http://journal.unoeste.br/index.php/ca/index
DOI: 10.5747/ca.2022.v18.n2.a483
ISSN on-line 1809-8215

Colloquium Agrariae

Submetido: 18/10/2021 Revisado: 31/03/2022 Aceito: 05/04/2022

Characterization of the antagonism of the mycorrhizal fungus *Waitea circinata* against *Magnaporthe oryzae*, *Cochliobolus miyabeanus*, *Monographella albescens* and *Sarocladium oryzae* rice pathogens

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Abstract

Rice production is affected by important pathogens such as *Magnaporthe oryzae*, *Cochliobolus miyabeanus*, *Monographella albescens* and *Sarocladium oryzae*, and orchid mycorrhizal fungi can contribute to the biocontrol of these diseases. The aim of this study was to characterize the antagonism mechanisms of *W. circinata* against rice pathogens. The indoleacetic acid (IAA) and lytic enzymes of *W. circinata* were quantified. The effectiveness of the bioagent and its metabolites was assessed *in vitro*. Antagonism *in vivo* against rice blast was also investigated.

Waitea circinata produced IAA (2.3 µg ml^{-1}), cellulase and polyphenol oxidase in a qualitative test. Glucanase, chitinase and protease were also produced in culture with cell walls and in co-culture with pathogens to explain antibiosis. *W. circinata* acts on direct antagonism by antibiosis and volatile metabolites. Mycelial suspension reduced *M. oryzae* conidial germination, appressorium formation, and rice blast by 61, 82 and 84%, respectively. *W. circinata*, a unique bioagent, controls four rice pathogens from mechanisms of parasitism and antibiosis. The results could contribute to new formulations containing this fungus, enzymes as well as its volatile metabolites.

Keywords: blast; biological control; mycorrhiza, lytic enzymes; volatile metabolites.

Caracterização do antagonismo do fungo micorrízico *Waitea circinata* contra os patógenos do arroz *Magnaporthe oryzae*, *Cochliobolus miyabeanus*, *Monographella albescens* e *Sarocladium oryzae*

Resumo

A produção de arroz é afetada por patógenos importantes como *Magnaporthe oryzae*, *Cochliobolus miyabeanus*, *Monographella albescens* e *Sarocladium oryzae*, e fungos micorrízicos de orquídeas podem contribuir para o biocontrole dessas doenças. O objetivo deste estudo foi caracterizar os mecanismos de antagonismo de *W. circinata* contra patógenos do arroz. O ácido indolacético (AIA) e as enzimas líticas de *W. circinata* foram quantificadas. A eficácia do bioagente e seus metabólitos foi avaliada *in vitro*. O antagonismo *in vivo* contra a brusone do arroz também foi investigado. *Waitea circinata* produziu AIA (2,3 µg ml^{-1}), celulase e polifenol oxidase em teste qualitativo. Glucanase, quitinase e protease também foram produzidas em cultura com paredes celulares e em co-cultura com patógenos para explicar a antibiose. *W. circinata* atua no antagonismo direto por antibiose e metabólitos voláteis. A suspensão micelial reduziu a germinação de conídios de *M. oryzae*, a formação de apressórios e a brusone do arroz em 61, 82 e 84%, respectivamente. *W. circinata*, um bioagente único, controla quatro patógenos do arroz a partir de mecanismos de parasitismo e antibiose. Os resultados podem contribuir para novas formulações contendo este fungo, enzimas, bem como seus metabólitos voláteis.

Palavras-chave: brusone; controle biológico; micorriza; enzimas líticas; metabólitos voláteis.

Introduction

Rice productivity is affected by rice blast (*Magnaporthe oryzae*), brown spot (*Cochliobolus miyabeanus*), leaf scald (*Monographella albescens*), and sheath rot (*Sarocladium oryzae*). Grain losses due to rice blast are approximately 30%, brown spot 3.7 - 52%, leaf scald 30%, and sheath rot 20-85%, (OU, 1985; MIA *et al*., 2001; CHAKRABARTI, 2001; NALLEY *et al*., 2016; SAKTHIVEL, 2001).

