

Article

Leaf Spot Disease Caused by Several Pathogenic Species of the Pleosporaceae Family on *Agave salmiana* and *Agave lechuguilla* Plants in Mexico, and Their Biocontrol Using the Indigenous *Trichoderma asperellum* Strain JEAB02

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Abstract

The genus *Agave* (family Asparagaceae) represents the second-most important group of plants in Mexico. Several fungal species have been identified as causal agents of leaf spot disease affecting *Agave salmiana* and *A. lechuguilla*, producing necrotic lesions that compromise plant health and productivity. Pathogenicity experiments were conducted under greenhouse conditions, field tests were performed, and in vitro antagonism using *Trichoderma asperellum* strain JEAB02 against selected pathogenic isolates was evaluated. Phylogenetic analysis of genomic DNA fragments allowed the identification of 26 fungal isolates belonging to *Curvularia lunata*, *C. verruculosa*, *Bipolaris zeae*, *Alternaria alternata*, *Fusarium lactis*, *Epicoccum sorghinum*, *Myrmaecium rubricosum*, *Penicillium diversum*, and *Aspergillus oryzae*. In pathogenicity assays under greenhouse conditions on *A. salmiana* and *A. lechuguilla*, treatments T5–T12 exhibited statistically similar levels of disease severity (33.10–37.29%), caused mainly by *C. verruculosa*, *A. alternata*, *B. zeae*, and *F. lactis*. In field tests, *Agave* plants inoculated with the selected pathogenic fungi (T4, T5, T7, T8, T10, and T11) showed 21.07–36.73% leaf damage after 75 days. The antagonistic effect of *T. asperellum* JEAB02 caused complete (100%) growth inhibition of the pathogenic isolate JCPN27 and inhibition levels from 99.81 to 99.98% for isolates JCPN18, JCPN24, JCPN28, JCPN29, JCPN31, and JCPN33, demonstrating its high potential as a biological control agent.

Keywords: *Curvularia*; *Bipolaris*; *Alternaria*; *A. salmiana*; *A. lechuguilla*; biocontrol; *Trichoderma asperellum*

Academic Editor: Eduardo Alves

Received: 16 September 2025

Revised: 13 October 2025

Accepted: 15 October 2025

Published: 16 October 2025

Citation: Aparicio-Burgos, J.E.; Romero-Cortes, T.; Armendáriz-Ontiveros, M.M.; Cuervo-Parra, J.A. Leaf Spot Disease Caused by Several Pathogenic Species of the Pleosporaceae Family on *Agave salmiana* and *Agave lechuguilla* Plants in Mexico, and Their Biocontrol Using the Indigenous *Trichoderma asperellum* Strain JEAB02. *Agronomy* **2025**, *15*, 2406. <https://doi.org/10.3390/agronomy15102406>

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1. Introduction

The genus *Agave* (family Asparagaceae) is the second-most important group of plants in Mexico for producing non-timber products such as tequila, mezcal, aguamiel, pulque, fibers, and probiotics. The genus comprises about 310 known species, with around 273 found in Mexico, 55% of which are endemic [1,2]. Mexico is regarded as the center of

origin and diversification of *Agave* species, which are mainly found in arid regions [3]. Therefore, they form a group of plants with significant economic importance due to their culinary [4,5], medicinal [6,7], industrial [8–10], and ornamental [11] uses, among others. Among all species, the most widely used include *Agave tequilana* [12], *A. americana* [13], *A. angustifolia* [14,15], *A. salmiana* [16], *A. victoria-reginae* [11], and *A. lechuguilla* [10]. Among these, *A. salmiana* Otto ex Salm-Dick ssp. *ferox* (Koch) Hochstätter [17], and *A. lechuguilla* Torr., have a wide distribution in the arid and semi-arid areas of the Llanos de Apan region, which includes the states of Hidalgo, Tlaxcala, Puebla, and the Estado de México [18,19]. Their resources are used by the inhabitants of this region, either directly from wild populations or from commercial plantations [20,21].

However, due to the slow growth of plants in this genus until they reach maturity and can be used [22,23], combined with their low rate of sexual and asexual reproduction [24], the presence of pests [25,26] and fungal diseases such as agave wilt [27], gray spot [28,29], and leaf spot [30–34], as well as growth delays caused by these factors [35], economic losses remain significant. However, despite the economic and ecological importance of *Agave* species, there is currently no scientific information available in Mexico regarding the economic losses caused by the causal agents of leaf spot disease in *A. salmiana* and *A. lechuguilla*, affecting both seedlings and mature plants. Moreover, the information available on control methods remains very limited. To date, only one study has reported the chemical control of *Bipolaris zeae* using silver nanoparticles in artificially inoculated *A. salmiana* plants [36]. This highlights the need for further research focused on the development of biological management strategies aimed at the sustainable control of these causal agents. Therefore, to identify which fungal species are responsible for the leaf spot symptoms observed in *A. salmiana* and *A. lechuguilla* plants in two municipalities in the state of Hidalgo, the following objectives were set: (i) molecularly characterize the causal agents of the “leaf spots” observed on *A. salmiana* and *A. lechuguilla* plants in these municipalities, (ii) evaluate the aggressiveness of the pathogens associated with the leaf spot symptoms in plants grown under both greenhouse and field conditions, and (iii) assess *in vitro* the effectiveness of a *Trichoderma asperellum* strain against the pathogenic fungi isolated from *Agave* plants.

To achieve these objectives, the study was structured in consecutive experimental phases. First, 26 fungal isolates were obtained from symptomatic leaves of *Agave salmiana* and *A. lechuguilla* collected in two contrasting environments—one natural (Mineral de la Reforma) and one disturbed (Apan). These isolates were subsequently subjected to morphological and molecular characterization to determine their taxonomic identity. In the second phase, *in vitro* confrontation assays were conducted using the *T. asperellum* JEAB02 strain to evaluate its antagonistic potential against the isolated fungi. Finally, pathogenicity tests were performed sequentially under greenhouse conditions (2023) and field conditions (2024), following Koch’s postulates, to identify which isolates were responsible for causing the leaf spot symptoms observed in *Agave* plants. Additionally, *Zea mays* plants were included as a comparative host species, since maize is an agriculturally and culturally important crop in Mexico and in the study region. Assessing the pathogenic potential of the isolates on maize provided a relevant reference for understanding cross-host infectivity and ecological adaptability of the fungi within the *Helminthosporium* complex.

2. Materials and Methods

2.1. The Fungal Strains

Twenty-six pathogenic fungal isolates were obtained from wild and cultivated populations of *Agave salmiana* and *A. lechuguilla* in the municipalities of Apan and Mineral de la Reforma, Hidalgo, Mexico, between 2019 and 2023 (Table 1). Based on macroscopic

and microscopic morphological characteristics (e.g., ascomata, conidiophores, macroconidia, conidia) observed during cultivation and through comparison with reference fungal isolates, the isolates were identified as belonging to nine genera. Nine strains were assigned to the genus *Bipolaris*, seven to *Curvularia*, and two each to *Penicillium*, *Fusarium*, *Alternaria*, and *Epicoccum*. The remaining two strains were identified as *Myrmaecium* and *Aspergillus*.

Table 1. Geographic origin of the strains isolated from *A. salmiana* and *A. lechuguilla*.

