Biological control of *Sclerotium rolfsii* Sacc. in *Stevia rebaudiana* using native isolates of *Trichoderma* spp. from Paraguay

Control biológico de *Sclerotium rolfsii* Sacc. en *Stevia rebaudiana* usando aislamientos nativos de *Trichoderma* spp. de Paraguay

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ABSTRACT

Stevia, *Stevia rebaudiana* [(Bertoni) Bertoni], is cultivated in Paraguay as a natural, non-nutritive sweetener, but yields are reduced because of wilting and plant death caused by the fungal pathogen *Sclerotium rolfsii*. This study aimed to evaluate the native fungi *Trichoderma* spp. isolates, individually and in mixtures, for controlling *S. rolfsii*. As a first step, ten *Trichoderma* isolates from agricultural soils of Paraguay were screened *in vitro* using dual culture tests against *S. rolfsii* to identify the most effective isolates. This preliminary phase allowed screening to select the most promising candidates before conducting *in planta* experiments under more realistic conditions. A greenhouse experiment with seven treatments and four replicates was carried out to assess their efficacy in controlling *S. rolfsii* in stevia. Treatments included commercial *Trichoderma viride,* three native isolates (3KH and TCAS, *T. asperellum*; MS28, *T. erinaceum*), a mixture of these isolates, a fungicide (azoxystrobin + cyproconazole), and a control. Stevia plants of the variety "Katupyry" were preventively treated with *Trichoderma* (1×10^7 spores ml^{-1}) twice before *S. rolfsii* inoculation. Fungicide and *Trichoderma* treatments were applied to the plant base and substrate after inoculation, followed by two weekly applications. Disease incidence and the area under the disease progress curve (AUDPC) were evaluated. The AUDPC for TCAS (7.67) was significantly lower than that in the control (13.27), the mixture (13.77), and the commercial *T. viride* (15.58), but not significantly different from the 3KH (9.15) or MS28 (10.47). Fungicide-treated plants had the lowest AUDPC (1.25). These results suggest that the *Trichoderma* isolate TCAS effectively manages *S. rolfsii* under greenhouse conditions and has potential for use in both organic and conventional stevia production.

Key words: sweeteners, stevia, biocontrol, mycology.

RESUMEN

Estevia, *Stevia rebaudiana* [(Bertoni) Bertoni], se cultiva en Paraguay como edulcorante natural no nutritivo, pero los rendimientos se ven afectados por el marchitamiento y la muerte de plantas causados por el hongo patógeno *Sclerotium rolfsii*. Este estudio tuvo como objetivo evaluar aislados nativos del hongo *Trichoderma* spp., individualmente y en mezcla, para el manejo de *S. rolfsii*. Como primer paso, se evaluaron *in vitro* diez aislamientos de *Trichoderma* provenientes de suelos agrícolas de Paraguay mediante pruebas de cultivo dual contra *S. rolfsii* para identificar los aislamientos más efectivos. Esta fase preliminar permitió filtrar y seleccionar los candidatos más prometedores antes de realizar experimentos *in planta* en condiciones más realistas. Un experimento en invernadero con siete tratamientos y cuatro repeticiones evaluó su eficacia. Los tratamientos incluyeron *Trichoderma viride* comercial, tres aislados nativos (3KH y TCAS, *T. asperellum*; MS28, *T. erinaceum*), una mezcla de estos aislados, un fungicida (azoxystrobin + cyproconazole) y un control. Las plantas de estevia de la variedad "Katupyry" fueron tratadas preventivamente con *Trichoderma* ($1x10^7$ esporas ml^{-1}) dos veces antes de la inoculación con *S. rolfsii*. Los tratamientos con fungicida y *Trichoderma* se aplicaron al sustrato y en la base de las plantas después de la inoculación, seguidos de dos aplicaciones semanales. Se evaluó la incidencia de la enfermedad y el área bajo la curva de progreso de la enfermedad (AUDPC). El AUDPC para TCAS (7,67) fue significativamente menor que en el control (13,27), en la mezcla de *Trichoderma* (13,77) y en el *T. viride* comercial (15,58), pero no difirió significativamente del 3KH (9,15) o MS28 (10,47). Las plantas tratadas con fungicida tuvieron el AUDPC más bajo (1,25). Los resultados sugieren que el aislado TCAS de *Trichoderma* maneja eficazmente a *S. rolfsii* en condiciones de invernadero y tiene potencial para su uso en la producción orgánica y convencional de estevia.

Palabras clave: edulcorantes, estevia, biocontrol, micología.

