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Hypocrea/Trichoderma viridescens ITV43 with potential for biocontrol of *Moniliophthora roreri* Cif & Par, *Phytophthora megasperma* and *Phytophthora capsici*

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A *HypocreaTrichoderma* strain was isolated from cacao (*Theobroma cacao* L.) pods from a cacao plantation in Huimanguillo, Tabasco, Mexico. The strain was identified as *H./T. viridescens* ITV43 by its morphological characteristics and DNA sequence analysis of ITS1-5.8s-ITS2 and was tested against *Moniliophthora* roreri Cif & Par and *Phytophthora* strains by confrontation experiments. The biocontrol of *H./T. viridescens* ITV43 measured as biocontrol index (BCI) at seven days against *M. roreri*(HT-ITV01, HT-ITV20, HT-ITV27), *P. megasperma* (HT-ITV08, HT-ITV15, HT-ITV37) and *P. capsici* (HT-ITV14, HT-ITV16, HT-ITV33) strains ranged from 78.28-81.63, 63.04-69.95 and 69.77-76.31%, respectively. After 31 days of incubation, the BCI values increased to 81.52-86.50 for *M. roreri* and 100% for *Phythopthora* strains. Samples taken from the interaction zone were observed by optical and scanning electron microscopy. The results show the effect of *H./T. viridescens* ITV43 strain on morphological deformations and disorganization of the *M. roreri* and *Phytophthora* cell wall structures. Highest BCI values obtained suggest that the *Hypocrea* strains isolated from *T. cocoa* could have the ability to antagonize more efficiently against fungal pathogens of this crop.

Key words: Antagonism, *Hypocrea*, mycoparasitism, *Moniliophthora*, *Phytophthora, Theobroma.*

INTRODUCTION

Cacao (*Theobroma cacao* L.) is one of the most important tropical crops since the seeds of its fruits are the raw material in the production of chocolate. However, diseases caused by fungi that attack cacao plantation have seriously affected cacao production. Among these, the witches broom caused by *Moniliophthora perniciosa* and the frosty pod rot caused by *Moniliophthora roreri*, are the most devastating. Losses are been estimated between 40 to 90% in several countries of America (Phillips-Mora and Wilkinson, 2007; Sánchez-Mora and Garces-Fiallos, 2012). On the other hand, black pod disease caused by species of *Phytophthora,* which are

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widely distributed in several countries of America, Asia and Africa cause losses in cacao plantation between 45 and 100% (Ploetz, 2007; Djocgoue et al., 2010). The control of these diseases is carried out by the application of fungicides, plant health practices and use of resistant cocoa varieties (Tondje et al., 2007). However, these conventional methods are not enough to completely stop the development of diseases in cocoa producing regions, being still necessary new tools to integrate into a comprehensive management program of cocoa diseases. An alternative strategy is the use of microorganisms isolated from the cacao tree or from its environment. For example, *Trichoderma ovalisporum* isolated from *Theobroma grandiflorum* and from *Banisteriopsis caapi* in Brazil and Ecuador, respectively demonstrated its ability to antagonize and parasitize *M. roreri* (Holmes et al., 2004). On the other hand, *Trichoderma theobromicola* and *Trichoderma paucisporum* isolated from healthy *Theobroma cacao* in Peru and Ecuador, respectively, showed an antibiotic effect against *M. roreri* on cocoa fruit *in situ* and *in vitro* experiments (Samuels et al., 2006). *Trichoderma martiale* isolated in Brazil from *Theobroma cacao* reduced the severity of symptoms caused by *P. palmivora* on cocoa fruit in the field (Hanada et al., 2008, 2009) and *Trichoderma stromaticum* isolated from *Theobroma grandiflorum*, was mycoparasitic on *Phytophthora palmivora* (Hanada et al., 2010) and is the active ingredient of 'Tricovab', a product that is being applied in the field to control black pod disease in Brazil (De Souza et al., 2008). Moreover, *Trichoderma asperellum* isolated from soil in Cameroon was mycoparasitic on *P. capsici*, *P. citrophthora* and *P. palmivora*, other causal agents of black pod worldwide (Tondje et al., 2007).

In a previous study, 128 fungal isolates were isolated from aerial plant tissues of cacao including tree trunks, stems and fruits with visual symptoms of frosty pod rot and black pod disease (Cuervo-Parra et al., 2011a). One of these isolates named ITV43 was identified to the genus level as *Hypocrea*/*Trichoderma*. The objectives of this study were to determine (i) the species of *Hypocrea*/*Trichoderma* strain ITV43 and (ii) its antagonistic activity *in vitro* against the plant pathogens *M. roreri*, *P. megasperma* and *P. capsici.*

MATERIALS AND METHODS

Fungal strains

Hypocrea/Trichoderma strain ITV43 was isolated from aerial plant tissues of cacao tree. *P. megasperma* (HT-ITV08, HT-ITV15, HT-ITV37) and *P. capsici* (HT-ITV14, HT-ITV16 and HT-ITV33) strains were isolated from cocoa fruits with symptoms of black pod from El Caobanal, La Noria and La Hacienda plantations, located in the municipality of Huimanguillo, Tabasco. *M. roreri* HQ231236, *M. roreri* JN241966 and *M. roreri* JN241967 strains were isolated previously from the same plantations (Cuervo-Parra et al., 2011a). The strains were maintained on potato dextrose agar medium (PDA) while the experiments were performed.

