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Efficiency and delivery methods of *Trichoderma harzianum* on biological control against southern blight in sweet pepper

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ABSTRACT: Soilborne pathogens are difficult to control with limited options, and it is important to seek effective alternatives for management. The objective of this study was to evaluate the effect of *Trichoderma harzianum* on the biological control of southern blight. The commercial product Trichodermil, containing the antagonist, was used for *in vitro* antagonism evaluation, followed by *in vivo* assays, with different forms of biocontrol application in *Capsicum annuum* seedlings inoculated with *Sclerotium rolfsii*. The biocontrol conditions used in the tests were: 1) without inoculation of the pathogen; 2) furrow spraying; 3) foliar spraying; 4) immersion of roots in spore suspension; 5) without biocontrol. The *T. harzianum* isolate tested produces antifungal metabolites that reduce mycelial growth and germination of the pathogen. In the *in vivo* tests, an effect of the application in the sowing furrow on the root mass of the plants was observed. However, none of the treatments prevented plant mortality. The biocontrol agent *T. harzianum* does not work as a stand-alone strategy for management of southern blight, but has potential for use in integrated management programs.

Key words: antagonism; biocontrol agent; *Capsicum annuum*; *Sclerotium rolfsii*

Eficiência e formas de aplicação de *Trichoderma harzianum* no controle biológico da podridão de esclerócio em pimentão

RESUMO: Os patógenos radiculares têm um controle difícil e com opções limitadas, sendo importante buscar alternativas eficazes para o manejo. O objetivo deste trabalho foi avaliar o efeito de *Trichoderma harzianum* no controle biológico da podridão de esclerócio. Foi utilizado o produto comercial Trichodermil contendo o antagonista, procedendo-se avaliação *in vitro* do antagonismo, seguida de ensaios *in vivo*, com diferentes formas de aplicação do biocontrole em mudas de *Capsicum annuum* inoculadas com *Sclerotium rolfsii*. As condições de biocontrole utilizadas nos testes foram: 1) sem inoculação do patógeno; 2) pulverização em sulco; 3) pulverização foliar; 4) imersão de raízes em suspensão de esporos; 5) sem biocontrole. O isolado de *T. harzianum* testado produz metabólitos antifúngicos que reduzem o crescimento micelial e germinação do patógeno. Nos testes *in vivo*, observou-se efeito da aplicação no sulco de semeadura sobre a massa radicular das plantas. Contudo, nenhum dos tratamentos impediu a mortalidade de plantas. O agente de biocontrole *T. harzianum* não funciona como estratégia isolada para manejo da podridão de esclerócio, mas tem potencial para utilização em programas de manejo integrado.

Palavras-chave: antagonismo; agente de biocontrole; *Capsicum annuum*; *Sclerotium rolfsii*

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Introduction

The occurrence of diseases is a limiting factor for bell pepper production, causing damage and losses in large proportions (Guigón-López, 2019). Root diseases, in particular, are some of the biggest management challenges facing farmers around the world.

Southern blight, caused by the fungus *Sclerotium rolfsii* Sacc., leads to wilting, root and collar rot, damping off, and death in more than 1,200 host plant species (Punja, 1985). This pathogen secretes non-specific toxins, pectolytic enzymes, and oxalic acid, which destroy the cell wall and tissues of the host (Muthukumar & Venkatesh, 2013). In addition, *S. rolfsii* has the ability to survive in the absence of the host, either through saprophytic colonization or by producing resistance structures called sclerotia (Punja, 1985).

Limitations to the management of *S. rolfsii* include a broad host spectrum, rapid mycelial growth, abundant sclerotia production, and high population variability, as well as its location below the soil surface (Punja, 1985). There are few options for genetic control against southern blight, crop rotation has little effect on the pathogen population, and chemical control relies on large volumes of syrup applied directly to the soil and/or use of fumigants. Both chemical control strategies are inefficient, costly and environmentally damaging, and have legislative restrictions (Md Meftaul et al., 2020; Souza et al., 2020). In this scenario, it is important to find viable alternatives for the management of root pathogens, especially *S. rolfsii*.

