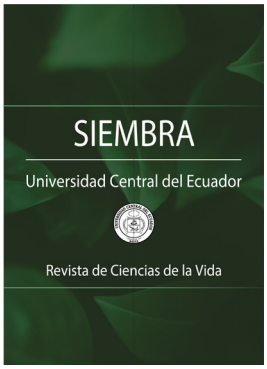
Synergy between biofumigation with Indian mustard and the antagonist fungus Trichoderma harzianum for the control of Phomopsis spp., under in vitro conditions



Sinergia entre la biofumigación con mostaza india y el hongo antagonista Trichoderma harzianum para el control de Phomopsis spp., bajo condiciones in vitro

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Abstract

This work aimed to evaluate the combined effect of two pest biocontrol strategies: biofumigation with *Brassica juncea* and inoculation of *Trichoderma harzianum*, on the *in vitro* growth of the pathogen *Phomopsis* spp., an etiological agent of canker disease in pecan crops (*Carya illinoinensis*). *B. juncea* plants were crushed and placed in polystyrene recipients in doses of 10 and 30 g. Then, a Petri dish was introduced to each recipient, with a disk with a grown mycelium of *Phomopsis* spp. or *T. harzianum,* or both fungi (dual culture). Treatments were incubated in a culture chamber. The parameters evaluated were colonies' surface area and percentage of mycelial inhibition of *Phomopsis* spp. Data were analyzed with non-parametric statistics using the Kruskal-Wallis test. The results indicated that: i) *B. juncea* did not affect the growth of *T. harzianum*; ii) the 30 g dose of *B. juncea* completely suppressed the growth of *Phomopsis* spp.; iii) *T. harzianum* significantly inhibited the growth of *Phomopsis* spp. colonies; iv) the combination of *T. harzianum* and biofumigation with 10 g of *B. juncea* showed synergy on the growth control of *Phomopsis* spp. Biofumigation with *B. juncea*, the application of *T. harzianum,* and the combination of both techniques could be promising alternatives for the biological control of *Phomopsis* spp.

Keywords: biocontrol, antagonism, *Brassica juncea, Carya illinoinensis*.

Resumen

El objetivo de este trabajo fue evaluar el efecto combinado de dos tácticas de biocontrol de plagas: la biofumigación con *Brassica juncea* y la inoculación de *Trichoderma harzianum*, sobre el crecimiento *in vitro* del patógeno *Phomopsis* spp., agente etiológico de la cancrosis en el cultivo de pecán (*Carya illinoinensis*). Se trituraron plantas de *B. juncea* y se colocaron en envases de poliestireno en dosis de 10 y 30 g. Luego, en cada uno de estos recipientes se introdujo una caja de Petri con un disco con micelio de *Phomopsis* spp. o *T. harzianum* o ambos hongos (cultivo dual). Se incubó en cámara de cultivo. Los parámetros evaluados fueron la superficie de las colonias y el porcentaje de inhibición miceliar de *Phomopsis* spp. Los datos se analizaron con estadística no paramétrica

mediante la prueba de Kruskal-Wallis. Se obtuvieron los siguientes resultados: i) *B. juncea* no afectó el crecimiento de *T. harzianum*; ii) la dosis de 30 g de *B. juncea* suprimió completamente el crecimiento de *Phomopsis* spp.; iii) *T. harzianum* inhibió significativamente el crecimiento de las colonias de *Phomopsis* spp.; iv) la combinación de *T. harzianum* y la biofumigación con 10 g de *B. juncea* mostró sinergia sobre el control del crecimiento de *Phomopsis* spp. La biofumigación con *B. juncea*, la aplicación de *T. harzianum* y la combinación de ambas técnicas podrían ser alternativas promisorias para el control biológico de *Phomopsis* spp.

Palabras clave: control biológico, antagonismo, *Brassica juncea*, *Carya illinoinensis*.