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Introduction

Stevia (*Stevia rebaudiana* [(Bertoni) Bertoni], also known as "ka'a he'ê" in the Guaraní language, is a plant grown to produce non-caloric sweeteners (Britos & Jongdae, 2016). It is a semi-perennial plant that grows naturally in the mountains of Mbaracayú and Amambay, primarily in Paraguay (Bogado-Villalba *et al*., 2021). Stevia is currently widely cultivated in many countries worldwide, such as Argentina, Colombia, Japan, Singapore, Taiwan, South Korea, China, and the USA (Ismail *et al*., 2020).

Stevia production can be significantly affected by plant pathogens such as the fungus *Sclerotium rolfsii* Sacc., the causal agent of stevia "stem rot" and also known as "white silk" disease (and informally "enfermedad del hongo blanco") in Paraguay (Britos & Jongdae, 2016). This disease causes significant losses since it causes the wilting and death of the plant and is difficult to control. The fungus can survive several years in the soil because of resistance structures called sclerotia (Koehler & Shew, 2014). This disease can be controlled by synthetic chemicals such as quinone outside inhibitors (QoI) fungicides (Koehler & Shew, 2017; Sanabria-Velazquez *et al*., 2023). However, due to the high demand for stevia free of chemical pesticides in international markets (Ismail *et al*., 2020), the application of a biological control agent that exhibits antagonism to *Sclerotium rolfsii* seems a promising alternative for managing stevia stem rot (Correa *et al*., 2007).

Biological control with antagonistic fungi is a promising option for the sustainable management of this disease since this technology is directly involved with the selection and reintroduction of an antagonist. A biological control agent can be established in the same ecological niche of the pathogen, and it can protect the root system of the stevia plant, avoiding greater disease intensity (Bull *et al*., 1991; Hoitink & Boehm, 1999; Mazzola, 2004; Panth *et al*., 2020).

One of the most studied and efficient antagonists in several pathosystems is the fungus *Trichoderma* spp. (Cai & Druzhinina, 2021; Holmes *et al*., 2004; Samuels, 2006). Trichoderma, as a biological control agent, can exert mechanisms of direct and indirect action on the pathogen through the production of volatile chemicals and antibiotics (Louzada *et al*., 2016), secretions of toxic enzymes (Galante *et al*., 1998), by penetration of hyphae (Sanabria Velázquez & Grabowski, 2016), competition for oxygen and nutrients as well as for space in the soil (Benítez *et al*., 2004) and as an inducer of resistance modifying the physiology of the plant (Rivera-Méndez *et al*., 2020). Native isolates of *Trichoderma* spp. are usually more effective than imported ones. These native isolates are better adapted to the local environment and can improve effectiveness in the edaphoclimatic soil conditions where they are introduced (Mukherjee *et al*., 2014; Sanabria Velázquez, 2020; Sanabria-Velázquez *et al*., 2023). Therefore, native *Trichoderma* isolates can help reduce stem rot incidence, promote plant growth, and increase stevia yields.

The main objective of this research was to select and evaluate native isolates of *Trichoderma* spp. for the management of *Sclerotium rolfsii* in stevia production. To optimize the identification of effective *Trichoderma* spp. isolates, a two-phase strategy was implemented. First, *in vitro* tests were carried out to evaluate the antagonistic capacity of each isolate against *S. rolfsii*. Subsequently, the isolates that demonstrated greater efficacy in these preliminary tests were subjected to *in planta* evaluations to validate their performance under more representative culture conditions. The specific objectives were to: i) evaluate *in vitro* the antagonism exerted by native isolates of *Trichoderma* spp. on *S. rolfsii* using the dual culture technique, and ii) assess the most promising native *Trichoderma* spp. isolates against *S. rolfsii in planta* to confirm their efficacy under greenhouse conditions.

Materials and methods

We carried out the experiments in the laboratory and greenhouses of the Olericultural Crops Research Program (PICO) of the Hernando Bertoni Research Center (CIHB) at the Paraguayan Institute of Agricultural Technology (IPTA), Cordillera, Paraguay (25°23'25.73" S; 57°11'98" W). The experimental period was between January 2015 and March 2018.

We collected samples from various stevia and horticultural production farms in Paraguay. To isolate the *Trichoderma* spp., we adapted the soil dilution method described by Fernández (1993). This method involved mixing 3 g of soil with 100 cc of sterile distilled water and shaking the mixture. Then, 1 cc of the suspension was placed in a sterilized Petri dish under a laminar flow chamber. We added PDA (Potato-Dextrose-Agar) + Oxytetracycline culture medium and gently shook the dish; and we incubated the plates at 28°C for 5 d in darkness. We labeled the pure cultures of *Trichoderma* spp. with an arbitrary nomenclature (Tab. 1).