The use of biological control agents can be an efficient strategy in southern blight management programs (Rani, 2017; Kamel et al., 2020). Fungi of the genus *Trichoderma* have physiological mechanisms that can aid in the control of root pathogens, including *S. rolfsii* (Kamel et al., 2020; Díaz-Gutiérrez et al., 2021). However, care must be taken with factors that affect the efficiency of biocontrol agents (Spadaro & Gullino, 2005). Biocontrol depends on factors such as population density, species-specific mode of action used, and mode of introduction of the biocontrol agent (Abdalla et al., 2014).

It is possible that microorganisms with biocontrol potential are underutilized in incorrect management systems (Spadaro & Gullino, 2005) or with inadequate applications (Blanco et al., 2021). Being composed of a microorganism and not a chemical formulation, biofungicides are more susceptible to factors involving application technology, such as pH, humidity and temperature, as well as being affected by the way they are introduced (Spadaro & Gullino, 2005). In this sense, it is important to investigate the interactions and forms of antagonism between the pathogen and the biocontrol agent, as well as the most effective application technologies for use in management. Therefore, the objective of this work was to evaluate the effect of the form of application on biocontrol of *Trichoderma harzianum* on *S. rolfsii* on sweet pepper.

Materials and Methods

In vitro and *in vivo* experiments were conducted to characterize the biocontrol of *T. harzianum* against southern blight. In the *in vitro* experiments, the antagonistic effects of *T. harzianum* on the growth of *S. rolfsii* were evaluated. In the *in vivo* experiments, the effect of different delivery methods of the biocontrol agent on the intensity of southern blight in sweet pepper plants (*Capsicum annuum*) was observed.

Isolation, growth and storage conditions

The isolate of *S. rolfsii* used in this study was obtained in the experimental area of the Universidade Estadual de Mato Grosso do Sul/Unidade Universitária de Aquidauana (UEMS/UUA) from bean roots (*Phaseolus vulgaris* L.) infected by the pathogen. After obtaining pure colonies on BDA culture medium, the plates were kept in a BOD at 27 ± 2 °C with a 12-hour photoperiod until the formation of sclerotia. Sclerotia were collected and stored in microtubes at 4 °C and used in the assays during all experiments. For the *in vitro* evaluations, colonies incubated for 3 days were used.

The *T. harzianum* isolate came from the commercial product Trichodermil (CEPA ESALQ 1306 - MAPA 2007 Registration). The commercial formulation containing spores (2×10^9 conidia per mL) of the antagonist was deposited in Petri dishes containing BDA culture medium, and incubated for seven days at 25 ± 2 °C temperature and 12-hour light photoperiod (Carvalho et al., 2014). After the incubation period, mycelium discs 0.7 cm in diameter were removed, assepsied, and re-incubated to maintain pure cultures. The conidia and conidiophores were examined under a light microscope to confirm the antagonist species. All antagonism tests were performed with the *T. harzianum* colonies at 7 days of incubation.

Biocontrol of *T. harzianum* on *S. rolfsii*

The *in vitro* characterization of the effect of *T. harzianum* on the mycelial growth of *S. rolfsii* was evaluated in four assays: i) growth kinetics; ii) paired growth; iii) production of non-volatile metabolites; and, iv) production of volatile metabolites. Assays iii and iv were also performed using sclerotia, to evaluate the effect of the antagonist on pathogen germination. All in vitro assays were conducted in an entirely randomized design with five repetitions.

The growth kinetics of the fungi was evaluated by the mycelial extension rate method. Mycelium discs were placed at the edge of Petri dishes (92 mm) containing BDA, one disc per plate, and incubated at 25 ± 2 °C and a 12-hour light photoperiod. For four days, colonies were measured from the center of the mycelium disk to the farthest edge of the mycelium. The growth kinetics test was performed for the pathogen and antagonist by calculating the growth rate (mm hour^{-1}) of both using Equation 1 (Silva et al., 2015):

$$\text{Growth rate} = \frac{C2 - C1}{T2 - T1} \quad (1)$$

where:

C2 - growth after 96 hours;
C1 - growth after 24 hours;
T2 - 96 h; and,
T1 - 24 h.