Morphological characterization of *Hypocrea/Trichoderma* ITV43

The *Hypocrea/Trichoderma* strain ITV43 was cultured on cornmeal agar with 2% (w/v) dextrose (CMD: cornmeal agar 17 g, dextrose 20 g, 1000 ml distilled water) and on synthetic low-nutrient agar (SNA: KH_2PO_4 1.0 g, KNO_3 1.0 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, KCl 0.5 g, glucose 0.2 g, sucrose 0.2 g, 1000 ml distilled water, agar 20.0 g). Petri dishes (9 cm-diam) were incubated under ambient laboratory conditions of light and dark at 25-28°C for 10 days. The morphology and color of the colony, formation and shape of pustules, diffusing pigment in the agar and odor were recorded. To analyze conidiophores, phialides and conidia, samples of pustules were placed in a drop (3% w/v KOH) and observed with a compound microscope (Leica DM 3000, Leica Microsystems GmbH Wetzlar, Germany) using bright field (BF) and phase contrast (PC). Chlamydospores were observed by examining the reverse of the colony grown on CMD with 20x objective. Thirty measurements for each of the following structures were made: conidium length, width, and length/width ratio (L/W), phialide length, width, length/width ratio (L/W), base of phialide and cells supporting the phialides, chlamydospore length, width, and length/width ratio (L/W). Measurements of the characters were taken from images using the software LAS 3.2.0 version Leica Microsystems (GmbH Wetzlar, Germany) and were reported as extremes in brackets separated by the means plus and minus the standard deviation. Also, the 95% confidence interval (CI) was calculated for each parameter. The ITV43 strain was identified using the key to species of *Hypocrea* with warted conidia (Jaklitsch et al., 2006).

Molecular characterization of *Hypocrea/Trichoderma* strain ITV43, *P. megasperma, P. capsici* strains and phylogram tree

Hypocrea/Trichoderma strain ITV43, *P. megasperma* (HT-ITV08, HT-ITV15, HT-ITV37) and *P. capsici* (HT-ITV14, HT-ITV16, HT-ITV33) strains were grown on potato broth medium (PD) at room temperature and 250 rpm. PD medium composition (expressed in g per liter in 1000 ml of distilled water) was as follows: glycerol, 20; polypeptone, 10 yeast extract, 10, glucose 5, and 100 ml of potato extract. The mycelium was grown for three days recovering and washing twice with TE buffer (TrisHCl 10 mM, pH 8.0, EDTA 1 mM, 2% SDS). The mycelium was ground and DNA was extracted by the method described by Kurzatkowski et al. (1996). A nuclear DNA region, containing the ITS I/5.8s/ITS II sequence, was amplified by PCR using the ITS1 (5′tccgtaggtgaacctgcgg3') and ITS4 (5′tcctccgcttattgatatgc3') primers (White et al., 1990). PCR amplification was performed in a final volume of 50 µl of reaction that contained: 10 µL of stamp 5x Colorless, 200 µMdNTPSs, 0.2 µM of each primer, 2,5 units of *Taq*DNA polimerase and 10-50 ng of template DNA. PCR reaction was placed in a thermocycler (Bio-Rad Gene CyclerTM series model No. 11453 Hercules, California, EE.UU.) under the following parameters: 5 min of initial denaturalization at 95°C, followed by 30 cycles of denaturalization of 1 min at 95°C, 1 min of alignment at 57°C, 1 min of extension at 72°C and a final extension period of 12 min at 72°C. The resulting product was purified with the kit Geneclean® II (Hercules, California, EE.UU.) according to the manufacturer's protocol and DNA was sequenced by the Biotechnology Institute, Cuernavaca, Morelos, México. Fungal DNA sequences were obtained with the program Chromas 1.45 (School of Health Science, Griffith University, Gold Coast Campus, Southport, Queensland, Australia) and a search was conducted with the BLAST (Zhang et al., 2000), for comparison of the homologies with the fungi sequences deposited in the GenBank (www.ncbi.nlm.nih.gov). The DNA sequence was aligned with other sequences through ClustalX (Thompson et al., 1997; Larkin et al., 2007) to identify the variable regions or base sequences. The distance matrixes between all pairs of sequence of multiple alignments were calculated and a tree

was generated by the Neighbor-Joining method developed by Saitou and Nei (1987). Two bootstrap analyses were performed with 1000 interactions with the program ClustalX (Thompson et al., 1997). Two phylogram trees were generated and rooted by importing the calculated values with the PhyloDraw program version.0.8 (Graphics Application Lab, 1999).

