El Colegio de la Frontera Sur

Efecto de esporas y sobrenadante de micoparásitos sobre el desarrollo de la roya del café

(*Hemileia vastatrix* Berk. & Br.)

TESIS
Presentada como requisito parcial para optar al grado de
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Por

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DEDICATORIA

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CONTENIDO

I. RESUMEN Y PALABRAS CLAVE 7
II. INTRODUCCIÓN 8
III. ARTÍCULO CIENTÍFICO 11
IV. CONCLUSIONES 39
V. LITERATURA CITADA 40

I. RESUMEN Y PALABRAS CLAVE

Existen diversos micoparásitos de la roya del café (*Hemileia vastatrix*). Sin embargo, la información sobre esta interacción biológica es limitada. A partir de una colecta de micoparásitos de *H. vastatrix* en la región Soconusco, Chiapas, México, se seleccionaron tres cepas para su caracterización. Se identificaron utilizando la región ITS del gen 18S ribosomal (primers ITS4/ITS5) y el gen para el Factor de Elongación 1 (primers EF1-F/EF1-R) como marcadores moleculares. Se determinó que dos cepas pertenecen a *Simplicillium lanosoniveum* (CERI-530 y CERI-542) y una correspondiente a *Akanthomyces muscarius* (Sinónimo de *Lecanicillium muscarius*) (CERI-701). Cada cepa se cultivó durante 10 días sobre uredosporas de *H. vastatrix* o sustrato inductor.

Diariamente se determinó actividad quitinasa, glucanasa (espectrofotometría) y proteasa (ensayo en placas) del sobrenadante de los cultivos. Se realizó electroforesis para la separación de proteínas (SDS-PAGE) y zimogramas para reportar quitinasa y glucanasa utilizando muestras del día 10. Se evaluó el efecto de la aplicación del sobrenadante o sus esporas sobre la germinación de *H. vastatrix.* Y utilizando Microscopía Electrónica de Barrido (MEB) se observó el efecto de estos micoparásitos sobre la integridad estructural de la uredospora de *H. vastatrix*

La cepa CERI-530 tuvo mayor actividad quitinasa, glucanasa y proteasa en presencia de *H. vastatrix*. En las tres cepas se detectó una banda común de quitinasas y glucanasas de 50 kDa. Sin embargo, CERI-701 presentó bandas adicionales de quitinasas en presencia de *H. vastatrix.* La aplicación de esporas y sobrenadante de los micoparásitos tuvo diferente efecto en la germinación y morfología de la uredospora de *H. vastatrix.*

Este trabajo aporta evidencias sobre el potencial de tres nuevas cepas de micoparásitos y sus metabólitos para el control biológico de *H. vastatrix.*

Palabras clave. Quitinasas, Glucanasas, Proteasas, *Simplicillium lanosoniveum*, *Acanthomyces muscarium.*

II. INTRODUCCIÓN

Actualmente la roya del café (*Hemileia vastatrix* Berkeley & Brome, 1869) es el problema fitosanitario más importante en el cultivo de café. Es un hongo parásito obligado que se alimenta de las hojas y en la planta ocasiona defoliación, pérdida de ramas (Avelino et al 2015) o disminución de hasta 35 % de la producción. A nivel mundial, anualmente se pierden hasta dos mil millones de dólares en el cultivo de café a causa de esta enfermedad (Talhinhas et al. 2017).

Aun cuando la enfermedad apareció en Centroamérica en los años 80´s, el control químico y la baja agresividad de la enfermedad, permitieron dejar a un lado la preocupación del peligro que podría causar en la cafeticultura. Sin embargo, la incidencia atípica ocurrida en el ciclo 2012/2013, representó pérdidas entre 10 y 90% para la mayoría de los países centroamericanos (Avelino et al. 2015).

El proceso infeccioso inicia cuando la uredospora de *H. vastatrix* se adhiere al envés de la hoja de café, posteriormente germina, forma apresorios sobre el estoma de la hoja, penetra y coloniza los espacios inter e intracelulares (Talhinhas et al. 2017). Inicialmente se observa clorosis en el haz de las hojas (1-3 mm), estas manchas cloróticas se expanden y en estas áreas del envés se forman nuevas uredosporas que en conjunto forman pústulas de aspecto pulverulento y color naranja. Finalmente, esa clorosis se convierte en necrosis y las uredosporas de la pústula se vuelven el nuevo inóculo de esta enfermedad (Zambolim 2016).

El uso de variedades con resistencia genética combinado con el control químico oportuno han permitido mantener bajo control a *H. vastatrix*. Aunque esto es una opción de largo plazo para productores convencionales (Henderson 2019), se requieren alternativas de control más ecológicas, principalmente para el sector de producción orgánica.

El control biológico representa una alternativa al control químico, a través del uso de enemigos naturales del fitopatógeno. Al respecto, se han reportado diversos hongos parasitando a *H. vastatrix* en campo. Esta interacción se conoce como micoparasitismo.

Entre las especies reportadas se encuentran *Lecanicillium leptobactrum* (Eskes et al. 1991), *Acremonium byssoides*, *Calcarisporium arbuscula*, *C. ovalisporum*, *Sporothrix guttuliformis*, *Fusarium pallidoroseum*, *L. lecanii* (Carrión y Rico 2002), *L. psalliotae* (Mahfud et al. 2006), *Simplicillium* sp. (Gómez-de la Cruz et al. 2018) e incluso se consideran como micoparásitos a *Passalora* sp., *Pseudocercospora* sp. y *Mycosphaerella* sp. (James et al. 2016).

El género más evaluado sobre *H. vastatrix* es *Lecanicillium* spp. Algunos autores sugieren que tanto sus esporas, como el micelio (Mahfud et al. 2006) y filtrados (Eskes et al. 1991; Leguizamon et al. 1989; González y Martínez 1996) disminuyen la germinación de *H. vastatrix,* así como su incidencia y severidad en plántulas de invernadero. Aunque se ha reportado que constantes aplicaciones de *L. lecanii* en campo, disminuyen ligeramente la defoliación (Carrión 1988), su establecimiento se encuentra limitado por las condiciones ambientales (Eskes et al. 1991).

Con los antecedentes mencionados resulta importante estudiar el potencial de otras especies de micoparásitos y sus metabolitos para el biocontrol de *H. vastatrix*.

Se ha determinado que *L. lecanii* produce quitinasas con efectos negativos sobre *Fusarium oxysporum* y *Rhizoctonia solani* (Nguyen et al. 2015).Y debido a que la pared celular de los hongos está compuesta principalmente por glicoproteínas, B-glucanos y quitina (Gow et al. 2017) es posible que los micoparásitos produzcan estas enzimas hidrolíticas o bien otros metabolitos de importancia para el biocontrol de *H. vastatrix.*

Se realizaron bioensayos en el laboratorio de Sanidad Vegetal del Instituto Nacional de Investigaciones Agrícolas y Pecuarias (Campo Experimental Rosario Izapa), los cuales indican algunos que micoparásitos provenientes de cafetales de la región Soconusco, Chiapas tienen potencial para micoparasitar a *H. vastatrix,* sin embargo, su efecto sobre la pústula es diferente entre cepas. Algunas colonizan directamente la pústula y otras previo a la colonización, liberan un material que cubre las uredosporas y sobre este material se desarrolla escaso micelio del micoparásito, esto sugiere que el mecanismo de acción sobre *H. vastatrix* varía entre cepas, sin embargo, la información sobre esta interacción biológica es limitada.

Debido a que la relación micoparasítica de estos hongos sobre *H. vastatrix* aún no se encuentra descrita y se desconoce qué efectos tienen estos micoparásitos sobre el desarrollo de este fitopatogeno; en este trabajo se planteó evaluar el efecto de las uredosporas de *H. vastatrix* sobre la actividad quitinasa, glucanasa y proteasa de tres cepas de micoparásitos. Además se determinó el efecto de la aplicación de sus esporas o sobrenadante sobre la germinación e integridad estructural de las uredosporas de *H. vastatrix.*

Para resolver los objetivos planteados se seleccionaron tres cepas de micoparásitos, se identificaron y se cultivaron durante 10 días en presencia de *H. vastatrix* o sustrato inductor. Diariamente se obtuvo su sobrenadante y a este, se le determinó actividad quitinasa, glucanasa y proteasa. También se aplicaron esporas o sobrenadante de estos micoparásitos en diferentes tiempos sobre uredosporas de *H. vastatrix* para determinar su efecto sobre la germinación y con ayuda del MEB se describió el efecto de estos micoparásitos o sus sobrenadante (mayor y menor a 10 kDa) sobre la integridad estructural de las uredosporas de *H. vastatrix.*

Se determinó que las cepas pertenecen a *Simplicillium lanosoniveum* (CERI-530 y CERI-542) y *Akanthomyces muscarius* (CERI-701). Las cepas presentaron diferente actividad enzimática en presencia del fitopatogeno, sin embargo, destaca la CERI-530 en actividad glucanasa, proteasa y quitinasa, la cepa CERI-542 inhibe la germinación y deforma a las uredosporas de *H. vastatrix* y la cepa CERI-701 presenta diferentes bandas de quitinasas inducidas por *H. vastatrix*.