Fungicides are the most widely used control method for these diseases, which may increase selection pressure, environmental pollution, incidence of cancer, poisoning, in addition to reducing the population of beneficial microorganisms (KHASKHELI *et al*., 2020; NASCIMENTO *et al*., 2020; BOZDOGAN, 2014). Thus, biological control against plant pathogens being used very successfully through live fungus and bioproducts to ensure sustainable agriculture (VAN LENTEREN *et al*., 2018).

Arbuscular mycorrhizal fungi and orchid mycorrhizal fungi interact symbiotically with plant species. Both groups promote tolerance against biotic and abiotic stresses when interacting with other hosts (MOSQUERA-ESPINOSA *et al*., 2013; CHAREESRI *et al*., 2020; PARVIN *et al*., 2020).

Waitea circinata (isolate En07), an example of an orchid mycorrhizal fungus (OMF) that can be grown in culture media, is a biological agent against *M*. *oryzae*, and an option for the formulation (CARVALHO *et al*., 2015). In this context, Carvalho *et al*. (2015) evaluated the antagonism of 10 orchid isolates against *M*. *oryzae*. *W*. *circinata* stood out in inhibition of the colony area and inhibition halo formation in the presence of *M*. *oryzae*. In addition, three extracts were developed from this isolate, which act as antagonists of *M*. *oryzae* by reducing mycelial growth, inhibiting conidial germination and appressorium formation, and suppressing leaf rice blast.

In another study, Carvalho *et al*. (2021) developed a new *W*. *circinata* extract that inhibited *M*. *oryzae*, *Cochliobolus miyabeanus*, *Monographella albescens* and *Sarocladium oryzae* and suppressed rice blast. However, the antagonist mechanisms of *W. circinata* fungus against rice pathogens remain unclear.

Our hypothesis is that *W. circinata* uses many mechanisms for control the pathogens, and for this we search answer which lytic enzymes can the bioagent produce when it is growing in conditions of nutrient deficiency enriched with pathogen cell walls? Do lytic enzymes produce the same pattern if the bioagent is grown together with the pathogen? Does the bioagent release volatile metabolites? Is it thermostable? Finally, to validate the laboratory results, the efficiency of a bioagent needs to be evaluated during plant/pathogen/bioagent interaction.

As such, the aims of this study were to characterize the antagonism mechanisms of *W*. *circinata* against *M. oryzae*, *C. miyabeanus*, *M. albescens*, and *S. oryzae*.

Material and methods

The isolates (*Waitea circinata*: En07 and rice pathogens: *M. oryzae*, *C*. *miyabeanus*, *M*. *albescens*, and *S*. *oryzae*) were cultured on potato dextrose agar (PDA - agar, 15 g; dextrose, 20 g; potato, 200 g; and water, 1 L). Petri plates containing fungi were incubated under continuous fluorescent light for seven days at room temperature (26 ± 2 °C).

Genomic DNA was extracted according to Dellaporta *et al*. (1983), with modifications (CHAIBUB *et al*., 2019a). The DNA was resuspended in 100 µL of ultrapure water, quantified using a spectrophotometer (NanoDrop 2000) by absorption measurements at 260 nm, diluted to a concentration of 50 ng μL^{-1} and stored at −20 °C. The internal transcribed spacer (ITS) region (ITS-1, 5.8S rDNA, ITS-2) was amplified using the primers ITS1 (5'-TCCG TAGGTGAACCTGCGG-3') and ITS4 (3'-TCCT CCGCTTATTGATATGC-5') (WHITE *et al*., 1990). Both strands (forward and reverse) were sequenced by Macrogen Inc. (South Korea).