Confrontation experiment of *Hypocrea/Trichoderma* against phytopathogenic fungi

The confrontation experiments of *Hypocrea/Trichoderma* strain ITV43 against *M. roreri*, *P. megasperma* and *P. capsici* strains were performed using the technique described by Szekeres et al. (2006). Petri dishes with PDA medium were seeded at equidistant points and were incubated at 25°C in the dark for 31 days. The antagonistic activity was recorded at 7 and 31 days of incubation. Digital images were taken with a camera Cyber-shot DSC-P72, using the same vertical distance from the camera to the Petri dish (18 cm). The percentage growth inhibition of the pathogenic fungi was calculated using the biocontrol index (BCI) according to the formula reported by Szekeres et al. (2006): BCI = [A / B] x 100. Where A is the area of the colony of *Hypocrea/Trichoderma* and B is the total area occupied by colonies of *Hypocrea/Trichoderma* and each pathogen. The software ImageJ (on line: http://rsbweb.nih.gov/) was used to calculate the area of the fungi colonies. From the digital images taken with the digital camera, the area was measured and its outline was drawn using the free tool of the program ImageJ. During the analysis, the scale was set to 28.346 pixels per centimeter and the corresponding unit area was calculated in cm^2. The experiments were made by triplicate and analysis of variance (ANOVA) was performed with the software Statistix9. Means were compared with Tukey test ($P \leq 0.05$). Samples obtained from the interaction area between *Hypocrea/Trichoderma*ITV-43 and the phytopathogenic fungi were observed with an optical and scanning electron microscope (JEOL, JSM-5600 LV).

RESULTS

Morphological characterization of *Hypocrea/-Trichoderma* strain ITV43

The *Hypocrea/Trichoderma* strain ITV43 covered the surface of CMD and SNA in 4-5 days. It produced a coconut odor and a diffusing yellow pigments were detectedon CMD. Pustules were cottony first white and then turned dark green distributed in 3 concentric rings on SNA (Figures 1A and B), and on CMD were scarce and developing around the edge of the colony. The formation of pustules was more quickly on SNA than on CMD medium being visible the long terminally fertile conidiophores at the surface (Figure 1D). Two types of conidiophores were seen depending on the culture medium: on SNA Type 2 and on CMD Type 3 (Figure 1E) as described by Jaklitsch et al. (2006). Intercalary phialides were observed in SNA medium. In the conidiophore Type 3 there were production of Type 2 lageniform straight, some curved or hooked, (3.79-)4.39-9.48(-13.20) µm long, CI = 6.03-7.85, n = 30; (1.54-)1.74-2.18(-2.38) µm wide in the widest point, CI=1.88-2.04, n=30; (0.93-)1.21-1.81(-2.01) µm at the base, CI=1.40-1.61, n=30; L/W=(0.98-)0.98-1.18(-1.50), CI=1.05-1.12, n=30; arising from a cell (1.09-)1.53-1.95(-2.12) µm wide, CI=1.66-1.81, n=30. Intercalary phialides were observed in SNA medium (Figure 1F). In the conidiophore Type 3, there was production of chains of proliferated phialides. Conidia on CMD with ornamentation warts, shape subglobose to ellipsoidal, (3.69-)4.01-4.85(-5.60) µm long, CI=4.28-4.58, n=30; (3.56-)3.84-4.31(-4.66) µm wide, CI=3.99-4.16, n=30; L/W=(0.98-)0.98-1.18(-1.50), CI=1.05-1.12, n=30 (Figure 1G, 1H). Chlamydospores on CMD (Figure 1I) were produced terminally and intercalary in hyphae, smooth, globose to subglobose (6.59-)8.21-11.01(-13.45) µm long, CI=9.11-10.11, n=30, (5.55-)7.47-10.50(-11.86) µm wide, CI=8.44-9.53, n=30, L/W= (0.93-)0.98-1.17(-1.39), CI=1.04-1.11, n=30. Based on the phenotype characteristics and the measurements described in the Table 1, the strain ITV43 was identified as *H./T. viridescens*.

Identification of fungal strain by ITS1-5.8s-ITS2 sequencing and phylogeny

Using ITS1 and ITS4 primers, the ribosomal DNA (rDNA) region containing the ITS1-5.8s-ITS2 sequence was amplified from each fungal strain. The amplified region of *H./T. viridesens* ITV43 had a size of 679 bp and was deposited in the NCBI GenBank (JX144329). The ITS1-5.8s-ITS2 sequence amplified showed the highest similarity of 100 and 94% with DNA *H. viridescens* sequence (GenBank EF534007) and *H. viridescens* (GenBank EU280104, EU280137), respectively. The first sequence was obtained from a strain isolated in the United States of America by Liu et al. (2008) and the other two from strains isolated in Colombia and Peru by Hoyos-Carvajal et al. (2009). Phylogenetic analysis of *H./T. viridescens* ITV43 and 11 representative ITS1-5.8s-ITS2 sequences of isolates of *Trichoderma*/*Hypocrea* generated a tree by the Neighbor Joining method and formed three majors groups (Figure 2). *H./T. viridescens* ITV43 grouped with *H. viridescens* (GU566274). This clade is affiliated with *H. viridescens* (EU280104 and EU280137) and close to *H. viridescens* (HQ833353).