Esta es la primera descripción del potencial de micoparasitosis sobre *H. vastatrix*, de tres cepas de micoparásitos aisladas en el Soconusco, Chiapas, México.

III. ARTÍCULO CIENTÍFICO

Enzyme activity of three mycoparasite strains and their effect on development of coffee rust (*Hemileia vastatrix* Berk. & Br.)

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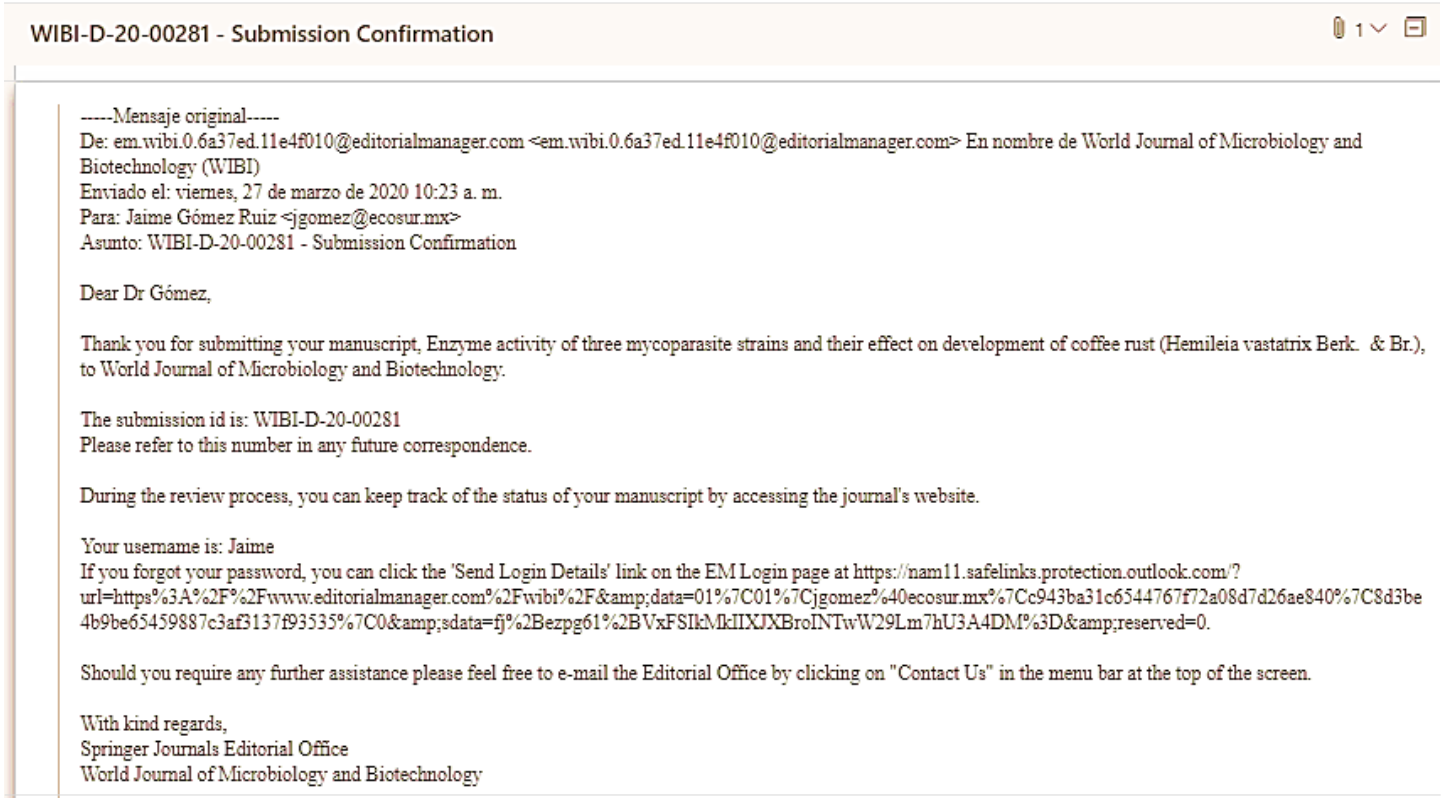

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Enzyme activity of three mycoparasite strains and their effect on development of coffee rust (*Hemileia vastatrix* Berk. & Br.)

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ABSTRACT

We evaluated chitinase, glucanase and protease activity of three strains (*Simplicilium lanosoniveum* CERI-530 and CERI-542 and *Akanthomyces muscarius* CERI-701) of mycoparasites growing for 10 d on an inducing substrate and on urediniospores of *H. vastatrix* collected in coffee plantations in Soconusco, Chiapas, Mexico. Determinations of enzyme activity indicate that, among the strains, *S. lanosoniveum* CERI-530 had more chitinase (38,178 $\pm$ 2,950 on day 3) and glucanase (9,720 $\pm$ 282 U/mg on day 6) activity in presence of *H. vastatrix*. Zymograms of chitinases and glucanases revealed a common band of 50 kDa in the three strains, while the strain CERI-701 revealed additional bands of chitinases of 40, 30 and 20 kDa in presence of *H. vastatrix*. Moreover, protease activity of the three strains is visualized on plates, even when the strains were cultured on *H. vastatrix*. Using scanning electron microscopy, the effect of the spores of the three strains and two fractions of the supernatant were compared (fraction 1 greater than 10 kDa, and fraction 2 less than 10 kDa) on *H. vastatrix* urediniospores. Differences in the infection process caused by the strains and fractions of the supernatant on *H. vastatrix* are discussed.

KEY WORDS

Akanthomyces muscarius, chitinases, glucanases, proteases, *Simplicium lanosoniveum*.

INTRODUCTION

Coffee rust (*Hemileia vastatrix*) is the most important disease of coffee (McCook 2006). When coffee was first introduced to the American continent in the 70s in Brazil and its later dissemination in Central America and Mexico (Barrera et al. 2013; Muller 1971), no impact of *H. vastatrix* was observed in these new sites. It was not until several years later that real impact of the disease was observed in 2008-2013 when there was an epidemic that affected several Mesoamerican countries causing losses of 10% and up to 54% (Avelino et al. 2015).

Copper-based fungicides have been used as preventive treatments and some systemic fungicides as curative treatments (Talhinhas et al. 2017). However, environmental repercussions attributed to chemical control have led to a search for alternatives, especially for certified organic growers. For control of this phytopathogen, Avelino et al. (2015) states that it is important to consider environmental and economic factors as well as management of the

coffee plantation, taking into account shade, nutrition, use of resistant varieties and use of biological control agents. Regarding biological control agents, one of the fungi that has often been found associated with *H. vastatrix* and has been most studied in relation to this disease is *Lecanicillium* sp. (Carrión 1988; Mahfud et al. 2006; James et al. 2016). Because of its entomopathogenic and mycoparasitic action (Jackson et al. 2012), its effectiveness in the field has been evaluated. However, its establishment has been unsuccessful, suggesting that environmental conditions are unfavorable and cause loss of viability (Eskes et al. 1991) and low incidence on the plants (Canjura-Sarabia et al. 2002). Moreover, field evaluations are difficult to measure because of natural infections of other mycoparasites (Carrion 1988).

On the other hand, little or nothing is known of the interactions that *H. vastatrix* has established with coffee microbiota or of the impact that they can cause on its population. One of the most evident is mycoparasitosis caused by fungi, among which *Lecanicillium lecanii* (Carrión 1988), *Leptobacillium leptobactrum* (Eskes et al. 1991), *Acremonium byssoides*, *Calcarisporium arbuscula*, *Calcarisporium ovalisporum*, *Sporothrix guttuliformis*, *Fusarium pallidoroseum* (Carrión and Rico 2002), *L. psalliotae* (Mahfud et al. 2006), *Simplicillium* sp. (Gómez-De La Cruz et al. 2018) and even *Passalora* sp., *Pseudocercospora* sp. and *Mycosphaerella* sp. were mentioned as mycoparasites of *H. vastatrix* (James et al. 2016).