1. Introduction

Canker disease caused by *Phomopsis* spp. affects pecan crops (*Carya illinoinensis* [Wangenh.] K. Koch) by inducing sunken and elongated wounds on branches, the root neck and the grafted zone between the scion and the rootstock, which can lead to plant wilting (Noelting et al., 2016b; Perniola et al., 2023). The disease was first reported in April 2024 in pecan orchards in the province of Buenos Aires, Argentina (Noelting et al., 2016a).

Preliminary *in vitro* studies were conducted to evaluate the biofumigation technique using Indian mustard (*Brassica juncea* L. Czerniak) and inoculation with the biocontrol fungus *Trichoderma harzianum* Rifai as environmentally friendly alternatives to prevent the disease caused by *Phomopsis* spp. These two agroecological techniques were selected for their potential use in disinfecting planting substrates at the nursery stage, aiming to prevent infections during the early growth stages of pecan.

Biofumigation was used as a biological control technique, and it consists of the release of substances originating from the decomposition of organic materials, called "biofumigants", into the soil (Gimsing & Kirkegaard, 2006). These biofumigants are a combination of agro-industrial and post-harvest residues, manure, fresh Brassicaceae and Poaceae plants, and others. When the biofumigant comes into contact with the soil, a series of chemical reactions are triggered, producing substances with biocidal activity: sulfur compounds, acetic acid, ammonium, and others. Likewise, if the biofumigant is made of fresh Brassicaceae plant residues (or an industrial by-product of the same origin), its decomposition produces different types of isothiocyanates with a variable degree of toxicity in soil organisms (Gowers, 2008; Harding & Wicks, 2001; Santos et al., 2021; Sarwar et al., 1998; Vandicke et al., 2020). This effect results from the degradation of glucosinolate compounds (present in some cells of Brassicaceae plants), which is catalyzed by myrosinase enzymes located in neighboring cells and come into contact with the glucosinolates after cell rupture (Chhajed et al., 2020; Kissen et al., 2009).

Indian mustard (*B. juncea*) is one of the most studied Brassicaceae plant species for biofumigation due to its fungicidal activity against various phytopathogenic fungi: *Rhizoctonia solani* Kühn (Abdallah et al., 2020; Baysal-Gurel et al., 2020), *Pythium ultimum* Trow, *Fusarium sambucinum* Fuckel, *Sclerotinia sclerotiorum* (Lib.) de Bary (Larkin & Griffin, 2007), *Fusarium oxysporum* Schlechtend.: Fr. f. sp. *lycopersici* (Sacc.) W.C. Snyder & H.N. Hansen (Mayton et al., 1996), *Verticillium dahliae* Kleb. (Debiase et al., 2008; Michel & Lazzeri, 2008), *Phytophthora capsici* Leonian (Mason et al., 2023), *Sclerotium rolfsii* Sacc. (Chorzempa et al., 2019; Garain et al., 2021), *Fusarium graminearum* Schwabe (Perniola et al., 2012; 2021), *Phytophthora nicotianae* Breda de Haan (Baysal-Gurel et al., 2020), among others.

The second biological control technique used in this study is the antagonism activity of *Trichoderma* spp. The use of this fungus as a biocontrol agent for pests is a widely studied agroecological tactic, due to its ease of cultivation and broad range of control over phytopathogens (Kullnig-Gradinger et al., 2002; Whipps, 2001) as a result of multiple mechanisms of action, such as mycoparasitism, competition for nutrients and space, antibiotic production, and induction of plant resistance (Guzmán-Guzmán et al., 2023; Poveda, 2021; Tyśkiewicz et al., 2022). Among the most effective biocontrol species is *T. harzianum*, with demonstrated action against numerous fungi: *R. solani* (Almeida et al., 2007), *Fusarium solani* (Mart.) Sacc. (Erazo et al., 2021), *Fusarium sudanense* SA Ahmed, Al-Hatmi & de Hoog (Larran et al., 2020), *Phytophtora nicotianae* Breda de Haan (Stefanova et al., 2004), *Colletotrichum dematium* (Pers. ex Fr.) Grove (Shovan et al., 2008), *Fusarium. oxysporum* f. sp. *asparagi* S.I. Cohen & Heald (Arriola et al., 2000), *F. oxysporum* f. sp. *lycopersici* (Zehra et al., 2017), *Alternaria cerealis* MT80847 (Mahmoud et al., 2021), *Alternaria burnsii* Uppal, Patel & Kamat, *Fusarium oxysporum* f. sp. *cumini* (Foc) (Deepak et al., 2008), *Fusarium ipomoeae, Fusarium oxysporum* Schlechtend: Fr., *F. solani*, *Penicillium citrinum* Thom, *Penicillium rotoruae* O'Callahan & Vaidya, *Asper-*

