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In vitro antagonism of *Trichoderma harzianum* on *Sclerotium cepivorum* Berk. and *S. rolfsii* Sacc., causal agents of onion rot

Antagonismo *in vitro* de *Trichoderma harzianum* sobre *Sclerotium cepivorum* Berk. y *S. rolfsii* Sacc., agentes causales de la pudrición en cebolla

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Abstract. *In vitro* tests were carried out, to evaluate the antagonist capacity of a strain of *Trichoderma* isolated from soil in La Cienega de Chapala, Michoacán, Mexico against *Sclerotium cepivorum* and *Sclerotium rolfsii*, casual agents of onion rot, an important disease in Mexico and the rest of the world. Percentages of radial growth inhibition (PRGI) were calculated every 24 h, until a rate of antagonism was obtained according to the Bell's scale, as well as the percentages of inhibition of the production of sclerotia and their parasitism. The PRGI was 17.4% against *S. rolfsii* and 22.2% against *S. cepivorum* after 48 and 72 h, respectively. *Trichoderma harzianum* obtained a degree of antagonism of 1 on *S. cepivorum* and two against *S. rolfsii*, on the Bell scale. On the other hand, *T. harzianum* reduced (P≤0.05) 95% the production of sclerotia of *S. cepivorum* and 78% that of *S. rolfsii*; the parasitism of the sclerotia of *S. cepivorum* and *S. rolfsii* was 91% and 54%, respectively.

Keywords: Biological control; *T. harzianum*; Mycoparasistism.

Resumen. Se llevaron a cabo pruebas in vitro para evaluar la capacidad antagonista de una cepa de *Trichoderma*, aislada de suelos de La Ciénega de Chapala, Michoacán, México frente a *Sclerotium cepivorum* y *S. rolfsii* agentes causales de la pudrición de la cebolla, enfermedad de importancia en México y el mundo. Se calcularon porcentajes de inhibición del crecimiento radial (PICR) cada 24 h, hasta obtener el grado de antagonismo de acuerdo a la escala de Bell, y los porcentajes de inhibición de la producción de esclerocios y su parasitismo. El PICR fue de 17,4% frente a *S. rolfsii* y 22,2% contra *S. cepivorum* a las 48 y 72 h, respectivamente. *Trichoderma harzianum* obtuvo un grado de antagonismo de 1 sobre *S. cepivorum* y 2 contra *S. rolfsii*, en la escala de Bell. Por otra parte, *T. harzianum* redujo (P≤0,05) un 95% la producción de esclerocios de *S. cepivorum* y un 78% la de *S. rolfsii*. El parasitismo de los esclerocios fue de 91% en *S. cepivorum* y 54% en *S. rolfsii*.

Palabras clave: Control biológico; *T. harzianum*; Micoparasistismo.

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INTRODUCTION

Diseases on onion cultivation are a limiting factor for obtaining good crops. Up to 50 diseases may affect this vegetable; 30 of these are caused by fungi that attack at any stage of crop cultivation, including the postcrop bing stage (Mohan & Schwartz, 2000).

In many parts of the world, *Sclerotium cepivorum* and *S. rolfsii* cause bulb rot, and damage to the root system and foliage (Pérez et al., 1994; Montes et al., 2003). The former fungus causes white rot, induces progressive yellowing from the top to the bottom of the oldest leaves until they wither and die; simultaneously, a white mycelium develops, which produces black sclerotia (Crowe et al., 1995). When the damage is severe, plants may be pulled out easily due to the deterioration of the root system. Humid and cold environments with soil temperatures between 10 °C and 23 °C, facilitate the development of symptoms (Pinzón, 2004). *Sclerotium rolfsii* is a disease known as southern blight, and its symptomatology can be very variable due to the fact that a large number of species are susceptible to its attack. In onion cultivation, the fungus causes yellowing and withering of basal leaves, until it leads to softening of the bulbs (Jenkins & Averre, 1986).