The results were analyzed and edited to obtain a consensus sequence using SeqScape V2.6 software (Applied Biosystems, CA). The identity of the sequence was then assessed using the BLAST tool at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). The alignment was performed with MUSCLE (Multiple Sequence Comparison by Log-Expectation) implemented in MEGA software version 7.0.26 with the En07 isolate (MG646336).

The *Waitea circinata* isolate CBS 473.82 (MH861519), which exhibits higher identity and has more accessions than other OMFs (*Ceratobasidium* sp. - GU206543 and JF273487, *Tulasnella* sp. - AY373264, and *Sebacina*

vermifera - JQ711843), and *Athelia rolfsii* (JN017199), was used as an outgroup. A maximum parsimony tree of 7 nucleotide sequences was generated, and the robustness of the branches determined with 1000 bootstrap replicates.

Morphological analyses of *W*. *circinata* were performed with an optical microscope equipped with an epifluorescence accessory and scanning electron microscopy (SEM), according to Sousa *et al*. (2019).

A completely randomized design with two treatments and three replicates was used for all *in vitro* assays.

Indoleacetic acid (IAA) quantification: three mycelial discs of the *W*. *circinata* isolate were placed in flasks containing 50 mL of potato dextrose (PD) medium (dextrose, 20 g; potato, 200 g; water, 1 L) and 100 mg L^{-1} L-tryptophan or no L-tryptophan (control). Mycelia were separated by centrifugation at 12,000 rpm for 15 min after 2, 4, 6 and 8 days of growth in a rotary shaker at 150 rpm and 26 ± 2 °C. One hundred microlitres of Salkowski reagent [$FeCl_3$ 0.5 mol L^{-1} + $HClO_4$ (35%)] was added to 150 µL of the supernatant, after which the solution was stored in the dark at 28 °C. Red color confirmed IAA production, and the hormone was quantified at 530 nm using a spectrophotometer. Concentrations (µg mL^{-1}) were calculated based on the standard curve with known concentrations of a synthetic form of the hormone (0 at 100 µg mL^{-1}) (3-IAA - Sigma®) (OLIVEIRA *et al*., 2012).

Pectinase (PEC): one disc of the *W*. *circinata* isolate was grown in three Petri dishes containing M9 medium (per L: sucrose, 2 g; Na_2HPO_4, 6 g; KH_2PO_4, 3 g; NaCl, 0.5 g; agar, 15 g; 0.01 M $CaCl_2$ solution, 10 mL; and 1 M $MgSO_4.7H_2O$ solution, 1 mL) after incubation at 28-30 °C for 5 days. Mycelia were removed by adding 10 mL of 2 N HCl, and colonies that formed a clear halo were considered to have produced PEC (CATTELAN *et al*., 1999).

Cellulase (CEL): one disc of *W*. *circinata* isolate was grown in three Petri dishes that contained tryptic soy agar [TSA, 1/10 (v/v)] supplemented with 10 g of powdered cellulose L^{-1}. The plates were incubated at 28-30 °C for 5 days. Mycelia were removed by the addition of 1 M NaCl solution. The production of CEL was confirmed by the formation of a clear zone around a colony (CATTELAN *et al*., 1999; STRAUSS *et al*., 2001).

Polyphenol oxidase (PPO, EC 1.10.3.1): *W*. *circinata* was grown on PDA for 7 days. PPO production was evaluated in Petri plates with yeast extract agar medium (ZELMER *et al*., 1996). The plates were incubated at 26 °C ± 2 °C under a 16/8-h light/dark photoperiod for 5 days, after which enzyme production was compared with that of the control. Enzyme production was detected by the presence of amber-colored halos around colonies.