Strains	Host	Sample *	Species	Location	Coordinates	Date
JCPN09	<i>A. salmiana</i>	M1	<i>Bipolaris zeae</i>	Apan	19°64'97" LN, 98°51'93" LW	2019
JCPN10	<i>A. salmiana</i>	M1	<i>Bipolaris zeae</i>	Apan	19°64'97" LN, 98°51'93" LW	2020
JCPN11	<i>A. salmiana</i>	M2	<i>Bipolaris zeae</i>	Apan	19°64'96" LN, 98°51'91" LW	2020
JCPN12	<i>A. salmiana</i>	M3	<i>Bipolaris zeae</i>	Apan	19°64'94" LN, 98°51'88" LW	2020
JCPN13	<i>A. salmiana</i>	M2	<i>Bipolaris zeae</i>	Apan	19°64'96" LN, 98°51'91" LW	2021
JCPN14	<i>A. salmiana</i>	M4	<i>Bipolaris zeae</i>	Apan	19°64'90" LN, 98°51'84" LW	2021
JCPN7	<i>A. salmiana</i>	M4	<i>Bipolaris zeae</i>	Apan	19°64' 90" LN, 98°51'84" LW	2022
JCPN16	<i>A. salmiana</i>	M5	<i>Fusarium lactis</i>	Mineral de la Reforma	20°07'60" LN, 98°72'43" LW	2022
JCPN18	<i>A. salmiana</i>	M6	<i>Curvularia lunata</i>	Mineral de la Reforma	20°07'78" LN, 98°72'00" LW	2022
JCPN19	<i>A. lechuguilla</i>	M7	<i>Curvularia lunata</i>	Mineral de la Reforma	20°07'81" LN, 98°72'16" LW	2022
JCPN22	<i>A. lechuguilla</i>	M8	<i>Alternaria alternata</i>	Mineral de la Reforma	20°07'67" LN, 98°72'06" LW	2022
JCPN24	<i>A. lechuguilla</i>	M9	<i>Curvularia verruculosa</i>	Mineral de la Reforma	20°07'51" LN, 98°72'50" LW	2022
JCPN27	<i>A. lechuguilla</i>	M10	<i>Penicillium diversum</i>	Mineral de la Reforma	20°07'85" LN, 98°71'57" LW	2022
JCPN28	<i>A. lechuguilla</i>	M11	<i>Epicoccum sorghinum</i>	Mineral de la Reforma	20°07'80" LN, 98°71'66" LW	2022
JCPN33	<i>A. salmiana</i>	M12	<i>Bipolaris zeae</i>	Mineral de la Reforma	20°07'93" LN, 98°71'35" LW	2022
JCPN15	<i>A. salmiana</i>	M13	<i>Fusarium lactis</i>	Mineral de la Reforma	20°07'84" LN, 98°71'78" LW	2023
JCPN17	<i>A. salmiana</i>	M14	<i>Penicillium diversum</i>	Mineral de la Reforma	20°07'89" LN, 98°72'18" LW	2023
JCPN20	<i>A. lechuguilla</i>	M11	<i>Epicoccum sorghinum</i>	Mineral de la Reforma	20°07'80" LN, 98°71'66" LW	2023
JCPN21	<i>A. lechuguilla</i>	M15	<i>Curvularia lunata</i>	Mineral de la Reforma	20°07'78" LN, 98°72'13" LW	2023
JCPN23	<i>A. lechuguilla</i>	M16	<i>Curvularia lunata</i>	Mineral de la Reforma	20°07'74" LN, 98°72'09" LW	2023
JCPN25	<i>A. lechuguilla</i>	M11	<i>Curvularia lunata</i>	Mineral de la Reforma	20°07'80" LN, 98°71'66" LW	2023
JCPN26	<i>A. lechuguilla</i>	M17	<i>Myrmaecium rubricosum</i>	Mineral de la Reforma	20°07'83" LN, 98°71'57" LW	2023
JCPN29	<i>A. lechuguilla</i>	M18	<i>Alternaria alternata</i>	Mineral de la Reforma	20°07'61" LN, 98°72'16" LW	2023
JCPN30	<i>A. salmiana</i>	M19	<i>Curvularia verruculosa</i>	Mineral de la Reforma	20°07'53" LN, 98°72'87" LW	2023
JCPN31	<i>A. salmiana</i>	M20	<i>Aspergillus oryzae</i>	Mineral de la Reforma	20°07'63" LN, 98°72'53" LW	2023
JCPN32	<i>A. salmiana</i>	M21	<i>Bipolaris zeae</i>	Mineral de la Reforma	20°07'85" LN, 98°71'37" LW	2023

* Percentage of occurrence of fungal isolates by municipality and *Agave* species. Apan: *A. salmiana*—*B. zeae* (100%). Mineral de la Reforma: *A. salmiana*—*B. zeae* (18.2%), *C. lunata* (36.3%), *C. verruculosa* (9.1%), *F. lactis* (18.2%), *P. diversum* (9.1%), *A. oryzae* (9.1%); *A. lechuguilla*—*C. lunata* (12.5%), *C. verruculosa* (12.5%), *A. alternata* (25%), *P. diversum* (12.5%), *E. sorghinum* (25%), *M. rubricosum* (12.5%).

All fungal isolates were obtained from leaves of sampled maguey plants showing symptoms of leaf spot disease. To process infected tissue, samples were surface sterilized for 1 min in a 3% sodium hypochlorite solution, followed by two rinses in sterile distilled water [37]. Subsequently, samples were allowed to dry and placed in Petri dishes with potato dextrose agar (PDA, BD Bioxon®) in a moist chamber. The fungi were incubated in the dark at 28 °C for one week [38] or until the isolates covered the entire culture surface [30]. Afterwards, each isolate obtained was subcultured on PDA under the same conditions. All fungal strains isolated from maguey leaves exhibiting leaf spot symptoms were maintained on slanted agar tubes at 4 °C and preserved as spore suspensions at −86 °C until further use for molecular characterization.

2.2. Molecular Characterization

Genomic DNA from 26 fungal strains isolated from the leaves of *A. salmiana* and *A. lechuguilla* showing leaf spot symptoms (Table 1) was sequenced using the Sanger sequencing method (Eurofins Genomics GmbH, Anzinger, Germany). DNA extraction was performed following the procedure described by Cuervo-Parra et al. [39]. Ribosomal DNA (rDNA) fragments were amplified by PCR as outlined by Romero-Cortes et al. [40],

using the primers ITS5 and ITS4B [41]. The amplicons obtained, containing the ITS1/5.8s/ITS2 region, were washed twice with TE buffer (Tris HCl 10 mM, EDTA 1 mM). Subsequently, the PCR products of each strain were analyzed with Chromas 1.45 software (School of Health Science, Griffith University, Gold Coast campus, Southport, Queensland, Australia). The sequences were then subjected to pairwise alignment using ElasticBLAST (v1.4.0) against the GenBank database of the National Center for Biotechnology Information (NCBI) [42].

Amplicon sequences were deposited in the GenBank database. Phylogenetic analysis of the 26 strains isolated in this study, together with 46 related fungal sequences from NCBI, was conducted by constructing a phylogenetic tree using the Neighbor-Joining (NJ) method, the Jukes–Cantor model, with bootstrap support on 100 replicates in MEGA X (v10.2) software [43]. The accessions OL840629 and OL840630 of *Irpex latemarginatus*, along with OL830229 and OL830230 of *Mucor circinelloides*, isolated from a textile industry effluent in Mexico, were used as an outgroup for the phylogenetic analysis [41].

2.3. Pathogenicity Tests for Agave Phytopathogens

2.3.1. Greenhouse Experiment Design and Treatments

To confirm which pathogenic fungi isolated from the maguey leaves (Table 1) developed the typical symptoms of leaf spot disease observed in the field on *A. salmiana* and *A. lechuguilla* plants, a preliminary experiment was carried out in 2023 at ESAP-UAEH facilities. This experiment aimed to evaluate, under greenhouse conditions, the fungus–plant interaction of one representative isolate per species of the nine fungal species isolated from infected *Agave* tissues showing leaf spot symptoms, in order to determine whether disease symptoms developed and thereby fulfill Koch’s postulates [44]. For the preliminary experiments, the isolate of each species that exhibited the greatest radial growth after one week of culture in PDA medium was used. The treatments were assigned using a completely randomized design with three replicates per treatment (Table 2).

Table 2. Experimental design for various pathological experiments evaluated.