gillus wentii Wehmer*, Mucor variicolumellatus* L. Wagner & G. Walther, *Macrophomina phaseolina* (Tassi) Goid. (Paul et al., 2021), *Colletotrichum truncatum* (Schweinitz) Andrus & WD Moore (Yadav et al., 2021), among other pathogens.

Regarding the combined use of biofumigation and *Trichoderma* spp., Kirkegaard and Matthiessen (2004) determined that low concentrations of isothiocyanates are required to achieve fungistatic action against pathogens like *Pythium* spp. or *Sclerotinia* spp., but to inhibit *Trichoderma* spp. growth, doses thirty times higher are needed. Previous studies have demonstrated that biofumigation with *B. juncea* supresses the *in vitro* growth of *Phomopsis* spp. (Perniola et al., 2023), but it does not affect the antagonist *Trichoderma* spp. (Perniola et. al., 2021). In addition, synergy between the two methods was observed for the *in vitro* control of *F. graminearum* (Perniola et al., 2014).

To determine the feasibility of the combined and simultaneous use of biofumigation with *B. juncea* and the application of *T. harzianum* as part of the integrated agroecological management of pecan canker, it is important to further study the effects of biofumigation on the beneficial fungus and the outcomes of the interaction between the two techniques on *Phomopsis* spp., the causal agent of this disease.

The aim of this study was to evaluate the combined effects of two biocontrol techniques: biofumigation with *B. juncea* and the inoculation of *T. harzianum* on the *in vitro* growth of the pathogen *Phomopsis* spp.

2. Materials and Methods

2.1. Materials

The biofumigant material was obtained from the aerial parts of Indian mustard (*B. juncea* cv. "SCOP-7"), an experimental variety developed in collaboration between the Santa Catalina Phytotechnical Institute, National University of La Plata [IFSC], and the Faculty of Agricultural Sciences, National University of Lomas de Zamora. The *B. juncea* crop was sown in the experimental field of the IFSC, Llavallol, Argentina, in May 2023. The entire aerial part, consisting of the main stem, branches, leaves and siliques were harvested when it reached the end of the fruiting stage (October of the same year).

The *Phomopsis* spp. strain used during this study was isolated from canker lesions found on infected pecan branches and identified and multiplied *in vitro* in the laboratories of the IFSC. The fungus *T. harzianum* was isolated from the soil of the experimental field in the IFSC.

2.2. Procedure

2.2.1 Study of the biofumigant effect of Indian mustard on *Phomopsis* spp. and *T. harzianum*

The evaluation of the biofumigant effect of Indian mustard on *Phomopsis* spp. and *T. harzianum* was based on a widely used methodology for evaluating the action of volatile substances generated by synthetic or natural fungicides (Mayton et al., 1996; Perniola et al., 2014; 2023; Richardson & Munnecke, 1964). The aerial part of the *B. juncea* plants was mowed, cut in shorter pieces of 2 cm of length and shredded in a processor for approximately one minute. The shredded material was placed in polystyrene containers (capacity: 900 mL) in two doses of 10 and 30 g (M10 and M30, respectively). Previously, the strains of *T. harzianum* y *Phomopsis* spp. were multiplied for fourteen and seven days, respectively, in 2 % potato glucose agar medium [PGA] at 25 ± 2 ºC in the dark. Disks of 5 mm in diameter were extracted from the respective fungal cultures using a hole punch and were translocated one by one to Petri dishes with 2 % PGA. Petri dishes with a disk of *Phomopsis* spp. or *T. harzianum* were placed one by one inside the containers, supported on plastic stands, and positioned 2 to 3 cm above the biofumigant. The containers were closed with plastic lids. The same methodology was used for the control treatment but no biofumigant was added.

