Abanico Boletín Técnico. ISSN: 3061-8452.
https://abanicoacademico.com/abanicoboletintecnico/index
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Abanico Boletín Técnico. Janaury-December, 2026; 5:1-13. Code: e2025-5.
Original Research. Received:23/08/2024. Accepted:10/02/2026. Publish:25/02/2026.
https://doi.org/10.21929/abanicoboletin/2026.4

Pathogenic potential of *Helminthosporium* sp. in chili (*Capsicum annumm*) and zucchini (*Cucurbita pepo*) seeds

Potencial patogénico *Helminthosporium* sp. en semillas de chile (*Capsicum annumm*) y calabacín (*Cucurbita pepo*)

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ABSTRACT

Mexico is center of origin and domestication for key crops such as chili peppers and zucchini, which are fundamental to agriculture and are part of the culture, especially in Mexican Indigenous communities. These communities are among the affected by the Mexican agri-food system and constitute 85% of small and medium-scale producers working in agriculture. However, these crops face threats from pathogenic microorganisms that affect their production. Therefore, the objective of this research is to determine the pathogenic potential of *Helminthosporium* sp. in chili pepper and zucchini seeds using *in vitro* and *in vivo* assays. The fungus under study was cultured, isolated, and purified in the laboratory of the General Local Livestock Association of Tapachula AC. An in vitro plate assay was performed to quantify the conidia. Dilutions with different concentrations of the fungus were prepared and applied by spraying to chili pepper and zucchini seeds. The incidence of *Helminthosporium* sp. was found. The concentration of $1x10^6$ was 10%, and differences were found between treatments ($p<0.05$), indicating that the *Helminthosporium* sp. treatments did not affect the physiological development of the seeds. This suggests that there is no risk to embryo imbibition, adaptability, survival, growth, or reproduction of chili peppers and zucchini.
Keywords: polyculture, antagonistic symbiosis, germination, fungal disease.

RESUMEN

México es reconocido como centro de origen y domesticación de diversos cultivos de importancia económica y cultural, entre los que destacan el chile (*Capsicum sp.*) y el calabacín (*Cucurbita pepo*). Estos cultivos constituyen una parte esencial de la agricultura y la identidad alimentaria nacional, especialmente en comunidades indígenas que representan aproximadamente el 85 % de los productores de pequeña y mediana escala del país. No obstante, ambos cultivos enfrentan amenazas fitosanitarias ocasionadas por microorganismos patógenos que pueden comprometer su productividad y calidad. El presente estudio tuvo como objetivo evaluar el potencial patogénico de *Helminthosporium* sp. en semillas de chile y calabacín mediante ensayos in vitro e in vivo. El aislamiento, cultivo y purificación del hongo se llevaron a cabo en el laboratorio de la Asociación Ganadera Local General de Tapachula A.C. En los ensayos in vitro, se

cuantificaron los conidios del hongo en placa y se prepararon diluciones a diferentes concentraciones. Estas suspensiones fueron aplicadas por aspersión sobre las semillas de ambos cultivos. Los resultados indicaron una incidencia de infección del 10 % a la concentración de 1×10^6 conidios. Se detectaron diferencias significativas entre tratamientos ($p < 0.05$); sin embargo, la presencia de *Helminthosporium* sp. no afectó significativamente el desarrollo fisiológico de las semillas. Estos hallazgos sugieren que el hongo no representa un riesgo relevante para los procesos de imbibición del embrión, ni para la adaptabilidad, supervivencia, crecimiento o reproducción de las plantas de chile y calabacín.
Palabras clave: policultivo, simbiosis antagónica, germinación, fungosis.

INTRODUCTION

Mexico is a center of origin, diversity, and domestication for several crops of agricultural and nutritional importance, including chili pepper and squash, which are widely cultivated in the region. These crops are deeply embedded in Mexican culture and constitute a fundamental component of Indigenous agricultural systems. Indigenous peoples represent one of the most significant sectors within the Mexican agri-food system (Boege & Chan, 2008), accounting for approximately 85% of small- and medium-scale farmers engaged in agricultural production (SADER, 2022). The states with the largest Indigenous populations are: Oaxaca (165,186), Chiapas (141,499), Veracruz (644,559), Puebla (601,680), Yucatán (537,516) and Guerrero (456,774) (INPI, 2022).