On the other hand, the amplified ITS1-5.8s-ITS2 rDNA of *Phytophthora* strains were sequenced and the strains were identified with the highest similarity of the sequences obtained with the Blast tool. The GenBank accession numbers obtained for the ITS1-5.8S-ITS2 sequences were: *P. megasperma*HT-ITV08 (KC753539), HT-ITV15 (KC753540), HT-ITV37 (KC753541), and *P. capsici*HT-ITV14 (KC753542), HT-ITV16 (KC753543), and HT-ITV33 (KC753544). The Neighbor Joining phylogenetic analysis with nine *Phythopthora* strains ITS1-5.8s-ITS2 sequences yielded a tree (Figure 3). *P. capsici* HT-ITV16 and *P. capsici* HT-ITV33 are affiliated with *P. capsici* AF467083. *P. capsici* HT-ITV14 grouped with *P. capsici* AF467085 and *P. megasperma* HT-ITV37 and HT-ITV15 are affiliated with *P. megasperma* (AF541899, AF54189 and AF541893) and close to *P. cryptogea* EU00073. On the other hand, *P. megasperma* HT-ITV08 is close to *P. megasperma* strains.

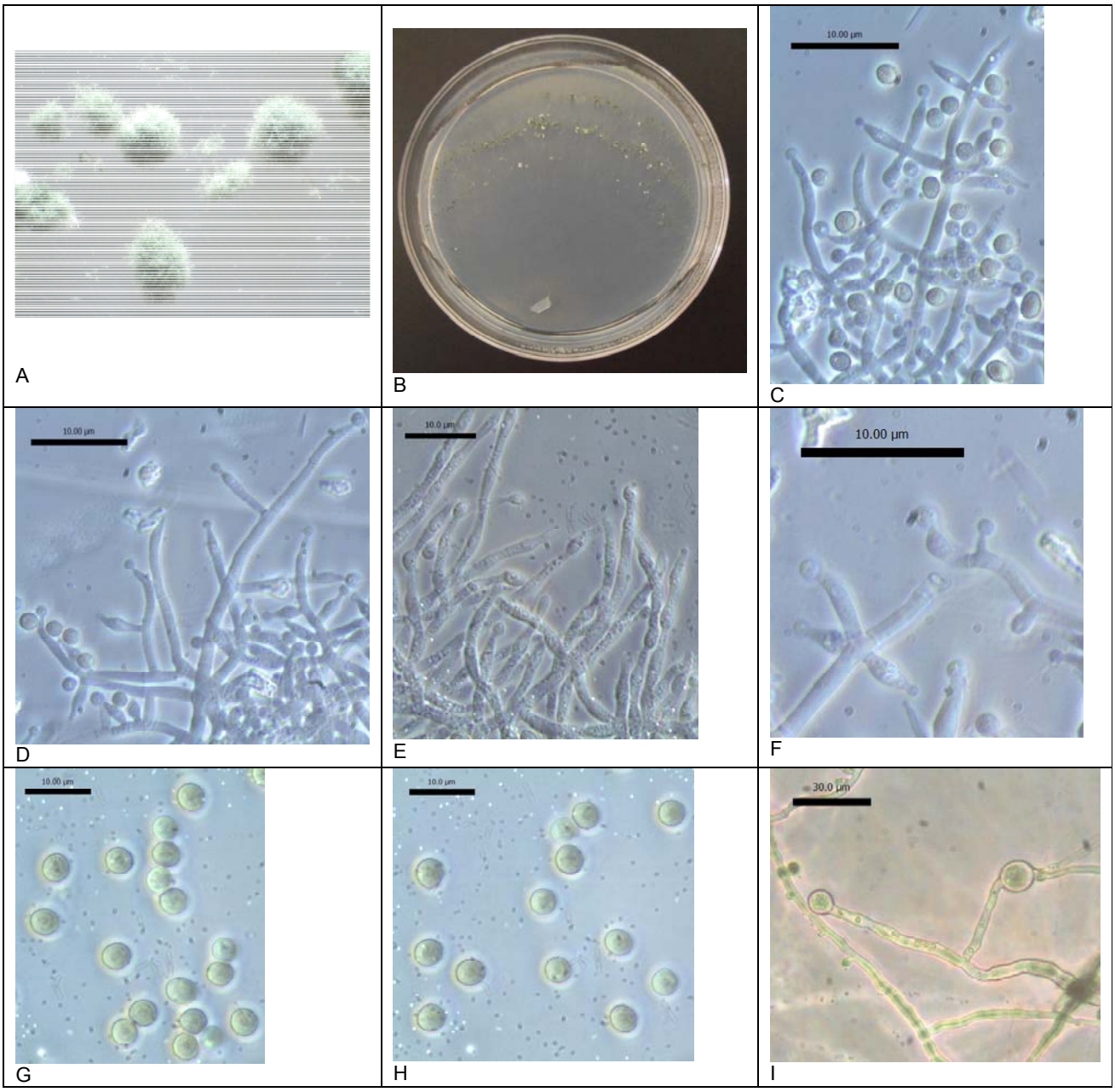


Figure 1. Morphology of *H. viridescens* ITV-43 *strain*. A. Pustules on SNA; B. colony grown on SNA after 10 days; C. Conidiophore Type 2 similar to *Trichoderma* on SNA; D. A long terminally fertile conidiophores; E. Type 3 conidiophore on CMD with chains of percurrently proliferating phialides; F. Intercalary phialide on SNA; G to H. Conidia warted on CMD; I. Chlamydospores on CMD. A. Magnification 25×. Scale bars: C-H = 10 µm, I = 30 µm.