The paired growth was performed by depositing mycelium discs of the pathogen and antagonist on opposite sides of the same Petri dish containing BDA, positioning them 1 cm from the edge of the plate, and incubated at a temperature of 25 ± 2 °C and photoperiod of 12-hours of measured light (Blanco et al., 2021). Plates containing *S. rolfsii* without antibiosis were incubated under the same conditions. After 3 days of incubation, the diameter of the colonies was measured in two directions. The diameters were used to calculate the area of the colonies, considering the ellipse area equation (Equation 2):

$$\text{Colony area} = \frac{\pi \times \text{Diameter } 1 \times \text{Diameter } 2}{4} \quad (2)$$

The presence of non-volatile metabolites was checked according to the cellophane method (Dennis & Webster, 1971b) by depositing a 10 cm diameter disc of cellophane on the surface of BDA culture medium. A disk of *T. harzianum* mycelium was deposited in the center of the cellophane for growth and production of metabolites, and incubated for 2 days at 25 ± 2 °C and 12-hour photoperiod. After incubation, the cellophane paper with the colony of the biocontrol agent was carefully removed and discarded, and the lid of the Petri dish was aseptically wiped clean. Then, a disk of *S. rolfsii* mycelium was placed in the center of the plates and incubated for 3 days at 25 ± 2 °C and 12-hour photoperiod. Plates containing *S. rolfsii* without antibiosis were incubated under the same conditions. At the end of the incubation, the diameter of the colonies was measured.

To verify the presence of volatile metabolites of *T. harzianum* antagonistic to *S. rolfsii*, the method of Dennis & Webster (1971a) was used. Mycelium discs of the pathogen and antagonist were individually arranged in the center of Petri dishes containing BDA culture medium and incubated for 24 hours at 25 ± 2 °C and 12-hour photoperiod. Then, the bases of the plates containing *T. harzianum* and *S. rolfsii* were joined together using PVC film and kept in incubation for 3 days. Plates containing *S. rolfsii* without antibiosis were incubated under the same conditions. At the end of the incubation, the diameter of the colonies was measured.

The antagonism tests on pathogen germination followed the same method used in the previous assays. However, a sclerotia was deposited in the center of each plate to evaluate colony growth under the antibiosis conditions. In this assay, the mycelial growth observed was considered as germination.

After the *in vitro* assays, the colony area data were used to determine the percentage of inhibition caused by biocontrol (Boat et al., 2020) using Equation 3:

$$\text{Inhibition} = \left(\frac{A_{sb} - A_{cb}}{A_{sb}} \right) \times 100 \quad (3)$$

where:

A_{sb} - area of the colony without the interference of the biocontrol agent; and,
A_{cb} - area observed in colonies under antibiosis.

The results obtained in the in vitro tests were, Levene test and Student t-test at 5%. The analyses were proceeded with the help of the Data Analysis tool from Microsoft Office Excel® 2019.

Biocontrol of *T. harzianum* on southern blight

The yellow sweet pepper (*Capsicum annuum* L. cv. Sf 134) seedlings were grown in polypropylene trays containing 160 cells in commercial Carolina Soil® substrate and kept in the greenhouse, being manually irrigated daily until they had two fully expanded true leaves, when they were transplanted into pots containing 1.5 L of Carolina Soil® substrate and biocontrol applications.

The seedlings were subjected to different antagonism conditions: i) without inoculation of the pathogen; ii) application of the antagonist by spraying the syrup over the open furrows in the pots before transplanting; iii) application of the antagonist by spraying the syrup onto the leaves until it runs off; iv) dipping (application of the antagonist via immersion of the root in the application mixture for 30 seconds, before transplanting); and, v) without biocontrol application. The commercial product Trichodermil was used at a dose of 0.25 L of product diluted in 20 L of water, as recommended by the manufacturer.