Tradicional agricultural systems of indigenous peoples combine polycultures (maíze, beans, squash, chili peppers, and other crops) with sustainable practices such as natural fertilizers, water conservation through terracing or canals, and no-till agriculture to enhace biodiversity. Polycultures reduce the likelihood of pest's infestations because they act as physical barriers and disorient insects through the changes in odor and color produced by the different plant species grown together (Leyva *et al*., 2021). Planting different crop species within the same field positively influences biodiversity through the presence of microorganism associated with each crop and the overall ecological balance achieved through interactions among the accompanying flora and fauna. Increasing biodiversity enhances system stability; therefore, the rate of pest spread and population growth is lower than under monocultures conditions. In addition, biodiversity contributes to reducing the economic and environmental costs associated with conservation and resource use, promote nutrient recycling, regulates the local microclimate, decreases pest's populations, conserves soils and water resources, and helps eliminate environmental contaminants. These benefits reduce the need for chemical inputs and the costs associated with purchasing potentially polluting agrochemicals (Ebel *et al*., 2017; Gómez & Gómez - Gonzales, 2006).

Chili pepper and zucchini are crops commonly included in the human diet; however, both are susceptible to fungal diseases (Perales & Aguirre, 2008). Chili pepper (*Capsicum annuum*) is a crop of great importance in Mexico, with a production of 3,324,260 tons in 2022 (GOB, MX). Nevertheless, its production is affected by fungal diseases caused by genera such as *Phytophthora*, *Fusarium*, *Alternaria*, and *Rhizoctonia*, which represent a serious threat to chili cultivation, causing significant yield losses and jeopardizing food security (Velásquez *et al*., 2007).

Zucchini (*Cucurbita pepo*) reached a production of 589,802 tons in 2022 (GOB, MX). Fungal diseases affecting zucchini crops are caused by genera such as *Phytophthora*, *Botrytis*, *Rhizopus*, *Fusarium*, powdery mildew, and *Colletotrichum* (Li *et al*., 2018; Keinath & DuBose, 2012).

These pathogenic fungi are known to cause damage at different stages of crop development, including seed storage, germination, seedling establishment, vegetative growth, and the reproductive phase. Infected seeds may fail to germinate and can serve as sources of disease transmission from seed to seedling and subsequently to growing plants (Ora *et al*., 2015). Fungi produce spores that remain viable until environmental conditions become favorable for their multiplication. Consequently, fungal pathogens are ubiquitous in nature and can be found in the air, soil, and water. As a result, crops grown in the Soconusco region of Chiapas are frequently affected, leading to crop losses that negatively impact both food availability and the local economy (Urbina, 2011).

The genus *Helminthosporium* comprises a group of mitosporic fungi that primarily infect cereal crops such as rice, wheat, and barley. It is also known to affect grasses and forage species, causing a disease commonly referred to as helminthosporiosis, which is characterized by necrotic lesions that can be observed on seedlings and leaves of infected plants (Khan *et al*., 2023; Imrani *et al*., 2017). *Helminthosporium* can be found in temperate, cold, and tropical climates, demonstrating its adaptability to a wide range of environmental conditions. Consequently, it is tolerant to high temperatures, has a broad geographical distribution, and its growth is favored by high humidity levels (Sanzo *et al*., 2023). In Mexico, particularly in the tropical region of Soconusco, Chiapas, relative humidity ranges from 60% to 80%, with an average annual temperature of 31 °C (SMN, 2023). These environmental conditions favor the proliferation of microorganisms and consequently increase the presence of pathogens, including viruses, bacteria, and fungi, in crops. Such pathogens can affect grasses and legumes that are commonly used as pastures and forage resources (Zayas *et al*., 2006).