Regarding the processes associated with mycoparasitism, the effect of *Trichoderma* sp on diverse hosts has been described in general. This mechanism of action involves processes such as chemotrophic growth of the mycoparasite, recognition of the host, adhesion, enrollment and lysis of the cell wall mediated by hydrolytic enzymes (Infante et al. 2009). In this respect, prior work conducted in our laboratory showed that some mycoparasites of *H. vastatrix* attack in different ways. Some isolations act directly on *H. vastatrix* pustules from growth of its hyphae to later development of mycelium on urediniospores of the rust. However, other strains germinated, and their hyphae produce a material that forms a white covering over the *H. vastatrix* pustules, deforming the urediniospores. Later, the mycoparasite develop scarce mycelium on the remains of the pustules (Data not published). It is known that cell wall-degrading enzymes, such as chitinases, glucanases and cellulases, intervene in the mycoparasitism process (Steyaert et al. 2003). For this reason, we evaluated the activity of chitinase, glucanase and protease from three mycoparasites when they grow in association with *H. vastatrix*. Moreover, we evaluated the effect of the spores and the supernatant of the mycoparasites on germination and development of the urediniospores to observe how these

enzymes or metabolites behave in presence of *H. vastatrix* urediniospores to select the strain that has the most potential for its control and choose between use of spores or of one of their metabolic products.

MATERIALS AND METHODS

Between 2015 and 2017, the Instituto Nacional de Investigaciones Forestales Agrícolas y Pecuarias (INIFAP), Rosario Izapa Experimental Station (CERI) began collection and isolation of *H. vastatrix* mycoparasites at different points in the Soconusco region, Chiapas, Mexico. Preliminary bioassays of antagonism were also conducted, and the strains that showed greater potential for biological control were conserved in the fungal strain collection of the same institute.

Three strains were selected for their high aggressiveness (<80% mycoparasitism on *H. vastatrix*) and because they exhibited differences in the host infection process. These strains were labeled with the identification numbers CERI-530, CERI-542 and CERI-701. DNA was then extracted from 0.2 g fresh mycelia following the method adapted from Allen et al., (2006), whose lysis buffer contains CTAB+ PVP (2%). From a 1:20 dilution of the extracted DNA, 1 µl was used for PCR with Master Mix 1X (Promega, Wisconsin USA), 0.2 pM of each primer in a final volume of 20 µL. The ITS region of the 18S ribosomal gene was amplified with the primers ITS4 (TCCTCCGCTTATTGATATGC) and ITS5 (GGAAGTAAAAGTCGTAACAAGG) (Schoch et al, 2012). PCR protocol was initiated with a 5 min denaturation at 95°C, followed by 35 cycles with a denaturation step of 95°C/1min, annealing of 55°C/1min and extension of 72°C/1min. Final extension was performed at 72°C/5min. PCR for transcriptional elongation factor 1-α (TEF1α) gene was performed with EF1-983F (GCYCCYGGHCAYCGTGAY TTYAT) / EF1-2218R (ATGACACCRACRGCRACRGTYTG) primers (Rehner and Buckley, 2005). A PCR touchdown procedure was followed on this case and it was initiated with a 3 min denaturation at 94°C, afterwards 10 cycles of denaturation step of 94°C/30seg, annealing of 68°C/30seg (with a 1°C/cycle decrease in temperature) and extension of 72°C/1min. Another 30 cycles were necessary (denaturation step of 94°C/30seg, annealing of 58°C/30seg and extensions of 72°C/1min, finishing with a final extension step of 72°C/5min.

Both ITS and TEF1α amplicons were sequenced with the Sanger method through the MACROGEN Company (Seoul, Korea). The consensus sequences were processed with the software MEGA (Molecular Evolutionary Genetic Analysis) version 7.0® (Kumar et al. 2016). Finally, the product was compared with the sequences in the GenBank and BOLD systems databases and deposited in the GenBank.

Effect of *H. vastatrix* urediniospores on chitinase, glucanase and protease activity of three mycoparasite strains

The three mycoparasite strains (CERI-530, CERI-542 and CERI-701) were inoculated ($1x10^5$/ml) in minimum medium (MM) with the following composition per liter: 1.4 g $(NH_4)_2SO_4$, 0.2 g KH_2PO_4, 6.9 g NaH_2PO_4. H_2O, $MgSO_4$. $7H_2O$, 1 g peptone, 2 g glucose, 0.3 g urea. This MM was enriched for enzyme determinations as described below.

To evaluate enzyme activity (chitinase, glucanase and protease) of the mycoparasites, an experiment was set up to evaluate the three strains separately (CERI-530, CERI-542 and CERI-701) on three growth substrates: i) MM (described above) adding 0.3% colloidal chitin or 0.1% laminarin, or 0.5% casein, depending on the enzyme activity to be evaluated, ii) MM + 0.1% *H. vastatrix* urediniospores, and iii) MM-control. Flasks containing 10 ml of the corresponding culture were inoculated with 1 ml mycoparasite conidia ($1x10^5$ spores/ml). All the treatments were cultured at 28 °C with shaking for one hour at 150 rpm. Every 24 hours for 10 days, a sample of 600 µl was collected and centrifuged for 5 min at 10,000 rpm. Specific enzyme activity of the supernatant was determined. All determinations were done in triplicate.

Chitinase activity was determined following the methodology proposed by Nguyen et al. (2015) with slight modifications. The reaction mixture contained 50 µl colloidal chitin (0.5% dissolved in 50 mM Tris-HCl buffer, pH 7) and 50 µl supernatant of the cultures. This was incubated for 2 h at 40 °C and shaken at 300 rpm; 100 µl DNS (3,5-dinitrosalicylic acid) was then added and the mixture was boiled for 10 min and cooled for 10 min. Absorbance was measured at 540 nm and, with these values, chitinase activity was determined using a N-acetyl-D-glucosamine curve as the standard. One unit of chitinase activity was considered to be the amount of enzyme required to release one µmol of N-acetyl-D-glucosamine under the conditions of the assay.

To evaluate glucanase activity, a reaction mixture was prepared with 50 µl laminarin (4 mg/ml dissolved in 50 mM acetate buffer, pH 5.5) and 50 µl supernatant from the cultures. The mixture was incubated for 30 min at 37 °C, then 100 µl DNS was added, and it was boiled for 5 min and cooled. Absorbance was measured at 540 nm and glucanase activity was determined using a glucose curve as the standard. One unit of glucanase activity was considered to be the amount of enzyme required to release µmol glucose under the conditions of the assay. With the data on chitinase and glucanase activity, mean activity for each strain was graphed.

For protease activity, 600 µl of the supernatant of the cultures were used every 24 h for 10 d. Thirty µl of supernatant were placed in the center of agar plates + 0.5% casein (dissolved in 0.1 M phosphate buffer, pH 5.8). These plates were incubated at 37 °C for 48 h and, after this time, the diameter of the halos formed by enzymatic degradation of the casein was measured. The hydrolysis halos were compared with those made by the curve of proteinase K on plates of agar + 0.5% casein. To determine the molecular size of the enzymes and corroborate the evaluated enzyme activity, zymograms and the corresponding SDS-PAGE gels were carried out, only of the supernatants of day 10 because on that day the strains were in full development. The gels were prepared following the methodology proposed by Laemmli (1970): 2 µg protein per sample was loaded and run at 200 V for 40 min, and bands were visualized using Dodeca^{TM} Silver Stain Kit (Cat. 161-0480). A broad-range molecule-sized marker (Prestained SDS Page Standards, Broad Range BioRad Cat. 161-0318) was used.

For chitinase, we followed the methodology proposed by Rocha-Pino et al. (2011) with slight modifications. A denaturalizing electrophoresis was run in 8 and 15% polyacrylamide gel that contained 0.01% glycol-chitin. The gel was then washed three times in distilled water for 15 min and incubated in a 0.1M phosphate buffer, pH6, with 1% Triton X-100 for 24 hours at 37 °C. Finally, the hydrolytic zones were observed using UV light.

For glucanase activity, we followed the methodology proposed by Rungrattanakasin et al. (2018). The 12% denaturalizing gel contained 0.5% carboxymethylcellulose. After electrophoresis, the gel was washed with deionized water for 15 min, then with 2.5% Triton X-100. It was then incubated in 50 mM acetate buffer, pH 5, for 24 h, and finally, the gel was dyed with 0.5% Congo red and immediately faded with 1 M NaCl until the hydrolysis bands appeared.

Effect of spores and supernatant of three strains of mycoparasites on *H. vastatrix* germination

An experiment was set up with a completely randomized factorial design with which we evaluated the three mycoparasite strains, two products (spores and supernatant) and two times of application of the products (one simultaneous with inoculation of *H. vastatrix* and the other 8 h after inoculating *H. vastatrix*). Twelve treatments and a control (application of sterile MM) were evaluated. The response variable was the number of germinated *H. vastatrix* urediniospores of 100 observed. Each treatment was replicated three times.

H. vastatrix urediniospores (100 µl) was applied at a concentration of $1x10^4$ urediniospores /ml on plates of agar water with 0.01% chloramphenicol and, according to the randomization, 100 µl mycoparasite conidia at a concentration of $1x10^5$ conidia/ml or 100 µl supernatant. These plates were incubated at 22 $\pm$ 1 °C in darkness for 24 h. At the end of this time, the number of germinated urediniospores was counted. A urediniospores was considered germinated when its germinative tube was longer than the urediniospores itself.