The inoculum of *S. rolfsii* was produced on milled rice grains according to the method of Blanco et al. (2021). Rice grains were immersed in distilled water for 2 hours and autoclaved. Subsequently, 5 mycelium fragments were inoculated into flasks containing 50 g of rice and incubated for 7 days (12-hour photoperiod at 27 ± 2 °C). Inoculation with the pathogen was performed 5 days after transplanting seedlings by incorporating contaminated rice into the substrate at a rate of 10 g L^{-1}.

The design was entirely randomized, with 5 repetitions in each treatment. Each repetition was composed of one pot containing 4 plants. Daily, for 8 days, symptoms and plant survival were evaluated. At the end of this period, the plants were removed from the substrate, washed, and dried. Subsequently, the plants were sectioned and the following growth variables were evaluated: green mass of the aerial part (GMAP) and root (GRM), and dry mass of the aerial part (DMAP) and root (RDM).

Analysis of the variables obtained in the *in vivo* tests included Levene test and visual-graphical evaluation of the data distributions and residuals. Plant mass data were subjected to logarithmic transformation

$$Y_{ij} = \log_{10} Y_{i-j} ,$$

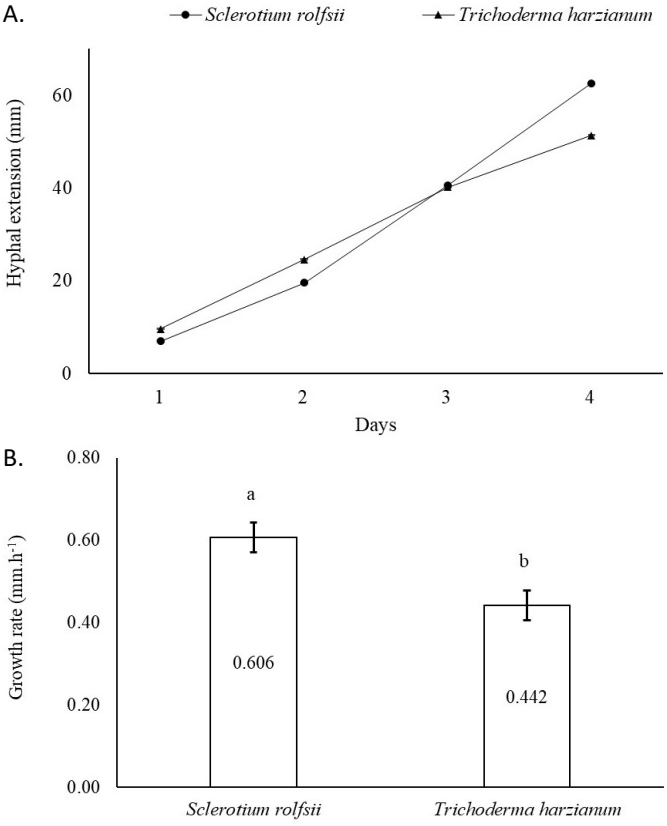
to control for variation between non-inoculated and inoculated plants. After the preliminary evaluations, analysis of variance was performed. When relevant, the comparison of averages was performed by Duncan test at 5%. The analyses were carried out with the help of the R statistical software.

Results and Discussion

The fungus *T. harzianum* has an antagonistic effect on the growth and germination of *S. rolfsii*, as observed in *in vitro* assays ($p < 0.05$). There was no colonization of the antagonist on the plates without added biocontrol, ruling out the occurrence of cross-contamination between treatments or repetitions.