Fungi belonging to the genus *Helminthosporium* include species such as *H. sativum*, which affects wheat, oat, and barley crops. This pathogen causes root rot and infects grains endophytically, resulting in either latent infections or black point disease. The black

discoloration of infected seeds reduces their germination capacity (Abdrassulova *et al.*, 2014). *Helminthosporium oryzae* produces spores that are transmitted through seeds and infect seedlings through the roots shortly after emergence. This pathogen negatively affects seed viability by causing seedling wilting and poor germination. It can also transmit diseases from seeds to seedlings and subsequently to growing plants, reducing rice yield and grain quality by up to 90% (Patricio, 2010). *Helminthosporium solani* primarily infects potato tubers through the lenticels of the periderm. The occurrence of silver scurf disease caused by this fungus can reduce potato quality by as much as 80% (Hoffman, 2009).

Currently, it is unknown whether a fungus belonging to the genus *Helminthosporium* can infect chili pepper and zucchini seeds. Plant pathogenic fungi generally exhibit host specificity, having evolved to infect only certain plant species through long-term host–pathogen interactions. Therefore, a fungal pathogen may affect a particular crop due to its natural affinity and adaptation to that host plant. The *Helminthosporium* sp. evaluated in this study was isolated from a mangrove ecosystem and a cacao plantation located in the tropical region of Soconusco, Chiapas, Mexico. Therefore, the objective of this study was to evaluate the pathogenic potential of *Helminthosporium* sp. on chili pepper (*Capsicum annuum*) and zucchini (*Cucurbita pepo*) seeds.

MATERIALS AND METHODS

Study area

The study was conducted in Tapachula de Córdova and Ordóñez, Chiapas, Mexico, at the Laboratory of the Asociación Ganadera Local General de Tapachula A.C., located at 7th North Avenue and 17th East Avenue No. 84, C.P. 30700 (14°54′13″ N, 92°15′26″ W), and at the Universidad Politécnica de Tapachula, located on the Tapachula–Puerto Madero Highway, Km 24, Tapachula, Chiapas, Mexico, postal code 30830.

Biological Material

Chili pepper (*Capsicum annuum*) and zucchini (*Cucurbita pepo*) seeds were obtained in Tapachula, Chiapas (14°55’01.7” N 92°15’59.5” W). The Helminthosporium sp. fungal strain was obtained from the culture collection of the Tapachula General Livestock Association Laboratory.

Reactivation of Helminthosporium spp.

The Helminthosporium sp. strain was reactivated on PDA medium (MCD Lab) at pH 6.5. This was done using two Bunsen burners under aseptic conditions and a bacteriological loop to collect a portion of the colony and subculture it onto another Petri dish. The dish was then incubated at 25°C for 7 days, as described by Rasyad *et al*., (2019).

Germination Tests

Ten seeds were used for each of the three crops under study and placed on Whatman #4 filter paper. Water was sprayed on the seeds every 24 hours. The length, width, thickness, hypocotyl, and epicotyl of the seeds were then measured daily to observe their growth until the emergence of the first pair of leaves. The seeds were observed before watering and after water absorption, noting the size of the epicotyl and hypocotyl in centimeters. In the case of chili peppers, the seeds were dried at 40°C for 2 hours in a drying oven (BiotechMR) to initiate enzymatic activity. Seed germination was then observed prior to the experiments with Helminthosporium sp. to establish a parameter for the number of seeds that germinated in a given batch and confirm their viability, according to the methodology described by Vishunavat *et al*., (2023).

Conidia Quantification in a Neubauer Chamber

One milliliter of sterile distilled water (SDW) was used to prepare the Helminthosporium sp. cultures. The colonies were agitated using glass beads to detach the mycelium from the culture medium. The resulting suspension was then filtered into a sterile beaker and transferred to a previously sterilized test tube. The suspension was filtered again using Whatman No. 4 filter paper to remove any remaining agar or mycelial fragments that could obstruct the flow of the suspension during spray inoculation, following the methodology described by Lemus *et al*. (2008). For conidia counting, a 1-milliliter aliquot of the suspension was taken with a micropipette and placed in the center of a Neubauer chamber. The sample was covered with a coverslip and observed under a light microscope at 40× magnification, counting the conidia present in the corresponding field. The following formula was used with the data obtained:

$$\frac{\text{X counted spores}}{\text{Y squares}} \times \frac{\text{No. camara pictures}}{\text{camera volume}} \times \frac{1000\ \text{mm3}}{1\ \text{cm3}} = \text{UFC/ml}$$

The averages for CFU/mL of each treatment were determined; this was multiplied by a constant that depends on the chamber to be used, and this product was adjusted to obtain the concentrations 1x105,6,7 in conidia/mL.