Key	Treatment *	Inoculation Site
T1	<i>A. salmiana</i> control	Without inoculants
T2	<i>A. lechuguilla</i> control	Without inoculants
T3	<i>C. lunata</i> vs. <i>A. salmiana</i>	Leaves and rhizosphere
T4	<i>C. lunata</i> vs. <i>A. lechuguilla</i>	Leaves and rhizosphere
T5	<i>C. verruculosa</i> vs. <i>A. salmiana</i>	Leaves and rhizosphere
T6	<i>C. verruculosa</i> vs. <i>A. lechuguilla</i>	Leaves and rhizosphere
T7	<i>B. zeae</i> vs. <i>A. salmiana</i>	Leaves and rhizosphere
T8	<i>B. zeae</i> vs. <i>A. lechuguilla</i>	Leaves and rhizosphere
T9	<i>A. alternata</i> vs. <i>A. salmiana</i>	Leaves and rhizosphere
T10	<i>A. alternata</i> vs. <i>A. lechuguilla</i>	Leaves and rhizosphere
T11	<i>F. lactis</i> vs. <i>A. salmiana</i>	Leaves and rhizosphere
T12	<i>F. lactis</i> vs. <i>A. lechuguilla</i>	Leaves and rhizosphere
T13	<i>E. sorghinum</i> vs. <i>A. salmiana</i>	Leaves and rhizosphere
T14	<i>E. sorghinum</i> vs. <i>A. lechuguilla</i>	Leaves and rhizosphere
T15	<i>M. rubricosum</i> vs. <i>A. salmiana</i>	Leaves and rhizosphere
T16	<i>M. rubricosum</i> vs. <i>A. lechuguilla</i>	Leaves and rhizosphere
T17	<i>P. diversum</i> vs. <i>A. salmiana</i>	Leaves and rhizosphere
T18	<i>P. diversum</i> vs. <i>A. lechuguilla</i>	Leaves and rhizosphere
T19	<i>A. oryzae</i> vs. <i>A. salmiana</i>	Leaves and rhizosphere
T20	<i>A. oryzae</i> vs. <i>A. lechuguilla</i>	Leaves and rhizosphere

* For greenhouse experiments, the isolate within each species that exhibited the highest radial growth after 7 days of cultivation was selected. The isolates chosen were: JCPN18 (*C. lunata*), JCPN24 (*C. verruculosa*), JCPN33 (*B. zea*), JCPN29 (*A. alternata*), JCPN16 (*F. lactis*), JCPN28 (*E. sorghinum*), JCPN26 (*M. rubricosum*), JCPN27 (*P. diversum*), and JCPN31 (*A. oryzae*).

Disease symptoms in greenhouse-grown *A. salmiana* and *A. lechuguilla* were evaluated through systematic weekly visual assessments. Symptoms' severity was quantified using a standardized 1–9 scale [34,45], which accounted for leaf spots, necrosis, chlorosis, and wilting. Disease incidence was calculated as the percentage of symptomatic plants, with non-inoculated controls included to ensure accurate symptom attribution.

Square plastic pots (11 × 11 × 11 cm; 1 kg capacity) were filled with a substrate consisting of 30 kg of sand, 45 kg of tepetate, and 75 kg of black soil. For each treatment, 15 healthy one-year-old maguey seedlings, obtained from seeds in 2022, each with 7–8 developed leaves, were used. A total of 150 seedlings were used for each of the two *Agave* species studied. The maguey plants were inoculated with pathogenic fungi isolated from *A. salmiana* and *A. lechuguilla* plants, using a 15 mL spore suspension containing equal volumes of conidia from each selected strain: *C. lunata*, *C. verruculosa*, *B. zea*, *A. alternata*, *F. lactis*, *E. sorghinum*, *M. rubricosum*, *P. diversum*, and *A. oryzae* (Table 2). The suspension was prepared in sterile distilled water adjusted to 5×10^6 conidia/mL and applied directly to the leaves and rhizosphere of each plant, in treatments T3 to T20. In the control treatments (T1 and T2), only sterile distilled water was applied to the leaves and rhizosphere near the base of each *A. salmiana* and *A. lechuguilla* plant.

Based on the results of the preliminary greenhouse pathology test and following Koch's postulates, an experimental design was developed to evaluate the pathogenic effect of the fungal species exhibiting the typical leaf spot symptoms observed under both field and greenhouse conditions.

2.3.2. Field Pathological Tests

To evaluate the impact of the selected pathogenic *Agave* fungi following Koch's postulates [44,46], healthy one-year-old rhizome seedlings of *A. salmiana* and *A. lechuguilla* obtained from healthy adult plants were planted under field conditions.

The experiment was conducted at the facilities of the Escuela Superior de Apan-UAEH, located at 19°65'48" N, 98°51'88" W, and at an elevation of 2488 m above sea level. This site has an average annual temperature of 14.4 °C and a total annual rainfall of 618.2 mm, in the Hidalgo state [47,48]. The total experimental plot was 96 m² (3 m wide by 32 m long), with treatments arranged in a completely randomized design with three replicates (Table 3). Each treatment consisted of 3 rows, each 3 m long, with 1 m spacing between rows and 0.60 m between plants. A 1 m gap was left between treatments to prevent crossover of results [49].

The maguey rhizome propagules used for the two *Agave* species were left to rest in the shade for a month before transplanting under field conditions in February 2024. Meanwhile, corn seeds were sown in pots under greenhouse conditions in January to ensure their germination and survival. Subsequently, after 15 days of germination, the seedlings were transplanted to the experimental plot together with the *A. salmiana* and *A. lechuguilla* seedlings.

Table 3. Experimental design of field pathological tests.

Key	Treatment *	Inoculation Site †
T1	<i>A. salmiana</i> control	Without inoculants
T2	<i>A. lechuguilla</i> control	Without inoculants
T3	<i>Z. mays</i> control	Without inoculants

T4	<i>B. zeae</i> vs. <i>A. salmiana</i>	Leaves and rhizosphere
T5	<i>B. zeae</i> vs. <i>A. lechuguilla</i>	Leaves and rhizosphere
T6	<i>B. zeae</i> vs. <i>Z. mays</i>	Leaves and rhizosphere
T7	<i>C. lunata</i> vs. <i>A. salmiana</i>	Leaves and rhizosphere
T8	<i>C. lunata</i> vs. <i>A. lechuguilla</i>	Leaves and rhizosphere
T9	<i>C. lunata</i> vs. <i>Z. mays</i>	Leaves and rhizosphere
T10	<i>C. verruculosa</i> vs. <i>A. salmiana</i>	Leaves and rhizosphere
T11	<i>C. verruculosa</i> vs. <i>A. lechuguilla</i>	Leaves and rhizosphere
T12	<i>C. verruculosa</i> vs. <i>Z. mays</i>	Leaves and rhizosphere

* Treatments involving *B. zeae*, *C. lunata*, and *C. verruculosa* correspond to the strains JCPN33, JCPN18, and JCPN24, based on their greater growth and pathogenic traits observed during the greenhouse experiment. † In the control treatments T1, T2, and T3, sterilized distilled water was used instead of each pathogenic strain.

For each treatment, 15 *Agave* and *Zea* seedlings with 7–8 fully developed leaves [50,51] were planted under field conditions in three replicates, using a total of 60 seedlings: *A. salmiana*, *A. lechuguilla*, and *Z. mays*. A substrate mixture of 120 kg sand, 120 kg tepetate, and 120 kg black soil was used, with 2 kg of substrate applied per maguey and corn seedling. Agronomic management of the experimental plot from February to July combined conventional practices with the application of chemical and organic fertilizers, following the methodology of Cuervo-Parra et al. [52]. During the dry period from February to April, flood irrigation was employed, whereas during the rainy period from May to July, a rainfed production system was used [53].

The maguey and corn plants were inoculated with each of the evaluated pathogenic fungi following a modified version of the technique described by Mata-Santoyo et al. [54]. For this, a 15 mL suspension containing equal parts of conidia from *C. lunata*, *C. verruculosa*, and *B. zeae* selected strains (Table 3) was prepared in sterile distilled water adjusted to 5×10^6 conidia/mL, directly onto the leaves and rhizosphere of plants in treatments T4 to T12. For the control treatments (T1 to T3), only sterile distilled water was applied to the leaves and rhizosphere near the base of each plant. After a five-day incubation period, evaluations were conducted using the disease incidence scale proposed by Perelló et al. [45] and Romero-Cortes et al. [34]: 1 = plants free of infection; 2 = plants with 1 to 5% leaf spots; 3 = 5 to 12%; 4 = 12 to 20%; 5 = 20 to 35%; 6 = 35 to 45%; 7 = 45 to 60%; 8 = 60 to 80%; and 9 = more than 80% of leaves showing leaf spots. Subsequently, the data were converted to percentages, and an ANOVA analysis was performed using Statistix 10 software [55]. Mean comparison was conducted using the Tukey HSD All-Pairwise test at a significance level of $p \leq 0.05$.

2.4. In Vitro Confrontation Tests

Antagonistic Activity of *Trichoderma asperellum* Strain JEAB02 Against Pathogenic Fungi of *Agave*

To determine the antagonistic effect of a strain of *Trichoderma asperellum*, isolated from the leaves and rhizosphere of mangosteen plants [56], confrontation experiments were conducted using dual culture. These involved the *Agave* fungi (*C. lunata*, *C. verruculosa*, *B. zeae*, *A. alternata*, *E. sorghinum*, *F. lactis*, *M. rubricosum*, *P. diversum*, and *A. oryzae*) and the JEAB02 strain of *T. asperellum* in 2024, following the methodology proposed by Cuervo-Parra et al. [37]. For the in vitro confrontation test, the strain with the greatest radial growth for each pathogenic fungal species was used, corresponding to the same strains selected for the pathogenicity experiment under greenhouse conditions (Table 2).