Confrontation experiments

The antagonistic ability of *H./T. viridesens* ITV43 strain was tested against *M. roreri* (HT-ITV01, HT-ITV20, HT-ITV27), *P. megasperma* (HT-ITV08, HT-ITV15, HT-ITV37) and *P. capsici* (HT-ITV14, HT-ITV16, HT-ITV33) strains by confrontation experiments in dual culture. The digital images taken on 7 and 31 days of incubation (Figure 4) were used to calculate the BCIs values. In the seven days of incubation, the BCI values of *H./T. viridescens*ITV43 against *M. roreri*, *P. megasperma*, and *P. capsici* strains ranged between 78.28-81.63, 63.02-69.95 and 69.77-76.31, respectively (Table 2).

According to Tukey´s tests, the highest BCI values were obtained against *M. roreri* HT-ITV20 (81.63), *P. megasperma* HT-ITV15 (69.95) and *P. capsici* HT-ITV16 (76.31). After 7 days, a progressive inhibition zone produced by *H./T. viridescens* ITV43 against all phytopathogenic fungi strains was observed and at 31 days of incubation, 100%

Table 1. Measurements for each structure of *H./T. viridescens* strain ITV43.

	Conidium characters	Phialide characters	Chlamydospore characters
Shape	Subglobose to ellipsoidal	Lageniform, straight, some curved or hooked	Globose to subglobose
Ornamentation	Warted	Smooth	Smooth
Length (µm)	(3.69-)4.01-4.85(-5.60)	(3.79-)4.39-9.48(-13.20)	(6.59-)8.21-11.01(-13.45)
95% CI	4.28-4.58	6.03-7.85	9.11-10.11
N	30	30	30
width (µm)	(3.56-)3.84-4.31(-4.66)	(1.54-)1.74-2.18(-2.38)	(5.55-)7.47-10.50(-11.86)
95% CI	3.99-4.16	1.88-2.04	8.44-9.53
N	30	30	30
L/W	(0.98-)0.98-1.18(-1.50)	(0.98-)0.98-1.18(-1.50)	(0.93-)0.98-1.17(-1.39),
95% CI	1.05-1.12	1.05-1.12	1.04-1.11
N	30	30	30.
Base of phialide (µm)		(0.93-)1.21-1.81(-2.01)	
95% CI		1.40-1.61	
N		30	
Cells supporting the phialides (µm)		(1.09-)1.53-1.95(-2.12)	
95% CI		1.66-1.81	
N		30	

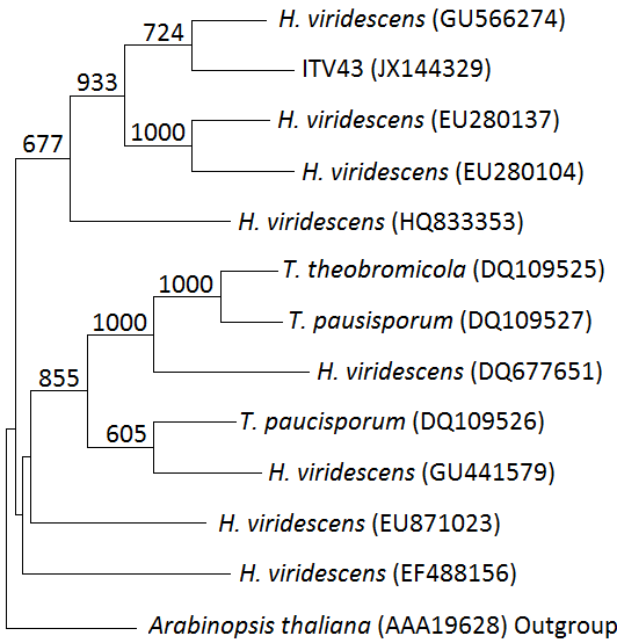


Figure 2. Phylogenetic tree for ITS1-5.8s-ITS2 region of rDNA, of *H. viridescens* ITV43 and the sequences from specimens related, obtained from GenBank (NCBI). The Kimura distance and the Neighbor-Joining method was used. The numbers adjacent to the branch points of ramification are bootstrap values of 1000 iterations.

inhibition was detected on *H./T. viridescens* ITV43-*Phytopthora* plates while *H./T. viridescens* ITV43-*M. roreri* plates produced BCI values between 81.52 and 86.50. The mycoparasitism was analyzed in the areas of interaction of *H./T. viridescens* ITV43-*M. roreri, H./T. viridescens* ITV43-*P. capsici* and *H./T. viridescens* ITV43-