The fungus *S. rolfsii* was previously isolated from symptomatic plants of the stevia experimental plot of the Ka'a he'ê and Medicinal Plants Research Program-CIHB,

TABLE 1. Georeferenced soil samples for obtaining *Trichoderma* spp. isolates.

Sample name	*Trichoderma* spp. isolates	Location	Coordinates
1KH	TKC14-01	Cordillera	S 25.3883413; W 57.1869743
3KH	TKC14-03	Cordillera	S 25.38807153; W 57.18603803
1 PICO 4	TFC14-04	Cordillera	S 25.38756; W 57.18980
1 PICO 6.1	TFC14-05	Cordillera	S 25.38763; W 57.18969
1 PICO 6.2	TFC14-06	Cordillera	S 25.38765; W 57.18960
TQUI	TFCe14-09	Central	S 25.33297699; W 57.35262201
TCAS	TFCe14-10	Central	S 25.32913; W 57.362126
K6	TKI14-11	Itapúa	S 26. 67623; W 57.08865
T9	TKI14-12	Itapúa	----------
MS28	TSESP14-15	San Pedro	S 24.148507; W 56.641762
C2 (Foreign sample)	TFLE-07	Ecuador	----------

Cordillera, Paraguay. We washed the roots collected from stevia plants with running water and cut them into 1 to 2-cm pieces, disinfesting them with a 70% alcohol solution and a 2% sodium hypochlorite solution, followed by triple rinsing in sterile distilled water. The plant tissue was dried and transferred to Petri dishes with a PDA culture medium under a laminar flow chamber. Subsequently, they were incubated at 28°C for 8 d. Pure cultures were maintained at 4°C until they were used in the experiments.

In vitro antagonism of *Trichoderma* spp. against *Sclerotium rolfsii*

We used the dual culture method to select the isolates of *Trichoderma* spp. with antagonistic capacity against *S. rolfsii*. The test consisted of facing a 9 cm Petri dish with PDA culture medium, two discs 5 mm in diameter with culture medium, and mycelium of *Trichoderma* spp. and *S. rolfsii,* separated by 7 cm from each other and incubated at 28°C until *S. rolfsii* treatment filled the plate. We evaluated the antagonistic activity of *Trichoderma* spp. isolates by quantifying the mycelial growth and production of S. *rolfsii* sclerotia in dual cultures. We employed a completely randomized design with 11 treatments, 10 being *Trichoderma* isolates, each confronted with *S. rolfsii*, and a control consisting of a disc with culture medium and mycelium of the pathogen without opposition to antagonists. Each treatment received five replicates, resulting in 55 experimental units (EU) consisting of one Petri dish each.

In planta antagonism of *Trichoderma* spp. against *Sclerotium rolfsii*

For the experiments, we used 90-d-old stevia seedlings of the cultivar "Katupyry", developed through genetic selection programs aimed at enhancing agronomic performance and disease resistance. This cultivar was selected for its adaptability to local environmental conditions, high steviol glycoside content, and its recognized tolerance to biotic and abiotic stresses, making it an ideal candidate for evaluating biological control strategies against *Sclerotium rolfsii*. The seedlings were provided by the Ka'a he'ê and Medicinal Plants Research Program-CIHB and were propagated through cuttings treated with indole butyric acid at a rate of 2 g L^{-1} of water. They were then transplanted into black polyethylene pots (60 µm, 20 cm x 20 cm) containing a sterilized substrate composed of sand, white sand, and humus in a 3:2:1 ratio. Substrate sterilization was performed using wood fire, maintaining a temperature of 250°C for 6 h to minimize microbial contamination. The stevia cuttings were transplanted on September 2017 and remained in polyethylene pots throughout the experiment.

The experimental design was completely randomized, with seven treatments and four replicates. The treatments consisted of treating the stevia plants with a *Trichoderma viride* isolate formulated as a soluble powder (TRIFESOL 1000 WP®, Biocultures, Ibagué, Colombia) with 1x conidia per gram. We used three native isolates of *Trichoderma* spp. (*T. asperellum* 3KH*, T. asperellum* TCAS, *and T. erinaceum* MS28) a mixture of the three native strains of *Trichoderma* spp., a synthetic fungicide azoxystrobin 200 g L^{-1} + cyproconazole 80 g L^{-1} (Priori Xtra® Syngenta, France) applied in a dose of approximately 5 ml L^{-1} water, and a control inoculated with the pathogen (Tab. 2). For the mixture, we combined individual suspensions of the native strains with concentrations of 10^7 spores mL^{-1} in equal proportions and diluted until the concentration of the mixture was equal to 10^7 spores mL^{-1}. The experimental unit (EU) consisted of nine pots with one plant each, using 252 stevia plants.

