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In vitro effect of Cuban strains of *Trichoderma* spp. on the growth of the pathogen *Phyllosticta citricarpa* (McAlpine) Aa

Efecto *in vitro* de cepas cubanas de *Trichoderma* spp., sobre el crecimiento del patógeno *Phyllosticta citricarpa* (McAlpine) Aa

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ABSTRACT: The objective of this research was to evaluate the *in vitro* effect of *Trichoderma* spp. on *Phyllosticta citricarpa* (McAlpine) Aa growth. A total of twenty *P. citricarpa* isolates from major citrus-producing regions in Cuba were included in the analysis. *Trichoderma harzianum* Rifai (strains LBAT-34 and LBAT-53) and *Trichoderma viride* Pers. (strain LBAT-TS3) were tested for their ability to inhibit mycelial growth of *P. citricarpa.* Inhibition was evaluated using the *in vitro* dual culture assay. Percentages of inhibition of radial growth (PIRG) were determined after 3, 5, and 7 days, and type and intensity of the antagonism of *Trichoderma* were also classified. Kruskal-Wallis and non-parametric multiple comparison tests were implemented for the statistical analysis. Results showed that the percentages of inhibition of radial growth ranged from 38.7% to 29.2% after 3 days of co-culture. After the fifth day, the antagonist strains overran the *P. citricarpa* colonies and inhibited their growth completely. Furthermore, it was found that the mycelial growth inhibition of *P. citricarpa* isolates by *Trichoderma* spp. was through mechanisms of antibiosis and hyperparasitism. This study is the first evaluation of the susceptibility of *P. citricarpa* to biological control agents in Cuba.

Keywords: antagonism, biocontrol agents, citrus black spot, *Phyllosticta citricarpa*, *Trichoderma* spp.

RESUMEN: El objetivo de este trabajo fue evaluar el efecto de *Trichoderma* spp. sobre el crecimiento *in vitro* de *Phyllosticta citricarpa* (McAlpine) Aa. En el análisis se incluyeron un total de 20 cepas de *P. citricarpa*, previamente aisladas de las principales regiones citrícolas de Cuba. Se evaluó la capacidad de *Trichoderma harzianum* Rifai (cepas LBAT-34 y LBAT-53) y *Trichoderma viride* Pers. (cepa LBAT-TS3) para inhibir el crecimiento micelial de *P. citricarpa*. La inhibición se evaluó mediante el ensayo de cultivo dual *in vitro*. Se determinaron los porcentajes de inhibición del crecimiento radial (PICR) luego de 3, 5 y 7 días y se clasificó el tipo e intensidad del antagonismo de *Trichoderma*. Para el análisis estadístico se implementaron las pruebas de Kruskal-Wallis y de comparación múltiple no paramétrica. Los resultados mostraron porcentajes de inhibición del crecimiento radial entre 38,7 % a 29,2 %, luego de tres días de co-cultivo. Posterior al quinto día, las cepas antagonistas invadieron las colonias de *P. citricarpa* e inhibieron completamente su crecimiento. Adicionalmente, se detectó que la acción antagonista de *Trichoderma* spp. se desarrolló mediante mecanismos de antibiosis e hiperparasitismo. Este estudio constituye la primera evaluación de la susceptibilidad de *P. citricarpa* frente a agentes de control biológico en Cuba.

Palabras clave: agentes de biocontrol, antagonismo, mancha negra de los cítricos, *Phyllosticta citricarpa*, *Trichoderma* spp.

Citrus black spot (CBS) is caused by the phytopathogenic fungus *Phyllosticta citricarpa* (McAlpine) van der Aa and affects *Citrus* spp. This disease is present in regions with summer rainfall climates of Africa, Asia, Australia, and the Americas (1). *P. citricarpa* causes damages on the appeal of the fruit, but the internal fruit quality remains unaffected. In addition, severe infections may cause premature fruit drop. The presence of CBS is related to production losses, high costs associated with management, and trade restrictions in the fresh produce

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market. Due to the potential economic impact, *P. citricarpa* is identified of quarantine importance in CBS-free citrus producing areas (2, 3).