Effect of the supernatant and spores of three mycoparasites strains on the structural integrity of *H. vastatrix* urediniospores

To evaluate the effect of the mycoparasite products *H. vastatrix* on urediniospores, the strains (CERI-530, CERI-542 and CERI-701) were cultured in MM for 14 d at 28 °C with one hour of shaking at 150 rpm daily to obtain spores and supernatant. The latter was separated by ultrafiltration at 2000 rpm using ultrafiltration units (Amicon® Cat. UFC 901008) with a cutting size of 10 kDa. Eight treatments were established: spores ($1x10^5$ spores/ml) of the three strains, supernatant with molecular size larger than 10 kDa (fraction 1) of the three strains and supernatant with molecular size smaller than 10 kDa (fraction 2) of two strains (CERI-530 and CERI-542).

Twenty µl of each treatment were applied to each *H. vastatrix* pustule on detached coffee leaves that were free of mycoparasitism, which was confirmed with a stereoscopic microscope. Per treatment, 20 pustules were inoculated at random. The control treatment was sterile distilled water.

In addition, samples (six pustules) of each treatment were processed after 72 h of incubation to be observed in the scanning electron microscope (SEM). To this end, we obtained 2 mm^2 fragments in the pustule, which were fixed for 24 h in a solution of formaldehyde (2%) and ethanol (70%) and dehydrated in a series of ethanol (70-100%). The samples were then dried to a critical point, mounted, metalized and observed in the SEM.

To understand the process of infection that occurred on the *H. vastatrix* pustules at different times of initial inoculation, the CERI-542 strain was selected because its behavior was different from that of the other two strains when they were observed after 72 h. For this observation, 20 µl conidia at a concentration of $1x10^5$ spores/ml was inoculated on *H. vastatrix* pustules free of mycoparasitism. Portions of tissue with pustules were collected 0, 24, 48 and 72 h after inoculation, processed and observed in the SEM.

RESULTS

Alignment of the DNA sequences obtained from the mycoparasites evaluated in our study suggest that the strains CERI-530 and CERI-542 belong to the species *Simplicillium lanosoniveum*, while the strain CERI-701 belongs to the species *Acanthomyces muscarius* (synonymous of *Lecanicillium muscarius*) (Kepler et al. 2017). The obtained sequences were deposited in the database of the GenBank (ITS accession numbers: MT102449, MT102450 and MT102451; EF1 accession numbers: MT131817, MT131818, MT131819, corresponding to the strains CERI-530, CERI-542 and CERI-701, respectively) (Table 1).

Effect of *H. vastatrix* urediniospores on chitinase, glucanase and protease activity of three mycoparasite strains

Chitinase activity. Daily enzyme activity of the two *S. lanosoniveum* strains peaked on day 6, while *A. muscarius* peaked on day 5 (Fig. 1A). The strain that had higher chitinase activity in the presence of *H. vastatrix* was *S. lanosoniveum* CERI-530 on day 3 (38,178 $\pm$ 2,950 U/mg), close to the chitinase activity with the inducing substrate (41,450 $\pm$ 5,396 U/mg).

Strain protein profiles differed according to whether they were grown in presence of colloidal chitin or *H. vastatrix* (Fig. 2A). In the case of *S. lanosoniveum* CERI-530, very similar patterns were observed in the different mediums, but this was not the case for *S. lanosoniveum* CERI-542 or *A. muscarius* CERI-701, which showed different protein profiles in SDS-PAGE. The zymogram indicated that *S. lanosoniveum* (CERI-542) produced a chitinase with a molecular weight of approximately 50 kDa, in presence of colloidal chitin and *H. vastatrix*. In contrast, in the presence of *H. vastatrix*, *A. muscarius* (CERI-701) presented a band of approximately 40 kDa and two others of 30 and 20 kDa (Fig. 2A). Although chitinase activity was detected by spectrophotometry for the strain CERI-530, no

band with activity was visualized in the zymogram. In none of the strains grown in MM were bands of chitinase activity observed.

Glucanase activity. Daily glucanase activity showed that the strain *S. lanosoniveum* CERI-530 was that with the most activity in presence of *H. vastatrix*, with a peak of 9,720 ± 282 U/mg on day 6, followed by *A. muscarius* CERI-701 with 8,333 ± 412 U/mg on day 5 and, lastly, the strain *S. lanosoniveum* CERI-542 with 5,506 ± 1,733 U/mg on day 4 (Fig. 1B). Between 1 and 5 days of culture, the three strains had the highest glucanase activity on *H. vastatrix*, relative to the inducing substrate, suggesting that this enzyme possibly participates in the mycoparasitic interaction of the strains with *H. vastatrix*.

The protein profile was different among strains. The zymogram (Fig. 2B) shows that the different treatments corresponding to *S. lanosoniveum* CERI-530 and CERI-542, in the different substrates evaluated, presented a band of approximately 50 kDa, in the presence of their inducer (laminarin) as well as *H. vastatrix*. In contrast, with the supernatant of *A. muscarius* CERI-701 and the treatments with MM, even when glucanases were determined by spectrophotometry, bands were not observed in the zymogram.

Protease activity. For all the treatments, protease activity on plates was most evident on day 10. However, the size and intensity of the hydrolysis halo in casein were different among treatments (Fig. 3). All the supernatants obtained from the culture of *S. lanosoniveum* CERI-530, *S. lanosoniveum* CERI-542 and *A. muscarius* CERI-701 in MM+ *H. vastatrix* urediniospores (Fig. 3-A,B,C) had lower protease activity than the equivalent of 0.1 U/mg proteinase K, while the supernatants from culture of the strains in casein (Fig. 3-D,E,F) developed a hydrolysis halo of a size similar to that developed with 0.1 and 0.3 U/mg proteinase K (Fig. 3-H, I).

Effect of spores and supernatant of three mycoparasite strains on *Hemileia vastatrix* germination

H. vastatrix germination was lower when treatments were applied simultaneously with the urediniospores. Application of *S. lanosoniveum* CERI-542 spores and supernatant, significantly decreased ($P<0.001$) *H. vastatrix* germination (less than 2%), relative to the control (9% germination) when applied at the same time (Fig. 4). Moreover, inoculation of *A. muscarius* CERI-701 spores reduced *H. vastatrix* germination to 2% when inoculation and urediniospores were simultaneous, but also when inoculation was 8 h after *H. vastatrix*, it was the only treatment that reduced germination to 3%.

Effect of the supernatant and spores of three mycoparasites strains on the structural integrity of *H. vastatrix* urediniospores

Seventy-two hours after application of spores and supernatant of the three strains, we observed that *S. lanosoniveum* CERI-530 (Fig. 5A) and *A. muscarius* CERI-701 (Fig. 5G) spores formed mycelium over the *H. vastatrix* pustule, while *S. lanosoniveum* CERI-542 spores and supernatant (Fraction 1 and 2) formed a layer that covered and deformed *H. vastatrix* urediniospores (Fig. 5D, E, F). This effect was similar to that of *S. lanosoniveum* CERI-530 (Fig. 5B and C) supernatant. However, CERI-542 was more aggressive: the urediniospores were more deformed than those of the treatment with CERI-530. Fraction 1 of *A. muscarius* CERI-701 (Fig. 5H) also slightly deformed *H. vastatrix* urediniospores, which became stuck together. During ultrafiltration of the *A. muscarius* CERI-701 supernatant, we were not able to obtain fraction 2 and therefore its effect on *H. vastatrix* was not evaluated. The urediniospores of the control did not exhibit any damage after this observation time (Fig. 5I).

Follow-up of *Simplicillium lanosoniveum* CERI-542 infection on *Hemileia vastatrix*

The strain *S. lanosoniveum* CERI-542, which deformed *H. vastatrix* urediniospores in the previous experiment, was observed at different times under the SEM. Before mycoparasite inoculation, *H. vastatrix* urediniospores were intact and characteristically equinulate and reniform (Fig. 6A). However, 24 h after inoculation, some hyphae elongated toward the *H. vastatrix* urediniospores and produced a material that formed a layer, which began to come together to cover the urediniospores (Fig. 6B). After 48 h, this structure almost totally covered the pustules (Fig. 6C). After 72 h, the covering completely deformed some urediniospores and continued advancing toward others (Fig. 6D). In addition, after 72 h, the mycoparasite developed scarce mycelia over the deformed urediniospores.

DISCUSSION

Several mycoparasites have been reported to be associated with *Hemileia vastatrix* in the field (Carrión 1988; Eskes et al., 1991; Carrión and Rico 2002; Mahfud et al. 2006; James et al., 2016). In our study, three strains belonging to *Simplicillium lanosoniveum* and *Akanthomyces muscarius* were selected for their different mycoparasitic and pathogenic characteristics. They showed chitinase, glucanase and protease activity when they grew on *H. vastatrix*.

