CT/PT)
1	0.7	0.7	0.7	0.7	1.00	1.00
2	1.34	1.16	1.46	1.04	1.16	1.40
3	2.18	2.22	2.36	2.02	0.98	1.17
4	2.9	4.24	2.86	2.08	0.68	1.38
5	3.76	5.54	3.74	2.58	0.68	1.45
6	4.68	7.04	4.64	3.08	0.66	1.51
7	5.42	8.02	5.5	3.7	0.68	1.49
8	6.5	8.54	6.74	4.22	0.76	1.60
9	7.7	9	7.6	4.7	0.86	1.62
10	8.94	9	9	5.34	0.99	1.69

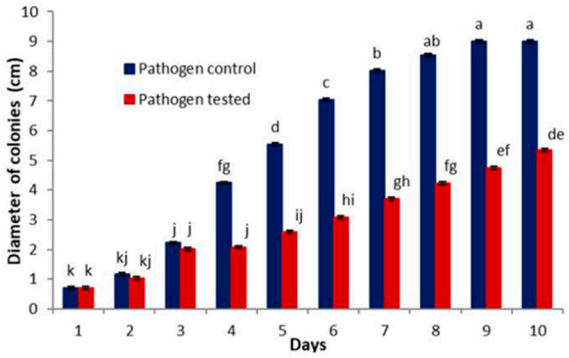

Figure 2. Mycelial growth of *F. chlamydosporum* (pathogen tested) against strain *C. virescens* and the control for ten days in dual culture. Mean and standard deviation is expressed in the error bar ($n = 5$). Different letters indicate a significant difference (Tukey test, $p \leq 0.05$).

Table 5. Competition for nutrients. In vitro inhibition activity of *C. virescens* on mycelial growth (cm) of *A. brassicicola* from day 1 to 10 on OA medium. Media proportion was calculated as: mycelial growth diameter of *C. virescens*/*A. brassicicola*. CT and Cc refer to tested and control *C. virescens*, respectively. Pc and PT refer to tested and control pathogen *A. brassicicola*, respectively.

Days	*Cladobotryum virescens* Control (Cc)	Pathogen Control (Pc)	*Cladobotryum virescens* Tested (CT)	Pathogen Tested (PT)	Media Proportion (Cc/Pc)	Media Proportion (CT/PT)
1	0.7	0.7	0.7	0.7	1.00	1.00
2	1.3	1.1	1.36	0.8	1.18	1.70
3	2.18	2.42	2.22	1.28	0.90	1.73
4	2.92	3.12	3.16	1.56	0.94	2.03
5	3.66	3.66	3.86	1.82	1.00	2.12
6	4.62	4.68	4.68	2.02	0.99	2.32
7	5.5	6.2	5.56	2.34	0.89	2.38
8	6.4	7.58	6.58	2.5	0.84	2.63
9	7.54	8.24	7.74	2.8	0.92	2.76
10	9	9	9	2.9	1.00	3.10

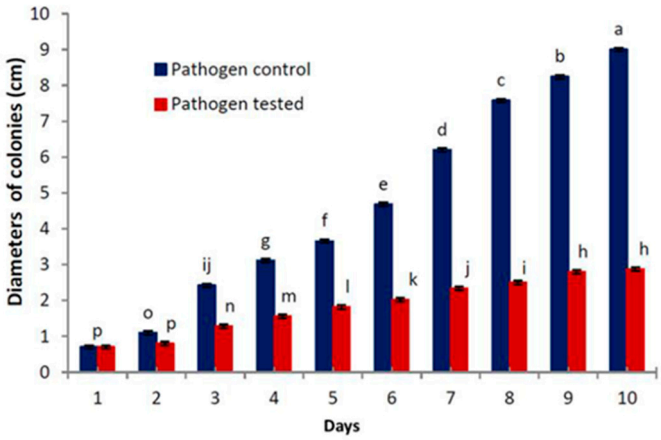

Figure 3. Mycelial growth of pathogen *A. brassicicola* confronted against strain *C. virescens* for 10 days. Mean and standard deviation is expressed in the error bar ($n = 5$). Different letters indicate a significant difference (Tukey test, $p \leq 0.05$).