Obtaining the supernatant

Part of the colony was taken with a bacteriological loop, to inoculate it in a potato dextrose broth medium at pH 6.5, it was left to incubate for 7 days and then centrifuged at 5000 rpm for 5 min in Falcon tubes to obtain the supernatant with a volumetric pipette (1mL, 500µl and 250µl).

In vivo tests

The three concentrations that showed the best results in the in vitro tests were subjected to in vivo trials. For this purpose, 3 seeds for corn, 5 for chili peppers, and 3 for zucchini were sown at a depth of 4 cm for corn and 2 cm for chili peppers and zucchini, in 2 kg nursery bags. The results of the highest pathogenicity from the in vitro test were obtained in vivo, using peat, perlite, and sterilized vermiculite as the substrate for the pots.

Experimental design

A completely randomized design with a 2x2x3 factorial arrangement was used. Twelve Petri dishes per treatment and three controls were used for seed germination. All Petri dishes were lined with Whatman #4 filter paper to absorb the fungal concentrations, which were sprayed using spray bottles (John Bee). The distribution is shown in Table 1. The tests were performed in triplicate, and the incidence of Helminthosporium sp. was calculated using the following formula.

$$\text{Incidence} = \frac{\text{number of infected seeds} \times 100}{\text{number of seeds on plate}}$$

Table 1. Distribution of treatments

Treatments, Conidia spores/ml	Chili (*Capsicum annuum)*	zucchini (*Cucurbita pepo)*
1	1x10*5	1x10*5
2	1x10*6	1x10*6
3	1x10*7	1x10*7
Supernatant Treatments	Chili (*Capsicum annuum)*	zucchini (*Cucurbita pepo)*
1	1mL	1mL
2	500µl	500µl
3	250µl	250µl
Control	0	0

Experimental desing for *in vivo* test

A completely randomized experimental design with a 2x3 factorial arrangement was used, and a mean comparison (Tukey α=0.05) was performed using the InfoStatMR2022 software.

RESULTS AND DISCUSION

Figures 1 and 2 show the germination of chili pepper and zucchini seeds. In the zucchini seed model, a germination rate of 100% was observed, with epicotyl lengths ranging from 0.4 to 1.4 cm and hypocotyl lengths ranging from 2.4 to 5.1 cm. In the chili pepper seed model, an 80% germination rate was obtained, with epicotyl lengths ranging from 0.8 to 1.9 cm and hypocotyl lengths between 2.4 and 4.3 cm. Our results are consistent with those reported by Sánchez – Pérez *et al.*, (2010) in corn, , for chili seeds with Prado–Urbina *et al*., (2015) and Lima-Lana *et al*., (2020) in zucchini seeds.

Figure 1. Germination of zucchini seeds up to the fourth day

Figure 2. Germination of chili seeds on days 7 and 12

Figures 3 and 4 present the means of the replicates for the treatments with and without the fungus. It was observed that, for the variables of seed length and width in zucchini, the presence of *Helminthosporium* sp. did not affect the imbibition process nor the initial stage of germination. In this regard, Lima-Lana *et al*., (2020) reported that when seeds do not suffer any damage and are not affected by microbial infections, the enzymatic and metabolic processes involved in germination are not altered.

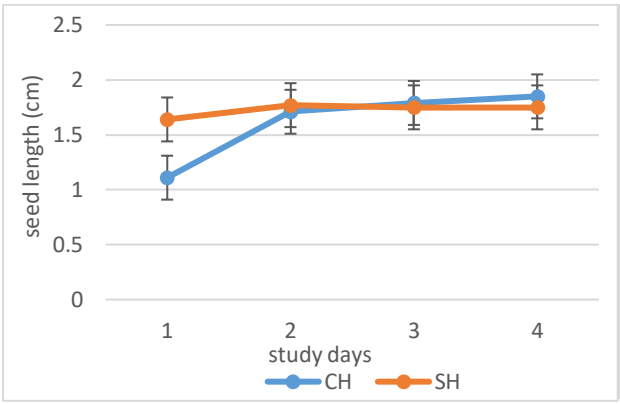

Figure 3. Effect of fungus on zucchini seed length. Note**:** CH = With fungus, SH = Without fungus.