TABLE 2. Description of treatments for the control of *Sclerotium rolfsii* in stevia.

Treatment	Description	Dose	Application
1	*Trichoderma* mix	10^7 spores mL^{-1}	Preventive
2	*T. asperellum* 3KH	10^7 spores mL^{-1}	Preventive
3	*T. asperellum* TCAS	10^7 spores mL^{-1}	Preventive
4	*T. erinaceum* MS28	10^7 spores mL^{-1}	Preventive
5	*T. viride*	10^7 spores mL^{-1}	Preventive
6	Azoxystrobin + cyproconazole	5 ml L^{-1}	Curative
7	Inoculated control	-	-

We carried out the preparation of the suspension of *Trichoderma* spp. following the methodology of Sanabria Velázquez (2020), described as follows: *Trichoderma* spp. isolates previously isolated from the culture of stevia were selected, and they were seeded in Petri dishes with PDA culture medium and incubated in an incubator chamber (Shimadzu Corporation, BITEC-300, Kyoto, Japan) at 25°C for 7 d. For the multiplication of *Trichoderma* spp., 1 kg of rice was used (Tio Nico®, Fine Long Glazed Rice Type 00000, Paraguay), soaked for one hour in a two-liter beaker; the water was allowed to drain for half an hour; 200 g of rice were loaded into polypropylene bags and autoclaved for 20 min at 120°C and 2 atm pressure.

For the colonization of the culture medium, 25 ml of sterilized water per plate was added; the surface was then scraped, creating a suspension of spores and hyphae. This solution was then placed in a magnetic stirrer for 20 min. To inoculate a bag of sterilized rice, 10 ml of spore suspension was taken, and 200 g of rice was dispensed in previously sterilized polypropylene bags. The bags were shaken manually to homogenize the distribution of the spore suspension and incubated at 28°C for 12 d in an incubator chamber.

To prepare *Trichoderma* spore suspension for *in planta* trials, the following steps were taken: 500 ml of sterilized distilled water was added to a polypropylene bag containing rice colonized by *Trichoderma* spp. and the mixture was stirred to extract as many conidia as possible and then poured into a beaker. To extract the rest of the spores, 500 ml of sterilized distilled water was added back into the polypropylene bag, stirred again, and poured into a beaker until 1000 ml of suspension with sterilized distilled water was obtained. Conidia were counted in the Neubauer chamber, and the concentration of conidia was adjusted to 10^7 spores mL^{-1}.

Spore suspensions of *Trichoderma* spp. were dissolved in water with a concentration of 10^7 spores mL^{-1}, and five applications were made. Each application differed from the previous one for one week. The spore suspensions in the respective pots were applied to the point of percolation. After 7 d of the second preventive application of the suspension of 10^7 spores mL^{-1} of *Trichoderma* spores coincided with the infestation with *S. rolfsii,* the chemical control (azoxystrobin + cyproconazole) was dispensed in recommended doses, applying 200 ml of the product in each pot. The suspension was applied directly to the pot's substrate and the base of the stevia plants.

Infestation with the phytopathogenic fungus *Sclerotium rolfsii*

We obtained inocula of *S. rolfsii* following the methodology of Sanabria-Velazquez *et al*. (2019) described as follows: an isolate of *S. rolfsii* previously isolated from symptomatic plants of the stevia culture was selected. This sample was seeded in Petri dishes with PDA culture medium and incubated at 25°C for 4 d. Potato slices with a diameter of approximately 5 cm and 2 cm in height were cut and autoclaved for 20 min at 120°C and 2 atm pressure. Under a laminar flow hood, discs of PDA (5 cm) colonized by *S. rolfsii* were extracted and placed on the previously sterilized potato slices, then incubated in an incubator chamber (Shimadzu Corporation, BITEC-300, Kyoto, Japan) at 28°C for 3 d.

We used a potato disc infested by *S. rolfsii* to infest a pot with stevia located 2 cm from the pot's center on the substrate surface. We treated the plants previously with two applications of *Trichoderma*. When the plants were treated with *S. rolfsii*, we applied all the treatments, including the chemical treatment described in the previous section. The surface of the plant was then covered with a polyethylene bag simulating a wet chamber. The plants were kept covered inside the greenhouse until the end of the experiment.