Different strategies have been used to control the fungi that cause onion rot: cultural (Singh & Dwivedi, 1991; Argüello et al., 2009), physical (Singh et al., 1990), or chemical control (Fullerton & Stewart, 1991; Hagan & Olive, 1999), and control by resistance (Crowe, 1995; Hunger et al., 2002). However, it has been reported that the efficiency of certain fungicides depends on the density of inoculum and the number of applications of the product (Delgadillo et al., 2002). However, some degree of resistance can occur as happened with various isolates of *S. rolfsii* growing *in vitro* in the presence of thiabendazole, procymidone and iprodione. Such isolates were obtained from fields with a continuous history of use of fungicides to control root diseases, in the states of Guanajuato, Colima and Morelos, Mexico (Pérez-Moreno et al., 2009). Thereafter, previous techniques are not effective when they are applied in an isolated manner. As a result, an integrated management should include the use of biological control agents (e.g., *Trichoderma harzianum*), aqueous plant extracts, calcium nitrate, solarization and even fungicides to reduce rot of *Allium* species (Ulacio et al., 2011).

Trichoderma harzianum is a major candidate for using as biological control, because it presents different mechanisms of action which combined, make it an excellent antagonist of these *Allium*'s pathogens (Harman, 2000; Yaqub & Shahzad, 2010; Martínez et al., 2013). Besides being effective in the control of pests and diseases, some species of *Trichoderma* have the ability to stimulate growth in some plants (Stefanova et al., 1999). *Trichoderma* species have shown great ecological plasticity, and made possible the manufacture of biological products with environmentally-friendly features (Martínez et al., 2013).

This research focused on the *in vitro* determination of the antagonist effect of *T. harzianum,* and its mechanism of action, on *S. cepivorum* and *S. rolfsii*, causal agents of onion rot.

MATERIALS AND METHODS

Study area. This research was conducted in the Phytopathology Laboratory of the Interdisciplinary Research Center for Integrated Regional Development of the National Polytechnic Institute, Unit Michoacán. Soil and bulb samples with advanced rot infections with mycelium and a large number of sclerotia of *S. cepivorum* and *S. rolfsii* were collected from productive plots of onions in La Palma, Michoacán, México (20° 08' 550.4" N; 102° 45' 36.4" W).

Isolation of pathogens. Using the sclerotia obtained from bulbs of infested onions collected in crop fields, both *S. cepivorum* and *S. rolfsii* were isolated. In both cases, sclerotia were disinfected with 2% sodium hypochlorite, rinsed five times with sterile distilled water, dried with sterile absorbent paper, sown in Petri dishes with medium Potato Dextrose Agar (PDA), and incubated at temperatures of 23 °C and 27 °C, respectively.

Isolation and identification of *Trichoderma harzianum*. The antagonist fungus was isolated using the decimal dilution technique. To do this, 10 g of soil were diluted in a glass flask with 90 mL of sterile distilled water. After shaking the mix for 10 minutes, 1 mL of the dilution was sampled and put in a test tube with a screw cap, which contained 9 mL of sterile distilled water. It was shaken for 15 seconds in a vortex and 0.3 mL were sampled and spread over a culture medium of PDA acidified with 85% lactic acid contained in Petri dishes. It was then incubated for four days at a temperature of 25 ± 2 °C. Then, those colonies with morphological characteristics described for *Trichoderma* (Rifai, 1969) were selected. The isolates were purified by the cutting edge hyphal tip technique and sent to Dra. Hilda Victoria Silva Rojas at the Postgraduate School of SAGARPA; she conducted the molecular identification. The comparison of the sequence obtained of the fungus was aligned with sequences of the *Hypocrea lixii* telemorph (access code: MIAE00042) corresponding to a *Trichoderma harzianum* with maximum identity (100%).

***In vitro* confrontation of *T. harzianum* on *S. cepivorum* and *S. rolfsii*.** The antagonist capacity of *T. harzianum* was evaluated through the dual culture technique (Chérif & Benhamou, 1990). Discs of PDA of 0.5 cm in diameter with *S. cepivorum* mycelium were sown in Petri dishes with PDA, 24 hours before the antagonist, because their growth is slower. Since both have similar growth, discs of *S. rolfsii* were sown at the same time as the antagonist. In the control samples, discs with mycelium of the pathogen without the antagonist were

placed in the center of a Petri dish. The fungi were incubated at the same temperature as the one they were isolated from (i.e., 23 °C: *S. cepivorum*, 27 °C: *S. rolfsii*). The percentages of radial growth inhibition were determined every 24 h, using the Samaniego formula, cited by Ezziyyani et al. (2004): PRGI = (R1-R2)/R1*100), where R1 is the radius of the control pathogen and R2 is the radius of the pathogen with the antagonist.