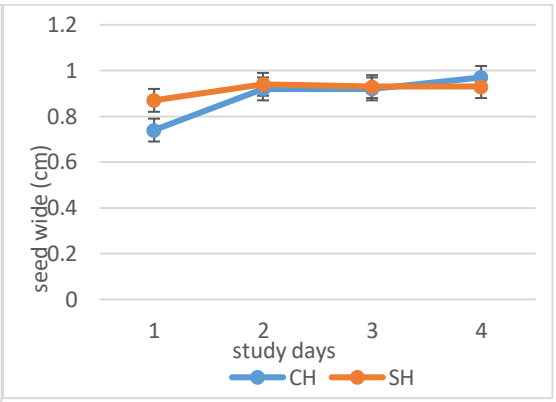

Figure 4. Effect of fungus on zucchini seed width. Note**:** CH = With fungus, SH = Without fungus.

Figures 5 and 6 show that the hypocotyl and epicotyl length in zucchini seeds, in the presence of *Helminthosporium* sp., did not cause any significant change in root formation, growth, or development. This suggests that this fungus is not potentially pathogenic to this crop at the germination and seedling emergence stages. These results differ from those reported by Khan *et al*., (2023), who observed damage caused by this fungal genus in susceptible plants, such as cereal crops.

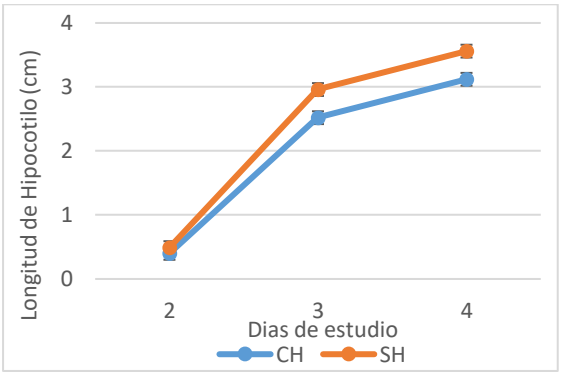

Figura 5. Efecto del hongo en hipocótilo de calabacín. Nota: CH= Con hongo, SH= Sin hongo.

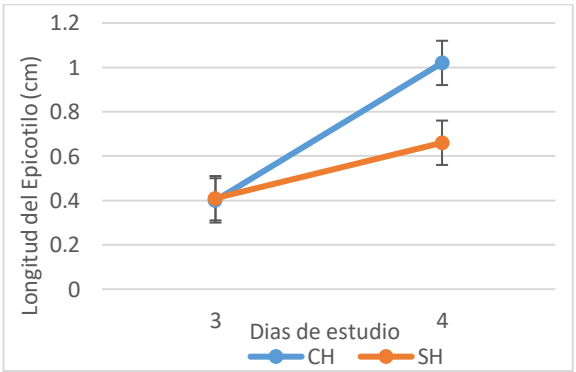

Figura 6. Efecto del hongo en epicótilo de calabacín. Nota: CH= Con hongo, SH= Sin hongo.

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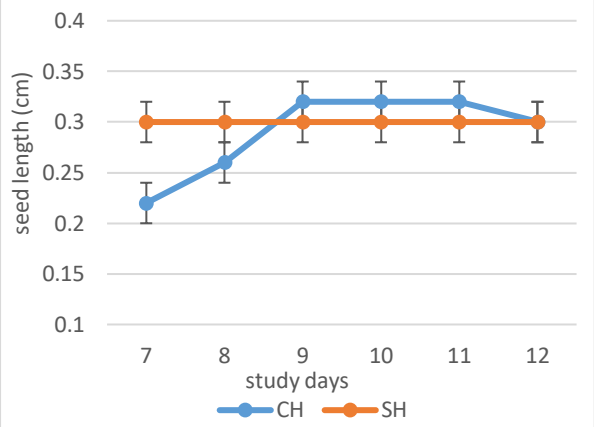
Figures 7 and 8 show the values obtained for the length and width of the chili seeds, noting that the presence of the fungus *Helminthosporium sp* did not affect the germination of the chili seeds.