2.2.2. Determination of the antagonism of *T. harzianum* against *Phomopsis* spp.

The dual culture technique (Morton & Stroube, 1955) was applied to determine the biocontrol potential of *T. harzianum* against *Phomopsis* spp. Two 5 mm-diameter disks of *Phomopsis* spp. and *T. harzianum*, respectively, were placed in Petri dishes with 2 % PAG at 4 cm of distance from each other. The Petri dishes were

placed inside the 900 mL containers, which were then sealed with plastic lids. The same control mentioned in section 2.2.1 was used.

2.2.3. Combination of *T. harzianum* and biofumigation with Indian mustard to control *Phomopsis* spp.

To study the combined effect of *T. harzianum* and biofumigation with Indian mustard on the growth of *Phomopsis* spp., the dual culture technique was combined with the previously described biofumigation technique.

2.2.4. Incubation

The incubation was carried out in a growth chamber at 25 ± 2 ºC and in the dark. The first stage of the assay (biofumigant stage) lasted for seven days, during which the experimental units containing *B. juncea* were exposed to the direct activity of the biofumigant gases. The biofumigant material was removed from the polystyrene containers on the seventh day, and incubation continued until day eleven (post-biofumigant stage) under the same temperature and darkness conditions previously described, with the aim of studying fungal growth after being exposed to the gases.

2.3. Evaluations

The measurements were carried out at the seventh and eleventh days when the biofumigant and post-biofumigant stages were finalized. The diameters of the colonies of both microorganisms and their surfaces were calculated. To evaluate the effect of different biocontrol treatments (biofumigation, *T. harzianum* and their combination) on the growth of the fungal pathogen, the percentage of mycelial inhibition of *Phomopsis* spp. [*I*] was calculated using equation [1] (Rekha et al., 2012, Vincent, 1947), where *C* is the diameter of the control colony of *Phomopsis* spp., and *T* is the diameter of the colony of *Phomopsis* spp. in the biocontrol treatment.

$$I = \frac{C-T}{C} x100 \quad [1]$$

The treatments were considered as "fungal suppressors" when the average colony surface was less than 50% of the control average (Mayton et al., 1996; Perniola et al., 2012).

2.4. Experimental design and statistical analysis

A completely randomized design with five repetitions per treatment was performed in all the procedures. Since the data did not meet the assumptions of normality and homoscedasticity, non-parametric statistics were applied using the Kruskal-Walli's test, and mean separation was performed with the pairwise multiple comparisons test for independent samples. Statistica 7 software was used.

3. Results and Discussions

3.1. Effect of the biofumigation with B. juncea on Phomopsis spp.

At the end of the biofumigation stage in M10, colonies of *Phomopsis* spp. exhibited limited growth, although there was no significant difference compared to the control. In contrast, no growth of *Phomopsis* spp. was observed in M30, and this result significantly differed from those obtained in M10 and the control treatments ($p = 0.0023$; $H = 12.13$) (Table 1; Figures 1A, 1D, 1G). Regarding the post-biofumigation stage, regrowth of *Phomopsis* spp. was observed in M10, whereas no growth was detected in M30 (Table 1; Figures 2A, 2D, 2G).

The biofumigation with 30 g of *B. juncea* completely supressed the growth of *Phomopsis* spp. causing fungal death.

In a previous study evaluating the biofumigation effect of *B. juncea* on *Phomopsis* spp. (Perniola et al., 2023), it was observed the *in vitro* suppression of the fungal growth at doses of 30 and 60 g of *B. juncea.* However, the reduction in growth was less pronounced when the biofumigant material was derived from *B. juncea* crops sown on suboptimal dates, which had lower glucosinolate content. This suggests that the effectiveness

of biofumigation with Indian mustard in reducing *Phomopsis* spp. growth may vary depending on the sowning date of *B. juncea* and its glucosinolate content.