Specific chitinase activity of the three strains was more than 10,000 U/mg protein, regardless of the substrate, indicating high activity compared with results of Ramírez-Coutiño et al. (2006), who purified chitinases from *Lecanicillium fungicola*, which had an activity of 747 U/mg protein.

The common chitinase band of 50 kDa found in *S. lanosoniveum* CERI-542 and *A. muscarius* CERI-701 was observed only in growth with the inducer (colloidal chitin) or *H. vastatrix*. This size does not coincide with the four bands of 23, 33, 85.5 and 123.1 kDa reported for *L. fungicola* (Ramírez-Coutiño et al. 2006) or that of 33 kDa reported in *L. lecanii* (Nguyen et al. 2015). Moreover, when the strain *A. muscarius* CERI-701 was cultured in presence of *H. vastatrix*, it had other bands of 40, 30 and 20 kDa, suggesting that this phytopathogen induces production of three different chitinases in *A. muscarius* in a way similar to what occurs in *T. harzianum* which has three bands (73, 52 y 33 kDa) in the presence of *R. solani* (Haran et al. 1996). This does not rule out that these bands are sub-units of the same chitinase, although generally chitinases from fungi are found as monomers or dimers (Hartl et al. 2012; Kidibule et al. 2018).

Our results point to an opportunity to evaluate these extracellular metabolites for biological control of *H. vastatrix*, given that field application of *L. lecanii* and *L. leptobactrum* has had little success (Eskes et al. 1991, Canjura-Sarabia et al. 2002). Also, the effect of chitinases from *Trichoderma* sp. on other phytopathogens such as *Sclerotium rolsfii* (El-Katatny et al. 2001) and *Botrytis cinerea* (De la Cruz et al. 1992) has been reported.

For the three strains, glucanase activity was observed in presence of the inducer, but the enzyme exhibited more activity during the first five days in presence of *H. vastatrix*. Moreover, with both the inducer and in presence of *H. vastatrix*, a common band of 50 kDa was visualized for *S. lanosoniveum* CERI-530 and CERI-542. This size differs from the 27 kDa band of 1,3 β-D-glucanase reported for *Trichoderma asperellum* in presence of *Rhizoctonia solani* (Da Silva et al. 2012); it also differs from the 66 kDa band reported for *T. harzianum* in presence of *Sacharomyces cerevisiae* and *Botrytis cinerea* (De la Cruz et al.1995). These data suggest that it is possible that these strains use the enzyme during mycoparasitism on *H. vastatrix* since its induction in the studied mycoparasites in presence of coffee rust urediniospores is evident.

Besides the chitinases and glucanases in our study, we also detected protease activity, mainly of *S. lanosoniveum* CERI-530 and *A. muscarius* CERI-701 when they were cultured in presence of *H. vastatrix*, suggesting that proteases also participated in this mycoparasitic interaction. This may be due to the fungus cell wall, which is made

up of glycoproteins, β-glucans and chitins (Gow et al. 2017), and the mycoparasites could require these enzymes to attack *H. vastatrix*, as observed with *Trichoderma* sp. in the biological control of *Rhizoctonia solani*, *Crinipellis perniciosa* (Kredics et al. 2005), *Fusarium oxysporum* and *F. subglutinans* (Michel-Aceves et al. 2005). This points to the opportunity of considering proteases in the biological control of *H. vastatrix*.

The results of our work indicate that *S. lanosoniveum* CERI-542 spores and supernatant, as well as *A. muscarius* CERI-701 spores significantly reduce *H. vastatrix* germination, relative to the control. The germination percentage (9%) of the control was low because urediniospores collected in the field were used and the low percentages are similar to those reported by other authors (Nutman et al. 1963). Despite this, the treatments evaluated in our study significantly decrease *H. vastatrix* germination, compared with the nine fungal isolates evaluated by Haddad et al. (2014) that were not able to reduce *H. vastatrix* germination.

It is important to consider the potential of *S. lanosoniveum* CERI-542 and *A. muscarius* CERI-701 spores, which diminished *H. vastatrix* germination, even when they were inoculated at lower concentrations (10^5) than those reported as effective for *L. lecanii* (10^7) (De González and Martínez 1996). Even *A. muscarius* CERI-701 decreased germination when it was inoculated after *H. vastatrix* germination, suggesting that it has control potential even if it is applied after deposition of the urediniospores on coffee leaves.

Scanning electron microscope images show that after 72 h of infection, the strain *A. muscarius* CERI-701 developed mycelia on *H. vastatrix* urediniospores. This mycelial formation has been reported for *L. lecanii* on *Uromyces dianthi* and *Puccinia recondita* (Spencer and Atkey 1981), as well as for *L. psalliotae* on *H. vastatrix* (Mahfud et al. 2006). Also reported was a slight deformation of *Uromyces dianthi* and *P. recondita* urediniospores caused by *L. lecanii* (Spencer and Atkey 1981), as well as of *Phakopsora pachyrhizi* uredospores caused by *S. lanosoniveum* (Ward et al. 2011). Nevertheless, as far as we have seen in the literature, formation of a covering with urediniospores deformation, which we observed in our study, caused by *S. lanosoniveum* CERI-542 conidia and supernatant (fraction 1 and 2), has not been previously reported.

Both fraction 1 and fraction 2 of *S. lanosoniveum* CERI-542 deformed *H. vastatrix* urediniospores. It is possible that fraction 2 contains metabolites such as antimicrobial volatiles (Gomes et al. 2018), non-ribosomal peptides, polyketides or terpenoids, which have been reported as secondary metabolites of mycoparasites (Karlsson et al. 2017).

The above suggests that both hydrolytic enzymes (chitinases and glucanases) that had a molecular weight of more than 10 kDa and the proteases, as well as other metabolites with molecular weight of less than 10 kDa, together could carry out infection and deformation of *H. vastatrix* urediniospores, as some authors have suggested in cases of necrotrophic mycoparasitism established by *Trihoderma* spp. and *Clonostachys rosea* (Köhl et al. 2019; Karlsson et al. 2017) on other hosts. However, more in-depth study on this biological interaction is necessary.

In this study we determined that the three evaluated strains have potential for biological control of *H. vastatrix*. However, it is possible that their mechanisms of action during mycoparasitism are different. For example, the strain *S. lanosoniveum* CERI-530 was outstanding for its glucanase, protease and chitinase activity, while *S. lanosoniveum* CERI-542 inhibited germination and deformed the uredospores even when fraction 2 of its supernatant was applied, and glucanase and chitinase activity were also present. The strain *A. muscarius* CERI-701 had three different bands of chitinases and also presented glucanase and protease activity. We present a graphic illustration of the sites of action of the three studied strains in Figure 7. It is known that the cell wall of the uredospores are constituted mainly of chitins, β-glucans (Talhinhas et al. 2017) and glycoproteins (Mendgen and Deising et al. 1992), and mycoparasites can use the chitinases, glucanases and proteases to hydrolyze these components and the metabolites may participate by attacking the plasma membrane or by inhibiting germination of the urediniospores, as has been mentioned by other authors who studied cases of mycoparasitism (Karlsson et al. 2017). This does not rule out the possibility that these metabolites act in conjunction during mycoparasitism.

We can conclude that the biological potential and mechanism of action of *S. lanosoniveum* on *H. vastatrix* is different from those of *A. muscarius*. However, it is necessary to conduct further study to identify their metabolites and their role in biological control of *H. vastatrix*.

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AUTHORS' CONTRIBUTIONS

IGC, JGR, KGN and MMB conceived the project. IGC and LVGF conducted the experiments. IGC wrote the manuscript. IGC, JGR, KGN, MMB and GHP participated in discussing the results, critiquing the scientific aspects, and proofreading the manuscript. IGC JGR, KGM, MMB, LGF and GHP approved the version to be published.