3.2.2. Antibiosis

The analysis of the PRGI values for the pathogens *F. chlamydosporum* and *A. brassicicola* (Figure 4) showed that *C. virescens* inhibited mycelial growth for both pathogens, thus for *F. chlamydosporum* values higher than 52% were obtained on the fifth day of incubation, while for *A. brassicicola* values higher than 40% were obtained on the third day of incubation of the pathogen and showed 50% on the fifth day of incubation. These results suggest that *C. virescens* can have a potential antibiotic effect.

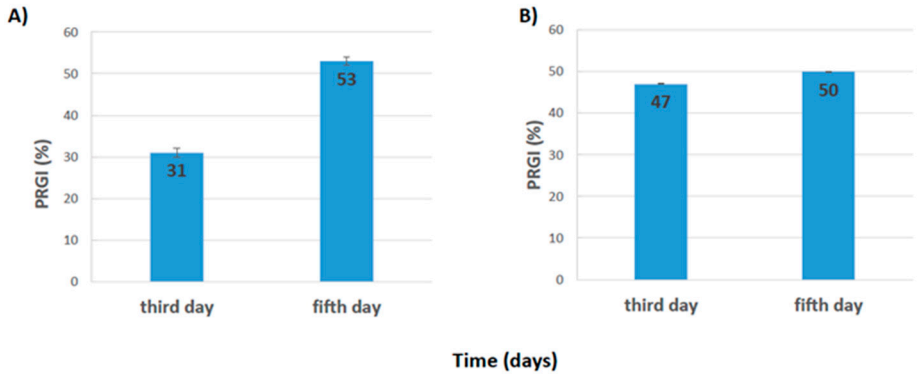

Figure 4. Inhibition percentage growth of pathogen *F. chlamydosporum* (**A**) and *A. brassicicola* (**B**) against *C. virescens* after three and five days of inoculation in dual culture on OA medium, respectively. Mean and standard deviation is expressed in the error bar (n = 5).

3.2.3. Mycoparasitism

Microscopic observations of *F. chlamydosporum* hyphae revealed vacuolization and whorls; no hyphal penetration or cellular lysis was detected. In addition, it was observed that *C. virescens* overgrew the tested pathogen (Figure S3). On the other hand, microscopy observations of *A. brassicicola* hyphae showed vacuolization, whorls, hyphal penetration, and cellular lysis. *C. virescens* overgrew the tested pathogen (Figure S4).

3.3. Testing of Ethyl Acetate Crude Extract from C. virescens

The crude extract showed antagonistic activity against *P. infestans*. The inhibition percent was higher than that of Epoxiconazole at all concentrations, while it was higher than Terbinafine in the range of 1.5–42 ppm. However, at 125 ppm the percentage was slightly lower than Terbinafine. (Figure 5).

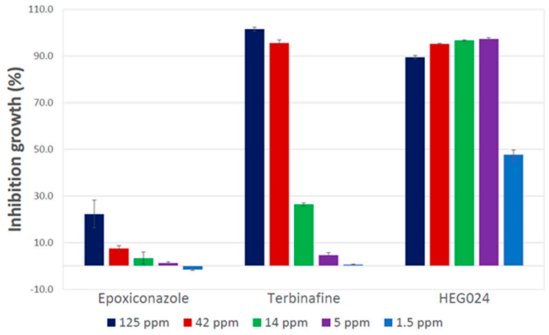

Figure 5. Inhibition percentage of growth of *P. infestans* by the crude extract (HEG024) from the culture broth of *C. virescens* in vitro at different concentrations in ppm, after eight days of pathogen inoculation. Epoxiconazole and Terbinafine were used as positive control. Mean and standard deviation is expressed in the error bar (n = 3).

The antagonistic effect of the crude ethyl acetate extract from the culture broth of *C. virescens* against *B. cinerea* is presented in Figure 6. The crude extract expressed low activity compared with Epoxiconazole and Terbinafine.