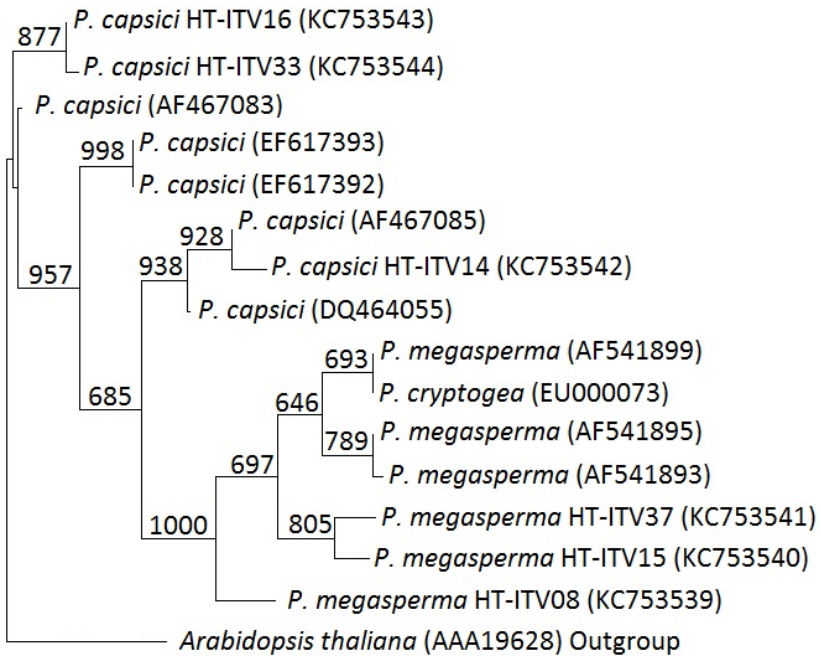


Figure 3. Phylogenetic tree for the ITS1-5.8S-ITS2 region of rDNA, for isolates of *P. megasperma*, *P. capsici* and related sequences of specimens obtained from GenBank (NCBI). The Kimura distance and the neighbor-joining method were used. Numbers adjacent to branch points are bootstrap values for 1000 iterations.

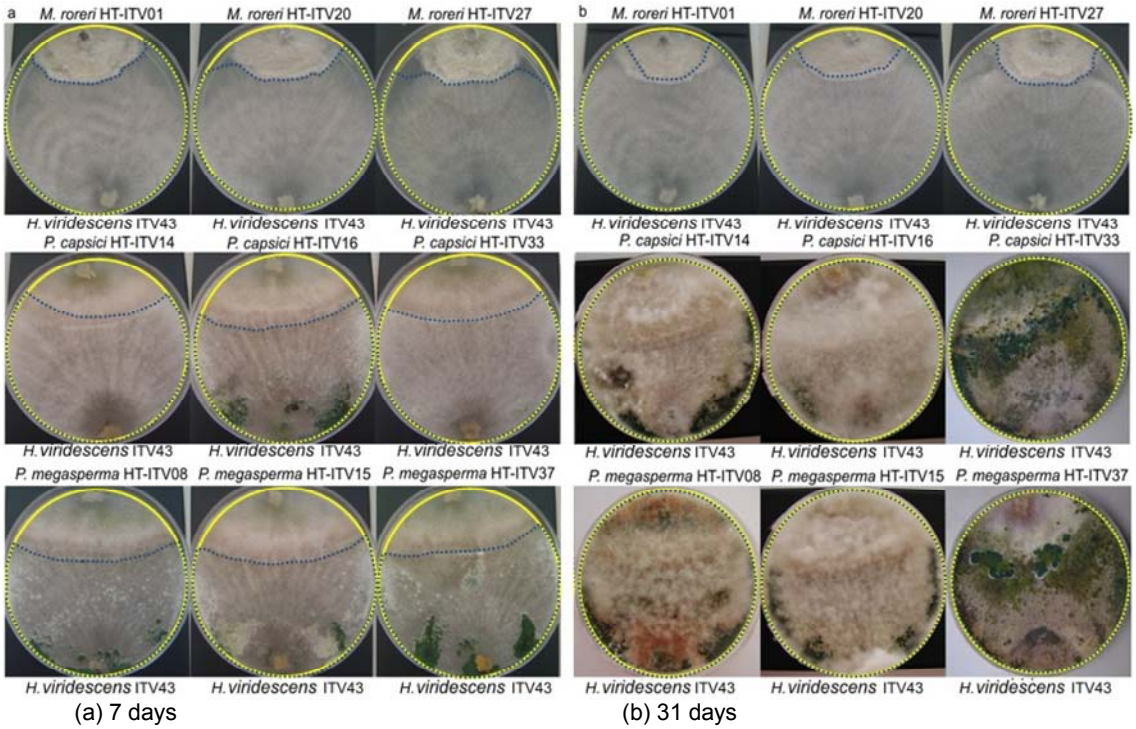
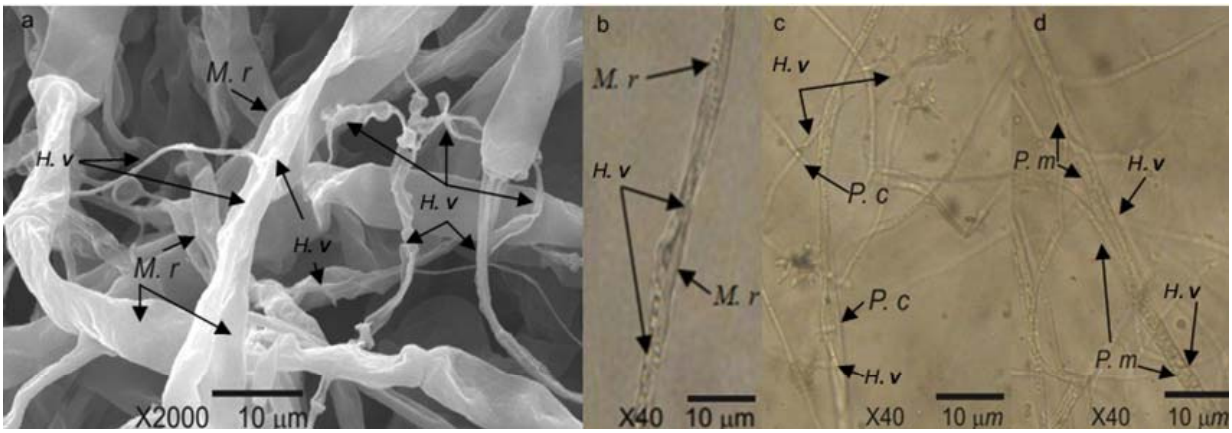