Until this day, there are no other studies on the effect of the biofumigation with *B. juncea* on *Phomopsis* spp. growth.

Table 1. Effect of biofumigation with *B. juncea* on the growth of *Phomopsis* spp. colonies.

Treatment	Average size of the colonies (cm^2) †	
	Biofumigation stage*	Post-biofumigation stage **
Control	63.62 a	63.62 a
B. juncea - 10 g	8.11 (87.25 %) ab	56.64 (10.97 %) a
B. juncea - 30 g	0.00 (100.00 %) b	0.00 (100.00 %) b

† Values in parentheses show the percentage of mycelial inhibition of *Phomopsis* spp. In each column, values with different letters indicate significant differences by Kruskal-Wallis test (* p value = 0.0023; H = *12.13 and ** p value = 0.0018; H = 12.67).*

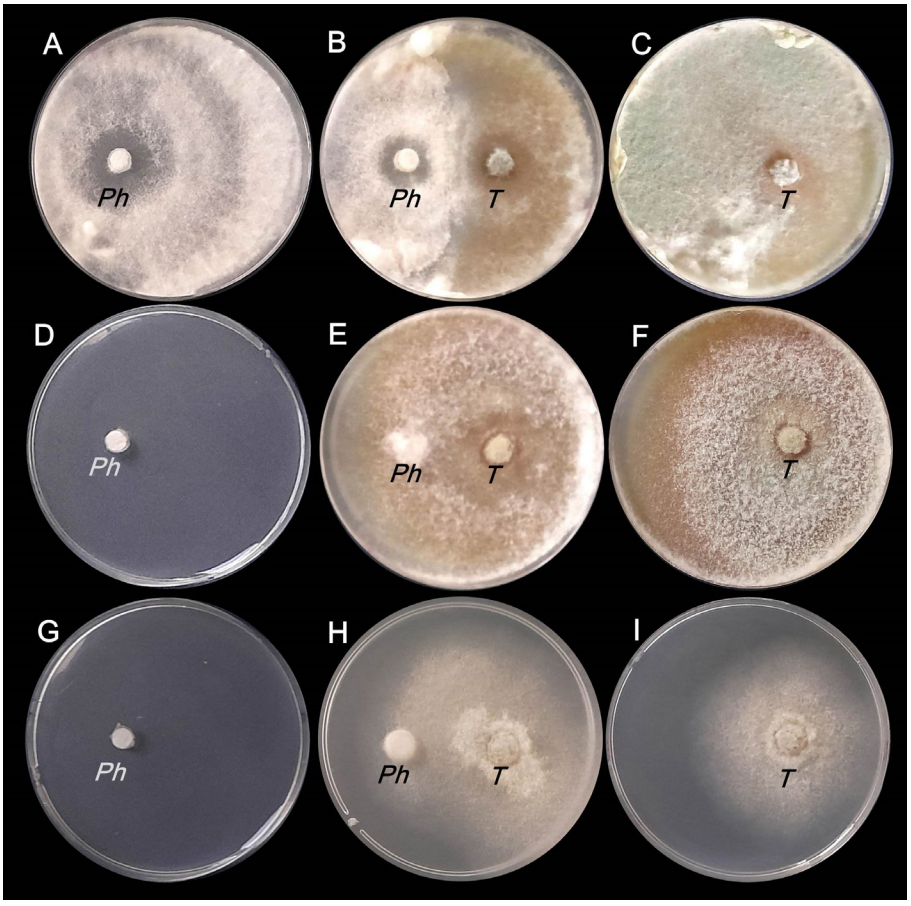

Figure 1. Colonies of *Phomopsis* spp. and *T. harzianum* at the end of the biofumigant stage (day 7 of incubation). A, B, C: in the absence of B. juncea; D, E, F: with 10 g of B. juncea; G, H, I: with 30 g of B. juncea; Ph: *Phomopsis* spp.*;* T: *T. harzianum.*

3.2. Effect of the biofumigation with B. juncea on T. harzianum

At the end of the biofumigant stage for the M10 treatment, the fungus *T. harzianum* had colonized almost the entire Petri dish, with no significant difference compared to the control treatment without fumigant. However, during the M30 treatment there was a significant reduction of the surface of the *T. harzianum* colonies compared to the control and the M10 treatment (*p value* = 0; H = 25) (Table 2; Figure 1C, 1F, 1I). Once the biofumigant was removed, the fungus resumed growth and colonized the entire Petri dish. No significant differences were observed between treatments at the post-biofumigant stage (*p value* = 1; H = 0) (Table 2; Figure 2C, 2F, 2I).

