

Characterization and control of the causal agent of leaf spot in strawberry (*Fragaria ananassa*) under greenhouse conditions

Caracterización y control del agente causal de la mancha foliar en fresa (*Fragaria ananassa*) en invernadero

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Abstract

Strawberry (*Fragaria ananassa*) plants showing symptoms of leaf spot were found in the producing areas of Manchay Bajo, Pachacamac District, Lima, Peru. The responsible pathogen was isolated and observed under a microscope to examine its morphological characteristics and the size of its structures, resulting in its identification as the fungus *Corynespora cassiicola*. Healthy strawberry plants that had been inoculated with *C. cassiicola* in a greenhouse developed lesions after 3 days and exhibited symptoms consisting of dark brown spots that had light brown centers and were surrounded by yellow halos, which coalesced and subsequently caused necrosis of the whole leaf. *In vitro* tests demonstrated that the fungus *Trichoderma harzianum* strain T-22 showed antagonistic activity against *C. cassiicola*, causing 41.8% mycelial growth inhibition, and the fungicides fosetyl-aluminum (Aliette[®]) and thiabendazole (Mertect^{®a}) inhibited mycelial growth by 100.00% and 96.32%, respectively. However, *in vivo*, 0.75 g/L of the fungicide azoxystrobin (Stronsil[®]) gave the best control of *C. cassiicola* and the lowest area under the disease progress curve (AUDPC), followed by 1.5 mL/L of thiabendazole (Mertect) under greenhouse conditions. The isolate was found to be pathogenic, and its morphological identification was confirmed.

Key words: Leaf spot, *Corynespora cassiicola*, Strawberry, *Fragaria ananassa*

Resumen

Plantas de fresa (*Fragaria ananassa*) mostrando síntomas de mancha foliar fueron encontradas en las áreas productoras de Manchay bajo, Distrito de Pachacamac, Lima. Después del aislamiento, observaciones bajo el microscopio de sus características morfológicas y tamaño de sus estructuras, permitieron identificar al patógeno como el hongo *Corynespora cassiicola*. Las plantas de fresa sanas inoculadas en invernadero, produjeron lesiones después de tres días. Los síntomas consistieron en manchas de color marrón oscuro con el centro de marrón claro, rodeados por un halo amarillo, que coalescen y posteriormente causaron la necrosis de toda la hoja. El hongo *Trichoderma harzianum* strain T-22 mostró una actividad antagonista contra *C. cassiicola* causando una inhibición de micelio en un 41.8 %. En los ensayos «In vitro», los fungicidas Fosetil aluminio (Aliette) y Tiabendazol (Mertect) inhibieron el crecimiento micelial de *C. cassiicola* en un 100 y 96.32 %, respectivamente. En invernadero, el fungicida Azoxystrobin (Stronsil) a una concentración de 0.75 g/L tuvo el mejor control sobre *C. cassiicola* y el menor AUDPC (área bajo la curva de progreso de enfermedad), seguido de Tiabendazol (Mertect) a una concentración de 1.5 ml/L.

Palabras clave: Mancha foliar, *Corynespora cassiicola*, Cultivo de Fresa (*Fragaria ananassa*).

Introduction

Strawberry (*Fragaria ananassa*) plants are grown in Pachacamac District, Peru, due to the favorable environmental conditions in this region. However, plants that grow here are also prone to a disease that is characterized by the presence of necrotic spots on the leaves, with brownish tones being acquired quickly under favorable conditions. These leaf spots appear as small,

circular, dark brown areas that have a light brown center and are surrounded by a purple or reddish halo, and infected plants also develop black lesions on the stems that are elongated and sunken in shape and surrounded by a reddish halo. The disease can affect any phenological stage of the plant and can eventually destroy the crop if a suitable control strategy is not carried out in time.

During the summer of 2015 in the area of Manchay Bajo,

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Pachacamac District, it was found that strawberry plants were dying from some unknown agent that caused purple-edged foliar spots. Therefore, the aims of this study were to identify the causal agent of leaf spot in strawberry and to determine the best biological or chemical control agent against this disease under greenhouse conditions.

Materials and methods

They were collected from random portions of leaves and petioles of five strawberry plants (cultivar 'Camino Real') with symptoms of leaf spot from a cultivation area located in Manchay low, Pachacamac District. Samples of leaves and petioles were placed in polyethylene bags duly identified with place and date of sampling, which were immediately transported to the laboratory. The laboratory and greenhouse phase were conducted from January to April 2016 and October 2016 to January 2017 at the Department of Phytopathology, National Agrarian University La Molina of Lima, Peru.

Isolation and identification of the pathogen

The leaves and petioles of diseased strawberry plants were washed with a continuous stream of water followed by three washes with sterile distilled water. The samples were placed inside an aseptic chamber and immersed in a 0.5% sodium hypochlorite solution for 3 minutes. The disinfected materials were then cut into small pieces taking into account the margins of advancement of the lesions, placed on sterile filter paper, and left to dry until they were dehydrated. The dehydrated materials were placed on plates containing a previously prepared potato dextrose agar (PDA) medium, which were sealed with parafilm and incubated at 28 °C–30 °C for 15 days. Once mycelium development of the causative agent of the disease had occurred, peels were made until a pure culture was obtained.