Interactions between *Trichoderma* and each confronted fungal pathogen were measured after one week. Digital images were captured at a fixed distance of 20 cm using an iPhone SE (Model MX9U2LZ/A, Mexico City, Mexico) equipped with iOS 17.2.1, a 12-megapixel single-lens camera, and a wide-angle lens with an $f/1.8$ aperture. The inhibition percentages of radial growth for each pathogenic fungus exposed to the *T. asperellum* (JEAB02) strain were reported as the biocontrol index (BCI), calculated using the formula $BCI = [T/C] \times 100$, as described by Cuervo-Parra et al. [37]. Where BCI represents the percentage of inhibition of the radial growth of the pathogenic fungal colony caused by its interaction with the antagonistic *Trichoderma* fungus, T denotes the area occupied by the *Trichoderma* colony, and C indicates the total area occupied by both fungi. To calculate the radial growth areas of the BCI, the ImageJ (v1.48q) software was used <http://rsbweb.nih.gov/> (accessed on 10 July 2024). Data from the dual culture experiments were analyzed using a completely randomized design with three replicates. Statistical analysis, including analysis of variance (ANOVA) and mean comparisons by the Tukey HSD All-Pairwise test (significance level set at $p \leq 0.05$), was conducted using Statistix (v10) software [55].

3. Results and Discussion

3.1. Molecular Characterization

In molecular biology, analysis of the ITS1/5.8s/ITS2 genetic regions is a valuable and straightforward tool for identifying DNA sequences from a wide range of organisms [57]. In this study, the molecular characterization of the ITS regions of the rDNA from 26 new sequences of phytopathogenic fungi isolated from plant tissues of *A. salmiana* and *A. lechuguilla* exhibiting disease symptoms was performed. These sequences were compared with related sequences obtained from the GenBank database of the National Center for Biotechnology Information (NCBI).

For the molecular identification of the rDNA region, which included the ITS1/5.8s/ITS2 sequence of each pathogenic fungus isolated in this study, the primers ITS5 and ITS4B were used [41]. Pairwise alignment of DNA fragments from fungal strain was performed using the ElasticBLAST (v1.4.0) program [58], by comparison with sequences of related pathogenic fungal accessions available in the NCBI GenBank database [59]. Similarity percentages for the 26 accessions corresponding to the nine genera of isolated fungi ranged from 99.43 to 100% when compared with related fungal sequences available in GenBank. The length of the amplified rDNA fragments from the fungal strains isolated in this study ranged from 497 to 578 base pairs. These sequences were subsequently deposited in the NCBI GenBank database with the following accession numbers: OR047532-OR047548, OR553111-OR553112, and OR553116-OR553122 (Table 4).

Table 4. Fungal strain's identity based on the ITS 1/5.8s/ITS II region of the rDNA.

Strain	GenBank Accession	Sequence-Based Association	Percent Identity	GenBank Closest Hit	References
JCPN7	OR553122	<i>B. zeae</i>	99.63%	MT505870	Visagie et al. [60]
JCPN09	OR553116	<i>B. zeae</i>	99.61%	MT505870	Visagie et al. [60]
JCPN10	OR553117	<i>B. zeae</i>	99.60%	MT505870	Visagie et al. [60]
JCPN11	OR553118	<i>B. zeae</i>	99.43%	MT505870	Visagie et al. [60]
JCPN12	OR553119	<i>B. zeae</i>	99.60%	MT505870	Visagie et al. [60]
JCPN13	OR553120	<i>B. zeae</i>	99.60%	MT505870	Visagie et al. [60]
JCPN14	OR553121	<i>B. zeae</i>	99.63%	MT505870	Visagie et al. [60]
JCPN15	OR047541	<i>F. lactis</i>	100%	MT279265	Camarena-Pozos et al. [61]
JCPN16	OR047542	<i>F. lactis</i>	100%	MT279265	Camarena-Pozos et al. [61]
JCPN17	OR047539	<i>P. diversum</i>	98.88%	HM469392	Jang et al. [62]
JCPN18	OR047532	<i>C. lunata</i>	100%	KY100122	Cristóbal et al. [63]
JCPN19	OR047533	<i>C. lunata</i>	99.82%	KY100122	Cristóbal et al. [63]

JCPN20	OR047543	<i>E. sorghinum</i>	100%	MH497563	Mora-Aguilera et al. [64]
JCPN21	OR047534	<i>C. lunata</i>	100%	KY100122	Cristóbal et al. [63]
JCPN22	OR047547	<i>A. alternata</i>	100%	MN615420	Zhang et al. [65]
JCPN23	OR047535	<i>C. lunata</i>	100%	HQ607991	Rodrigues et al. [66]
JCPN24	OR047537	<i>C. verruculosa</i>	99.45%	MH859788	Vu et al. [67]
JCPN25	OR047536	<i>C. lunata</i>	99.82%	KY100122	Cristóbal et al. [63]
JCPN26	OR047545	<i>M. rubricosum</i>	100%	MK041894	Campos-Rivero et al. [68]
JCPN27	OR047540	<i>P. diversum</i>	98.91%	HM469392	Jang et al. [62]
JCPN28	OR047544	<i>E. sorghinum</i>	100%	MH497563	Mora-Aguilera et al. [64]
JCPN29	OR047546	<i>A. alternata</i>	100%	MN481948	Ma and Wu [69]
JCPN30	OR047538	<i>C. verruculosa</i>	99.46%	MH859788	Vu et al. [67]
JCPN31	OR047548	<i>A. oryzae</i>	100%	OQ202044	Martínez-Pérez et al. [70]
JCPN32	OR553111	<i>B. zeae</i>	99.62%	MT505870	Visagie et al. [60]
JCPN33	OR553112	<i>B. zeae</i>	100%	MT505870	Visagie et al. [60]

When comparing the ITS regions of the 26 fungal isolates obtained from field-infected maguey plants, the data from this study are consistent with previously reported results for *E. sorghinum* from *Agave angustifolia* and *A. tequilana* plants [64,68,71]; *C. lunata* [63,72,73]; *C. verruculosa* [67,74]; *F. lactis* [61]; *M. rubricosum* [75]; *A. alternata* [69,76–78]; *P. diversum* [62]; and *B. zeae* [34,60,79,80].

Phylogenetic analysis of 26 fungal sequences from maguey plants and 46 related sequences from GenBank was performed, resulting in a phylogenetic tree constructed using the Neighbor-Joining (NJ) method. The tree was rooted with DNA sequences from accessions OL840629 and OL840630 of *Irpex latemarginatus*, and OL830229 of *Mucor circinelloides*, which were used as outgroups to root the tree and infer phylogenetic relationships [41]. Analysis of the NJ tree revealed three main clades (I, II, and III). Clade I was represented by sequences from the families Pleosporaceae and Didymellaceae, clade II by sequences from Nectriaceae, Valsariaceae, and Trichocomaceae, and clade III, serving as the external group, included sequences from Irpicaceae and Mucoraceae. All clades were supported by a similarity value of $\geq 97\%$.

Among the ITS regions, the ITS2 was the most conserved across all rDNA sequences examined in the multiple sequence alignment in this study. The optimal phylogenetic tree, generated using the NJ method with a total branch length of 1.72543294, is shown. Phylogenetic analysis using the NJ method indicated that the rDNA sequences from the 26 accessions, representing nine pathogenic fungal species isolated from *A. salmiana* and *A. lechuguilla*, grouped separately on different branches of the phylogenetic tree (Figure 1).

At the top of the phylogenetic tree, in clade I, were the accessions of the genera *Curvularia*, *Bipolaris*, and *Alternaria*, all belonging to the Pleosporaceae family [81], and supported by a bootstrap value of 100. Within these accessions, those with a bootstrap support value of 92 at the top, included MN688814 (*C. pseudobrachyspora*), KJ922372 (*C. brachyspora*), MH858446 (*C. lunata* var. *lunata*), KP340066 (*C. aerea*), KX610322 (*C. lunata*), MF490823 (*C. variabilis*), KF018919 (*C. hawaiiensis*), and MH863648 (*C. spicifera*), all obtained from different sources and geographical regions [67,73,82–86]. In a sister branch with a bootstrap value of 62, the *C. lunata* accessions OR047532, OR047533, OR047534, OR047535, and OR047536 reported in this work were grouped with NCBI *C. lunata* accessions KY100122 and GQ280375, which were isolated from sugarcane and *Agave fourcroydes* samples [63,72]. In another branch near the *C. lunata* accessions, with a bootstrap support value of 100, the *C. verruculosa* accessions OR047537 and OR047538 were grouped with GenBank accessions KJ748697 and MH859788 of *C. verruculosa* [67,74].