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interest: The authors have no confict of interest to declare

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TABLES

Table 1 ITS and EF1 accession numbers of three mycoparasite strains of *H. vastatrix*

Strain	Name	E-value	ITS Genebank accesion number (% Identity)	TEF1α Genebank accesion number (% Identity)
CERI-530	*Simplicillium lanosoniveum*	0.0	MT102449 (99.6)	MT131817 (99.12)
CERI-542	*Simplicillium lanosoniveum*	0.0	MT102450 (99.33)	MT131818 (99.67)
CERI-701	*Acanthomyces muscarius*	0.0	MT102451(98.20)	MT131819 (99.42)

FIGURE LEGENDS

Fig. 1 Kinetics of the chitinase (A) and glucanase (B) activity of three strains of mycoparasites on different media: MM + Laminarin, MM + *H. vastatrix* and MM

Fig. 2 Silver SDS-PAGE (left) and zymogram (right) of chitinases stained with white calcoflouride (A) and SDS-PAGE (left) and zymogram (right) of glucanases stained with congo red (B). The lines correspond to supernatant samples from day 10 of culture of three mycoparasites on different media: MM + inductor substrate (colloidal chitin or laminarine); MM + *H. vastatrix* and MM. M, molecular size marker; CC, colloidal chitin; Lam: laminarine; M, minimal medium. The arrows indicate the height at which the chitinase band are found in the zymogram according to the molecular size

Fig. 3 Representative images of the protease activity of the supernatant of three strains of mycoparasites (*S. lanosoniveum* CERI-530, *S. lanosoniveum* CERI-542 y *A. muscarium* CERI-701) on agar plates + casein at 0.5 % (day 10) and of the proteinase K curve (H-I) with concentrations of 0.1 and 0.3 U/mg

Fig. 4 Effect of spores and supernatant of three mycoparasites (*S. lanosoniveum* CERI-530, *S. lanosoniveum* CERI-542 and *A. muscarium* CERI-701) and two application times (simultaneous to *H. vastatrix* and 8 hours after inoculating it) on *H. vastatrix* percentage germination in agar water plates. The dotted line in the graph represents the control germination percentage

Fig. 5 SEM images of *H. vastatrix* pustules after 72 hours of spore application ($1x10^5$ spores/ml) or two different fractions supernatant from three strains of mycoparasites on arabica coffee leaves. The different treatments applied were: mycoparasite spore solution: fraction 1, metabolites >10 kDa; fraction 2, metabolites <10 kDa; control, sterile distilled water. MH, mycoparasite hyphae; C, coverage; DU, deformed urediniospores

Fig. 6 *S. lanosoniveum* CERI-542 infection process on *H. vastatrix* pustules. A, 0 hours; B, 24 hours; C, 48 hours; D, 72 hours. MH, mycoparasite hyphae; C, coverage

Fig. 7 A hypothetical scheme representing the possible action sites of three strains of mycoparasites (*S. lanosoniveum* CERI-530, *S. lanosoniveum* CERI-542 and *A. muscarium* CERI-701) on *H. vastatrix*

FIGURES

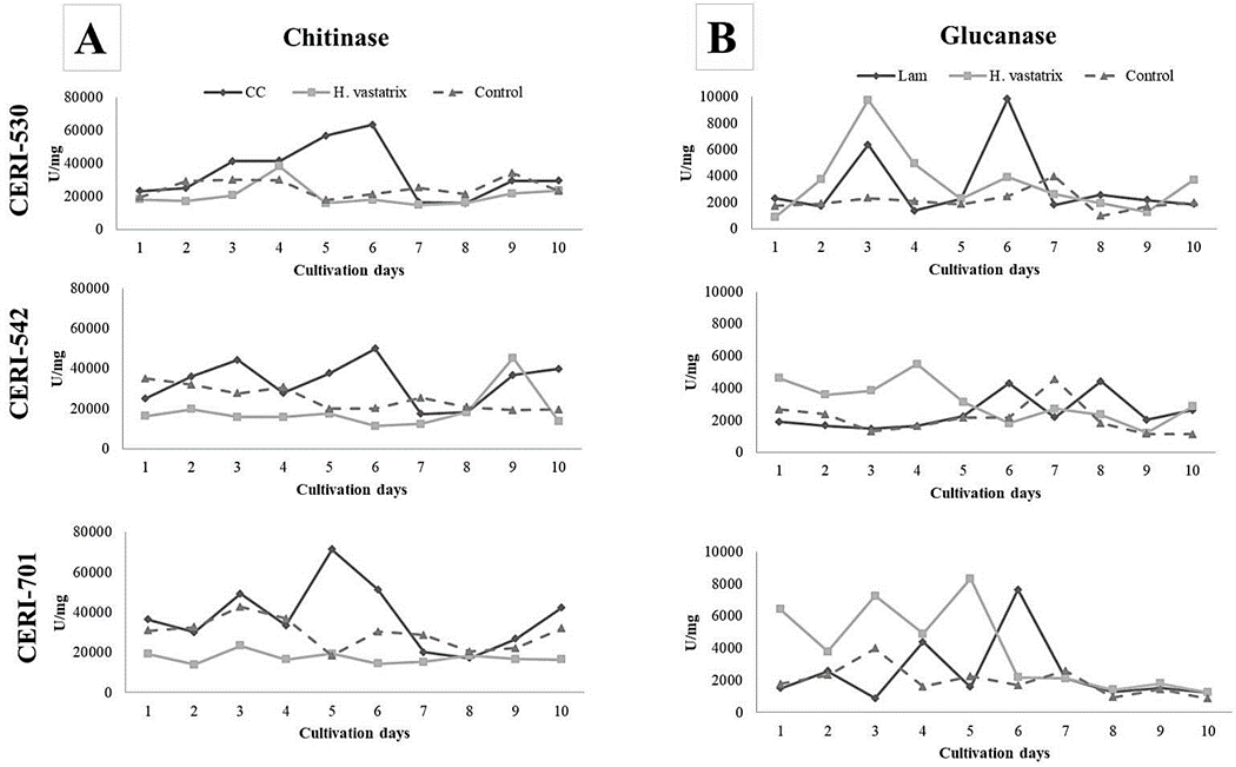

Fig. 1

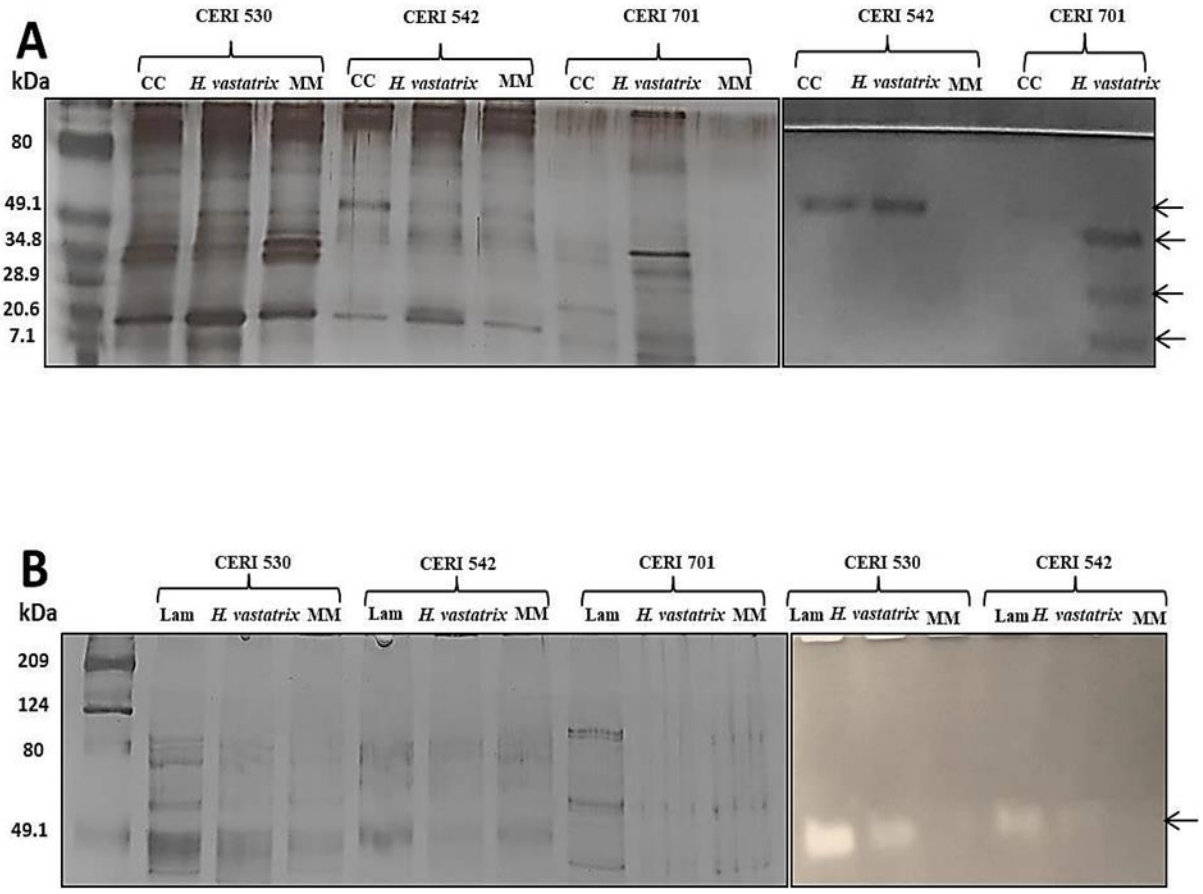
A
kDa
CERI 530
CERI 542
CERI 701
CERI 542
CERI 701
CC
H. vastatrix
MM
80
49.1
34.8
28.9
20.6
7.1
B
kDa
CERI 530
CERI 542
CERI 701
CERI 530
CERI 542
Lam
H. vastatrix
MM
209
124
80
49.1