F Figure 7. Effect of fungus on chili seed length. Note: CH = With fungus, SH = Without fungus.

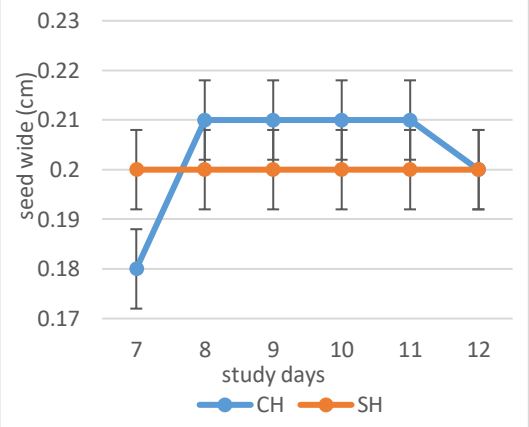


Figura 8. Efecto del hongo en ancho de semilla de chile. Nota: CH= Con hongo, SH= Sin hongo.

Figures 9 and 10 show that the treatment applied with the fungus *Helminthosporium sp*. did not have a detrimental effect on the length of the hypocotyl and epicotyl in chili seeds. This suggests that, as in the zucchini model, the fungus does not have the potential to act as a phytopathogen in chili seeds.

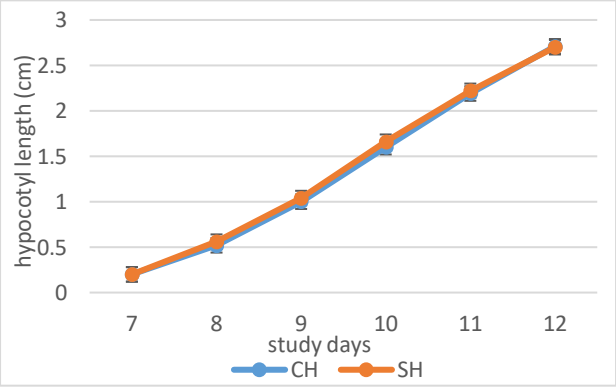


Figure 9. Effect of the fungus on the hypocotyl of chili pepper. Note: CH= With fungus, SH= Without fungus.

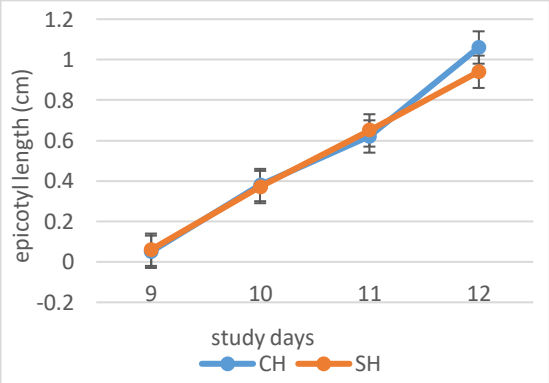


Figure 10. Effect of the fungus on the epicotyl of chili pepper. Note: CH= With fungus, SH= Without fungus.

The results presented show that no negative effects were observed on root water imbibition, embryo development, or stem germination in chili and Zucchi. Our results agree Lima–Lanna *et al*., (2020), but contrast with those reported by Khan *et al*., (2023), and Abdrassulova *et al.*, (2010), who found that *Helminthosporium sativum* affected wheat, oat, and barley seeds, causing root rot and a loss of germination capacity in affected seeds.

CONCLUSION

The fungus Helminthosporium did not inhibit seed germination, embryo development, epicotyl and hypocotyl growth, or root generation and development in chili pepper and zucchini seeds. This fungal strain does not pose a phytopathogenic threat to these two crops.

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