In the upper part of clade I, all accessions of the genus *Curvularia* grouped together with those of the genus *Bipolaris*, supported by a bootstrap value of 98 at the node joining these sequences. The top accessions included OR553112 and OR553118 of *B. zeae* reported

in this work, together with MT505867, MT505870, MT645704, and ON630341 of *B. zeae* from GenBank [34,60,79,80]. The *B. zeae* accessions OR553111, OR553116, OR553117, and OR553119-OR553122 reported in this work were located adjacent to *B. zeae* accessions ON630338 and ON630344, which were isolated from *A. salmiana* plants in Mexico exhibiting leaf spot disease symptoms [34]. Another branch contained the accessions OR047546 and OR047547 of *A. alternata* isolated from *A. lechuguilla* plants in this study, along with the accessions KX115418, MN481948, MG255300, MN596827, and MN615420 of *A. alternata* from GenBank, with a bootstrap value of 100 [65,69,76–78]. At the bottom of clade I, along with all Pleosporaceae accessions considered in this analysis and supported by a bootstrap value of 100, are OR047543 and OR047544 of *E. sorghinum* isolated from *A. lechuguilla* plants in Mexico, together with GenBank accessions FJ427068, MH497563, OQ346146, and OR388091 of *E. sorghinum* isolated from *A. angustifolia* and *A. tequilana* samples in Mexico [33,64,68,71].

The sequences of OR047541 and OR047542 of the genus *Fusarium*, isolated from *A. salmiana* samples in Mexico, clustered at the top of clade II in the phylogenetic tree, together with MT279265 (*F. lactis*), ON229472 (*F. verticillioide*), OR426451 (*F. fujikuroi*; a former synonym of *F. verticillioide* within the *Gibberella fujikuroi* species complex [87]), and ON329680 (*F. oxysporum*) [61,88–90], all supported by a bootstrap value of 100 (Figure 1). In addition, OR047545 of *M. rubricosum*, isolated from *A. lechuguilla* in this study, was closely aligned with MK041893, MK041894, and OQ975458 of *M. rubricosum*, which were isolated from *A. tequilana* and *Pinus* sp. samples in Mexico [68,75], as well as KP687862 of *M. fulvopruinatum* from acacia bark in Taiwan [91]. At the bottom of clade II, with a bootstrap value of 97, were the sequences of *Penicillium* and *Aspergillus* accessions. OR047539 and OR047540 of *P. diversum*, isolated from *A. salmiana* and *A. lechuguilla*, were located near HM469392 (*P. diversum*) and KY966028 (*P. verrucosum*) from Korea and India [62,92], supported by a bootstrap value of 100. OR047548 of *A. oryzae*, isolated from *A. salmiana* in this study, aligned with OQ202044 of *A. oryzae*, isolated in Mexico from *A. salmiana* [70]. On a nearby branch with a bootstrap value of 100 were OR397993 (*A. niger*), OK012380 (*A. minisclerotigenes*), and MT529296 (*A. flavus*) [93–95]. Finally, in clade III, OL840629 and OL840630 of *I. latemarginatus* and OL830229 and OL830230 of *M. circinelloides* were positioned as an outgroup [41]. The high bootstrap support values obtained in this phylogenetic analysis corroborate the macroscopic identification and microscopic characterization of each fungal isolate, providing new records of pathogenic fungi associated with maguey plants in Hidalgo, Mexico.

3.2. Pathogenicity Tests

3.2.1. Greenhouse Tests

After completing the pathogenicity experiment under greenhouse conditions, treatments T5–T12 were statistically similar, showing the highest disease severity on *A. salmiana* and *A. lechuguilla*, with mean values ranging from 33.10 to 37.29%, caused by the pathogenic fungi *C. verruculosa*, *A. alternata*, *F. lactis*, and *B. zeae*. The second-highest severity was seen in plants from treatments T3 and T4, with mean values of 24.20 and 25.29%, caused by *C. lunata*. Treatments T13 and T14, associated with *E. sorghinum*, showed severity values of 18.24 to 18.26%. The fourth-highest severity was caused by *M. rubricosum*, with mean values of 11.20 to 11.28% on *A. salmiana* and *A. lechuguilla*. The lowest severity, ranging from 7.13 to 7.38% was recorded in treatments T17–T18, associated with *P. diversum*. Meanwhile, treatments T19 and T20 of *A. oryzae*, as well as the control treatments T1 and T2, showed no disease symptoms (0.0%) throughout the experimental period. Notably, the treatments that included *C. verruculosa*, *A. alternata*, *F. lactis*, and *B. zeae* exhibited the highest levels of damage (Table 5).

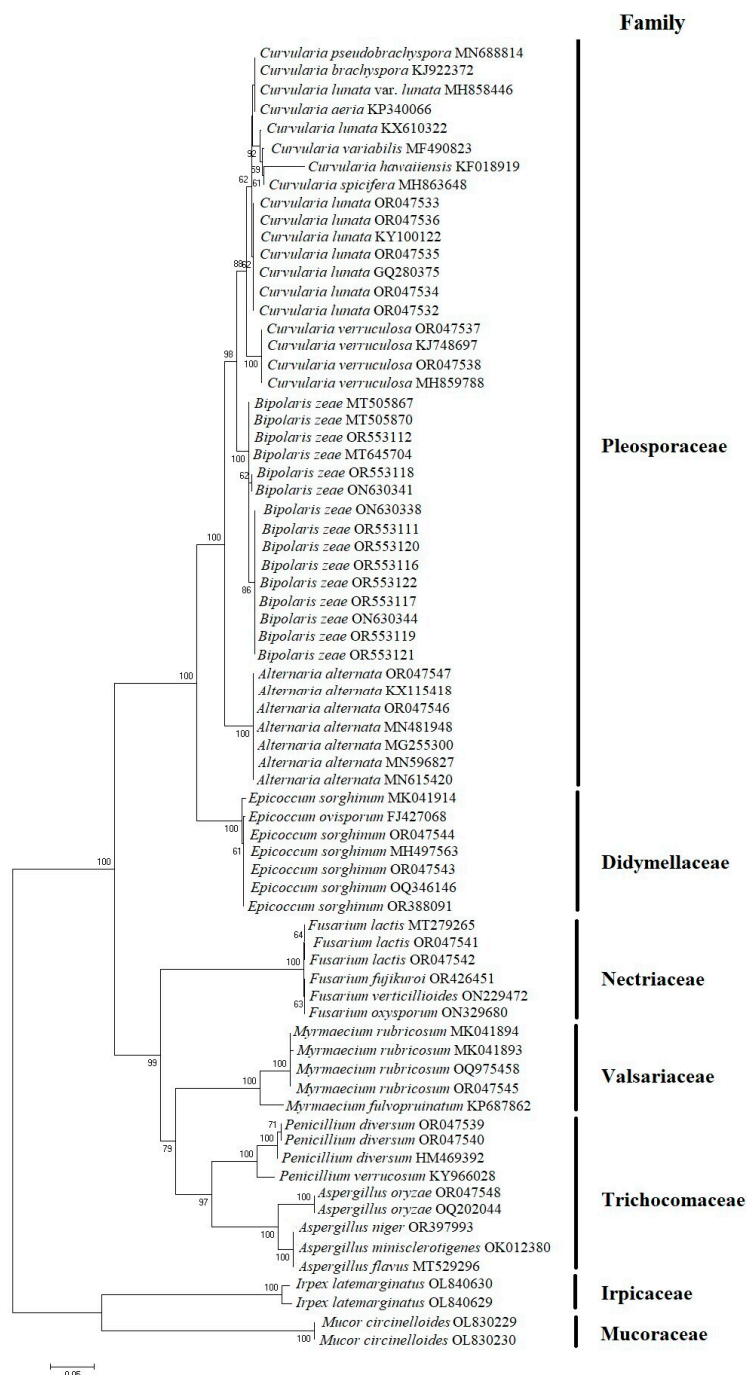


Figure 1. Neighbor-Joining phylogenetic analysis of pathogenic fungi based on ITS alignment. Numbers at nodes indicate bootstrap values. Three main clades were observed: I (Pleosporaceae, Didymellaceae), II (Nectriaceae, Valsariaceae, Trichocomaceae), and III (Irpiceae, Mucoraceae), all supported by $\geq 97\%$ similarity.