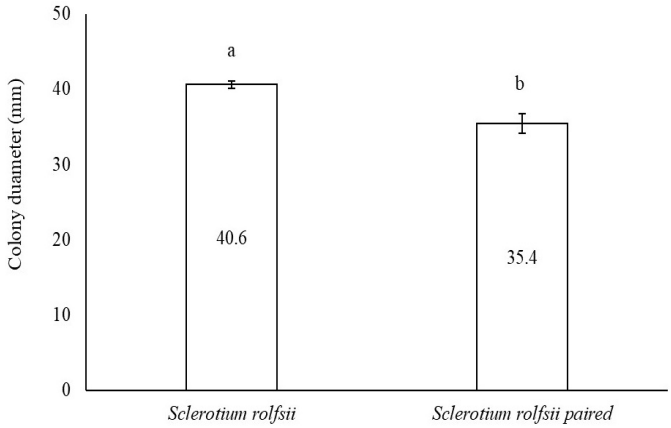
There was a significant difference ($p = 0.004$) between the growth rates of the antagonist and pathogen, with faster growth of *S. rolfsii* (Figure 1). The lower growth rate observed in *T. harzianum* suggests that its efficiency will be higher when it is introduced into the area before the arrival of the pathogen (Díaz-Gutiérrez et al., 2021).

In the paired growth test, the antagonist reduced the growth of *S. rolfsii* (Figure 2). Although there is a higher growth rate of the pathogen compared to the biocontrol agent, this result indicates that *T. harzianum* has antagonistic potential against the pathogen.



Averages followed by different letters in the columns differ statistically by Student t test ($p = 0.0061$).

Figure 1. (A) Mycelial growth of *S. rolfsii* and *T. harzianum* incubated for 4 days. Standard error of the average for each of the evaluations less than 0.15. (B) Mycelial growth rate of *S. rolfsii* and *T. harzianum* incubated for 4 days.

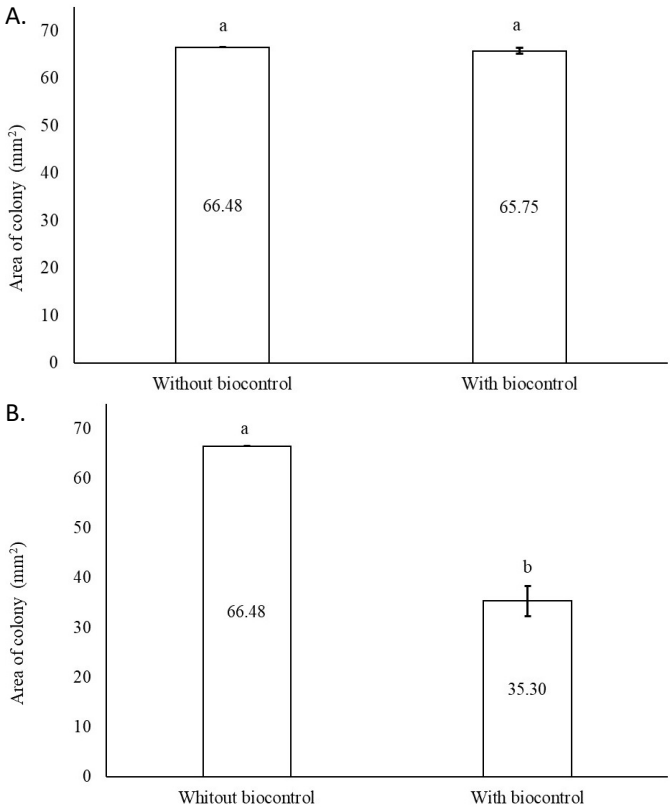


Averages followed by different letters in the columns differ statistically by Student t-test ($p = 0.0028$). The error bar represents the standard error.

Figure 2. Mycelial growth of *S. rolfsii* grown in pairing with *T. harzianum* after 4 days of incubation.

The antifungal metabolite test of *T. harzianum* showed that the non-volatile metabolites (Figure 3A) produced have no antagonistic effect on the mycelial growth of the pathogen ($p = 0.1164$). On the other hand, volatile metabolites (Figure 3B) reduced the mycelial growth of *S. rolfsii* ($p > 0.0001$).

The inhibition of *S. rolfsii* provided in the paired, non-volatile and volatile metabolite test evaluations were 12.81, 1.1, and 46.9%, respectively. The level of inhibition with the greatest effect on mycelial growth was that provided by the volatile metabolites.