Micoparasitism and antagonist classification of *T. harzianum*. The degree of antagonism was established through the contact between both fungi, measuring the progress of the antagonist over the pathogen, according to the Bell scale (Bell et al., 1982), 12 days after the evaluation started for *S. cepivorum* and 15 days for *S. rolfsii*. It included five categories: 1) *Trichoderma* covered the surface of the middle of the Petri dish, and grew totally on the pathogen; 2) *Trichoderma* grew in 2/3 parts of the Petri dish; 3) both *Trichoderma* and the pathogen colonized half of the Petri dish each; 4) the pathogen colonized 2/3 parts of the Petri dish, and 5) the pathogen completely covered the Petri dish and grew over *Trichoderma*.

Wet samples were prepared using the technique of imprinting with scotch tape to observe the interaction and parasitism of the hyphae antagonist on both pathogens. In this process, a drop of blue cotton was placed on a slide, and then a piece of tape of approximately 5 cm long and 1.5 cm wide was cut, which was placed in a previously sterilized mycological handle lighter. After this, a sample was carefully taken from the dual cultures (three replicates per Petri dish) in the area of interaction between the antagonist and each pathogen (the second and third days) lightly pressing the handle with scotch tape. Finally, a coverslip was placed on it, and it was observed in a compound microscope with 40X and 100X magnification. Photographs were obtained with a Kodak digital camera (optical 14 mega pixels).

Parasitism of *T. harzianum* on sclerotia of *S. cepivorum* and *S. rolfsii*. The production of sclerotia in the dual tests was quantified once the antagonist had colonized 100% of the mycelium of *S. cepivorum* and observed the maximum growth of *Trichoderma* on *S. rolfsii* (12 and 30 days, respectively). The percentage of inhibition was obtained by differentiating the number of sclerotia formed after comparisons with the control samples. After they were taken out of the Petri dish, they were rinsed with sterile distilled water, treated with 2% sodium hypochlorite for one minute, rinsed five times with sterile water, and dried with absorbent paper under a laminar flow hood. The test for parasitized sclerotia was carried out by choosing 20 samples at random, both of each pathogen, and samples from control; all were sown in PDA and incubated at 23 °C (*S. cepivorum*) and 27 °C (*S. rolfsii*). Four days after sowing, the percentage of parasitized sclerotia was determined through null growth and/or viable sclerotia.

The bioassays of sclerotia production were designed in blocks totally at random with four repetitions per treatment and were carried out twice in order to confirm the reproducibility of the results. One-way analysis of variance was conducted by the GLM (General Lineal Model) and the comparison of media through the Tukey test (P≤0.05) with the Statistic Analysis System program (SAS Institute, 1988).

RESULTS AND DISCUSSION

***In vitro* confrontation of *T. harzianum* on *S. cepivorum*.** *Trichoderma harzianum* acquired better control in less time on *S. cepivorum* than on *S. rolfsii*. This effect was possibly related to the fast growth that characterizes the genus *Thichoderma* (Martínez et al., 2008) or the degree of specificity of the antagonist against each pathogen (Hoyos et al., 2008). The contact among the hyphae of both microorganisms was observed at 72 h, with a PRGI of 22.2% (Fig. 1) at a temperature of 23 °C. In another study performed by Hernández et al. (2011) it was obtained a PRCI among 50.9% and 81.5%, with different species of *Trichoderma*. However, Hernández performed his study at 27 °C, which is not an optimum temperature for growth of *S. cepivorum*. The optimum conditions to obtain the highest percentage of eruptive sclerotia and hyphal germination of this fungus were at 22 °C (Rodríguez et al., 2011).

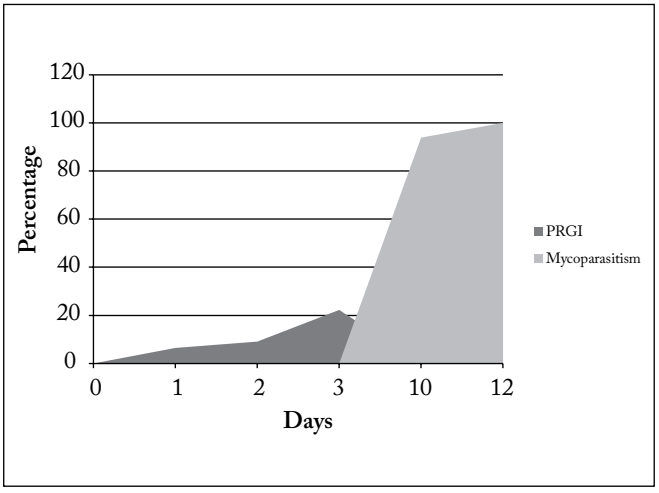

Fig. 1. Percentage of radial growth inhibition (PRGI) of mycelium, after 72 h, and mycoparasitism (%) according to Bell scale, of *T. harzianum* on *S. cepivorum* in the dual culture, at 12 days from the test assembly.