Fig. 2

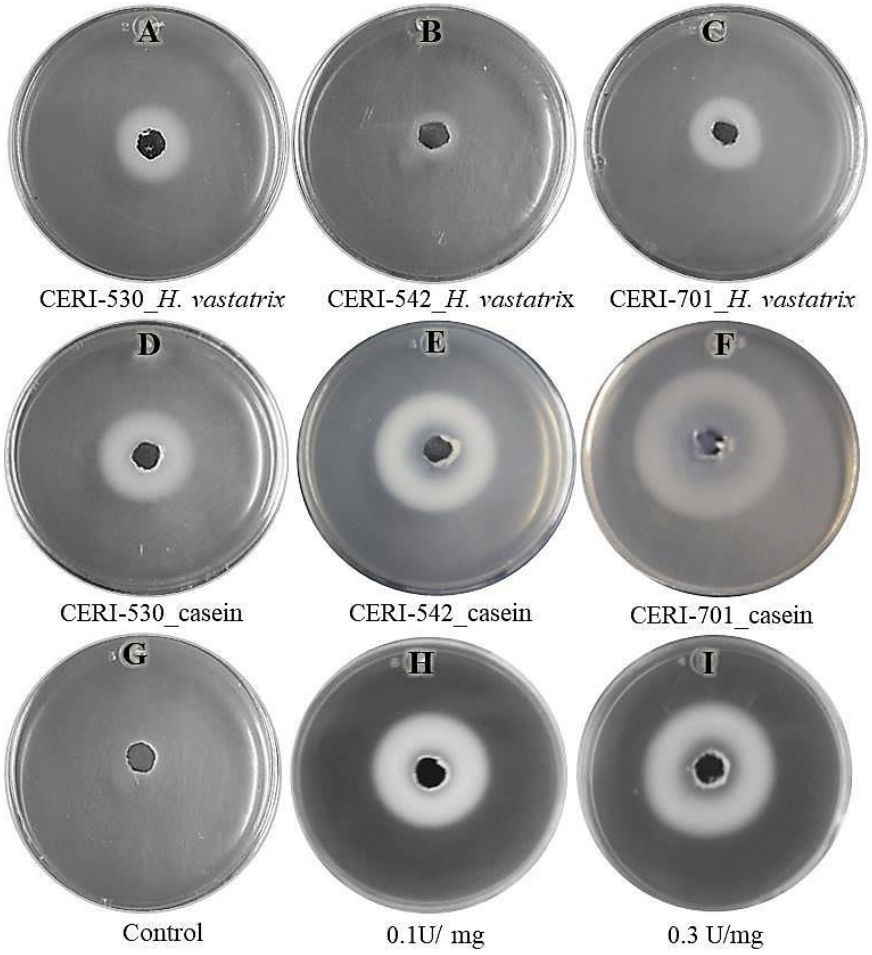
A
B
C
CERI-530_H. vastatrix
CERI-542_H. vastatrix
CERI-701_H. vastatrix
D
E
F
CERI-530_casein
CERI-542_casein
CERI-701_casein
G
H
I
Control
0.1U/ mg
0.3 U/mg

Fig. 3

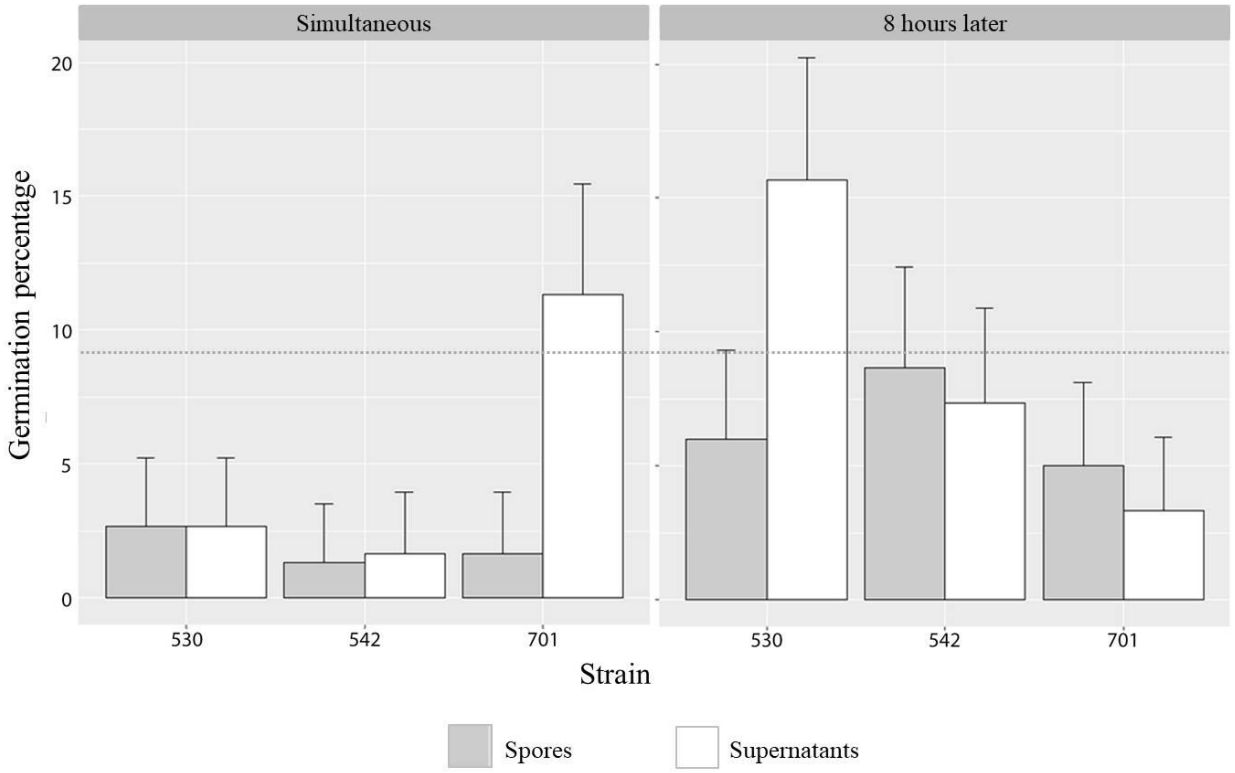
Simultaneous
8 hours later
Germination percentage
20
15
10
5
0
530
542
701
530
542
701
Strain
Spores
Supernatants

Fig. 4

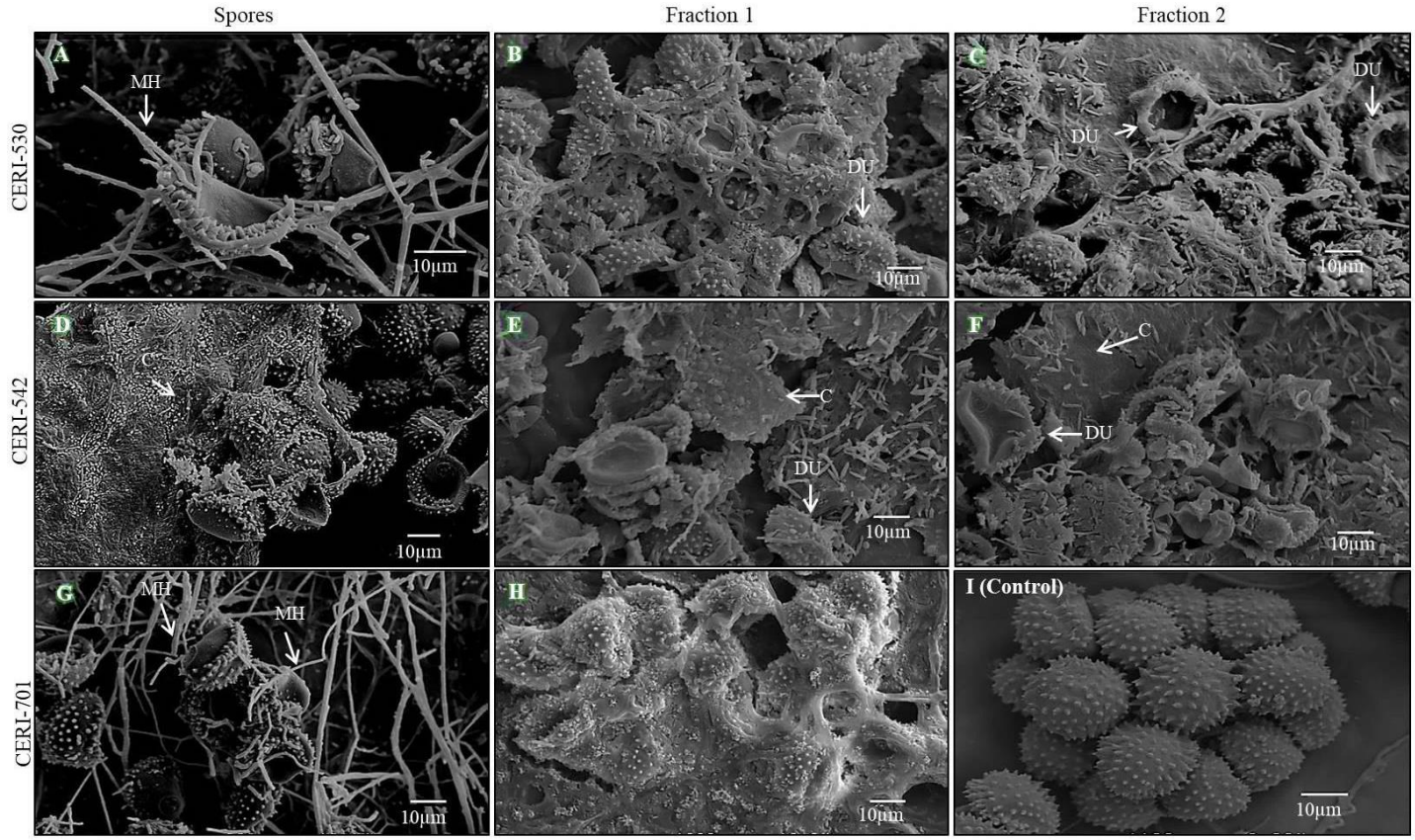
Spores
Fraction 1
Fraction 2
CERI-530
CERI-542
CERI-701
A
MH
10µm
B
DU
10µm
C
DU
DU
10µm
D
C
10µm
E
C
DU
10µm
F
C
DU
10µm
G
MH
MH
10µm
H
10µm
I (Control)
10µm