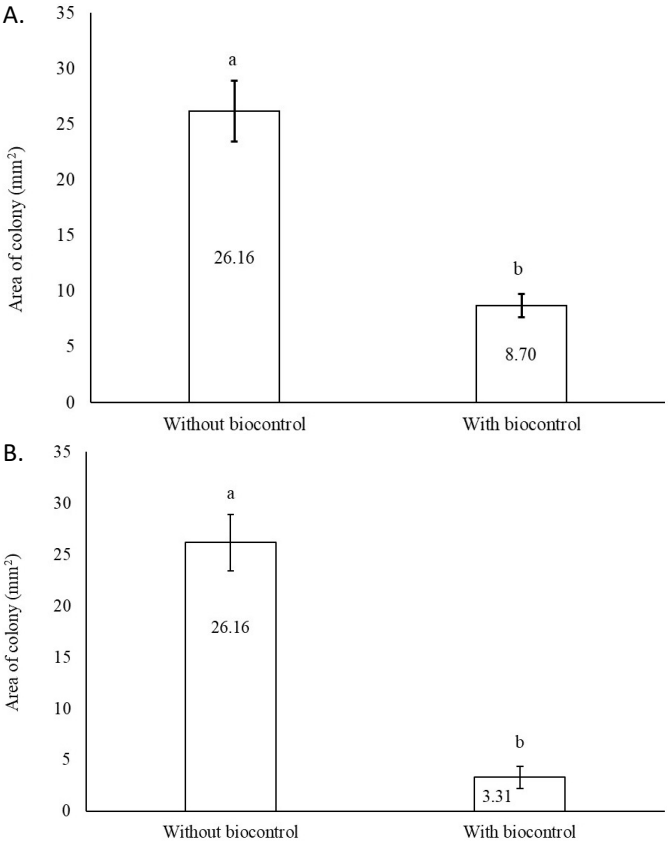
Averages followed by different letters in the columns differ statistically by Student t test. The error bar represents the standard error of the average.

Figure 3. Mycelial growth of *S. rolfsii* grown under antibiosis with (A) non-volatile ($p = 0.1164$) metabolites of *T. harzianum* and (B) volatile ($p > 0.0001$) after 3 days of incubation.

The antifungal metabolites significantly ($p > 0.0001$) reduced mycelial growth in colonies from sclerotia (Figure 4). This inhibition may indicate an effect of the antagonist on the germination of the pathogen. The inhibitions of sclerotia germination provided by the non-volatile and volatile metabolites were 66.74 and 87.35%, respectively.

This isolate of *T. harzianum* grew more slowly than *S. rolfsii*, but produces metabolites capable of reducing pathogen development. The data suggest that the effect of the antagonist may be better utilized when used in a preventive manner. Other *Trichoderma* species have similar behavior and provided greater control of *Macrophomina phaseolina* and *Fusarium solani* when introduced prior to pathogen arrival (Pastrana et al., 2016; Díaz-Gutiérrez et al., 2021). The lower growth rate of the antagonist relative to the pathogen can make biocontrol difficult in curative applications.

In the in vivo experiment, inoculation with the fungus *S. rolfsii* had an effect on the plants and led to the appearance of symptoms. The symptoms observed were: wilt, collar and root rot, damping off, yellowing, and death (Figure 5). In some plants, defoliation was observed. In addition, signs of the pathogen appeared on the surface of the substrate: profuse, white, cottony mycelium with developing sclerotia. All the symptoms observed are typical of southern blight. The non-inoculated plants did not show any symptoms of *S. rolfsii* infection, ruling out the occurrence of cross-contamination between treatments.



Averages followed by different letters in the columns differ statistically by Student t-test ($p = 0.0028$). The error bar represents the standard error of the average.

Figure 4. Colony area (cm^2) arising from sclerotia of *S. rolfsii* grown under antibiosis with (A) non-volatile ($p > 0.0001$) metabolites of *T. harzianum* and (B) volatile ($p > 0.0001$) metabolites after 3 days of incubation.