Identification of the pathogen was carried out in three stages. First, the pathogen was identified to the genus level using the key of [Barnett and Hunter \(1972\)](#). Second, it was identified to species level based on the morphological characteristics of its fruiting structures using the key of [Ellis \(1971\)](#). To determine the dimensions of spore-bearing structures, the length, diameter, and number of proliferations were measured in 50 conidiophores, and the length, diameter, truncated base diameter, and number of pseudosepta were measured in 50 conidia. Third, the identity of the pathogen was confirmed by biomolecular DNA analysis at the Institute of Molecular Biotechnology of the National Agrarian University La Molina according to the protocol developed by [Aamir, Sutar, Singh, and Baghela \(2015\)](#) with some modifications.

Molecular identification

The species-specific molecular identification of the strain was based on an analysis of the internal transcribed spacer (ITS) 1-5.8s-ITS2 region of the rDNA ([White, Bruns,](#)

[Lee, and Taylor 1990](#)). For the extraction of DNA, the mycelium of a fungus that had been grown in a liquid culture based on potato dextrose for 5 days at 28 °C was collected and the mycelial mass was recovered by filtration through gauze. The DNA was then obtained following the methodology of [Aamir et al. \(2015\)](#).

The DNA integrity was verified by electrophoresis in 1% (w/v) agarose gel. The internal regions ITS1 and ITS2 between ribosomal genes (rDNA) 18S-5.8S and 28S ([White et al., 1990](#)) were amplified by polymerase chain reaction (PCR) with the primers ITS1 (5'-CCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTC-CGCTTATTGATATGC-3'). The reaction mixture consisted of PCR buffer (1X), the ITS1 and ITS4 primers (1 mM), MgCl₂ (3 mM), deoxyribonucleotide triphosphate (dNTP; 0.2 mM), Taq DNA polymerase (2.5 units), and DNA (100 ng) to give a final volume of 50 mL. The PCR amplification was carried out in a thermal cycler (TECHNE®-312, Minnesota, USA) under the following conditions: initial denaturation at 95 °C for 2 min, followed by 30 cycles of denaturation at 94 °C for 1 min, alignment at 54 °C for 30 s, and extension at 72 °C for 1 min, with a final extension at 72 °C for 5 min ([Aamir et al., 2015](#)).

The amplification products were visualized by electrophoresis using 2% (w/v) agarose gel (SIGMA®), and the amplified PCR products were sequenced in the Laboratory Psomagen Inc: Humanizing Phenomics 120 (www.macrogenusa.com). The consensus sequence was constructed using BioEdit Sequence Alignment Editor version 7.2.5 (<http://www.mbio.ncsu.edu/BioEdit/bioedit.html>). Then, this sequence was compared using the GenBank database of the [National Center for Biotechnology Information](#) (<http://blast.ncbi.nlm.gov>) through the BLAST program, and these are presented as percent identity in this study. Then, the sequences were aligned using MEGA program version 7.0 (<https://www.megasoftware.net/>) with the help of the MrBayes version 3.2.6 program (<http://mrbayes.sourceforge.net/>) and they were analyzed by Bayesian inference after obtaining information on distribution and arrangement using a phylogenetic tree, which was built using FigTree version 1.4.3 (<https://beast.community/figtree>).

Pathogenicity test and inoculation of strawberry plants for the greenhouse experiment

A pathogenicity test and inoculations were carried out on 5-week-old strawberry plants of the 'Camino Real' cultivar under greenhouse conditions. For the pathogenicity test, five plants were inoculated and five were left as uninoculated controls, while for the greenhouse experiment, 120 plants were inoculated to test the effects of nine treatments compared with an untreated control. In all cases, the plants were inoculated by sprinkling a spore suspension that contained 1.8×10^4 conidia/mL in a volume of 8–10 cc/plant over the entire leaf area of each plant. For all uninoculated control plants, only water was sprayed on the leaves. Subsequently, each of the plants was covered with

a polypropylene bag containing holes for 2 days to provide the necessary humidity for developing disease symptoms.

Reisolation of the pathogen

Samples were taken from inoculated plants that showed symptoms of leaf spot. These samples were obtained from the border of the necrotic and healthy tissue on leaves with lesions and petioles with symptoms of canker. The samples were disinfected, dehydrated, and planted in APG medium, following which mycelium purification was performed and the developed colony was placed under alternating periods of 12 hours of artificial light and 12 hours of darkness to induce sporulation.

In vitro evaluation of the effectiveness of biocontrol agents

The biocontrol agents used included *Bacillus subtilis* (5 g/L), *Pseudomonas fluorescens* (2.5 g/L), and *Trichoderma harzianum* strain T-22 (5 g/L). The effectiveness of biocontrol agents was examined using the dual sowing method: bacteria *B. subtilis* and *P. fluorescens* as well as fungus *T. harzianum* strain T-22. Each biocontrol agent was sown on four plates facing the isolated pathogen, using agar, potato, sugar, peptone as the culture medium for the bacteria and PDA for the fungus. All of the confrontation plates were sealed with parafilm and incubated at 28 °C–30 °C.