Figure 4. Digital images taken at: (a) 7 and (b) 31 days of confrontation of *H. viridescens* ITV43 against *T. cacao* phytopathogenic fungi (*M. roreri, P. capsici* and *P. megasperma*) in PDA medium. Areas of *H. viridescens* ITV43 colonies (Area T, line blue dots) and the total area occupied by colonies of *H. viridescens* ITV43 and the phytopathogenic fungi (Area T + P, yellow line).

Table 2. Biocontrol index (BCI) of *H. viridescens* ITV43 (inhibition zone) against *T. cacao* phytopathogenic fungi.

Phytopathogenic fungi	BCI ± sd* (7 days)	BCI ± sd* (31 days)
M. roreri HT-ITV01	80.68[a] ± 0.01	84.85[a] ± 0.01
M. roreri HT-ITV20	81.63[b] ± 0.02	86.50[b] ± 0.01
M. roreri HT-ITV27	78.28[c] ± 0.01	81.52[c] ± 0.02
P. megasperma HT-ITV08	65.73[d] ± 0.02	100.00[d] ± 0.00
P. megasperma HT-ITV15	69.95[e] ± 0.01	100.00[d] ± 0.00
P. megasperma HT-ITV37	63.02[f] ± 0.03	100.00[d] ± 0.00
P.capsici HT-ITV14	69.77[g] ± 0.06	100.00[d] ± 0.00
P. capsici HT-ITV16	76.31[h] ± 0.03	100.00[d] ± 0.00
P. capsici HT-ITV33	70.90[i] ± 0.01	100.00[d] ± 0.00

*Standard deviation; Different letter within the column indicate significant differences ($P < 0.05$, ANOVA and Tukey´s tests).



(a) (b) (c) (d)

Figure 5. Images showing the effect of *H. viridescens* ITV43 on phytopathogenic fungi with the scanning electron microscope (a) and light microscope (b, c, d) in PDA medium: (a) *H. viridescens* (*H.v*) parasitizing mycelium of *M. roreri* (*M.r*) and inducing the lysis and deformation of the pathogen hyphae, (b) mycoparasitism by wrapping the hyphae of *M. roreri* by *H. viridescens*, (c) mycoparasitism of hyphae of *P. capsici* (*P.c*) by hyphae of *H. viridescens*, (d) parallel hyphal growth of *H. viridescens* and *P. megasperma* (*P.m*).

P. megasperma by scanning electron and optical microscopy. The effect of *H./T. viridescens* ITV43 on morphological deformations and disorganization in the structure of the *M. roreri* cell walls was observed by scanning electron microscope (Figure 5a) and by optical microscopy for *M. roreri* (Figure 5b), *P. capsici* (Figure 5c) and *P. megasperma* (Figure 5d).