Fungicides have been shown to be effective in reducing damage by *P. citricarpa* and increase yields of quality fruit (2, 3, 4). Nevertheless, incorrect use of chemicals can lead to selection of resistant fungal strains (5). In this sense, due to increasing concerns about the environment, human health issues and high costs associated with fungicides, biological-based control strategies are studied as promising alternatives. The control of *P. citricarpa* with biological agents, such as *Trichoderma* spp., were examined with encouraging results by treatments with highly suppressive effects on the *in vitro* pathogen growth (≥ 30%) (6, 7). *Trichoderma* spp. have been previously reported to be an effective biological control agent of plant pathogens by mechanisms of antibiosis, mycoparasitism, competition for nutrients and space, or induction of host resistance and promotion of plant growth (8).

In Cuba, *P. citricarpa*, which has affected different citrus-productive regions, was first reported in 2007 (3, 9). The management of CBS in Cuba is based on a combination of chemical control and cultural practices. In addition, it should be noted that, although biocontrol agents are part of the management toolbox for production of tobacco (*Nicotiana tabacum* L.), vegetables, and ornamental plants in our country (10), this approach have not yet been tested for CBS control. Thus, the objective of this research was to evaluate *in vitro* of *Trichoderma* spp. on *P. citricarpa* growth.

A total of twenty *Phyllosticta citricarpa* strains (IIFT A1, IIFT A2, IIFT A4 to IIFT A11, IIFT B1 to IIFT B4, IIFT B6, IIFT C1 to C3, IIFT D1, and IIFT D2) were included in this study. They were collected from major Cuban citrus-producing provinces (Matanzas, Cienfuegos, Camagüey and Santiago de Cuba) and identified using a polyphasic taxonomy approach (Table 1) (9). The strains of *Trichoderma* spp., *Trichoderma harzianum* Rifai (strains LBAT-34 and LBAT-53) and *Trichoderma viride* Pers. (strain LBAT-TS3), were provided by the Department of Technologies for Biological Media Production at the Plant Health Research Institute (INISAV), located in Havana, Cuba.

Single spore cultures of each fungal isolate were stored on dried filter paper at 4°C and -20°C, as described by Silva-Junior *et al.* (2). When needed, small fragments of these colonized filter papers were placed on PDA. Incubation was performed in the dark for 7 days at 27°C. Fungal agar plugs (5 mm diameter), taken from the edge of actively growing mycelium, were used as inoculums for the experiments (9).

Growth inhibition of *P. citricarpa* by *Trichoderma* spp. was evaluated using the *in vitro* dual culture assay (7). A 5 mm diameter mycelium-agar plug of each *P. citricarpa* isolate was placed 1 cm from the edge of a fresh PDA plate. After 5 days of incubation (dark, 27°C), plugs of each *Trichoderma* spp. were transferred to the opposite edge of the plates. Dual cultures were incubated for 7 days and radial growth of *P. citricarpa* was measured after 3, 5, and 7 days. Percentage of inhibition of radial growth (PIRG) was determined by $\text{PIRG}(\%) = \frac{(\text{R1} - \text{R2})}{\text{R1}} \; x \; 100$, where R1: radial growth of the pathogen on PDA (control treatment) and R2: radial growth of the pathogen on dual culture (11). Type and intensity of the antagonism of *Trichoderma* spp. was classified following Davet *et al*. (12).

Table 1. Collection details of the *Phyllosticta citricarpa* isolates included in this study (9) / Detalles de la colección de las cepas de *Phyllosticta citricarpa* empleadas en este estudio (9).