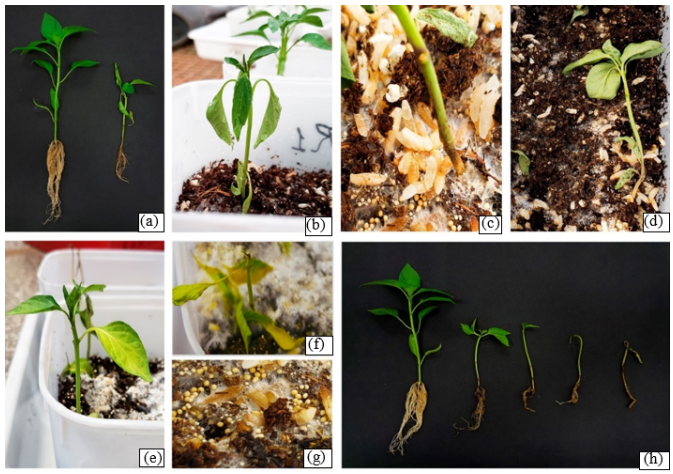


Figure 5. Southern blight in peppers: (a) plants without and with inoculation of *S. rolfsii*; (b) wilting symptom; (c) neck rot; (d) tumbling; (e) yellowing; (f) defoliated plant; (g) mycelium and sclerotia on the substrate; (h) (right to left) non-inoculated, furrow-applied biological control, foliar application, dipping, and no biological control.

In vivo evaluations indicate that there is an effect of inoculation with the antagonist ($p > 0.05$) and that different forms of application may be an important factor in the efficacy of biological control. Although none of the applications prevented the occurrence of southern blight, the direct application in the transplanting furrow showed an effect on plant mass (Table 1).

The non-inoculated plants presented the highest averages in all the variables evaluated. On the other hand, the plants inoculated with the pathogen and without *T. harzianum* application did not perform the worst. The application via dipping showed variables with lower averages than those observed without antibiosis.

The dipping application treatment showed no effect on southern blight under the conditions of the experiment. In experiments with *T. asperellum*, preventive application via dipping reduced infection of *M. faseolina* and *F. solani*

Table 1. Green mass of the aerial part (GMAP), green root mass (GRM), dry mass of the aerial part (DMAP), and root dry mass (RDM) of sweet pepper plants inoculated with *S. rolfsii* and treated with *T. harzianum* in different forms of application.

Application	GMAP	GRM	DMAP	RDM
Non-inoculated	1,810.05 ^{a}	935.85 ^{a}	164.64 ^{a}	144.28 ^{a}
Furrow spraying	598.80 ^{b}	133.3 ^{b}	82.7 ^{b}	27.31 ^{b}
Foliar application	250.45 ^{bc}	36.6 ^{c}	44.59 ^{bc}	19.34 ^{c}
Dipping	116.80 ^{c}	11.51 ^{d}	41.56 ^{c}	8.260 ^{e}
No antibiosis	222.15 ^{c}	23.42 ^{cd}	58.50 ^{bc}	14.80 ^{d}
F	17.02	11.76	38.37	29.8
P	3.18×10^{-6}	4.1×10^{-9}	4.49×10^{-5}	3.60×10^{-8}
CV (%)	19.27	42.37	14.63	34.70

Averages followed by equal letters in the same column do not differ statistically by Duncan test ($p \leq 0.05$). CV - Coefficient of variation.

(Pastrana et al., 2016), however, for *S. rolfsii*, this delivery method was less effective and less cost-effective than other control strategies (Konjengbam & Devi, 2020).

It is possible that the application via dipping promoted higher rhizosphere colonization and led to a relationship between *T. harzianum* and the substrate. In high-carbon environments, various microorganisms, including fungi of the genus *Trichoderma*, can compete with plants for nutrients and immobilize them, preventing them from being taken up by the root system (Zhang et al., 2018; Lin et al., 2021). The commercial substrate with high carbon content (Amaral et al., 2017) may have stimulated this phenomenon.

Foliar application gave intermediate averages in all cases, while furrow application gave higher green and dry root mass averages. The data show that there is no difference between the effect of the two sprays on aerial part mass, being both better than dipping and no biological control. However, it is possible that furrow application provides better control.