For enzymatic activity with cell wall the pathogens preparation with three mycelial discs (5 mm) of each pathogen were transferred to three Erlenmeyer flasks (500 mL) containing 250 mL of TLE liquid medium (per L: KH_2PO_4, 2 g; $(NH_4)_2SO_4$, 1.4 g; $MgSO_4$, 0.3 g; $CaCl_2$, 0.15 g; $FeSO_4$, 0.005 g; $MnSO_4$, 0.016 g; and $ZnSO_4$, 0.014 g). The flasks were incubated in a rotary shaker at 28 °C and 140 rpm for 3 days. The liquid medium was filtered and the mycelial mass was recovered, frozen, lyophilized and macerated in liquid nitrogen to obtain a powder. The powder was subsequently rinsed with autoclaved distilled water four times until no protein was present in the sample. Total protein concentration was quantified according to Bradford (1976). After being washed, the powder was autoclaved, frozen and lyophilized to obtain the cell wall of the pathogens (adapted from ALMEIDA *et al*., 2007; GERALDINE *et al*., 2013).

W. *circinata* mycelia grown in PDA plates were removed with a scalpel. One gram of mycorrhizal mycelium was inoculated in 500 mL Erlenmeyer flasks containing 250 mL of TLE medium with or without 0.125 g of cell wall tissue from *M*. *oryzae*. The flasks were incubated in a rotary shaker at 28 °C and 140 rpm, and aliquots were collected every 24 h up to 144 h of incubation. The culture was then filtered and used as a source of enzymes (adapted from ALMEIDA *et al*., 2007; GERALDINE *et al*., 2013).

At enzymatic activity in co-culture *W*. *circinata* and pathogen mycelia were obtained as explained above. One gram of each mycelial sample was inoculated in a 500 mL Erlenmeyer flask containing 250 mL of TLE. As a control, 1 g of *W. circinata* mycelia was placed in TLE. The flasks were incubated in a rotary shaker at 28 °C and 140 rpm, and aliquots were collected every 24 h up to 144 h of incubation. The culture was then filtered and used as a source of enzymes.

The activity of β-1,3-glucanase (GLU, EC 3.2.1.6) was determined using laminarin in sodium acetate buffer (50 mM, pH 5.0) as a

substrate. The activity of chitinase (CHI, EC 3.2.1.14) was determined with colloidal chitin as a substrate under the same conditions. After incubation at 50 °C for 10 min, 100 µL of 3,5-dinitrosalicylic acid (DNS) was added, and the samples were again incubated at 95 °C for 5 min. The amount of reducing sugars was determined based on absorbance at 540 nm using a spectrophotometer. One unit of enzyme activity was defined as the amount of enzyme needed to release 1 mmol reducing sugar min^{-1} (ALMEIDA *et al*., 2007).

Protease (PRO) activity was determined using 0.25% azocasein in 50 mM phosphate buffer (pH 5.0) as a substrate. The samples were incubated at 37 °C for 30 min; 100 µL of 10% (w/v) trichloroacetic acid (TCA) was added, and the mixtures were subsequently re-incubated at 4 °C for 10 min. The samples were then centrifuged at 2500 rpm for 30 min, after which 100 µL of the supernatant was transferred to microplates containing 100 µL of 1 M NaOH. The PRO concentration was determined at 450 nm using a spectrophotometer. One unit of enzyme activity was defined as the amount of enzyme required to generate 1 U absorbance min^{-1} (GERALDINE *et al*., 2013).

The experiments were performed for each enzyme and pathogen in a completely randomized design with three treatments and 3 replicates h^{-1} for each enzyme.

In the antagonism between *W*. *circinata* and rice pathogens all assays were performed using a completely randomized design and repeated three times.

Dual-plate assays: An experiment involving direct antagonism between En07 and *M*. *oryzae* was performed according to Carvalho *et al*. (2015). Assays for the other pathogens (*C*. *miyabeanus*, *M*. *albescens*, and *S*. *oryzae*) were carried out with two treatments and 20 replicates per pathogen. The treatments were as follows: T1 (*W*. *circinata* + pathogen) and T2 (pathogen only). Mycelial discs (5 mm) from the two treatments were placed in the center of Petri dishes containing PDA and then placed on opposite sides, approximately 3 cm apart.