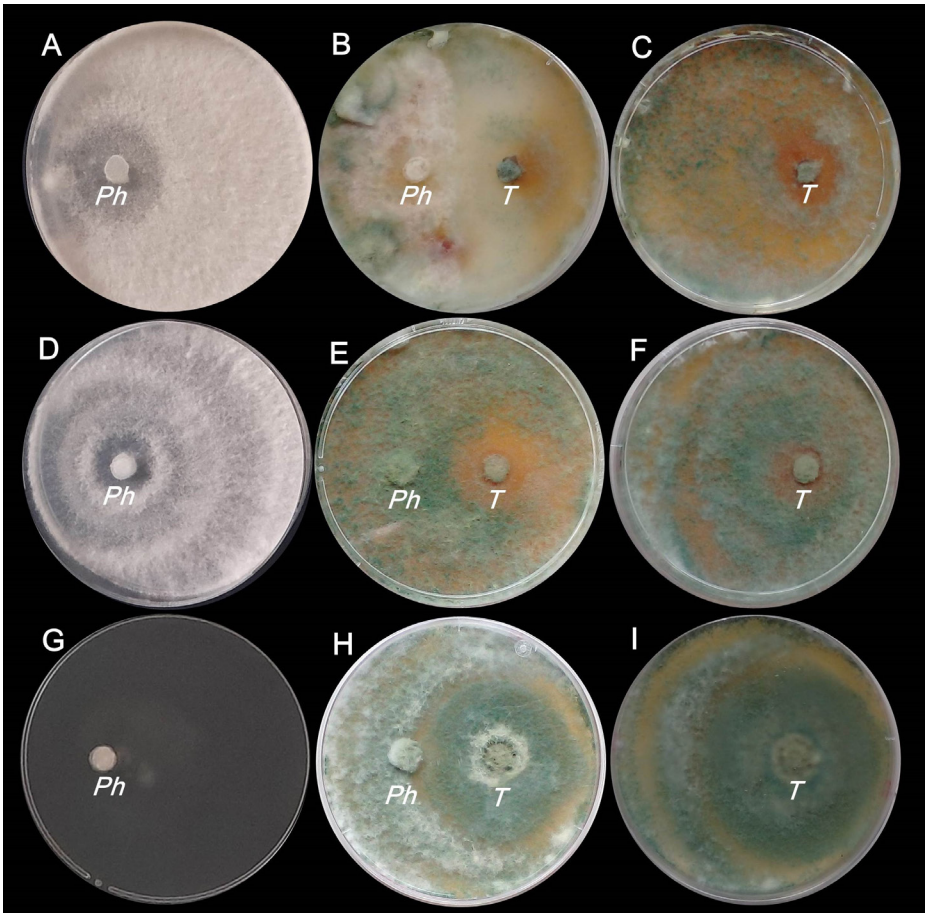

Figure 2. Colonies of *Phomopsis* spp. and *T. harzianum* at the end of the post-biofumigant stage (day 11 of incubation). A, B, C: in the absence of B. juncea; D, E, F: with 10 g of B. juncea; G, H, I: with 30 g of B. juncea; Ph: *Phomopsis* spp.; T: *T. harzianum.*

Table 2. Effect of biofumigation with *B. juncea* on the growth of *T. harzianum* of colonies.

Treatment	Average size of the colonies (cm^2) †	
	Biofumigation stage*	Post-biofumigation stage**
Control	63.62 a	63.62 a
B. juncea - 10 g	62.18 a	63.62 a
B. juncea - 30 g	32.46 b	63.62 a

† In each column, values with different letters indicate significant differences by Kruskal-Wallis test (* p value = 0; H = 25 y ** p value = 1; H = 0).