During sowing for the biocontrol bacteria and biocontrol fungus, pathogen colonies were observed; daily measurements of mycelium were performed and compared with the data obtained for controls. The effectiveness of each biocontrol agent was then determined by calculating the percentage of mycelial inhibition (PI) using the following equation of Pandey et al. (Jaramillo, Barrezueta, Luna, & Castillo, 2017):

$$PI = ((dc - dt) / dt) \times 100$$

where dc is the average diameter of the control colony and dt is the average diameter of the treated colony. The average growth rate was determined by dividing the radial mycelial growth by the number of days elapsed. The significance of any differences in growth between treatments was analyzed using analysis of variance (ANOVA) and Tukey's multiple comparison test with a significance level of $\alpha = 0.05$.

In vitro evaluation of the effectiveness of fungicides

The active ingredients of fungicides used were azoxystrobin (0.75 g/L), chlorothalonil (1.5 mL/L), difenoconazole (0.15 mL/L), thiabendazole (1.5 mL/L), and fosetyl-aluminum (3 g/L). The efficacy of fungicides was evaluated using the Poisoned Food Test. Four plates containing PDA medium were used with the corresponding fungicide and isolated pathogen undergoing active growth and for the controls, whereas 4 plates containing PDA medium were used without fungicides but with the isolated pathogen. All plates were sealed with parafilm and incubated at 28 °C–30

°C. The average rate of growth was then determined and the efficacy of each fungicide was assessed by calculating the PI. The significance of any differences in growth between treatments were analyzed using ANOVA and Tukey's multiple comparison test with a significance level of $\alpha = 0.05$.

In vivo evaluation of the effectiveness of biocontrol agents and fungicides

Strawberry seedlings were moved from a field in the lower Manchay area to the greenhouse in October 2016 when they were 3 weeks old and were planted in a mixture of compost, sand, and farmland soil at a ratio of 1:1:1. A total of 130 seedlings were transplanted, 10 of which were used for the pathogenicity test (five for inoculation and five for the uninoculated control). The remaining 120 plants were used to test the effectiveness of the biocontrol agents and fungicides *in vivo*.

A completely randomized experimental design was used, with a total of nine treatments plus a control, four replicates per treatment, and three subsamples per replicate ($10 \times 4 \times 3 = 120$ plants). The same biocontrol agents and fungicides were tested as in the *in vitro* tests outlined above.

The biocontrol agents and fungicides were applied every 7 days in the afternoon when the daytime temperatures were lower. Before each application, the progression of the disease was evaluated using the following scale: 0% = healthy plant, 0%–5% = appearance of small spots, 5%–25% = spots covering up to 25% of the plant; 25%–50% = spots covering up to 50% of the plant; 50%–75% = spots covering up to 75% of the plant, and 75%–100% = spots affecting the entire plant. At the start of the experiment, all plants had 0% infection and the percentages were recorded as foliar disease progressed. The area under the disease progress curve (AUDPC) was then calculated for each treatment using the formula described by Pedroza-Sandoval and Samaniego (2009). The significance of differences between treatments was assessed using ANOVA followed by Tukey's multiple comparison test in the SAS 8.0 statistical program with a significance level of $\alpha = 0.05$.

Results

Isolation and identification of the pathogen

After 15 days of incubation, the development of a mycelium was observed on each tissue sample exhibiting leaf spot symptoms. Each mycelium produced solitary, monopodial conidiophores bearing conidia that were solitary or in chains, which together formed a fluffy layer that covered the entire leaf spot area (Figure 1). The colony had a large cotton-like mycelium that was light green in color at 4 days after sowing in PDA medium and developed a cottony, dark brown mycelium at 13 days after sowing (Figure 2).