Evaluation of variables

Disease incidence was obtained by counts of wilted plants with symptoms of *S. rolfsii*. The incidence of the disease was recorded from 2 d after inoculation (DAI) with the pathogen until all control plants presented symptoms. Using the mean percentage of disease at each estimated date of incidence for each experimental unit, the area under the disease progression curve (AUDPC) was calculated using the formula:

$$\text{AUDPC} = \sum_{i=1}^{Ni-1} \left(\frac{y_i + y_{i+1}}{2}\right)(t_{i+1} - t_i) \quad (1)$$

where

AUDPC = area under the disease progression curve for EU;

y_i = percentage of disease incidence at each assessment date for each EU;

t_i = each evaluation date.

The percentage of disease control for the different treatments was calculated using the following formula:

$$\text{Control} = 100\% - [(\frac{PC}{TC}) \times 100] \quad (2)$$

where

Control: percentage control of disease progression (%);

PC: area under the progression curve of the EU disease with the highest incidence;

TC: area under the disease progression curve of each EU.

Data analysis

Analysis of variance (ANOVA) was used for data results; the means of the treatments were compared using the Fisher LSD test at a 5% probability of error. Statistical analysis was performed with the statistical package InfoStat® (Di Rienzo *et al*., 2008). For all studies, normality of distributions was checked earlier for analysis. Untransformed means and confidence intervals were reported.

Results and discussion

Table 3 shows the production of *S. rolfsii* sclerotia compared to the 10 *Trichoderma* spp. isolates and control (only the pathogen) after 72 h of incubation.

TABLE 3. Production of *Sclerotium rolfsii* sclerotia in dual culture with *Trichoderma* spp. isolates during *in vitro* antagonism tests.

Treatments	Number of sclerotia*
TCAS	0.00 C
1KH	0.00 C
3KH	0.00 C
1 PICO 4	19 B
1 PICO 6.1	33 B
1 PICO 6.2	20 B
TQUI	19 B
K6	30 B
C2	40 B
T9	124 A
Control	178 A

* Means with a common letter are not significantly different ($P>0.05$) based on the Fisher LSD (Least significant difference) test.

Significant differences in sclerotia production were observed when *Sclerotium rolfsii* was confronted with various *Trichoderma* spp. isolates (Fig. 1). The number of sclerotia per Petri plate ranged from 0 to 178. Notably, the TCAS, 1KH, and 3KH isolates significantly inhibited sclerotia formation compared to other *Trichoderma* isolates and the control (Tab. 3). In contrast, isolates 1 PICO 4, 1 PICO 6.1, 1 PICO 6.2, K6, and C2 showed no significant differences in sclerotia production, with values ranging from 19 to 40 sclerotia per plate. The T9 isolate exhibited a low capacity to inhibit sclerotia production, yielding 124 sclerotia per plate, which was not statistically different from the control. The variability in sclerotia inhibition among different *Trichoderma* isolates underscores the complexity of their antagonistic interactions with *S. rolfsii*, suggesting that some isolates may be more effective biological control agents than others.

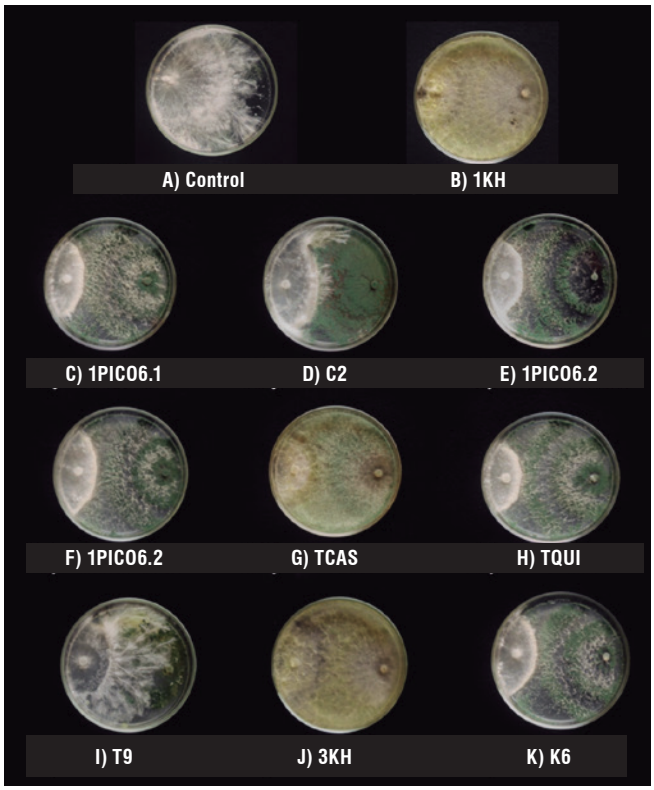

FIGURE 1. Inhibition of growth and production of sclerotia of *Sclerotium rolfsii* in dual culture: *Sclerotium rolfsii* (left) and *Trichoderma* spp. (right).