Fig. 1. Porcentaje de inhibición del crecimiento radial (PICR) del micelio, a las 72 h, y micoparasitismo (%) de acuerdo a la escala de Bell, de *T. harzianum* sobre *S. cepivorum* en los cultivos duales, a los 12 días desde el montaje del ensayo.

***In vitro* confrontation of *T. harzianum* on *S. rolfsii*.** During the confrontation between pathogens and the antagonist, a halo of inhibition was formed 44 h after being sown in the PDA medium. Later, the mycelium turned brown in the contact zone between both microorganisms. Under these conditions, there was competition for space, nutrients, and possibly oxygen (Sivan & Chet, 1989; Inbar & Chet, 1997). After 48 h, the PRGI was 17.4% at 27 °C (Fig. 2). In contrast, another study which did not indicate the temperature at which the measurements were made, *Trichoderma* sp. inhibited the radial growth of *S. rolfsii* 62.5% at 96 h of the evaluation in dual culture (Folgueras et al., 2008). Temperature is an important feature in this type of evaluations. *Trichoderma harzianum* demonstrated a better antagonistic effect against S. *rolfsii* at 25 °C than at 30 °C; even at this temperature, it is difficult to control the pathogen (Rollan et al., 1999).

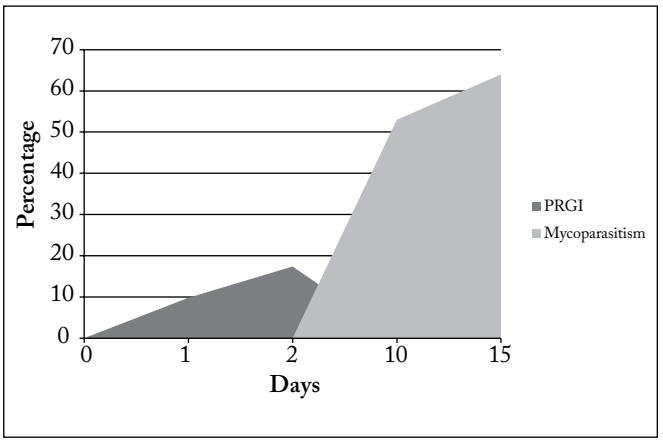

Fig. 2. Percentage of radial growth inhibition (PRGI) of mycelium, after 48 h, and mycoparasitism (%) according to Bell scale, of *T. harzianum* on *S. rolfsii* in the dual culture, at 15 days from the test assembly.
Fig. 2. Porcentaje de inhibición del crecimiento radial (PICR) del micelio a las 48 h, y micoparasitismo (%) de acuerdo a la escala de Bell, de *T. harzianum* sobre *S. rolfsii* en los cultivos duales, a los 15 días desde el montaje del ensayo.

Mycoparasitism and antagonist classification of *T. harzianum*. According to Bell's scale (Bell et al., 1982), *T. harzianum* obtained a degree of antagonism of 1 against *S. cepivorum* because it successfully colonized the entire Petri dish (only spent 12 days). It grew on the pathogen and maintained a strong sporulation from its sclerotia; the mycoparasitism of sclerotia of *S. cepivorum* also has been reported by McLean et al. (2001). Nevertheless, *T. harzianum* only achieved a degree of antagonism of 2 against *S. rolfsii*, by colonizing 2/3 parts of the Petri dish; changes were not significant from 15 to 30 days (Fig. 3). Another study evaluated 20 native strains of *Trichoderma* against *S. rolfsii*, five of which achieved a degree of antagonism of 1 after 120 h of confrontation, but at a temperature of 25 °C (Corrêa et al., 2007). This pathogen is considered to exhibit a rapid and aggressive growth because the mycelium confronts in less than 48 h, and in addition, it has a higher degree of resistance against the antagonist. This is because it forms rhizomorphs, allowing it to compete with the antagonist, although after three days these rhizomorphs were destroyed by *T. harzianum*. In addition, through the excretion of secondary metabolites such as trichozianine, *T. harzianum* is capable of controlling the growth of mycelium and the formation of pathogen sclerotia (Correa et al., 1996; Alvarado et al., 2011).