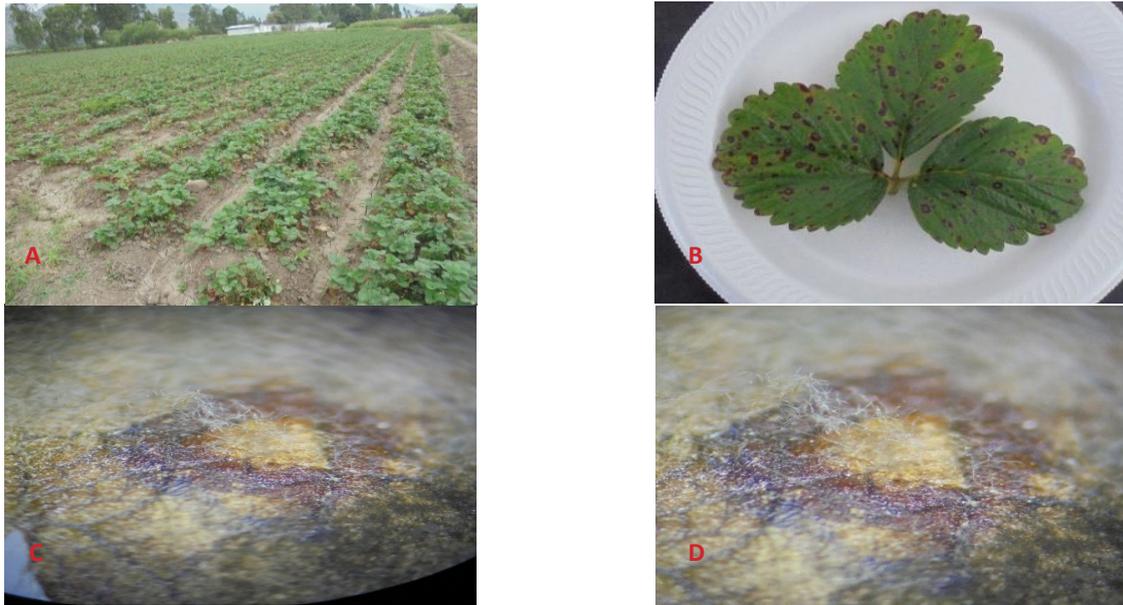


Figure 1. Leaf spot in strawberry (*Fragaria ananassa*) plants. A) Strawberry field infected with leaf spot. B) Leaves exhibiting symptoms of leaf spot. C) Symptoms of leaf spot after 15 days of incubation in a humid chamber. D) Pathogen structures developed on an area of leaf showing symptoms of leaf spot.

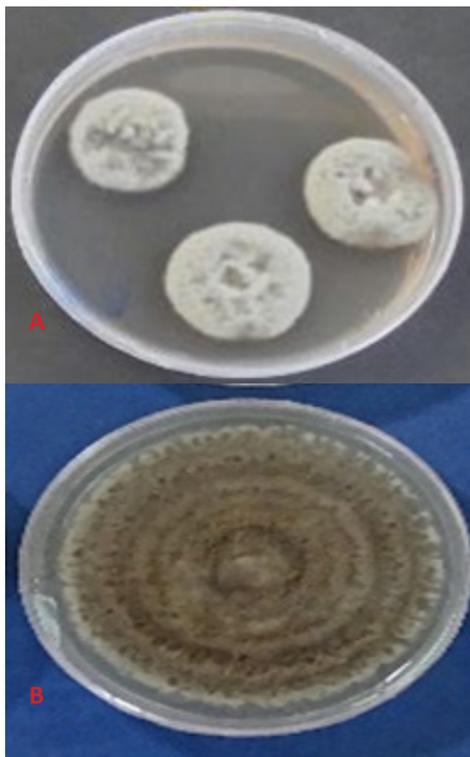


Figure 2. Leaf samples sown in potato dextrose agar (PDA) medium. A) Fungal mycelia isolated 4 days after sowing. B) Pure culture in PDA medium 13 days after sowing.



Figure 3. Fruiting structures of *C. cassiicola* observed under a microscope: A) Conidiophore and conidia in formation. B) Conidia with transverse septa.

Observation of the pathogen under a compound microscope showed the presence of a mycelium, conidiophores, and conidia with dark pigmentation. Each cylindrical conidiophore was simple, short or long, and bore exogenous, straight or curved conidia that were ellipsoidal or obclavate, fragmospore type, solitary with a thick wall, and had a rounded apex without an apical

appendix. The conidia emerged from an apical pore on the conidiophore either individually or in short chains and developed acropetally. Based on these characteristics and the key of [Barnett and Hunter \(1972\)](#), the fungus was identified as belonging to the genus *Corynespora*. Based on these dimensions ([Table 1](#)), the macroscopic and microscopic characteristics described above, and the

Table 1. Dimensions of the conidiophore and conidia of the isolated *Corynespora* sp. obtained from leaf spots on strawberry (*Fragaria ananassa*) leaves.

Value	Conidiophore			Conidia			
	Length (μ)	Diameter (μ)	No. proliferations	Length (μ)	Diameter (μ)	Diameter (μ) Truncate base	No. pseudosepta
Minimum	111	5	3	41	9	4	4
Maximum	363	11	9	116	12	5	8
Average	164	6	5	59	10	5	5
Published range, (Ellis, 1971)	110–850	4–11	2–9	40–20	9–22	4–8	4–20

Table 2. Percentage identity of the isolated strain with sequences of *Corynespora cassiicola* accessions from different parts of the world.

Code of strain	Morphological identification	Reported country	Molecular identification		
			Homology	Identity (%)	Accession number
AEE (Peru)	<i>C. cassiicola</i>	Argentina	<i>C. cassiicola</i>	100	KX786348
		Florida (USA)	<i>C. cassiicola</i>	100	KU593529
		India	<i>C. cassiicola</i>	100	KM458795
		China	<i>C. cassiicola</i>	100	KT002182
		Mexico	<i>C. cassiicola</i>	100	GU461298