Fig. 5

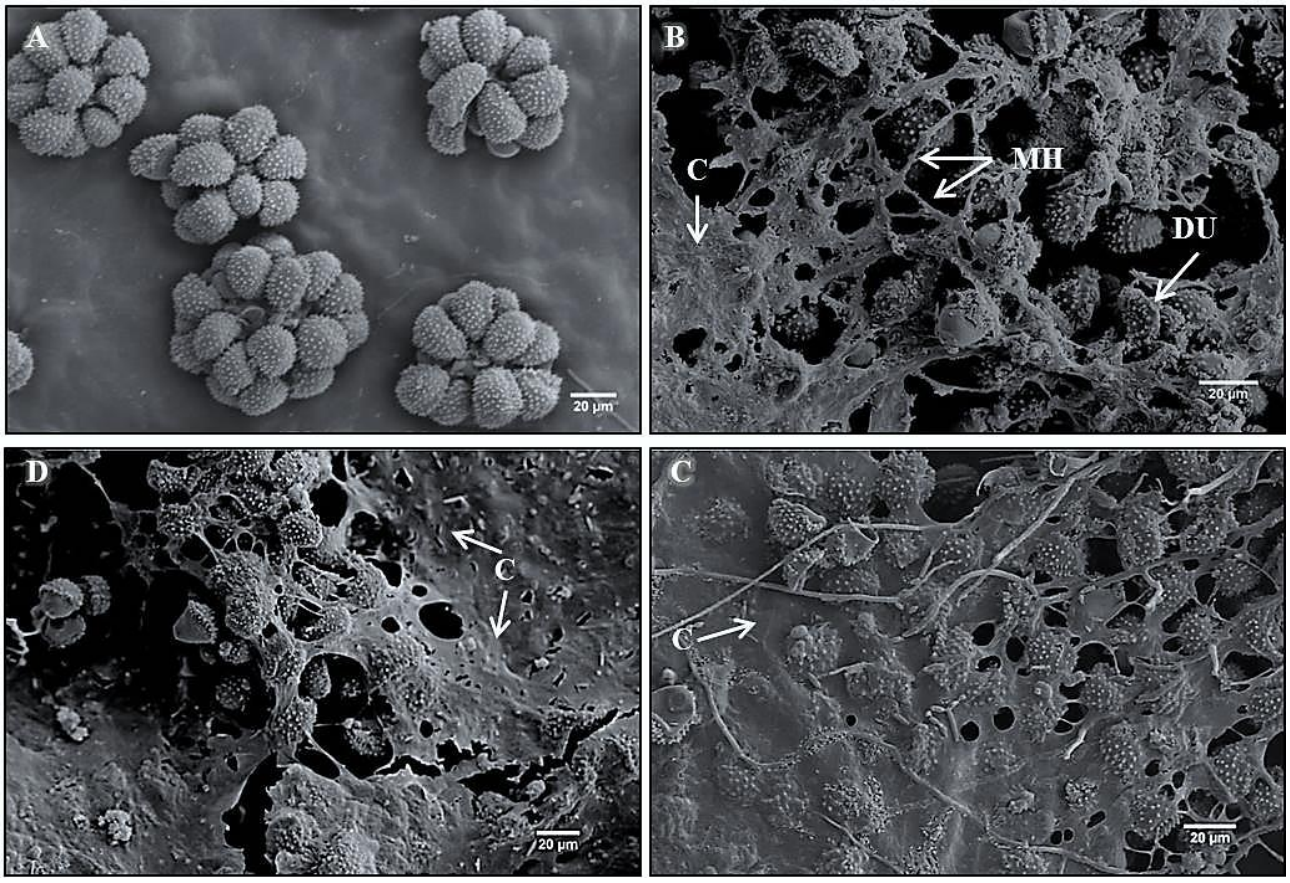

Fig. 6

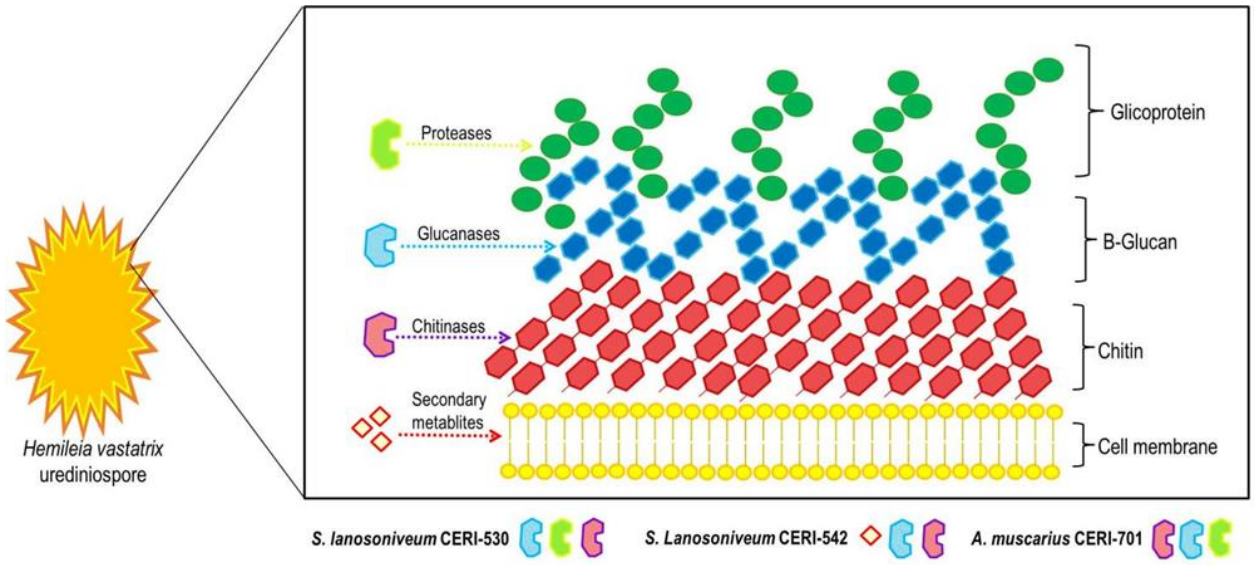
Hemileia vastatrix
urediniospore
Proteases
Glucanases
Chitinases
Secondary
metablites
Glicoprotein
B-Glucan
Chitin
Cell membrane
S. lanosoniveum CERI-530
S. Lanosoniveum CERI-542
A. muscarius CERI-701

Fig. 7

IV. CONCLUSIONES

1. Se determinó que *Simplicillium lanosoniveum* CERI-530 presentó mayor actividad quitinasa, glucanasa y proteasa durante los primeros cinco días de cultivo en presencia de *H. vastatrix.*
2. La roya del café (*H. vastatrix)* indujo la producción de tres diferentes bandas de quitinasas en *A. muscarius* (CERI-701).
3. La aplicación de esporas y sobrenadante de *Simplicillium lanosoniveum* CERI-542, así como la aplicación de esporas de CERI-701 disminuyeron significativamente la germinación de *H. vastatrix.*
4. El mecanismo de acción sobre *H. vastatrix* posiblemente fue diferente entre cepas, la cepa CERI-701 colonizó las pústulas y su sobrenadante deformó ligeramente las uredosporas, sin embargo, las cepas CERI-542 y CERI-530 produjeron una cubierta que deformó a las uredosporas.
5. Las tres cepas evaluadas en este trabajo tienen potencial para el control biológico de *H. vastatrix*, sin embargo, es necesario realizar posteriores investigaciones para profundizar en el conocimiento de sus interacciones.

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