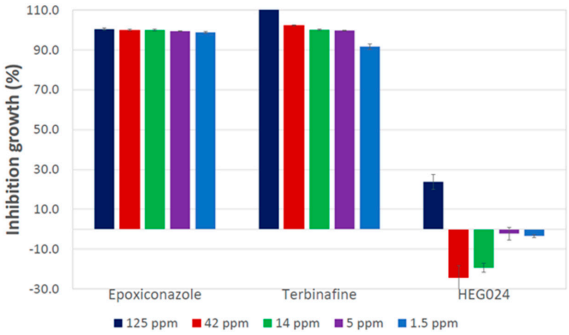

Figure 6. Inhibition percentage of growth of *B. cinerea* by the crude extract (HEG024) from the culture broth of *C. virescens* in vitro at different concentrations in ppm, after eight days of pathogen inoculation. Epoxiconazole and Terbinafine were used as positive control. Mean and standard deviation is expressed in the error bar (n = 3).

Figure 7 shows the antagonistic effect of crude ethyl acetate extract from the culture broth of *C. virescens* against *S. tritici*. The crude showed stronger inhibition at concentrations of 125 and 42 ppm.

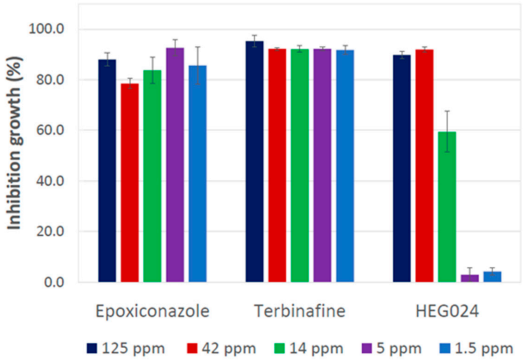

Figure 7. Inhibition percentage of growth of *S. tritici* by the crude extract (HEG024) from the culture broth of *C. virescens* in vitro at different concentrations in ppm, after eight days of pathogen inoculation. Epoxiconazole and Terbinafine were used as positive control. Mean and standard deviation is expressed in the error bar (n = 3).

4. Discussion

In this study, we analyzed the cultural characteristics of *C. virescens* on nine different culture media in order to define nutritional pattern and factors influencing the growth of the strain and select the most suitable medium for its development. Additionally, the antagonistic effect against five pathogens that caused significant damage in different crops was analyzed. Conidia characteristics have been used to distinguish the species in correlation to other factors [28,29,32]. In general, the morphology of conidia in synthetic culture media can be changed according to slight or drastic variations [33,34]. It should also be considered that fungi require carbon nutrients to produce energy and secondary metabolites; therefore, this study was conducted in a detailed manner to determine the best growth conditions [35–37]. To the best of our knowledge, this is the first report of the qualitative characterization of *C. virescens* on nine culture media. Exponential growth is connected to the physiological characteristics of the fungi and the differences between substrates and incubation conditions. The strain showed the highest growth in oat agar media. Similar results were found by Shirouzou et al. [38] for the best growth of fungal strains, such as *Beltraniella botryospora* Shirouzu and Tokum., in artificial culture media with OA.

In addition, *C. virescens* exhibited a faster growth compared with the pathogen *F. chlamydosporum* and inhibited the mycelial growth of *A. brassicicola*. Values of in vitro inhibition of the tested phytopathogens are the result of the high competition for nutrients and space. In addition, the excretion of metabolites with significant fungistatic action over the pathogens was also evidenced. Similar results were found by Santos et al. [39] when evaluated 24 isolates of *Cladobotryum mycophilum* against eight phytopathogens [40,41]. *Thrichoderma* species are the most studied and used for the control of plant disease [41]. Khaledi et al. (2016) studied the biocontrol mechanisms of 11 *Trichoderma* spp. isolates against *M. phaseolina*, and the results revealed that all isolates inhibited the mycelial growth of the pathogen from 20.2 to 58.7% in the dual culture tests [42]. The PRGI reported in this study are in a range of the values reported by these authors.