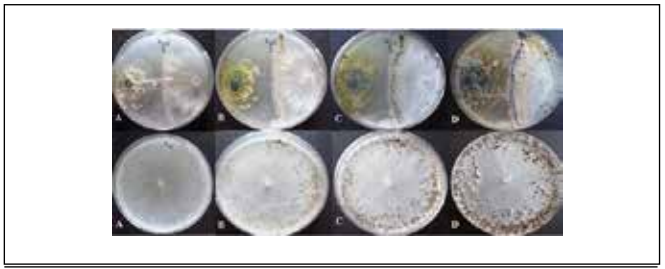

Fig. 3. Dual tests and controls of *T. harzianum* against *S. rolfsii* (A=4, B=7, C=10 and D=30 days of confrontation).
Fig. 3. Pruebas duales y controles de *T. harzianum* contra *S. rolfsii* (A=4, B=7, C=10 y D=30 días de enfrentamiento).

Parasitism of *T. harzianum* on mycelium of *S. cepivorum* and *S. rolfsii*. The antagonistic strain showed an excellent capacity to parasitize the mycelium of both pathogens. Observations under a microscope confirmed the interaction of mycelium, showing winding of hyphae, distortion, detachment of mycelium and, in advanced stages, lysis and degradation of the pathogen hyphae (Fig. 4 A, B, C); similar results were reported by various authors (García et al., 2006; Martínez et al., 2008; Shaigan et al., 2008; Yaqub & Shahzad, 2010; Alvarado et al., 2011). In such cases, hydrolytic enzymes, such as chitinases and β-1-3 glucanases, caused the disintegration of the hyphae cellular wall on the pathogen, and were later parasitized by *Trichoderma* (Benhamou & Chet, 1996; González et al., 2010).

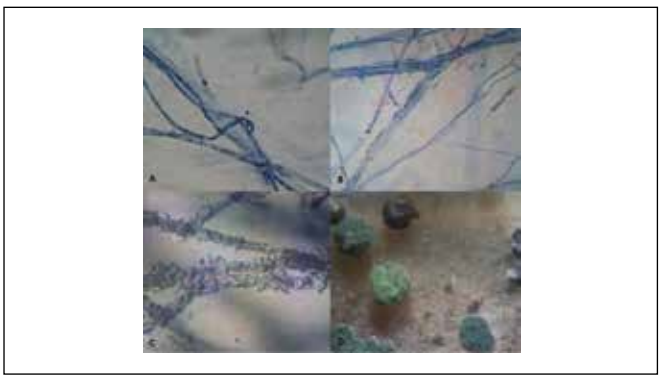

Fig. 4. Mycoparasitism of *T. harzianum* on *S. rolfsii*; A) curl of hyphae of antagonist "a" on the hyphae of *S. rolfsii* "b" (40X), B) conidia of *T. harzianum* on mycelium of pathogen (40X), C) lysis of mycelium of *S. rolfsii* (100X), D) sclerotia of *S. rolfsii* colonized by *T. harzianum*.
Fig. 4. Micoparasitismo de *T. harzianum* sobre *S. rolfsii;* A) enrollamiento de hifas del antagonista "a" sobre hifas de S. rolfsii "b" (40X), B) conidios de *T. harzianum* sobre micelio del patógeno (40X), C) lisis del micelio de *S. rolfsii* (100X), D) esclerocios de *S. rolfsii* colonizados por *T. harzianum*.

Parasitism of *T. harzianum* on sclerotia of *S. cepivorum* and *S. rolfsii*. The formation of sclerotia of *S. cepivorum* and *S. rolfsii* was delayed 24 and 48 h, respectively. *Trichoderma harzianum* was able to efficiently inhibit the number of sclerotia of both pathogens; there were significant differences (P≤0.05) in comparison to control samples. *Trichoderma* had a greater effect on the inhibition of sclerotia of *S. cepivorum* than on those of *S. rolfsii* (Table 1). These results coincide with another study which also showed a delay in the production and number of sclerotia (Alvarado et al., 2011). This fungus was also able to grow and sporulate on the sclerotia of both pathogens (Fig. 4 D). Nevertheless, the parasitism in sclerotia of *S. cepivorum* was higher (91%) in comparison to *S. rolfsii* (54%) (Fig. 5). Other studies reported high percentages of parasitism in both species with *T. harzianum* and *T. koningii* (Mónaco et al., 1998).