Inoculation with *S. rolfsii* caused plant death starting at 4 days after inoculation (Table 2). At 6 days after inoculation (Table 2), plants with biological control applied via foliar and in furrow provided survival that did not differ from that observed in non-inoculated plants ($p = 0.0204$). At 8 days after inoculation, a 35% survival rate was observed in plants with furrow application and only 5% in plants treated via dipping ($p > 0.001$).

None of the treatments fully guaranteed the survival of inoculated plants (Table 2). The effect of applications was only observed on plant mass, indicating that the biological control agent used can mitigate damage by the pathogen, but does not fully control the disease. The pathogen inoculum concentration used is a factor that may be linked to the low biocontrol efficiency. The increase in the concentration of *S. rolfsii* inoculum accelerates the development of symptoms (Sri et al., 2020), which may have hindered the action of the biocontrol agent.

It is possible that the preventive application of *T. harzianum* contributed to the establishment of the control agent by mitigating the fact that its growth is slower than that of the pathogen.

Sowing furrow spraying is, in some respects, more efficient than foliar application. The introduction of *Trichoderma* via soil application often provides a greater effect on the biological control of soilborne pathogens (Kamel et al., 2020). Possibly, this method of introduction provided a better establishment of the antagonist in the substrate. Importantly, biological control by *Trichoderma* is not limited to the production of metabolites, but is also a result of competition in the soil and by their interaction with the environment (Rani, 2017; Sunpapao et al., 2018; Kamel et al., 2020).

The use of *T. harzianum* in biological control of *S. rolfsii* can be potentiated by using it together with other antagonists or even with agrochemicals (Spadaro & Gullino, 2005), if there is compatibility. Combining biological control with cultural methods (Funahashi & Parke, 2016) may also be an alternative to increase the effect of both control strategies. It is necessary to overcome the outdated concept of control strategies that magically work, especially when it comes to soilborne pathogens. Management of these fungi faces a number of limitations, so combined methods are needed in the pursuit of healthy soils (Spadaro & Gullino, 2005; Lal, 2016; Rani, 2017; Sunpapao et al., 2018).

The evidence from the experiments suggests that, under the conditions tested, biological control with *T. harzianum* has low efficacy as a stand-alone strategy for management of southern blight in bell pepper plants. However, it is important to note that all management strategies will be ineffective when used as a sole method. Biological control is a strategy that can have its effect increased in combination with other strategies. In this sense, the preventive application of *T. harzianum* has potential for use in integrated management programs.

Table 2. Survival (%) of sweet pepper plants inoculated with *S. rolfsii* and treated with *T. harzianum* in different forms of application.

Antibiosis condition	2 DAI ^{ns}	4 DAI	6 DAI	8 DAI
Non-inoculated	100	100 ^{a}	100 ^{a}	100 ^{a}
Furrow spraying	100	100 ^{a}	65 ^{ab}	35 ^{b}
Foliar application	100	100 ^{a}	75 ^{ab}	20 ^{bc}
Dipping	100	85 ^{b}	35 ^{b}	5 ^{c}
No antibiosis	100	100 ^{a}	50 ^{b}	15 ^{bc}
F	-	6	3.71	19.17
P	-	0.00243	0.0204	1.28×10^{-6}
CV (%)	-	6.31	44.19	55.33

DAI - Days after inoculation. * Averages followed by equal letters in the same column do not differ statistically by Duncan test ($p \leq 0.05$). ns - not significant. CV - Coefficient of variation. P - descriptive level (H_0 is rejected if $p \leq 0.05$).

Compliance with Ethical Standards

Author contributions: Conceptualization: NHMB, CGM, FASG; Data curation: NHMB; Formal analysis: NHMB, FASG; Methodology: NHMB, FASG; Supervision: FASG, CGM; Investigation: NHMB; Project administration: NHMB; Visualization: NHMB; Writing - original draft: NHMB, FASG; Writing - review and editing: NHMB, FASG.

Conflict of interest: The authors declare that there are no conflicts of interest.

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