Several authors have described *Cladobotryum* spp. as mycoparasitic on many pathogens [43]. This genus is known to cause hyphal lysis by producing a wide variety of secondary metabolites with marked antifungal as well as antibacterial activities and repressive effects on cancer cells [44–46]. *C. virescens* showed antifungal effects against *F. chlamydosporum*. The strain exhibited strong competition for the substrate and high mycoparasitism (mainly vacuolization and enrollment). Different authors refer to these kinds of hyphal interaction for *Trichoderma*, considering them as a potential for use as biocontrol agent [47].

Microscopic observations of *A. brassicicola* hyphae showed vacuolization, enrollment, hyphal penetration and cellular lysis. *C. virescens* was able to cover the pathogen and inhibit the development and growth of *A. brassicicola*. Different types of interactions are important characteristics when selecting a biocontrol agent. The greater the probability that fungi will manifest several modes of action, the more efficient and long-lasting the control on the pathogen will be [48,49]. We provided evidence of three modes of action present in *C. virescens* when it is confronted by phytopathogenic fungi. These results are important to reduce risks of pathogen resistance. The competition with pathogens for space and nutrients and the production of antifungal compounds are important mechanisms in biocontrol activity [50]; therefore, this strain should be considered as a potential biocontrol agent to be used for fungal disease control.

Taking into account the results obtained by Ortiz et al. [17] when evaluating the antagonistic effect of the ethyl acetate crude of *C. virescens* against *Corynespora cassiicola* by the qualitative bioautographic TLC method, it was decided to evaluate the ethyl acetate crude against *P. infestans*, *S. tritici*, and *B. cinerea* through the microtiter plate method at five different concentrations. The crude extract exhibited strong growth inhibitory activity against the oomycete *P. infestans*. Additionally, the extract inhibited the growth at concentrations as low as 1.5 ppm, i.e., it was more active than the positive controls

Terbinafine and Epoxiconazole. Most likely, this is caused by the presence of secondary metabolites produced by the strain. In contrast, modest inhibition was shown against the ascomycete *S. tritici*, and no inhibition effect was observed against *B. cinerea.* The antifungal effect is determined by the presence of metabolites produced by *C. virescens* under the fermentation conditions used, which are not able to inhibit the growth of *B. cinerea* on this in vitro assay. In the literature, various secondary metabolites from the genus *Cladobotryum* have been reported, mainly polyketides and α-pyrone derivatives, with antifungal effects against saprophytic (wood-rotting) fungi, such as *Ganoderma lucidum* (Curtis) P. Karst [51].

5. Conclusions

This work shows evidence of the differences in terms of the cultural characteristics of *C. virescens* in nine different culture media. Furthermore, *C. virescens* showed an inhibitory effect on the mycelial growth of the selected phytopathogens, *F. chlamydosporum* and *A. brassicicola*. The crude extract showed inhibition of the growth of *P. infestans* at low concentrations and is a promising candidate as a biocontrol agent against diseases caused by *P. infestans*. Investigations on the isolation and purification of the active constituents present in ethyl acetate crude extract are in progress in our laboratory.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy13020389/s1, Figure S1: Confrontation of the colonies of *Cladobotryum virescens* and *Fusarium chlamydosporum*; Figure S2: Confrontation of the colonies of *Cladobotryum virescens* and *Alternaria brassicicola.*