Species and Culture no[1]	Host		Province, Country
	***Citrus* species, plant sample**	***Citrus* cultivars**	
P. citricarpa IIFT A1	*Citrus sinensis*, fruit	'Valencia' orange	Cienfuegos, Cuba
P. citricarpa IIFT A2	*Citrus sinensis*, fruit	'Valencia' orange	Cienfuegos, Cuba
P. citricarpa IIFT A4	*Citrus sinensis*, fruit	'Valencia' orange	Cienfuegos, Cuba
P. citricarpa IIFT A5	*Citrus sinensis*, fruit	'Valencia' orange	Cienfuegos, Cuba
P. citricarpa IIFT A6	*Citrus sinensis*, fruit	'Valencia' orange	Cienfuegos, Cuba
P. citricarpa IIFT A7	*Citrus sinensis*, fruit	'Valencia' orange	Cienfuegos, Cuba
P. citricarpa IIFT A8	*Citrus sinensis*, fruit	'Valencia' orange	Cienfuegos, Cuba
P. citricarpa IIFT A9	*Citrus sinensis*, fruit	'Valencia' orange	Cienfuegos, Cuba
P. citricarpa IIFT A10	*Citrus sinensis*, fruit	'Valencia' orange	Cienfuegos, Cuba
P. citricarpa IIFT A11	*Citrus sinensis*, fruit	'Valencia' orange	Cienfuegos, Cuba
P. citricarpa IIFT B1	*Citrus sinensis*, fruit	'Valencia' orange	Matanzas, Cuba
P. citricarpa IIFT B2	*Citrus sinensis*, fruit	'Valencia' orange	Matanzas, Cuba
P. citricarpa IIFT B3	*Citrus sinensis*, fruit	'Valencia' orange	Matanzas, Cuba
P. citricarpa IIFT B4	*Citrus sinensis*, fruit	'Valencia' orange	Matanzas, Cuba
P. citricarpa IIFT B6	*Citrus paradisi*, fruit	'Marsh Jibarito' grapefruit	Matanzas, Cuba
P. citricarpa IIFT C1	*Citrus sinensis*, fruit	'Valencia' orange	Camagüey, Cuba
P. citricarpa IIFT C2	*Citrus sinensis*, fruit	'Valencia' orange	Camagüey, Cuba
P. citricarpa IIFT C3	*Citrus paradisi*, fruit	'Marsh Jibarito' grapefruit	Camagüey, Cuba
P. citricarpa IIFT D1	*Citrus sinensis*, fruit	'Valencia' orange	Santiago de Cuba, Cuba
P. citricarpa IIFT D2	*Citrus reticulata*, fruit	'Dancy' tangerine	Santiago de Cuba, Cuba

[1]IIFT: Instituto de Investigaciones en Fruticultura Tropical (Research Institute for Tropical Fruit Crops).

Randomized designs were applied to evaluate the effect of each *Trichoderma* spp. on *P. citricarpa* growth. Each experiment with five replicas per treatment was carried out twice. Two colony diameter measurements were taken perpendicular to each other. PIRG data were analyzed by Kruskal-Wallis and non-parametric multiple comparison tests with STATISTICA version 14 software (13).

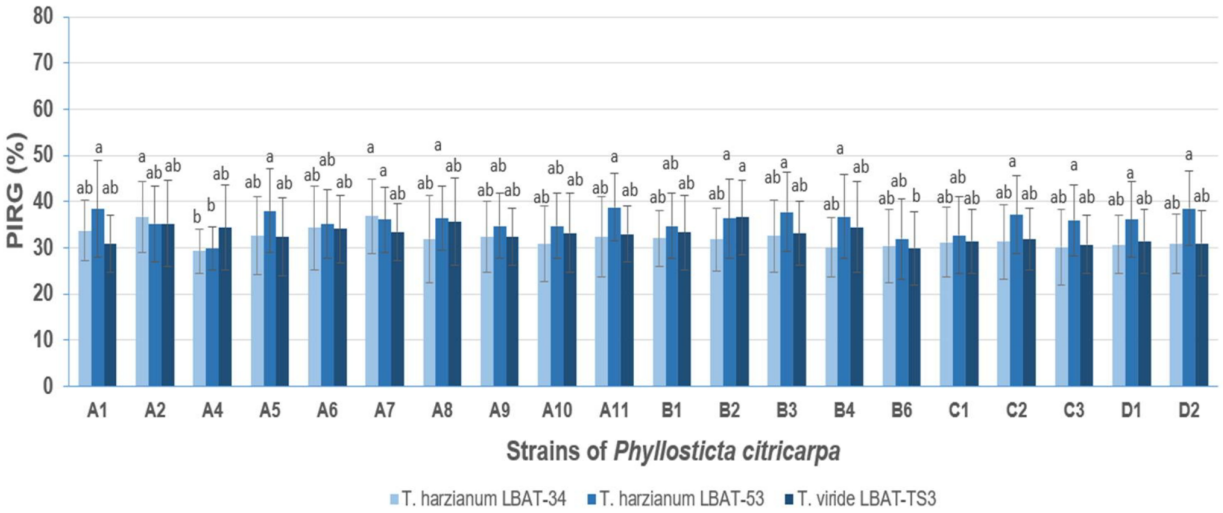
As results, dual culture tests showed that *Trichoderma* spp. inhibited the growth of *P. citricarpa* with significant statistical differences between the PIRGs achieved by the antagonists ($H=173.6626$; $P < 0.001$) (Fig. 1).