These results emphasize the importance of screening multiple isolates to identify the most effective candidates for biological control, as their performance can vary significantly, even within the same species or geographic origin. Similar levels of inhibition when selecting 12 out of 20 strains of *Trichoderma* spp. from agricultural areas in Brasilia, Brazil, were observed against *S. rolfsii*, inhibiting the pathogen in a range of 18.97 to 44.12% *in vitro* (Correa

et al., 2007). Isolates of *Trichoderma* spp. interfere with the survival and development of sclerotia through various antagonistic mechanisms (Hoyos-Carvajal *et al*., 2008). Similarly, several studies have demonstrated the effectiveness of using *Trichoderma* spp. strains against *S. rolfsii* (Ram *et al*., 2020; Rodriguez-Paez & Jaraba-Navas, 2023).

Incidence of stem rot caused by *Sclerotium rolfsii*

Significant differences were observed between plants treated with *Trichoderma* TCAS isolate and those treated with azoxystrobin + cyproconazole in terms of reduction of wilt caused by *S. rolfsii* compared to untreated control plants (Tab. 4).

TABLE 4. Effect of different treatments with *Trichoderma* spp. and azoxystrobin + cyproconazole isolates on the area of the disease incidence progress curve (AUDPC) in *Stevia rebaudiana* plants inoculated with *Sclerotium rolfsii* under greenhouse conditions.

Treatments	AUDPC[w] (Mean ± standard deviation)		Confidence Interval 95%[y] (%)	
T. viride (commercial)	15.58 ±1.28	A[x]	12.55	18.62
Trichoderma mix	13.77 ±3.73	AB	10.74	16.8
Control	13.27 ±2.36	ABC	10.24	16.3
MS28	10.47 ±2.75	BCD	7.44	13.51
3KH	9.15 ±4.40	CD	6.12	12.19
TCAS	7.67±2.91	D	4.63	10.69
Azoxystrobin + cyproconazole	1.25 ±1.73	E	-1.78	4.28

[w]Area under disease progress curve obtained based on the progress of the incidence of the disease during 20 d after inoculation. [x]Means with a common letter are not significantly different ($P>0.05$) based on the Fisher LSD (Least significant difference) test. LSD=4.29. [y]AUDPC 95% confidence interval.

Among the *Trichoderma*-based treatments, only the TCAS isolate showed a significant difference compared to the control. Plants treated with the TCAS isolate had a disease progression area of 7.67. In contrast, those treated with the 3KH isolate had a progression area of 9.15, which was not statistically different from the disease progression observed in plants treated with the MS28 isolate (AUDPC 10.47). Plants treated with the mixture of isolates (TCAS, 3KH, and MS28) had an AUDPC of 13.77, which was significantly higher than that of plants treated solely with the TCAS isolate. Meanwhile, plants treated with the *T. viride* formulation showed a disease progression area of 15.58, which was not significantly different from the control treatment.

The biological control agent *Trichoderma* TCAS effectively reduced the progress of *S. rolfsii*-induced wilt under greenhouse conditions. It could directly attack and parasitize the sclerotia of the pathogen (Fig. 2). These results are consistent with Rawat and Tewari (2010), who studied the interaction between *T. harzianum* and *S. rolfsii* sclerotia using light and transmission electron microscopy. This research observed and confirmed this parasitism of *Trichoderma* on the sclerotia of *S. rolfsii*. The lysis and deformation of the cell wall of hyphae, the degradation and disappearance of cytoplasmic contents, and the loss of cell integrity in *S. rolfsii* sclerotia parasitized by *T. harzianum* were evident from transmission electron micrographs.

Sclerotia are the disease's primary inoculum, and reducing their viability can significantly reduce the progression of the disease. Therefore, the TCAS isolate presents excellent potential for managing stevia stem rot (Lourenço Jr *et al*., 2018). In our study, no significant differences were observed in disease suppression among *Trichoderma* isolates of the same species. However, TCAS (*T. asperellum*) presented the least progress of disease incidence per area.

Hoyos-Carvajal *et al*. (2008) demonstrated the ability of *Trichoderma to* reduce the incidence of bean wilt (*Phaseolus vulgaris* L.) caused by *S. rolfsii* and cotton wilt (*Gossypium barbadense* L.) by *Rhizoctonia solani* under nursery conditions. Their results showed that the antagonistic capacity of the isolates varied depending on the targeted pathogen. Consistent with the results of this study, they found no direct correlation between taxonomic classification and antagonistic activity. Instead, significant variation was observed within the same species, emphasizing the need for careful selection of isolates in phytopathogen control programs.