The results had shown that the biofumigation with 10 g of *B. juncea* did not supress the growth of *T. harzianum* colonies. However, higher doses of 30 g caused a temporal inhibition of the fungal growth without resulting in death. *T. harzianum* had resumed growth once the biofumigation finished.

These results are consistent with those reported in previous studies. Perniola et al. (2014; 2016) observed, in experiments conducted using methodologies comparable to the present study, that the biofumigation with 5 to 55 g of *B. juncea* (at the end of fruiting stage) did not affect the *in vitro* growth of *Trichoderma* spp. Chorzempa et al. (2019) found that biofumigation in field conditions with 2.26 kg m^{-2} of fresh crushed *B. juncea* crops, at the end of fruiting stage, did not supress the antagonist fungus *T. harzianum*. In addition, Garain et al. (2021) observed that native isolates of *Trichoderma* spp. T-Nam were highly tolerant to biofumigant treatments with fresh macerated leaves of *B. juncea*, var. Pusa Mahak (inhibiting concentration value of 99%: 9,46 g per 127 mL of aerial space, equivalent to 67.04 g per 900 mL). Prasad et al. (2018) determined that *T. harzianum* was less sensible when exposed to the volatile gases from various species of the genus *Brassica* (including *B. juncea*) compared to different assay pathogens (*R. solani*, *S. rolfsii*, *Fusarium oxysporum* f. sp. *ciceris* (Padwick) Matuo & K. Sato, and *S. sclerotiorum*).

3.3. Interction between T. harzianum and Phomopsis spp. in the dual culture without biofumigation

During the dual culture without biofumigation, colony surface values similar to those observed at 7 and 11 days of incubation were recorded for both fungi. The colonies of *Phomopsis* spp. had a significant smaller diameter to those observed in the individual culture (*p value* = 0,0053; H = 7,76). In contrast, the colonies of *T. harzianum* showed the same diameter as those observed in the individual culture (*p value* = 1; H = 0) (Table 3, Figure 1A, 1B, 1C & Figure 2A, 2B, 2C).

Table 3. Antagonistic effects of *T. harzianum* on *Phomopsis* spp.

Treatment	Average size of the colonies (cm^2) †	
	Phomopsis* spp.	***T. harzianum*** **
Individual culture	63,62 a	63,62 a
Dual culture	32,80 (48,44 %) b	63,62 a

† Values in parentheses show the percentage of mycelial inhibition of *Phomopsis* spp. In each column, values with different letters indicate significant differences by Kruskal-Wallis test (* p value = 0.0053; H = 7.76 y ** p value = 1; H = 0).

T. harzianum significantly inhibited the growth of *Phomopsis* spp. but there was not a suppressing effect because the pathogen colonies in the dual culture increased 50% of the average surface media of the control, with a mycelial inhibition percentage of *Phomopsis* spp. of 48.44 % (Table 3). The mycelium of *T. harzianum* spread over that of *Phomopsis* spp. and covered the entire surface of the Petri dish. However, this behaviour was barely noticeable to the naked eye on day 7 of incubation (Figure 1B). It was clearly noticeable on the day 11 due to the production of green conidia (Figure 2B).

Other studies have reported higher inhibition percentages on the growth of *Phomopsis* spp. in dual cultures with *T. harzianum*, with values varying depending on the pathogen species and the strain of the antagonist. Crovo and Clemente (2015) observed a 50 % inhibition of *Phomopsis* spp. growth after 3.5 days of incubation, with this percentage increasing to over 60% by day 7. Jakatimath et al. (2017) reported variable mycelial inhibition percentages in *Phomopsis vexans* (Sacc. & Syd.) ranging from 56.33% to 70.66%, depending on the strain of *T. harzianum* used to antagonize the pathogen. López-López et al. (2023) observed 82.2% inhibition of *Phomopsis perseae* Zerova when controlled with the *T. harzianum* strain TSONM6.