After 3 days of incubation, the strains of *Trichoderma* spp. showed PIRG values that ranged from 38.7 % to 29.2% (Fig. 1). At this stage of the assay, the highest percentages of radial growth inhibition of *P. citricarpa* (≥ 35.8%) were obtained with *T. harzianum* strain LBAT-53 against 12 strains of *P. citricarpa* (IIFT A1, IIFT A5, IIFT A7, IIFT A8, IIFT A11, IIFT B2 to B4, IIFT C2, IIFT C3, IIFT D1, and IIFT D2); *T. harzianum* strain LBAT-34 against the IIFT A2 and A7 strains, and *T. viride* LBAT-TS3 against *P. citricarpa* strain IIFT B2. The lowest inhibition values (≤ 29.9%) were recorded with *T. harzianum* strain LBAT-34 against *P. citricarpa* strain IIFT A4, *T. harzianum* strain LBAT-53 against strain IIFT A4, and *T. viride* LBAT-TS3 against strain IIFT B6. As the PIRG values of the remaining treatments did not differ significantly from those mentioned above (Fig. 1), they were considered of intermediate effects.

Interestingly, after 5 and 7 days of co-culture, the antagonist strains overran the *P. citricarpa* colonies (Fig. 2) and completely inhibited their growth (100% of PIRG). These inhibition values achieved by the strains of *Trichoderma* spp. did not show statistical differences between them, but they significantly differed from those achieved after 3 days of incubation (Fig. 1).

T. harzianum LBAT-34 and LBAT-53 were previously characterized for the control of the phytopathogenic fungi *Rhizoctonia solani* J.G. Kühn, *Fusarium* spp., *Agroathelia rolfsii* (Sacc). Redhead & Millineux, and *Bipolaris oryzae* (Breda de Haan) Shoemaker (14, 15), while *T. viride* LBAT-TS3 showed to be antagonistic against nematodes (16). The mentioned *Trichoderma* strains are the basis of the commercially available bio-products TRICOSAVE 34, TRICOSAVE 53, and TRICOSAVE TS3. These products are produced in Cuba to be applied under greenhouse and field conditions as part of an integrated system to control fungi and oomycetes in the production of tobacco, vegetables, and ornamental plants (10).

Several mechanisms have been reported in the suppression of pathogenic fungi by *Trichoderma* spp. These include competition for nutrients and space, antibiosis, and hyperparasitism. In the present work, the growth inhibition of *P. citricarpa* in dual culture prior to physical contact with the antagonist suggested an antibiosis mechanism of *Trichoderma* spp. This mechanism can be attributed to bioactive compounds of volatile or non-volatile nature produced by the antagonists (10). Additionally, a hyperparasitic effect was manifested by *Trichoderma* spp. as observed from the rapid colonization (after 5 days of incubation) and the production of reproductive structures on the pathogen colony. The antagonism of *Trichoderma* spp. in this study could be classified as of high intensity (++) since the PIRG values were > 25% (12). In this regard, Kupper *et al*. (6) concluded that *Trichoderma* spp. was a promising antagonist against *P. citricarpa*, with PIRG values of 31, 37, and 50% after 5 days of co-incubation.



*Average of two independent experiments ± SD: standard deviation. Different letters indicate significant differences ($p < 0.001$) by non-parametric multiple comparison tests / Promedio de dos experimentos independientes ± SD: desviación estándar. Letras diferentes indican diferencias significativas ($p < 0.001$) según prueba no paramétrica de comparación múltiple.

Figura 1. *In vitro* sensitivity of twenty *Phyllosticta citricarpa* isolates to biological control agents. Bars represent percentages of inhibition of radial growth (PIRG) for each strain of *Phyllosticta citricarpa* after 3 days of dual culture with *Trichoderma* spp. / Sensibilidad *in vitro* de 20 cepas de *Phyllosticta citricarpa* frente a agentes de control biológico. Las barras representan los porcentajes de inhibición del crecimiento radial (PICR) para cada cepa de *P. citricarpa* después de 3 días de cultivo dual con *Trichoderma* spp.