Combining native *Trichoderma* isolates in a mixture was no more effective than applying each isolate separately (Tab. 4). This may be due to incompatibility among the isolates, which could have compromised their antagonistic action against the pathogen. Similarly, Ortuño *et al*. (2013) observed an antagonistic effect between different *Trichoderma* isolates by forming a defense barrier between incompatible strains within a shared growth area through the secretion of antifungal substances.

The plants treated with the formulated *T. viride* preparation exhibited the highest disease incidence. Likely, non-native *Trichoderma* isolates do not adapt well to Paraguayan conditions. Previous work demonstrated that highly effective *Trichoderma* isolates introduced from Ecuador exhibited a low level of inhibition of *Colletotrichum* spp. during *in vitro* and field tests (Sanabria Velázquez, 2020). However, native *Trichoderma* isolates have been relatively effective against soil pathogens such as *Macrophomina phaseolina* in soybeans (Franco Ortellado & Orrego Fuente, 2013)

FIGURE 2. *Stevia rebaudiana* plants inoculated with *Sclerotium rolfsii* under greenhouse conditions. A) Wilt caused by *S. rolfsii* in the control treatment. B) Mycelium of the pathogen *S. rolfsii* growing in the control treatment. C) Healthy plants treated with *Trichoderma* suspension. D) White and green colonies corresponding to *Trichoderma* spp. parasitizing sclerotia of *S. rolfsii* in treated plants.

and *Rosellinia* spp. in macadamia (Sanabria Velázquez & Grabowski, 2016). However, despite these promising results, the absence of formulation methodologies has hindered the commercial development of products based on native *Trichoderma* strains.

Percentage control of stem rot caused by *Sclerotium rolfsii*

The azoxystrobin + cyproconazole treatment reduced disease incidence by 92.7% compared to the control (24.8%) and was significantly more effective than treatments with *Trichoderma* spp. (Tab. 5). The fungus *S. rolfsii* can rapidly infect both the roots and stems of stevia, emphasizing the importance of curative treatments with fungicides in stevia production. The most effective control of *S. rolfsii* in stevia was attained with the fungicides azoxystrobin + cyproconazole. This finding aligns with previous research, which reported that azoxystrobin was the only fungicide applied as a transplant water treatment with significantly lower AUDPC values from stevia stem rot caused by *S. rolfsii* in stevia crops in North Carolina, USA (Koehler & Shew, 2017).

In plants treated with the TCAS isolate, the area of disease progression was 7.67%, corresponding to a 55.48% reduction compared to plants with 100% incidence. The 3KH isolate achieved a 44.1% control, which was statistically similar to TCAS, although it exhibited greater variability, with a 95% confidence interval ranging from –4.53% to 92.73%. The MS28 isolate provided an average control of 38.85%, with a confidence interval between 13.29% and 64.40%. The control treatment did not show the highest incidence, and the disease control achieved with the *Trichoderma* mixture (18.27%) and the formulated *T. viride* (8.84%) treatments was 24%. However, since there were no significant statistical differences between these treatments, their effectiveness can be considered equivalent.

TABLE 5. Percentage control of the disease caused by *Sclerotium rolfsii* for different treatments with *Trichoderma* spp. and azoxystrobin + cyproconazole isolates under greenhouse conditions.

Treatments	Control (%) (Mean ± standard deviation)		Confidence Interval 95%[y] (%)	
T. viride (commercial)	8.84 ±07.27	A[x]	-2.72	20.41
*Trichoderm*a mix	18.27 ±21.25	AB	-15.55	52.08
Control	24.80 ±17.49	AB	-3.03	52.62
MS28	38.85 ±16.06	BC	13.29	64.40
3KH	44.10 ±30.56	BC	-4.53	92.73
TCAS	55.48 ±16.81	C	28.73	82.23
Azoxystrobin + cyproconazole	92.7 ±10.10	D	76.63	108.77

[x]Means with a common letter are not significantly different ($P>0.05$) based on the Fisher LSD (Least Significant Difference) test. LSD=26.03. [y]95% confidence interval of the percentage control of the disease caused by *Sclerotium rolfsii* under greenhouse conditions.