Table 5. Greenhouse pathogenicity experiment.

Key	Treatments *	Damage Percentage $\pm$ SE **
T1	<i>A. salmiana</i> control	0.00 $\pm$ 0.00 f
T2	<i>A. lechuguilla</i> control	0.00 $\pm$ 0.00 f
T3	<i>C. lunata</i> vs. <i>A. salmiana</i>	25.29 $\pm$ 0.37 b
T4	<i>C. lunata</i> vs. <i>A. lechuguilla</i>	24.20 $\pm$ 0.18 b

T5	<i>C. verruculosa</i> vs. <i>A. salmiana</i>	37.29 ± 1.79 a
T6	<i>C. verruculosa</i> vs. <i>A. lechuguilla</i>	36.36 ± 0.32 a
T7	<i>B. zeae</i> vs. <i>A. salmiana</i>	34.23 ± 1.27 a
T8	<i>B. zeae</i> vs. <i>A. lechuguilla</i>	33.38 ± 0.27 a
T9	<i>A. alternata</i> vs. <i>A. salmiana</i>	36.32 ± 0.29 a
T10	<i>A. alternata</i> vs. <i>A. lechuguilla</i>	35.64 ± 0.21 a
T11	<i>F. lactis</i> vs. <i>A. salmiana</i>	33.10 ± 0.11 a
T12	<i>F. lactis</i> vs. <i>A. lechuguilla</i>	34.53 ± 1.66 a
T13	<i>E. sorghinum</i> vs. <i>A. salmiana</i>	18.26 ± 0.19 c
T14	<i>E. sorghinum</i> vs. <i>A. lechuguilla</i>	18.28 ± 0.22 c
T15	<i>M. rubricosum</i> vs. <i>A. salmiana</i>	11.20 ± 0.23 d
T16	<i>M. rubricosum</i> vs. <i>A. lechuguilla</i>	11.28 ± 0.08 d
T17	<i>P. diversum</i> vs. <i>A. salmiana</i>	7.38 ± 1.21 e
T18	<i>P. diversum</i> vs. <i>A. lechuguilla</i>	7.13 ± 0.19 e
T19	<i>A. oryzae</i> vs. <i>A. salmiana</i>	0.00 ± 0.00 f
T20	<i>A. oryzae</i> vs. <i>A. lechuguilla</i>	0.00 ± 0.00 f

* Treatments involving *B. zeae*, *C. lunata*, and *C. verruculosa* correspond to the strains JCPN33, JCPN18, and JCPN24, based on their enhanced growth and pathological traits observed during morphological characterization and greenhouse experiment treatment. ** Means sharing the same letter indicate no significant differences ($p < 0.05$, ANOVA and Tukey tests); ± Standard error.

The symptoms caused by each pathogenic fungus on infected leaves of *A. salmiana* and *A. lechuguilla* were as follows. For *A. alternata*, initial signs were chlorotic marginal spots, especially at the leaf tips, which progressed to tip wilting and ultimately necrosis. These symptoms align with previous reports in *Agave tequilana* [96,97], a species of significant economic importance in Mexico, particularly in the states of Jalisco, Michoacán, Nayarit, Guanajuato, and Tamaulipas [98]. The isolation of this fungus from *A. salmiana* and *A. lechuguilla* in this study is not surprising, as it is an opportunistic pathogen known to infect more than 380 plant species [65,76,77,99]. In the case of *F. lactis*, the fungus primarily caused root rot and leaf blight, which led to bud wilting and drying of the leaf tips. These symptoms have also been reported in commercial plantations of *A. tequilana* [96,100,101], *Agave potatorum* [102], and sweet pepper [103].

In plants infected with *E. sorghinum*, irregularly shaped lesions were observed, sometimes surrounded by a yellow halo. These lesions became sunken, forming necrotic depressions. The results of this study align with reports for other crops such as rice [104–106], cocksfoot grass [107], sugarcane [108], chrysanthemum [109], *A. angustifolia* [33,64], and *A. tequilana* [68,71]. In plants infected with *M. rubricosum*, leaf blisters were observed, which eventually caused wilting and rotting of affected leaves. In some cases, leaf deformation was also observed. These symptoms are consistent with reports for *Pinus patula* [110,111] and *A. tequilana* [68,112]. In plants infected with *P. diversum*, brown spots developed. Over time, these spots caused leaves wilting and stunted growth in affected specimens. Similar symptoms have been reported in several crops of economic importance [113–115]. This group of fungi is commonly found on a wide variety of crops and, under favorable humidity and temperature conditions, typically causes the diseases described here, as well as chlorosis and water-soaked tissues that emit a moldy odor [116,117]. Although, *A. oryzae* is not classified as a direct plant pathogen, it can indirectly affect their plant health as an opportunistic microorganism that thrives under high humidity [118]. No disease symptoms were observed on the leaves and roots of *A. salmiana* and *A. lechuguilla* inoculated with this fungus. Instead, improved growth was observed in these plants. These results are consistent with previous studies reporting *A. oryzae* as a microorganism with potential biotechnological [119] and agricultural [120]

applications, and it is classified by the FDA as a food additive within the Generally Recognized As Safe (GRAS) category [121,122].

The fungi *C. lunata*, *C. verruculosa*, and *B. zeae*, which are part of the *Helminthosporium* complex of pathogenic fungi associated with various crops worldwide [63,82,83,123,124], caused black leaf spots on *A. salmiana* and *A. lechuguilla* leaves, consistent with field observations of infected tissues. Additionally, the leaf spot symptoms observed in this study match reports for *A. salmiana* plants [34,36,125] and *A. fourcroydes* [72]. *C. lunata* is recognized as an opportunistic pathogenic microorganism, frequently found in food, decaying plant matter, and soil [126]. It mainly affects cereals [63,82,127,128], cacao [83], and, more recently, maguey plants [125], where it causes leaf spot disease [129]. For *C. verruculosa*, although specific data on the extent of damage and symptoms in *Agave* species are not yet available, this fungus is known to infect cultivated grasses such as rice and corn [130–132], as well as wild grasses [133–135], and legumes [136], leading to significant economic losses. *B. zeae* is another pathogen responsible for various plant diseases, including seedling death, leaf and stem wilting, and black leaf spots [137]. This pathogen is frequently reported in economically important crops such as grasses [138,139], in polygonaceous plants [140], often cultivated alongside magueys under production systems like metepantle [2,141], and in several wild Poaceae species growing near maguey plantations [142–144]. This ecological context may explain the presence of these fungi in the sampled maguey plants, which may have been infected through biotic and abiotic vectors that transported spores of the *Helminthosporium* complex [145] from infected wild or cultivated grasses to a new host, in this case *A. salmiana* and *A. lechuguilla*.

3.2.2. Field Tests

Field evaluations were carried out to confirm the pathogenicity of *B. zeae*, *C. lunata*, and *C. verruculosa*—the fungal species previously identified as causal agents of leaf spot disease under greenhouse conditions (Table 5). Under natural field conditions, disease development was monitored in *A. salmiana*, *A. lechuguilla*, and *Zea mays* plants corresponding to treatments T4–T12 (Table 6). Characteristic leaf spot symptoms appeared 75 days after inoculation in *Agave* plants and 15 days in maize, with foliar damage ranging from 21.07% to 41.50%. These results confirmed the pathogenic capacity of the three fungi in both hosts and revealed interspecific differences in susceptibility, which are likely associated with structural and physiological adaptations of *Agave* species to arid environments that delay pathogen penetration and symptom expression.

Under field conditions, plants of the genus *Agave* show adaptations to Mexico's arid and semi-arid climates, such as a thick cuticle, wax layers, and complex stomata that help protect them from water loss [31,146]. These adaptations are some of the mechanisms that enable these plants to defend against pathogen attacks. They also explain the long time (75 days) it takes for the three pathogenic fungi to penetrate these biological barriers [34], in contrast to the very short time (15 days) required for the same pathogens to penetrate and cause leaf spot symptoms in the corn plants. Similarly, the tissue of corn leaves, which is formed from thin-walled parenchymal cells with numerous perforations [147], could have facilitated the hyphae of pathogens of the genera *Bipolaris* and *Curvularia* evaluated in this study to penetrate and infect the plant tissues of corn in a considerably shorter time (Table 6).