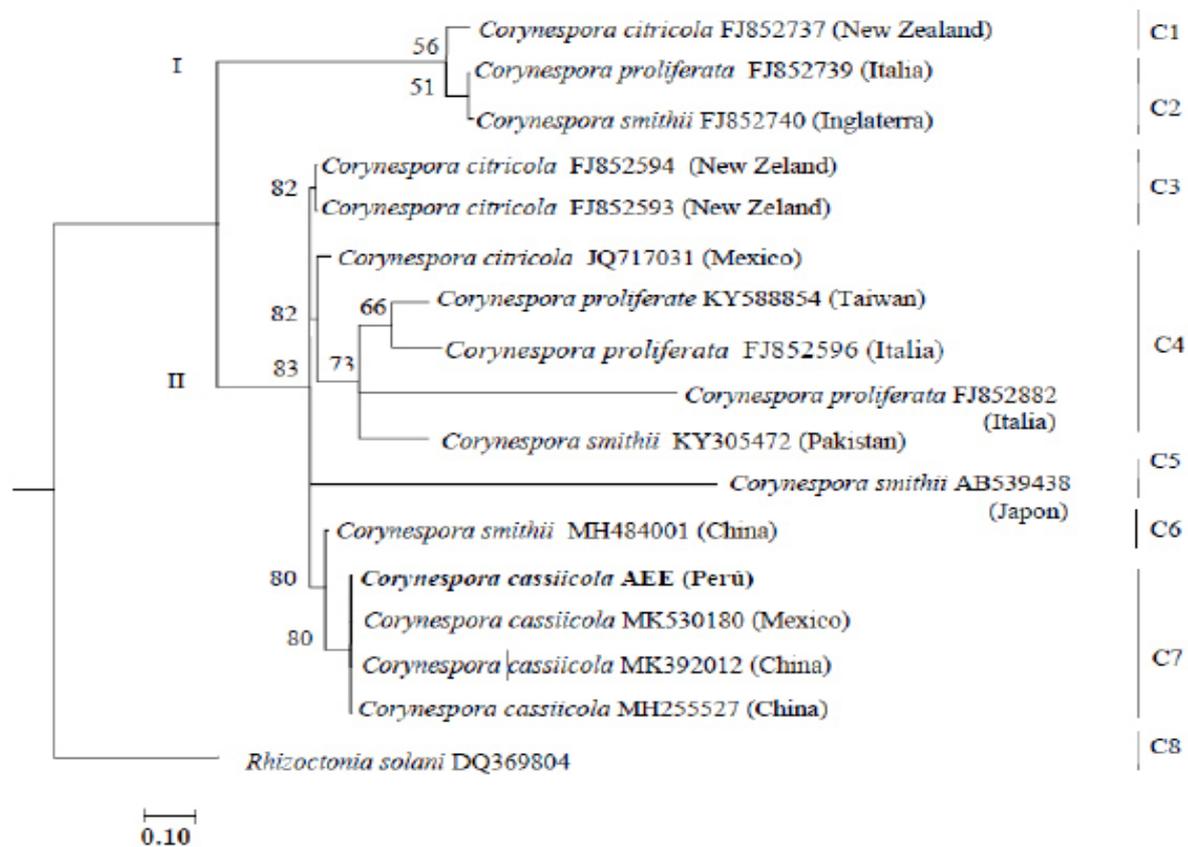


Figure 4. Phylogenetic tree constructed by Bayesian inference wherein it shows the relationships between the isolate under study *Corynespora cassiicola* AEE (Peru) and *Corynespora cassiicola* species.

identification keys of [Barnett and Hunter \(1972\)](#) and [Ellis \(1971\)](#), it was confirmed that the fungus belonged to the genus *Corynespora*, with similar characteristics to the species *Corynespora cassiicola* ([Figure 3](#)).

Molecular identification

Comparative analysis of the sequence using GenBank allowed the identification of the strain with 100% identity indexes with the compared species, reported in different parts of the world ([Table 2](#)). Phylogenetic analysis of the sequence of *C. cassiicola* strains isolated from strawberry and other 15 sequences of *Corynespora* derived from GenBank generated a tree by Bayesian inference, which revealed two major groups (I and II) formed by subgroups (bootstrap values: 51–83). In group I, two subgroups (C1 and C2) were grouped. Subgroup C1 included the isolate FJ852737 corresponding to the sequences of *C. citricola* (bootstrap value: 56). Subgroup C2 included the isolates FJ852739 and FJ852740, with the former corresponding to the sequences of *C. proliferata* and latter to those of *C. smithii* (bootstrap value: 51). In group II, six subgroups were grouped (C3, C4, C5, C6, C7, and C8). Subgroup C3 included the isolates FJ852594 and FJ852593 corresponding to the sequences of *C. citricola* (bootstrap value: 82). Subgroup C4 included the isolates JQ717031, KY588854, FJ852596, FJ852882, and KY305472 corresponding to the sequences of *C. citricola*, *C. proliferata*, *C. proliferata*, *C. proliferata*, and *C. smithii*, respectively (bootstrap value: 82). Subgroup C5 included the isolate AB539438 corresponding to the sequence of *C. smithii* (bootstrap value: 83). Subgroup C6 included the isolate MH484001 corresponding to the sequence of *C. smithii* (bootstrap value: 80).

Subgroup C7 included the isolate under study, i.e., AEE of *C. cassiicola* aligned with the ailamientos MK530180, MK392012, and MH255527 corresponding to the sequences of *C. cassiicola* isolated from Mexico and China (bootstrap value: 80). The alignment of the isolate AEE among the species of *C. cassiicola* within subgroup C7 showed the probability of it belonging to this species. Subgroup C8 included the isolate DQ369804 corresponding to the sequence of *Rhizoctonia solani*, which was only used to anchor the phylogenetic tree ([Figure 4](#)).