The effectiveness of native *Trichoderma* isolates, such as TCAS, in suppressing *Sclerotium rolfsii* highlights the importance of selecting locally adapted strains for biological control. Native isolates are more likely to be well-adapted to the environmental and soil conditions where they are applied, potentially leading to greater persistence and efficacy in the field. Their use aligns with sustainable disease management strategies, reducing reliance on synthetic fungicides and minimizing ecological disruption. The variability observed among *Trichoderma* isolates reinforces the need for comprehensive screening programs to identify the most effective strains for specific agricultural settings.

The findings of this study have practical implications for stevia producers, who face significant losses due to *S. rolfsii*. The observed 55.48% pathogen control by TCAS in greenhouse trials suggests that *Trichoderma* based biocontrol strategies can contribute to disease suppression. However, integrating additional management strategies is likely to enhance control levels further. Similar improvements have been documented in sugar beet production, where *T. harzianum* achieved up to 88% disease control when applied to *S. rolfsii*-infested soil across two growth cycles (Upadhyay & Mukhopadhyay, 1986). Additionally, combining *T. harzianum* with the fungicide PCNB significantly reduced disease incidence and increased crop yield. A more recent reference documenting the effectiveness of *T. harzianum* in controlling *S. rolfsii* in sugar beet production is the study by Ellatif *et al*. (2019). This study found that *T. harzianum* (strain KJ831197) significantly suppressed the radial growth of *S. rolfsii* with an efficiency ranging from 77.77% to 91.11% in dual culture techniques. Additionally, the combination of *T. harzianum* with β-glucanase enzymes increased disease control effectiveness and improved sucrose content in sugar beet (Ellatif *et al*., 2019). Other studies have demonstrated that *T. asperellum* BCC1 reduced white rot-related mortality by 74% in greenhouse conditions (Rivera-Méndez *et al*., 2020), while *T. asperellum* NVTA2 suppressed disease incidence by up to 69% and enhanced plant growth (Vinodkumar *et al*., 2017). These findings suggest that an integrated disease management approach, combining *Trichoderma* with compatible fungicides or cultural practices, may be the most effective strategy for managing *S. rolfsii* in stevia cultivation.

Despite the promising results, this study has limitations that should be acknowledged. The trials were conducted under controlled greenhouse conditions, which may not fully represent the environmental variability encountered in field settings. Factors such as soil microbiome interactions, temperature fluctuations, and moisture levels could influence the performance of *Trichoderma* isolates in commercial stevia production. Future research should focus on validating these findings in field trials, optimizing application methods, and evaluating the long-term persistence of *Trichoderma* in soil. Additionally, exploring the potential of combining *Trichoderma* with organic amendments or other biocontrol agents could further enhance disease suppression. Understanding the mechanisms underlying the differential effectiveness of *Trichoderma* isolates will also contribute to the development of more targeted and reliable biocontrol strategies. By addressing these research gaps, the application of *Trichoderma* spp. can be optimized to provide stevia producers with a sustainable and effective solution for *S. rolfsii* management.

Conclusions

This study demonstrated the potential of native *Trichoderma* spp. isolates as effective biological control agents against *Sclerotium rolfsii*, a major pathogen affecting *Stevia rebaudiana* production in Paraguay. The two-phase evaluation strategy identified *T. asperellum* TCAS as the most effective isolate, reducing disease incidence by 55.48% under greenhouse conditions. However, applying *Trichoderma* as a mixture did not enhance control, suggesting possible antagonistic interactions among strains. The findings emphasize the advantages of using native isolates, which are better adapted to local conditions and offer a sustainable alternative to synthetic fungicides. While these results provide valuable insights for integrating *Trichoderma*-based biocontrol into stevia production systems, further field validation is needed to assess its long-term efficacy, persistence, and potential synergies with other management strategies. Optimizing application methods and formulation techniques will be crucial for maximizing the practical benefits of *Trichoderma*, reducing reliance on chemical control, and enhancing sustainable disease management in stevia cultivation.

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Conflict of interest statement

The authors declare that there is no conflict of interests regarding the publication of this article.

Author´s contributions

ADSV conceptualized and designed the study, supervised the experimental work, performed data analysis, and drafted the manuscript. HKBV assisted with the design and execution of greenhouse experiments, contributed to data collection, and revised the manuscript. GM conducted laboratory work, including the isolation and maintenance of fungal cultures, and contributed to manuscript

preparation. FC performed statistical analyses, contributed to data visualization, and assisted in results interpretation and manuscript review. RB coordinated the collection of soil samples, conducted in vitro assays, and contributed to manuscript preparation. CO participated in greenhouse trials, monitored disease progression, and assisted with data recording and manuscript revision. HJSO provided technical guidance, facilitated access to research facilities, supported experimental design and execution, and reviewed the manuscript. All authors approved the final version of the manuscript.

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