3.4. Joint activity of T. harzianum and the biofumigation with Indian mustard on Phomopsis spp.

The treatments that combined biofumigation and *T. harzianum* revealed a mycelial inhibition percentage in *Phomopsis* spp. significantly higher than that obtained in the treatment with *T. harzianum* without biofumigant. No significant differences were observed between the different doses of *B. juncea*, both in the biofumigation stage and the post-biofumigation stage (*p value* = 0,0013; H = 13,29) (Table 4, Figure 1B, 1E, 1H and Figure 2B, 2E ; 2H).

At the end of the assay, the combination of the antagonist *T. harzianum* and the biofumigation with 10 g of *B. juncea* had a synergetic effect on the control of *Phomopsis* spp. growth. The percentage of mycelial inhibition in the treatment that combined the two techniques was significantly higher than that observed during the treatments with separated biocontrol techniques (Tables 4, 1 and 3).

Table 4. Joint effects of *T. harzianum* and Indian mustard biofumigation (*Brassica juncea*) on *Phomopsis* spp.

Treatment	Percentage mycelial inhibition of *Phomopsis spp.* †	
	Biofumigation stage	**Post-biofumigation stage**
T. harzianum	48.44 % a	48.44 % a
T. harzianum + *B. juncea* - 10 g	100.00 % b	100.00 % b
T. harzianum + *B. juncea* - 30 g	100.00 % b	100.00 % b

† *Values with different letters indicate significant differences by Kruskal-Wallis test (p value = 0.0013; H = 13.29).*

We could not find previous literature on the combined activity of *T. harzianum* and biofumigation with *B. juncea* against *Phomopsis* spp. However, there are previous studies on the simultaneous application of both biocontrol techniques against other pathogens. In a previous study where the combined in vitro effect of *Trichoderma* spp. and biofumigation with fresh Indian mustard plants (at the late fruiting stage) on *Fusarium graminearum* was evaluated, a synergistic effect was observed in controlling the pathogen's growth, with doses of 5 and 10 g of biofumigant (Perniola et al., 2014). Other studies have observed favorable effects when using biofumigation in combination with *Trichoderma* spp. Garain et al. (2021) reported a 95.66 % reduction in the incidence of rot induced by *S. rolfsii* on *Piper betle L.* due to the combined effect of biofumigation with *B. juncea* and the incorporation of *Trichoderma* spp. into the soil.

4. Conclusions

The results from this experiment are consistent with those obtained from previous *in vitro* and field trials and confirm that the biocontrol technique with the antagonist fungus *T. harzianum* can be considered compatible with the biofumigation with *B. juncea.* The growth of *T. harzianum* and its potential biocontrol effect against *Phomopsis* spp. were not affected by the biofumigation with *B. juncea.*

The combined use of *T. harzianum* and biofumigation with 10 g of *B. juncea* had a synergetic effect on controlling the growth of *Phomopsis* spp.

In addition, the dose of 30 g of *B. juncea* is sufficient to reach a complete suppression of the pathogen in conditions presented in this study.

The biofumigation with *B. juncea*, the application of *T. harzianum*, and the combination of both techniques could be seen as promising alternatives for the biological control of *Phomopsis* spp.

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Contributor roles

- Omar Salvador Perniola: conceptualization, formal analysis, investigation, methodology, project administration, validation, visualization, writing – original draft, writing – review & editing.
- María Cristina Isabel Noelting: conceptualization, investigation, methodology, project administration, validation, visualization, writing – review & editing.
- Silvia Elena Chorzempa: conceptualization, formal analysis, funding acquisition, investigation, methodology, project administration, resources, writing – review & editing.
- Mónica Beatriz Aulicino: formal analysis, resources, writing – review & editing.
- Guillermo Martín Mantz: investigation, writing – review & editing.
- Marta Mónica Astiz Gassó: conceptualization, investigation, methodology, supervision, resources, writing – review & editing.

Ethical implications

Ethics approval Not applicable.

Conflicts of interest

The authors declare that they have no affiliation with any organization with a direct or indirect financial interest that could have appeared to influence the work reported.

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