Pathogenicity test

Strawberry plants began to show symptoms of leaf spot on the upper and lower sides of young and mature leaves 3 days after being inoculated with *C. cassiicola*, whereas sporadic petioles showed signs of infection 4–5 days after inoculation. Both young and mature leaves exhibited spots and circular patches of brown that were 2–6 mm in diameter and had a light brown circle in the center. As time progressed, these spots enlarged until they coalesced with each other. In addition, a thick, yellow halo appeared around the spots, which expanded once they had joined together and subsequently caused necrosis of the leaf ([Figure 5A](#)). On the petiole, the disease appeared as a small black spot

surrounded by a reddish halo, which subsequently enlarged to become a canker ([Figure 5B](#)). The control plants did not show any symptoms of leaf spot ([Figure 5C](#)).



Figure 5. Disease symptoms in strawberry (*Fragaria ananassa*) plants. A) Strawberry plant showing necrotic spots and yellowing at 11 days after inoculation with *Corynespora cassiicola*. B) Symptoms of leaf spot on the petiole. C) Uninoculated control plant.

Reisolation of the pathogen

The mycelium that developed from the leaf spot and canker sores were similar to those found in the pathogen isolate. Both developed light green-gray cotton mycelium, and the mycelium gradually acquired a dark brown tone. Subsequently, measurements of the conidiophore and conidia were performed, and the results are shown in [Table 3](#).

In vitro evaluation of the biocontrol agents

B. subtilis (Biosplent) exhibited 24.60% mycelial inhibition against the pathogen, while *P. fluorescens* (Biodelta) achieved 12.00% inhibition compared with 0.00% inhibition for the control (PDA). ANOVA indicated that there was a significant difference between treatments, with a coefficient of variability of 7.57% ([Table 4](#)), and Tukey's multiple comparison test showed that *B. subtilis* had a significantly higher percentage inhibition against

Table 3. Average measurements of the conidiophore and conidia that were reisolated from strawberry (*Fragaria ananassa*) plants in the pathogenicity test.

Value	Conidiophore			Conidia			
	Long Length (μ)	Diameter (μ)	N°Proliferations	Long (μ)	Diameter (μ)	Diameter (μ) Truncate base	No. pseudosepta
Minimum	118.58	4.84	2	43.56	6.66	3.63	3
Maximum	219.01	6.05	6	82.28	10.89	4.84	7
Average	167.22	5.28	4.16	57.79	9.32	4.65	4.28
Published range (Ellis, 1971)	110–850	4–11	2–9	40–220	9–22	4–8	4–20

Table 4. Results of the analysis of variance for the percentage mycelial inhibition of *Bacillus subtilis* and *Pseudomonas fluorescens* against *Corynespora cassiicola*.

Source of variability	Degrees of freedom	Sum of squares	Mean square	F	P
Treatment	2	0.12005000	0.06002500	697.06	<0.0001 **
Error	9	0.00077500	0.00008611		
Total	11	0.12082500			

C.V. = 7.57% α = 0.05 ** = highly significant

Table 5. Results of the Tukey's multiple comparison test for the percentage mycelial inhibition of *Bacillus subtilis* and *Pseudomonas fluorescens* against *Corynespora cassiicola*.

Treatment	Description	Average	Level of significance*
T2	<i>B. subtilis</i> against <i>C. cassiicola</i>	0.245000	A
T1	<i>P. fluorescens</i> against <i>C. cassiicola</i>	0.122500	B
T0	Control	0.000000	C

*Rows with different letters are significantly different ($p < 0.05$)

Table 7. Results of the Tukey's multiple comparison test for the percentage mycelial inhibition of *Trichoderma harzianum* strain T-22 against *Corynespora cassiicola*.

Treatment	Description	Average	Level of significance
T1	<i>Trichoderma harzianum</i> strain T-22 against <i>C. cassiicola</i> .	0.41750	A
T0	<i>C. cassiicola</i> (control)	0.00000	B

*Rows with different letters are significantly different ($p < 0.05$)

Table 6. Results of the analysis of variance for the percentage mycelial inhibition of *Trichoderma harzianum* strain T-22 against *Corynespora cassiicola*.

Source of variability	Degrees of freedom	Sum of squares	Mean square	F	P
Treatment	1	0.34861250	0.34861250	919.42	<0.0001 **
Error	6	0.00227500	0.00037917		
Total	7	0.35088750			

C.V. = 9.32 α = 0.05 ** = highly significant

C. cassiicola than the other treatments at the 0.05 level (Table 5). *T. harzianum* biocontrol strain T-22 inhibited the growth of *C. cassiicola* mycelia by 41.8% compared with 0.0% for the control. ANOVA indicated that there was a significant difference between treatments, with a coefficient of variability of 9.32% (Table 6), and Tukey's multiple comparison test showed that *T. harzianum* strain T-22 significantly inhibited the growth of *C. cassiicola* compared with the control at the 0.05 level (Table 7).

In vitro evaluation of the fungicides

The fungicides tiabendazole (Mertect®; T4) and

fosetyl-aluminum (Aliette®; T5) achieved the greatest percentage inhibition of *C. cassiicola* mycelia, with rates of 96% and 100%, respectively, while the fungicides azoxystrobin (Stroasil®; T1), chlorothalonil (Zetron® 720; T2), and difenoconazole (Difeconasil®; T3) inhibited the development of the colony by 31%, 51%, and 68%, respectively. ANOVA indicated that there was a significant difference between treatments, with a coefficient of variability of 4.69% (Table 8), and Tukey's multiple comparison test showed that fosetyl-aluminum and tiabendazole achieved significantly greater levels of inhibition than the other treatments at the 0.05 level (Table 9).