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References

1. Yan, K.; Han, G.; Ren, C.; Zhao, S.; Wu, X.; Bian, T. *Fusarium solani* infection depressed photosystem performance by inducing foliage wilting in apple seedlings. *Front. Plant Sci.* **2018**, *9*, 479. [CrossRef] [PubMed]
2. Ibrahim, N.F.; Mohd, M.H.; Izham, N.M.; Zakaria, L. Pathogenicity of *Fusarium semitectum* and *Fusarium chlamydosporum* associated with pineapple fusariosis. *Malays. J. Microbiol.* **2016**, *12*, 164–170. [CrossRef]
3. Macioszek, V.K.; Wielanek, M.; Morkunas, I.; Ciereszko, I.; Kononowicz, A.K. Leaf position-dependent effect of *Alternaria brassicicola* development on host cell death, photosynthesis and secondary metabolites in *Brassica juncea*. *Physiol. Plant* **2020**, *168*, 601–606. [CrossRef]
4. Deep, S.; Sharma, P.; Behera, N.; Chowdappa, P. Diversity in India of *Alternaria brassicicola* (Schwein) Wiltsshire, causing black leaf spot diseases in Cauliflower. *Plant Pathol. J.* **2014**, *13*, 232–245. [CrossRef]
5. Tomas-Grau, R.H.; Hael-Conrad, V.; Requena-Serra, F.J.; Perato, S.M.; Caro, M.d.P.; Salazar, S.M.; Díaz-Ricci, J.C. Biological control of strawberry grey mold disease caused by *Botrytis cinerea* mediated by *Colletotrichum acutatum* extracts. *BioControl* **2020**, *65*, 461–473. [CrossRef]
6. Petrasch, S.; Knapp, S.J.; Van Kan, J.A.L.; Blanco-Ulate, B. Grey mould of strawberry, a devastating disease caused by the ubiquitous necrotrophic fungal pathogen *Botrytis cinerea*. *Mol. Plant Path.* **2019**, *20*, 877–892. [PubMed]
7. Collins, F.; Bancal, P.; Spink, J.; Kock, P.; Smith, J.; Paveley, N.D.; Bancal, M.-O.; Foulkes, M.J. Wheat lines exhibiting variation in tolerance of Septoria tritici blotch differentiated by grain source limitation. *Field Crops Res.* **2018**, *217*, 1–10. [CrossRef]
8. Brennan, C.J.; Benbow, H.R.; Mullins, E.; Doohan, F.M. A review of the known unknowns in the early stages of Septoria tritici blotch disease of wheat. *Plant Pathol.* **2019**, *68*, 1427–1438. [CrossRef]