Table 6. Field pathogenicity in maguey and maize plants.

Key	Treatments *	Damage Percentage $\pm$ SE **
T1	<i>A. salmiana</i> control	0.00 $\pm$ 0.00 e
T2	<i>A. lechuguilla</i> control	0.00 $\pm$ 0.00 e
T3	<i>Z. mays</i> control	0.00 $\pm$ 0.00 e

T4	<i>B. zeae</i> vs. <i>A. salmiana</i>	33.27 ± 0.41 c
T5	<i>B. zeae</i> vs. <i>A. lechuguilla</i>	31.73 ± 0.11 c
T6	<i>B. zeae</i> vs. <i>Z. mays</i>	41.50 ± 0.94 a
T7	<i>C. lunata</i> vs. <i>A. salmiana</i>	23.67 ± 0.61 d
T8	<i>C. lunata</i> vs. <i>A. lechuguilla</i>	21.07 ± 0.46 d
T9	<i>C. lunata</i> vs. <i>Z. mays</i>	32.66 ± 1.88 c
T10	<i>C. verruculosa</i> vs. <i>A. salmiana</i>	36.73 ± 0.61 b
T11	<i>C. verruculosa</i> vs. <i>A. lechuguilla</i>	34.07 ± 0.30 c
T12	<i>C. verruculosa</i> vs. <i>Z. mays</i>	38.08 ± 0.35 b

* Treatments involving *B. zeae*, *C. lunata*, and *C. verruculosa* correspond to strains JCPN33, JCPN18, and JCPN24, respectively, based on their enhanced growth and pathogenic traits observed during morphological characterization and greenhouse experiments. ** Means sharing the same letter indicate no significant differences ($p < 0.05$, ANOVA and Tukey tests); ± Standard error.

When analyzing results by species, significant differences were observed among treatments T6, T9, and T12, with the highest percentage of foliar damage (41.50%) in corn leaves in treatment T6, which was inoculated with the fungus *B. zeae*. In second place were corn plants from treatment T9, inoculated with the fungus *C. verruculosa*, with a leaf damage percentage of 38.08%. In third place were corn plants from treatment T12, with 32.66% leaf damage. These results fall within the damage range reported by other authors for various corn varieties affected by *B. setariae* [148,149], *B. zeicola*, *B. cynodontis*, *B. oryzae*, *B. saccharicola*, *Curvularia* spp. [148], *C. lunata* [127,150], and *C. verruculosa* [131].

Damage percentages higher than those reported in this study have been noted for the fungus *C. verruculosa*, with damage reaching up to 60% in bean plants [136], and for the phytopathogen *B. maydis*, with damage exceeding 70% in corn crops [148,151]. Therefore, the damage caused by fungi of the *Helminthosporium* complex in corn crops varies and depends on multiple factors, such as environmental conditions, the susceptibility of the corn variety, and the virulence of the pathogen [127,152,153].

For treatments T4, T7, and T10, significant differences were also observed among the evaluated treatments. The highest percentage of damage was recorded at 36.73% for *A. salmiana* plants inoculated with the fungus *C. verruculosa*, followed closely by plants in treatment T4 with 33.27% damage caused by *B. zeae*, and a lower percentage of 23.67% for maguey plants inoculated with *C. lunata*. On the other hand, for *A. lechuguilla* plants in treatments T5 and T11, inoculated with the fungi *C. verruculosa* and *B. zeae*, no significant differences were observed, with damage values of 34.07 and 31.73%. For *A. lechuguilla* plants in treatment T8, the lowest percentage of damage among all treatments was recorded, at 21.07%. Finally, plants in treatments T1, T2, and T3 did not exhibit any disease symptoms throughout the entire duration of the pathogenicity experiment (Table 6).

Information on disease symptoms caused by *Bipolaris* and *Curvularia* species in agave leaves is scarce, particularly for maguey [34,125]. Moreover, no reports exist on the pathogenicity of *C. verruculosa* in agaves. Therefore, our data can only be compared with previous studies on *B. zeae*, a pathogen of *A. salmiana* in Hidalgo, Mexico. In this study, *B. zeae* caused 33.27% foliar damage, which is higher than the 10.92% reported for the JCP N07 strain of *B. zeae* in *A. salmiana* [34]. This difference may be explained by the experimental conditions: our work was conducted during the rainy season (lower temperatures, higher humidity), whereas Romero-Cortes et al. [34] performed their study in the dry season (higher temperatures, lower humidity). Such climatic conditions are critical for fungal pathogens to complete their infection cycle [152,154,155]. In particular, reduced moisture decreases nutrient availability and absorption, which lowers fungal colony density [156,157] and delays the development of somatic (mycelium) and reproductive (asci and conidia) structures. Under dry conditions, this delay extended the

appearance of disease symptoms in *A. salmiana* plants to 90 days. By contrast, our results for treatments T4–T12 (inoculated with *C. lunata*, *C. verruculosa*, and *B. zea*) showed lower tissue damage than the 66.34% reported by [36] for *B. zea* on *A. salmiana*. The latter difference is attributable to methodology: in that study, plants were artificially wounded prior to inoculation, which altered natural infection processes and bypassed the plant's defense mechanisms [31].

Once the pathogenicity experiment was concluded, a confirmation step was carried out to verify that the leaf spot symptoms observed in maguey (75 days) and corn (15 days) were caused by conidia of the fungi *B. zea*, *C. lunata*, and *C. verruculosa*. A total of 9 samples of infected tissue from each host species were incubated in PDA medium. The process led to the isolation of pure colonies of the three pathogenic fungi evaluated under field conditions, namely *B. zea*, *C. lunata*, and *C. verruculosa*. These isolates exhibited the same macroscopic and microscopic morphological characteristics previously reported from maguey, rice, oil palm, and tule plants [34,125,132,134,158]. Subsequently, each isolate was genetically characterized to confirm the identity of each pathogen recovered from the plants of *A. salmiana*, *A. lechuguilla*, and *Z. mays* (Table 7).

Table 7. Molecular identification of the causal agents of leaf spots in maguey and corn.

Strain	Strain Accession	Specie	Host	Query Cover (%)	Accession Closest Hit	Reference
JCPN34	PV875568	<i>C. lunata</i>	<i>A. salmiana</i>	100	OR047534	Cuervo-Parra et al. [159]
JCPN35	PV875569	<i>C. verruculosa</i>	<i>A. salmiana</i>	100	OR047538	Cuervo-Parra et al. [160]
JCPN36	PV875570	<i>B. zea</i>	<i>A. salmiana</i>	100	MT505867	Visagie et al. [79]
JCPN37	PV875624	<i>C. lunata</i>	<i>A. lechuguilla</i>	100	OR047534	Cuervo-Parra et al. [159]
JCPN38	PV875625	<i>C. verruculosa</i>	<i>A. lechuguilla</i>	100	OR047538	Cuervo-Parra et al. [160]
JCPN39	PV875626	<i>B. zea</i>	<i>A. lechuguilla</i>	100	MT505867	Visagie et al. [79]
JCPN40	PV875637	<i>C. lunata</i>	<i>Z. mays</i>	100	OR047534	Cuervo-Parra et al. [159]
JCPN41	PV875638	<i>C. verruculosa</i>	<i>Z. mays</i>	100	OR047538	Cuervo-Parra et al. [160]
JCPN42	PV875639	<i>B. zea</i>	<i>Z. mays</i>	99	MT505867	Visagie et al. [79]

The results of pairwise sequence alignment using the NCBI BLAST program for the 9 DNA sequences obtained from infected tissues of *A. salmiana*, *A. lechuguilla*, and *Z. mays* plants showed identity percentages of 99 to 100% with sequences from closely related fungal accessions in the GenBank database. Isolates were then identified to the species level using the ITS1/5.8s/ITS2 rRNA region, and the data were deposited in GenBank (Table 7). Based on molecular characterization and the fulfillment of Koch's postulates with the fungi isolated from leaf spots on infected maguey and corn tissues, the pathogenicity of *B. zea*, *C. lunata*, and *C. verruculosa* was confirmed as the causal agents of leaf spot symptoms in the maguey and corn plants analyzed in this work. This is also the first report of these Helminthosporium complex fungi species as pathogenic microorganisms associated with *A. salmiana* ssp. *ferox* and *A. lechuguilla* plants in field conditions.