Table 8. Results of the analysis of variance for the *in vitro* evaluation of fungicides against *Corynespora cassiicola*.

Source of variability	Degrees of freedom	Sum of squares	Mean square	F	P
Treatment	5	30429.66337	608.593.267	810.57	<0001 **
Error	18	135.14753	750.820		
Total	23	30564.81090			

C.V. = 4.69% $\alpha = 0.05$ ** = highly significant

Table 9. Results of Tukey's multiple comparison test for the *in vitro* evaluation of fungicides against *Corynespora cassiicola*.

Treatment	Commercial fungicide	Active ingredient	Average	Level of significance
T5	Aliette®	Fosetyl-aluminum	100.000	A
T4	Mertect®	Tiabendazol	97.893	A
T3	Difeconasil®	Difeconazol	68.405	B
T2	Zetron® 720	Clorotalonil	53.053	C
T1	Strosil®	Azoxystrobin	30.508	D
T6	PDA (control)	Potato, dextrose, agar	0.000	E

***In vivo* evaluation of the biocontrol agents and fungicides**

Strawberry plants that were cultivated under greenhouse conditions exhibited the lowest leaf spot severity when treated with azoxystrobin (Strosil), which had a lower AUDPC value compared with the other treatments (Table 10 and Figure 6). ANOVA indicated that there was a significant difference between treatments, with a variability coefficient of 14.34% (Table 11), and Tukey's multiple comparison test showed that azoxystrobin resulted in a significantly lower AUDPC than the other treatments at the 0.05 level (Table 12).

Table 10. Average area under the disease progress curve (AUDPC) for leaf spot in strawberry (*Fragaria ananassa*) plants following the application of nine treatments and in an uninoculated control under greenhouse conditions.

Treatment	Active ingredient	AUDPC
T1	<i>Bacillus subtilis</i>	487.02
T2	<i>Trichoderma harzianum</i> strain T-22	490.61
T3	<i>Pseudomonas fluorescens</i>	383.09
T4	Azoxystrobin	194.10
T5	Clorotalonil	543.82
T6	Difenoconazol	409.05
T7	Tiabendazol	358.48
T8	Fosetyl-aluminum	494.39
T9	Inoculation	555.68
T10	Without inoculation	0.00

Discussion

The morphological characteristics of the fungus that was

isolated from strawberry plants showing symptoms of leaf spot, such as the color of the colony and the characteristics of the conidiophore (cylindrical, simple, short or long) and conidia (exogenous, straight or curved, ellipsoidal or obclaved, fragmospore type, individual or in short chains, and acropetal formation) coincided with reports by Barnett and Hunter (1972) and Jayasuriya and Thennakoon (2007) for the genus *Corynespora*. The microscopic characteristics of this fungus, such as the dimensions of the conidiophores and conidia, together with the macroscopic characteristics resembled the species *C. cassiicola* (Barnett and Hunter, 1972; Ellis, 1971).

'Camino Real' strawberry plants developed disease symptoms on the leaves and petioles 3 days after inoculation with isolated *C. cassiicola* under greenhouse conditions. Similarly, Breton et al. and Lamotte (Déon et al., 2012) stated that *C. cassiicola* infection occurs 1–2 days after inoculation due to secretion of the toxin cassiicolina, which is a glycosylated protein of 27 amino acids. The symptoms of leaf spot in the inoculated leaves were characterized by dark brown spots with a light brown center surrounded by a yellow halo. This matches the description of Coelho, Pino, Fonseca and Muniz (2013) who reported that the disease originating from *C. cassiicola* causes lesions on the leaves that initially have a yellow halo and then progress to develop a beige or light brown area in the center. In addition, they mention that the spores of this pathogen are mainly dispersed by wind, irrigation water, sprinkler systems, and rain. Tan (Manju, 2011) indicates that symptoms of leaf spot can occur on leaves, petioles, and twigs throughout the year.

Infected leaves of strawberry plants that were collected from the field exhibited a reddish halo around the leaf spots, whereas those that were inoculated in the greenhouse

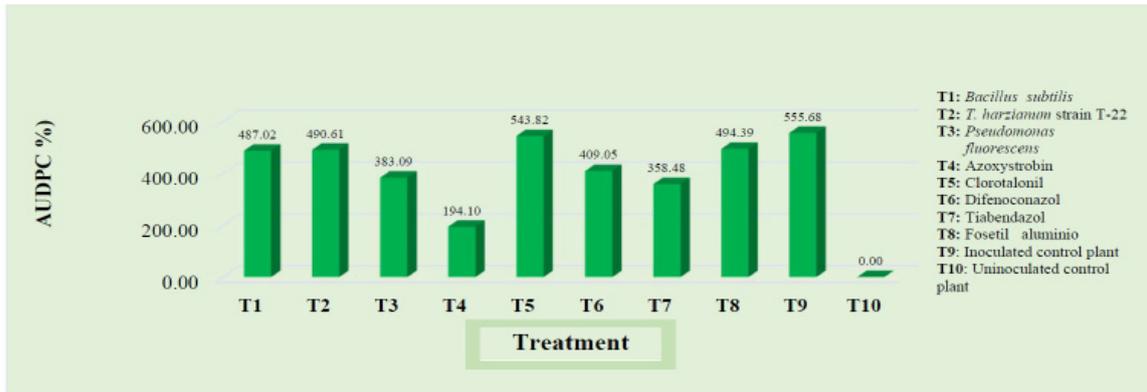


Figure 6. Average area under the disease progress curve (AUDPC) for different treatments used to control leaf spot in strawberry (*Fragaria ananassa*) under greenhouse conditions..