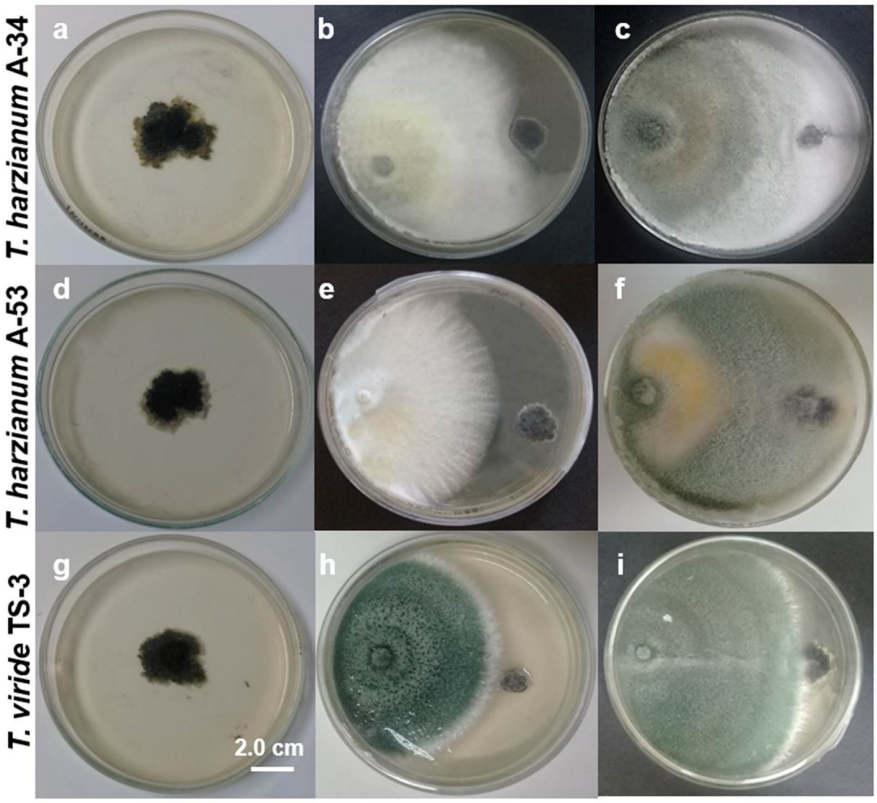


Figura 2. Dual culture of *Phyllosticta citricarpa* IIFT A1 with *Trichoderma harzianum* LBAT-34, *T. harzianum* LBAT-53, and *T. viride* LBAT-TS3. (a, d, g) Control treatment: *P. citricarpa* IIFT A1 on PDA, after 8 days of incubation. (b, e, h) *P. citricarpa* IIFT A1 + *Trichoderma* spp., after 3 days and (c, f, i) 5 days of co-culture. / Cultivo dual de *Phyllosticta citricarpa* IIFT A1 con *Trichoderma harzianum* LBAT-34, *T. harzianum* LBAT-53 y *T. viride* LBAT-TS3. (a, d, g) Control: *P. citricarpa* IIFT A1 en PDA, luego de 8 días de incubación. (b, e, h) *P. citricarpa* IIFT A1 + agentes de control biológico a los 3 días y (c, f, i) 5 días de co-cultivo.

Moreover, the *P. citricarpa* isolates evaluated in this study included isolates from diverse geographic regions of Cuba, where, also, most of the Cuban citrus is produced. These analyses provide a broader view of the effects of the biological control agents on the *P. citricarpa* populations present in Cuba.

It should be noted that, although *Trichoderma* spp. are commonly found in the soil associated with the plant root ecosystem, it can be used successfully for foliar fungal disease management (8, 17). The presence of *Trichoderma* in the rhizosphere of the plant can induce the systemic resistance mechanism and, therefore, promote the protection of other organs besides the roots (17, 18). Several formulations of *Trichoderma*, which may include active spores of this antagonist and/or metabolite extracts, have been shown to be effective for foliar pathogen management. Also, these formulations are recommended for foliar applications or for being directly applied to the soil (8, 18).

Apparently, this is the first study in Cuba that assesses the *in vitro* susceptibility of *P. citricarpa* to biological control agents. Taken together, these results suggest a potential new tool to be included in the management of CBS together with the chemical and cultural strategies currently used. Nevertheless, further evaluations need to be conducted to validate the biocontrol efficacy of *Trichoderma* against *P. citricarpa* under production conditions.

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