Table 1. Percentage of inhibition of the production of esclerotia of *S. cepivorum* and *S. rolfsii* by effect of *T. harzianum*.
Tabla 1. Porcentaje de inhibición de la producción de esclerocios de *S. cepivorum* y *S. rolfsii* por efecto de *T. harzianum*.

Strains (Individual and dual cultures)	Number of esclerotia	Inhibition of the esclerotia
S. cepivorum	4292 a	95%
S. cepivorum/T. harzianum	211 b	
S. rolfsii	381 a	78%
S. rolfsii/T. harzianum	83 b	

Means with different letters within a column are significantly different (P≤0.05).

CONCLUSIONS

Trichoderma harzianum showed accelerated growth *in vitro* cultures, with high antagonistic capacity against both pathogens that were analyzed. However, the control appeared to be greater on *S. cepivorum* than on *S. rolfsii*. It was evident that the mycoparasitism inhibited not only the growth of the mycelium, but also the production of sclerotia and the parasitizing of these structures.

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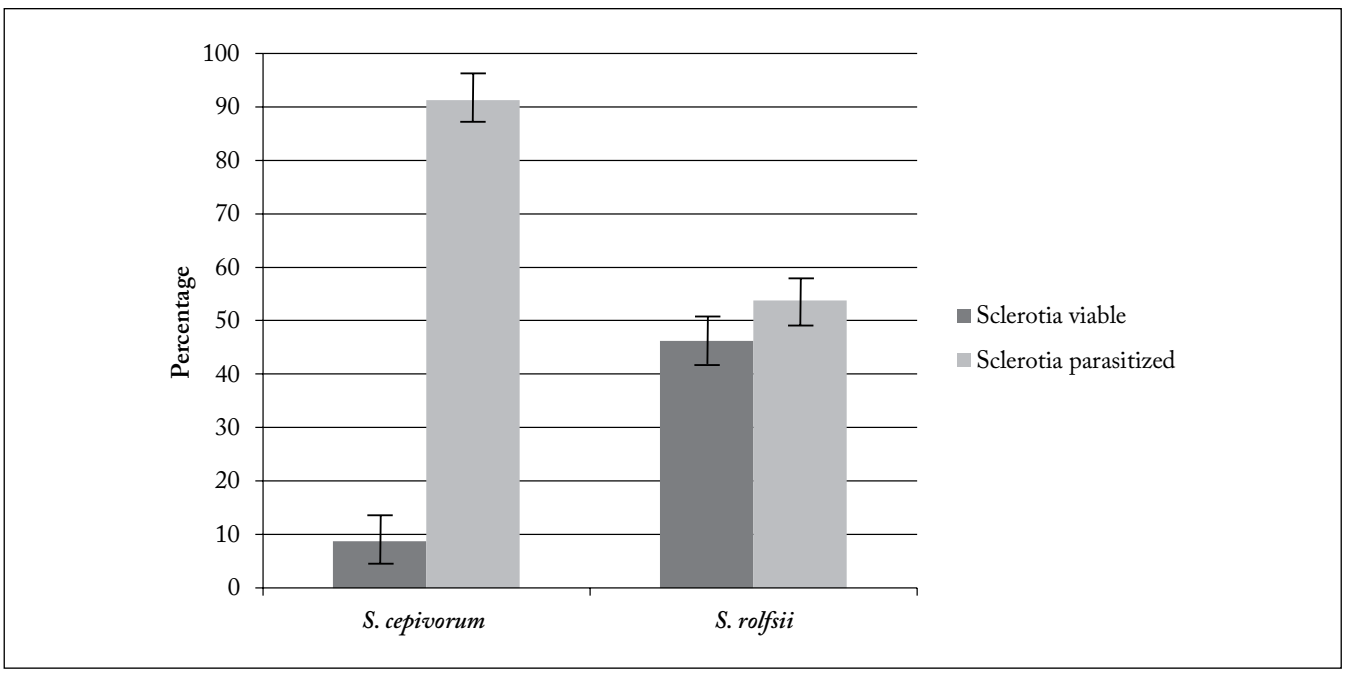

Fig. 5. Percentage of parasitized sclerotia of *S. cepivorum* and *S. rolfsii* by *T. harzianum*.
Fig. 5. Porcentaje de esclerocios parasitados de *S. cepivorum* y *S. rolfsii* por *T. harzianum*.

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