Table 11. Results of the analysis of variance for the average values of the area under the disease progress curve (AUDPC) for leaf spot in strawberry (*Fragaria ananassa*) plants with different treatments under greenhouse conditions..

Source of variability	Degrees of freedom	Sum of squares	Mean square	F	P
Treatment	9	1093589.571	121509.952	38.52	<0001 **
Error	30	94627.650	3154.255		
Total	39	1188217.220			

C.V. = 14.34% α = 0.05 ** = highly significant

Table 12. Results of the Tukey's multiple comparison test for the average values of the area under the disease progress curve (AUDPC) for leaf spot in strawberry (*Fragaria ananassa*) plants with different treatments under greenhouse conditions.

Treatment	Active ingredient	Fungicide	Average	Level of significance
T9	-----	Inoculation	555.69	A
T5	Clorotalonil	Zetron® 720	543.82	A B
T8	Fosetyl-aluminum	Aliette®	494.39	A B C
T2	<i>Trichoderma harzianum</i> strain T-22	T-22	490.61	A B C D
T1	<i>Bacillus subtilis</i>	Biosplent	487.02	A B C D
T6	Difenconazolof	Difeconasil®	409.05	B C D
T3	<i>Pseudomonas fluorescens</i>	Biodelta	383.09	C D
T7	Tiabendazol	Mertect®	358.49	D
T4	Azoxystrobin	Stronsil®	194.1	E
T10	-----	No inoculation	0	F

did not. It is important to note that the plants from which leaves were collected in the field were around 16 months of age, whereas those that were used in the pathogenicity test were only 5 weeks old. In addition, the field crop was grown under a temperature range of 15.9 to 25.1 °C with a relative humidity of 87.3%, whereas the greenhouse had a temperature range of 23.7 to 38.7 °C and a relative humidity of 78%. Jacob (Manju, 2011) previously reported that the symptoms originating from *C. cassiicola* infection vary widely between different clones, localities, and leaf stages at the time the infection established.

Furthermore, Miller and Alfieri (1974) found that the symptoms that developed on variegated and non-variegated *Ligustrum sinense* plants following inoculation with *C. cassiicola* isolated from variegated plants were indistinguishable from those found in naturally infected plants, with tiny reddish, circular spots that enlarged into light brown lesions with purple margins being formed in the variegated plants, and brown lesions with yellow halos being produced in the non-variegated plants. Therefore, it is very likely that age and environmental conditions influenced the expression of the symptoms in the present

study.

The experimental inoculations gave positive results and the final characteristics of the fungus that was isolated from leaves and petioles exhibiting symptoms were the same as those of the pathogen that was initially isolated, fulfilling Koch's postulates. Consequently, it is considered that *C. cassiicola* is the causal agent of leaf spot in strawberry.

The fungus *T. harzianum* strain T-22 was able to control *C. cassiicola* growth *in vitro*, achieving 41.8% mycelial inhibition. Leal (2000; Lorenzo, 2001) indicated that *Trichoderma* species have a direct action against phytopathogenic fungi and use different mechanisms to regulate their development, with the main mechanisms being competition for space and nutrients, mycoparasitism, and antibiosis. Among the fungicides that were tested, fosetyl-aluminum (Aliette) had the greatest efficacy against *C. cassiicola* *in vitro*, inhibiting mycelial growth by 100%. This inhibition was caused by the acidic environment it created (pH 2.3) when mixed with the culture medium. The fungicide tiabendazol (Mertect) showed the second highest level of control *in vitro*, achieving 96.32% inhibition of the mycelium. By contrast, azoxystrobin achieved the greatest control of leaf spot disease in strawberry plants under greenhouse conditions, with the lowest AUDPC value. Similarly, Carmona, Gally, Sautua, Abello, and Lopez (2011) found that the application of azoxystrobin to soybean (*Glyxine max*) exhibiting ringed spots originating from *C. cassiicola* controlled the disease and increased yield, the 1000-seed weight, and, in some cases, the number of grains per square meter.

Conclusions

The causal agent of leaf spot in strawberry was identified morphologically and biomolecularly as *C. cassiicola*. The biological control agent *T. harzianum* strain T-22 achieved significant control of *C. cassiicola* *in vitro*, with 41.8% mycelial inhibition, compared with 24.6% for the bacterium *B. subtilis* (Biosplent) and 12.0% for the bacterium *P. fluorescens* (Biodelta). In addition, the fungicides tiabendazol (Mertect) and fosetyl-aluminum (Aliette) achieved control levels of 96.32% and 100.00%, respectively, *in vitro*. However, the fungicide azoxystrobin (Stronsil) was the best treatment for strawberry leaf spot *in vivo* under greenhouse conditions, with fosetyl-aluminum (Aliette) having no effect on this disease under the same conditions.

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