9. Morales, J.G.; Gaviria, A.E.; Gilchrist, E. Allelic variation and selection in effector genes of *Phytophthora infestans* (Mont.) de Bary. *Pathogens* **2020**, *9*, 551. [CrossRef]
10. Seifu, Y.W. Reducing severity of late blight (*Phytophthora infestans*) and improving potato (*Solanum tuberosum* L.) tuber yield with pre-harvest application of calcium nutrients. *Agronomy* **2017**, *7*, 69. [CrossRef]
11. Abbey, J.A.; Percival, D.; Lord, A.; Asiedu, S.K.; Prithiviraj, B.; Schilder, A. Biofungicides as alternative to synthetic fungicide control of grey mould (*Botrytis cinerea*)—Prospects and challenges. *Biocontrol Sci. Technol.* **2018**, *29*, 207–228. [CrossRef]
12. Moura, R.D.; de Castro, L.A.M.; Culik, M.P.; Fernandes, A.A.R.; Fernandes, P.M.B.; Ventura, J.A. Culture medium for improved production of conidia for identification and systematic studies of *Fusarium* pathogens. *J. Microbiol. Methods* **2020**, *173*, 105915. [CrossRef] [PubMed]
13. Bills, G.F.; Platas, G.; Overy, J.; Fillola, A.; Jiménez, M.R.; Collado, J.; Vicente, F.; Martín, J.; Gonzalez, A.; Burzimmermann, J.; et al. Enhancement of antibiotic and secondary metabolite detection from filamentous fungi by growth on nutritional arrays. *J. Appl. Microbiol.* **2008**, *104*, 1644–1658. [CrossRef]
14. Uzcátegui, N.M.C.; Serrano, J.A.; Panizo, M.M. Condiciones de cultivo que fomentan la producción de sustancias antimicrobianas en actinomicetos patógenos y del suelo. *Rev. Soc. Venez. Microbiol.* **2013**, *33*, 134–139.
15. Back, C.G.; Lee, C.H.Y.; Seo, G.S.; Jung, H.Y. Characterization of species of *Cladobotryum* which cause cobweb disease in endible mushrooms grown in Korea. *Mycobiology* **2012**, *40*, 189–194. [CrossRef] [PubMed]
16. Breinholt, J.; Jensen, H.C.; Kajaer, A.; Olsen, C.E.; Rassing, B.R.; Rosendahl, C.N.; Sotofte, I. Cladobotryal: A fungal metabolite with a novel ring system. *Acta Chem. Scand.* **1998**, *52*, 631–63417. [CrossRef] [PubMed]
17. Ortiz, Y.; Ramos, B.; Álvarez, M.E.; Valdés, R.; Lorenzo, Y.; Plana, L.; Marrero, I.; García, D.; Aguado, Y.; Ruenes, M.E.; et al. Actividad fungicida de cepas del género *Cladobotryum* sobre *Corynespora cassiicola*. *Agrotec. Cuba* **2016**, *40*, 71–83.
18. Ramos, B. Actividad fungicida de cepas de *Cladobotryum* spp., para el control de hongos patógenos presentes en modalidades productivas de la agricultura urbana. *Agrotec. Cuba* **2018**, *42*, 98–99.
19. Põldmaa, K. Tropical species of *Cladobotryum* and *Hypomyces* producing red pigments. *Stud. Mycol.* **2011**, *68*, 1–34. [CrossRef] [PubMed]
20. Index Fumgorum Fungal Names Search. Available online: http//:www.indexfungorum.org/names/Names.asp2021 (accessed on 24 September 2021).
21. Centraalbureau Voor Schimmelcutures (CBS). *List of Cultures*; Fungal Biodiversity Center: Utrecht, The Netherlands, 2001; p. 687.
22. Stammler, G.; Speakman, J. Microtiter Method to Test the Sensitivity of *Botrytis cinerea* to Boscalid. *J. Phytopathol.* **2006**, *154*, 508–510. [CrossRef]