DISCUSSION

Fungi of the genus *Trichoderma* (*Hypocrea*) have been widely studied due to their antagonistic activity on phytopathogenic fungi in agriculture and are an alternative to synthetic chemical products (Mukherjee et al., 2013). Most *Trichoderma* species has been isolated from soil, however there are reports of *Trichoderma* isolates from other sources such as bark, decorticated wood, roots, decaying wood, mushroom compost, leaf litter, as an endophyte from trunks and aerial parts of plants (Harman et al., 2004). In the biological control of cocoa (*T. cacao*) pod diseases, mycoparasites isolated from cocoa fruits, aerial plant parts and rhizospheric soil from plot planted with cocoa trees have shown great promise in the control of *M. roreri* (Villamil et al., 2012), causal agent of black pod rot and *Phytophthora* spp. (Hanada et al., 2009), causal agent of black pod. Only a small number of the vast microbial diversity associated with cacao has been studied and endophytic and epiphytic microbes are candidates for biocontrol of cacao

diseases. In this study, we have isolated a *Hypocrea/Trichoderma* strain from cacao pod with typical symptoms of frosty pod rot and our results show that the strain has strong antagonism against six *Phytophthora* and three *M. roreri* strains. *Phytophthora* and *M. roreri* strains were also isolated from cocoa pod with typical symptoms of frosty pod rot (Cuervo-Parra et al., 2011a). We suggested that *H./T.viridescens* ITV43 could have developed an antagonism against *Phythopthora* and *M. roreri* strains. BCI at 7 days of *H./T. viridescens* was higher on *M. roreri* strains (average 80.2) than on *Phytophthora* strains (average 69.3) and BCI values are higher as compared to other biological control agents. For example, Cuervo-Parra et al. (2011b) tested a *Trichoderma harzianum* strain (VSL291) by confrontation experiments against *M. roreri* and *Phytophthora* strains. *T. harzianum* VSL291 was isolated from soil samples obtained from *Agave tequilana* crop in the State of Jalisco, Mexico (Sánchez and Rebolledo, 2010). In 7 days, the strain inhibited 72.72, 20.77 and 28.60% growth of *M. roreri* HT-ITV01, *P. megasperma* HT-ITV08 and *P. capsici* HT-ITV14, respectively. In contrast to this study, the same strains were tested and the inhibition percentages were 80.68, 65.73 and 69.77%, respectively. BCI was higher: 7.94, 44.96 and 41.17%, respectively. These results strongly suggest that species associated with *T. cacao* can potentially be used as effective biocontrol agents. Although these percentages obtained *in vitro* cannot be translated directly to biocontrol of phytopathogenic fungi in plant, these results are useful to select candidate strains for use as biocontrol agents. Our results show a better inhibition on *Phythophthora* strains as compared to report by Etebarian et al. (2000). In that study, *in vitro* mycelia growth of *P. erythroseptica* was reduced by 49-71 and 49-54% by *T. virens* DAR 74290 and *T. harzianum* T39, respectively. On the other hand, Mejia et al. (2008) isolated fungal endophytes from healthy *Theobroma cacao* tissues and tested their antagonism *in vitro* against *M. roreri, P. palmivora* and *M. perniciosa*. The antagonism percentage obtained by this group was 40, 65 and 27%, respectively.

According to their morphological characteristics, the strain ITV43 was identified as *H./T. viridescens* that match the descriptions reported by Jaklitsch et al. (2006) and we suggest that this identification is reliable. *H./T. viridescens* belongs to Viride clade that includes *Trichoderma viride, Trichoderma atroviride, Trichoderma koningiopsis* and others (Samuels and Ismaiel, 2009). The characteristic of coconut aroma attributed to 6-PAP (6-pentyl-a-pyrone) was detected in *H./T. viridescens* ITV43 and the antifungal compound 6-PAP (Collins and Halim, 1972; Cutler et al., 1986) is a distinctive feature of many members of the Viride clade (Samuels et al., 2006). Analysis by BLAST of ITS1-5.8s-ITS2 DNA sequence of *H./T. viridescens* ITV43 showed high degree similarity (99-100%) with *H. viridescens* strains (EU280104, EU280137) and phylogenetic tree (Figure 2) locate *H./T. viridescens* ITV43 close to *H. viridescens* (GU441579, GU566274) and *T. asperellum* (AY380912) strains and located away from *T. theobromicola* (DQ109525) and *T. paucisporum* (DQ109527, DQ109526) strains. Although ITS1-5.8s-ITS2 DNA sequences are useful for identifying and studying the evolution of microorganisms, do not appear to be useful in identifying some *Trichoderma/Hypocrea* species (Jaklitsch et al., 2006). Druzhinina et al. (2005) developed the Trich OKEY program for the quick molecular identification of *Hypocrea/Trichoderma* at the genus and species levels based on an oligonucleotide barcode for the internal transcribed spacers 1 and 2 ((www.isth.info). To identify *H./T. viridescens* ITV43, we used the ITS1-5.8s-ITS2 DNA sequence and the program was not able to identify the strain. Similar results were obtained by Torres-Palacios (2010) who used the program to identify 16 *Trichoderma* strains isolated near the Nevado de Toluca volcano, Mexico. In that study, 19% of the strains were identify entirely, 19% were identify as new species and 62% were ambiguous mainly *viridescens-konongii*. Other cases reported by Druzhinina et al. (2005) in which the software initiate ambiguities in *Trichoderma/Hypocrea* strains are: *cerinum=tomentosum, crassum=longipile* and *orientalis=cerebriformis=longibrachiatum*.

In conclusion, the strain ITV43 was identified as *H./T.viridescens* and was mycoparasitic on *M. roreri*, *P. megasperma* and *P. capsici.*

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