3.3. In Vitro Antagonism of Trichoderma Against Pathogenic Fungi in Agave

The antagonistic effect of *T. asperellum* strain JEAB02 on pathogenic fungi isolated from *A. salmiana* and *A. lechuguilla* plants in this study was assessed through dual culture confrontation experiments. BCI values were calculated from digital images taken 7 days after confronting all tested pathogenic strains. The interaction of *T. asperellum* strain JEAB02 in the *in vitro* antagonism experiment resulted in significantly different levels of inhibition of the *Agave* phytopathogens tested. The most susceptible isolate was the strain JCPN27, with 100% growth inhibition. It was followed by isolates from strains JCPN18, JCPN24, JCPN28, JCPN29, JCPN31, and JCPN33, which showed growth inhibition

percentages ranging from 99.81% to 99.98%. In contrast, isolates from strains JCPN16 and JCPN26 exhibited the lowest inhibition values (Table 8).

Regarding this topic, the ability of fungi to grow rapidly, compete for space and nutrients with antagonistic fungi, and coexist with phytopathogenic microorganisms is an advantageous trait when using them as biological control agents [124,161]. Although the JEAB02 strain of *T. asperellum* grew quickly in most Petri dishes, the BCI values recorded against the different strains of pathogenic fungi tested ranged from 89.55 to 100%. In this context, studies evaluating the genetic variations observed in isolates of the same species from different geographical regions have reported that the spatiotemporal distribution of a species across a large geographical area could explain the differences in virulence observed between strains of the same fungal species [162–164]. This information is particularly relevant when considering that most of the fungal species reported in this work have a global distribution [36,85,112,148,165–171].

Table 8. *Trichoderma asperellum* biocontrol index against *Agave* pathogenic fungi.

Pathogenic Fungi	Pathogenic Strain	<i>Trichoderma</i> Strain	BCI $\epsilon \pm$ SE *
<i>Penicillium diversum</i>	JCPN27	JEAB02	100 $\pm$ 0.0 a
<i>Curvularia lunata</i>	JCPN18	JEAB02	99.98 $\pm$ 0.01 b
<i>Bipolaris zeae</i>	JCPN33	JEAB02	99.89 $\pm$ 0.08 b
<i>Curvularia verruculosa</i>	JCPN24	JEAB02	99.86 $\pm$ 0.07 b
<i>Alternaria alternata</i>	JCPN29	JEAB02	99.81 $\pm$ 0.006 b
<i>Aspergillus oryzae</i>	JCPN31	JEAB02	99.81 $\pm$ 0.007 b
<i>Epicoccum sorghinum</i>	JCPN28	JEAB02	99.81 $\pm$ 0.006 b
<i>Fusarium lactis</i>	JCPN16	JEAB02	96.01 $\pm$ 0.25 c
<i>Myrmaecium rubricosum</i>	JCPN26	JEAB02	89.55 $\pm$ 0.97 d

* Equal letters in the same column indicate that the means did not differ significantly ($p < 0.05$, ANOVA and Tukey tests) at a confidence level of 95%; $\pm$ Standard error. ϵ BCI = Percentage of inhibition of the radial growth of the pathogenic fungal colony due to the antagonistic effect of *T. asperellum*.

It has been reported that some pathogenic strains of the genus *Fusarium*, by releasing mycotoxins into the culture medium, can affect genes related to the mycoparasitism process of *Trichoderma* species [172]. These results align with the lower growth inhibition values recorded in the JCPN16 *F. lactis* strain evaluated in this study. Although some variation was observed in the biocontrol effect exerted by the *T. asperellum* strain JEAB02 assessed in this research, most of the pathogenic strains tested showed growth inhibition percentages of 99% or higher, and *Trichoderma* colonized the surface of the pathogenic strains, which were sporulating on them. According to the classification by Ezziyyani et al. [173], the JEAB02 strain can be considered a strong candidate for biological control of pathogenic fungi that attack *Agave* plants.

On the other hand, when comparing the results obtained in this study for the interaction of the *T. asperellum* strain JEAB02 with each pathogenic fungus tested, our data are consistent with reports from other authors on different *T. asperellum* strains for the pathogenic fungi *C. lunata*, *Bipolaris oryzae*, *Fusarium oxysporum* [174,175], *Fusarium* sp. [176], *A. niger* [177], and *Colletotrichum* spp. [178]. Conversely, some studies have reported lower inhibition rates, ranging from 41.73 and 61.66% for different strains of the *Trichoderma* genus when confronted with the *Curvularia eragrostidis* pathogen affecting pineapple crops [179].

Similarly, in studies with other *Trichoderma* species, Arispe et al. [180] reported antagonistic effects greater than 67.74% for strains of *T. harzianum* and *T. longibrachiatum* against several pathogens associated with corn crops, including *Alternaria arborescens*,

Bipolaris shoemakeri, *Bipolaris victoriae*, *Exserohilum sorghinum*, *Exserohilum longirostratum*, *Fusarium brevicatenulatum*, *Penicillium polonicum*, *Phaeocytostroma ambiguum*, and *Fusarium equiseti*. In addition, strains of *T. viride* and *T. harzianum* showed inhibition percentages of 75.04 and 67.83%, respectively, against *A. alternata*, the causal agent of black point disease in wheat [181]. In another experiment, the biocontrol effect of three antagonistic *Trichoderma* strains (*T. asperellum*, *T. harzianum*, and *T. lignorum*) was tested individually and in combination against *F. oxysporum* and *Rhizoctonia solani*, the pathogens responsible for chili wilt. Disease incidence decreased by 31.35–71.34% under field conditions, with the strongest effect observed when all three antagonistic species were applied together [182]. Since there are no previous reports for most of the species isolated in this study, our results provide the first data on the pathogenicity of these fungal species in *A. salmiana* and *A. lechuguilla* plants in Hidalgo, Mexico.

4. Conclusions

Pathogenicity assays supported by Koch's postulates demonstrated that leaf spot disease in *A. salmiana* and *A. lechuguilla* is caused by fungal isolates belonging to the genera *Curvularia* and *Bipolaris*, while molecular characterization confirmed the taxonomic identity of these pathogenic species within the group of nine fungal species isolated from field samples. This disease is currently widespread across several municipalities in Hidalgo, Mexico. To date, two *Curvularia* species (*C. lunata* and *C. verruculosa*) and one *Bipolaris* species (*B. zeae*) have been identified as responsible for leaf spots on maguey and corn plants. Notably, this study represents the first report of *C. lunata* and *C. verruculosa* infecting *A. lechuguilla* in Mexico. Among the three species, *B. zeae* is the most prevalent, occurring in the greatest number of municipalities, while *C. verruculosa* exhibits the highest virulence in the field pathogenicity experiments. Dual culture confrontation assays demonstrated that *Trichoderma asperellum* strain JEAB02 is an effective biological control agent against these leaf spot pathogens. Consequently, this strain could be employed to manage leaf spot disease in commercial *A. salmiana* plantations in Mexico.

Author Contributions: Conceptualization, J.A.C.-P. and J.E.A.-B.; methodology, J.A.C.-P.; software, J.A.C.-P.; validation, J.A.C.-P., J.E.A.-B., M.M.A.-O. and T.R.-C.; formal analysis, J.A.C.-P.; investigation, J.A.C.-P., J.E.A.-B. and T.R.-C.; resources, J.A.C.-P., J.E.A.-B. and T.R.-C.; data curation, J.A.C.-P. and J.E.A.-B.; writing—original draft preparation, J.A.C.-P.; writing—review and editing, J.A.C.-P., J.E.A.-B., M.M.A.-O. and T.R.-C.; visualization and supervision, J.A.C.-P. and J.E.A.-B.; project administration, J.A.C.-P. and J.E.A.-B. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Data Availability Statement: Data are contained within the article.

Acknowledgments: All the authors thank the staff of the Escuela Superior de Apan-UAEH for providing all the facilities needed to carry out the field and laboratory experiments. We also appreciate M.M. Armendáriz-Ontiveros's support in checking and improving the English language of the final manuscript.

Conflicts of Interest: The authors declare no conflicts of interest, and the funders had no role in designing the study, collecting, analyzing, or interpreting data, writing the manuscript, or deciding to publish the results.

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