23. Stammler, G. *Phytophthora infestans* Microtiter Method with Sporangia. Available online: http://www.frac.info/docs/default-source/monitoring-methods/approved-methods/phytin-microtiter-method-sporangia-basf-2006-v1.pdf?sfvrsn=4. (accessed on 21 April 2021).
24. Stammler, G.; Semar, M. Sensitivity of *Mycosphaerella graminicola* (anamorph: *Septoria tritici*) to DMI fungicides across Europe and impact on field performance. *Bull. OEPP* **2011**, *41*, 149–155. [CrossRef]
25. Crous, P.W.; Verkley, G.J.M.; Groenewald, J.Z.; Samsom, R.A. *Fungal Biodiversity*; CBS Laboratory Manual Series; CBS-KNAW Fungal Biodiversity Centre: Utrecht, The Netherlands, 2009; Volume 1, p. 269.
26. Frey, D.; Oldfield, R.J.; Bridger, R.C. *A Color Atlas of Pathogenic Fungi*; Barry, G., Ed.; Wolfe Medical Publication Ltd London: London, UK, 1979; p. 168. Available online: https://catalogue.nla.gov.au/Record/2917010 (accessed on 31 May 2019).
27. Bernal, A.; Martínez, B.; Castellanos, M.D. Caracterización Cultural de Tres Aislamientos de *Cladosporium fulvum* Cooke. *Cent. Agríc.* **2005**, *32*, 93–94. Available online: https://scholar.google.com/scholar?hl=es&as_sdt=0%2C5&q=Bernal%2C+A.%3B+Mart%C3%ADnez%2C+B.%3B+Castellanos%2C+M.D.+Caracterizaci%C3%B3n+cultural+de+tres+aislamientos+de+Cladosporium+fulvum+Cooke.+Centro+421+Agr%C3%ADcola+2005%2C+32%2C+93%E2%80%9394.&btnG=. (accessed on 31 May 2019).
28. Martínez, B.; Reyes, Y.; Infante, D.; González, E.; Baños, H.; Cruz, A. Selección de aislamientos de *Trichoderma* spp. candidatos a biofungicidas para el control de *Rhizoctonia* sp. en arroz. *Rev. Prot. Veg.* **2008**, *23*, 118–125.
29. Baiyee, B.; Ito, S.; Sunpapao, A. *Trichoderma asperellum* T1 mediated antifungal activity and induced defense response against leaf spot fungi in lettuce (*Lactuca sativa* L.). *Physiol. Mol. Plant Pathol.* **2019**, *106*, 96–101. [CrossRef]
30. Reyes, Y.; Martínez, B.; Infante, D. Evaluación de la actividad antagónica de trece aislamientos de *Trichoderma* spp. sobre *Rhizoctonia* sp. *Rev. Prot. Veg.* **2008**, *23*, 112–117.
31. Ren, G.C.; Wanasinghe, D.N.; Monkai, J.; Mortimer, P.E.; Hyde, K.D.; Xu, J.C.; Pang, A.; Gui, H. Novel saprobic *Hermatomyces* species (*Hermatomycetaceae*, *Pleosporales*) from China (Yunnan Province) and Thailand. *MycoKeys* **2021**, *82*, 57–79. [CrossRef]
32. Sun, J.Z.; Liu, X.Z.; Jeewon, R.; Li, Y.L.; Lin, C.G.; Tian, Q.; Zhao, Q.; Xiao, X.P.; Hyde, K.D.; Nilthong, S. Fifteen fungicolous Ascomycetes on edible and medicinal mushrooms in China and Thailand. *Asian J. Mycol.* **2019**, *2*, 129–169. [CrossRef]
33. Konopická, J.; Bohatá, A.; Zemek, R.; Čurn, V. The effects of natural substrates and artificial media on the production of conidiospores and blastospores of the entomopathogenic fungus *Isaria fumosorosea*, strain CCM 8367. *Microb. Nematode Control. Invertebr. Pests IOBC-WPRS Bull.* **2017**, *129*, 58–64.
34. Gandarilla-Pacheco, F.L.; Arévalo-Niño, K.; Galán-Wong, L.J.; Sandoval-Coronado, C.F.; Quintero-Zapata, I. Evaluation of conidia production and mycelial growth in solid culture media from native strains of entomopathogenic fungi isolated from citrus-growing areas of México. *Afr. J. Biotech.* **2012**, *11*, 14453–14460. [CrossRef]

35. Chithra, J.; Kulkarni, S.; Gururaj Sunkad, S.B.; Amresh, Y.S.; Shekhar, P. Effect of different media, temperature, pH and illumination on the growth of *Fusarium oxysporum* f.sp. *zingiberi* causing wet rot of ginger. *J. Pharmacogn. Phytochem.* **2020**, *9*, 786–790.
36. Assefa, A.; Abate, D. Evaluation of nutritional requirements of medicinal fungus, *Pyrofomes demidoffii* under submerged fermentation. *Biocatal. Agric. Biotech.* **2020**, *28*, 101765. [CrossRef]
37. Ulrich, S.; Schäfer, C. Toxin production by *Stachybotrys chartarum* genotype S on different culture media. *J. Fungi* **2020**, *6*, 159. [CrossRef]
38. Shirouzou, T.; Hirose, D.; Tokumasu, S.; To-Anum, C.; Maekawa, N. Host affinity and phylogenetic position of new anamorphic fungus *Beltraniella botryospora* from living and fallen leaves of evergreen oaks. *Fungal Divers.* **2010**, *43*, 85–92. [CrossRef]
39. Santos, M.; Diánez, F.; Moreno-Gavíra, A.; Sánchez-Montesinos, B.; Gea, F.J. *Cladobotryum mycophilum* as Potential Biocontrol Agent. *Agronomy* **2019**, *9*, 891. [CrossRef]
40. Sood, M.; Kapoor, D.; Kumar, V.; Sheteiwy, M.S.; Ramakrishnan, M.; Landi, M.; Araniti, F.; Sharma, A. Trichoderma: The "Secrets" of a Multitalented Biocontrol Agent. *Plants* **2020**, *9*, 762. [CrossRef] [PubMed]
41. Khan, M.R.; Mohiddin, F.A. Trichoderma: Its multifarious utility in crop improvement. In *Crop Improvement trough Microbial Biotechnology. New and Future Developments in Microbial Biotechnology and Bioengineering*; Aligarh Muslim University: Aligarh, India; Srinagar, India, 2018; Chapter 13; pp. 263–291. [CrossRef]
42. Khaledi, N.; Taheri, P. Biocontrol mechanisms of *Trichoderma harziamam* against soybean charcoal rot caused by *Macrophomina phaseolina*. *J. Plant Prot. Res.* **2016**, *56*, 21–31. [CrossRef]
43. Fadhilah, N.; Kheng, Y.; Jiat, H.; Keng, Y.; Joo, K. Evaluation on the cultural characteristics and antagonistic activities of *Cladobotryum semicirculare* against *Ganoderma boninense* in vitro. *J. Oil Palm Res.* **2015**, *27*, 326–338.
44. Zhou, T.; Katsuragawa, M.; Xing, T.; Fukaya, K.; Okuda, T.; Tokiwa, T.; Tashiro, E.; Imoto, M.; Oku, N.; Urabe, D.; et al. Cyclopeptides from the Mushroom Pathogen Fungus *Cladobotryum varium*. *J. Nat. Prod.* **2021**, *84*, 327–338. [CrossRef]
45. Sakemi, S.; Bordner, J.; De Costa, D.L.; Dekker, K.A.; Hirai, H.; Inagaki, T.; Kim, Y.J.; Kojima, N.; Sims, C.J.; Siugie, Y.; et al. CJ-15,696 and its analogs, new furopyridine antibiotics from the fungus *Cladobotryum varium*: Fermentation, isolation, structural elucidation, biotransformation and antibacterial activities. *J. Antibiot.* **2002**, *55*, 6–18. [CrossRef]
46. Mitova, M.I.; Lang, G.; Blunt, J.W.; Cummings, N.J.; Cole, A.L.; Robinson, W.T.; Munro, M.H. Cladobotric acids A-F: New cytotoxic polyketides from a New Zealand *Cladobotryum* sp. *J. Org. Chem.* **2006**, *71*, 492–497. [CrossRef]
47. El Komy, M.H.; Saleh, A.A.; Eranthodi, A.; Molan, Y.Y. Characterization of Novel *Trichoderma asperellum* Isolates to Select Effective Biocontrol Agents Against Tomato Fusarium Wilt. *Plant Pathol. J.* **2015**, *31*, 50–60. [CrossRef]
48. Infante, D.; Martínez, B.; González, N.; Reyes, Y. Mecanismos de acción de *Trichoderma* frente a hongos fitopatógenos. *Rev. Prot. Veg.* **2009**, *24*, 14–21.
49. Singh, S.; Bhagat, S.; Singh, V.; Hashmi, S.; Bhagel, M. Evaluation of biocontrol agents against *Phytophthora drechsleri* f.sp. *cajani* (Leaf Blight) in Pigeonpea. *Int. J. Curr. Microbiol. Appl. Sci.* **2020**, *9*, 3536–3543. [CrossRef]
50. Savazzini, F.; Oliveira, C.M.; Pertot, I. Impact of the biocontrol agent *Trichoderma atroviride* SC1 on soil microbial communities of a vineyard in northern Italy. *Soil Biol. Biochem.* **2009**, *41*, 1457–1465. [CrossRef]
51. Tezuka, Y.; Huang, Q.; Kikuchi, T.; Nishi, A.; Tubaki, K. Studies on the metabolites of mycoparasitic fungi. l. Metabolites of *Cladobotryum varium*. *Chem. Pharm. Bull.* **1994**, *42*, 2612–2617. [CrossRef]