Volatiles: two treatments (T1: *W*. *circinata* + pathogen, and T2: pathogen) and 50 replicates per pathogen were used. To avoid contact with *W*. *circinata*, each pathogen was placed in an upside-down lid of a Petri dish, and the bottom half, in which *W*. *circinata* was growing, placed on top. The control consisted of a base that contained the pathogen disc, and the other base only a PDA disc (QUALHATO *et al*., 2013).

For both assays, the plates were incubated in a biological oxygen demand (BOD) chamber at 27 °C for 15 days under continuous light.

Thermostability: seven treatments and five replicates for each pathogen were used. Mycorrhizal mycelium was removed and weighed, and 2, 5 and 10 g of mycelia transferred to six Erlenmeyer flasks containing PDA (mycorrhiza-PDA). Three samples of each mycorrhiza-PDA combination were autoclaved (A) and subsequently labelled A2g, A5g, and A10g; non-autoclaved (S) samples (three of each) were labelled S2g, S5g, and S10g. The mycorrhiza-PDA combinations (with and without sterilization) were transferred to Petri dishes and incorporated into the PDA. Next, a 5-mm mycelial disc of the pathogen was transferred to the center of the plates. The control consisted of pathogens cultured on PDA in the absence of mycorrhiza-PDA. The treatments were subsequently incubated in a BOD chamber at 27 °C for 11 days under a 12/12-h (light/dark) photoperiod.

Assay evaluations: when the control colony reached the edge of its dish, the horizontal and vertical diameters of the pathogen colonies in both assays were measured using a digital pachymeter in a laminar flow chamber at 27 °C under continuous white light. The colony area and amount of reduction were calculated according to Carvalho *et al*. (2015).

The conidial germination and appressorium formation of *M. oryzae* was performed after growth on PDA, *W*. *circinata* mycelia were scraped, weighed and diluted in autoclaved distilled water to obtain three suspensions at concentrations of 2, 5 and 10 g L^{-1}.

M. oryzae isolate BRM 10900 was grown for 7 days in Petri dishes that contained PDA and then transferred to dextrose agar. For colony growth, dishes were kept under continuous white light at 25 °C for 7 days. The colonies were then induced to produce conidia according to Filippi e Prabhu (2001). The resulting conidial suspension was then filtered and adjusted to 1×10^5 conidia mL^{-1}.

Assays were conducted in a completely randomized design with four treatments and three replicates and organized into microscopic slides in Petri dishes previously lined with paper towels moistened with sterile water to provide a high-humidity environment. A 10 µL aliquot of each treatment was separately placed on the

hydrophobic side of each previously sterilized piece of parafilm; next, the aliquots and 10 µL of *M. oryzae* conidial suspension were mixed. Conidial germination and appressorium formation were evaluated after 3, 6 and 24 h by observing the slides under a light microscope (BelAnalyzer MicroImage software was also used). The control consisted of 20 µL of the *M. oryzae* conidial suspension placed on the same type of support under the same conditions as described above, but mixed with water (CARVALHO *et al*., 2015).

The assay on greenhouse conditions was conducted in a completely randomized design with four treatments and three replicates, under artificial spray inoculation. Planting and inoculation were performed according to Carvalho *et al*. (2015).

Three mycorrhizal suspensions (2, 5, and 10 g L^{-1}) were used. In each treatment, 21-day-old rice plants were sprayed with *M*. *oryzae* (M.o.) conidial suspension (3×10^5 conidia mL^{-1}). The treatments consisted of the following: control (only M.o), 2 g of *W*. *circinata* + M.o, 5 g of *W*. *circinata* + M.o., and 10 g of *W*. *circinata* + M.o.

Leaf blast severity (LBS) was based on a 10-point scale according to Notteghem (1981) at 2-day intervals to determine the area under the disease progress curve (AUDPC). We also calculated the reduction in LBS and AUDPC in relation to the control. The severity data were transformed using $\sqrt{x + 0.5}$

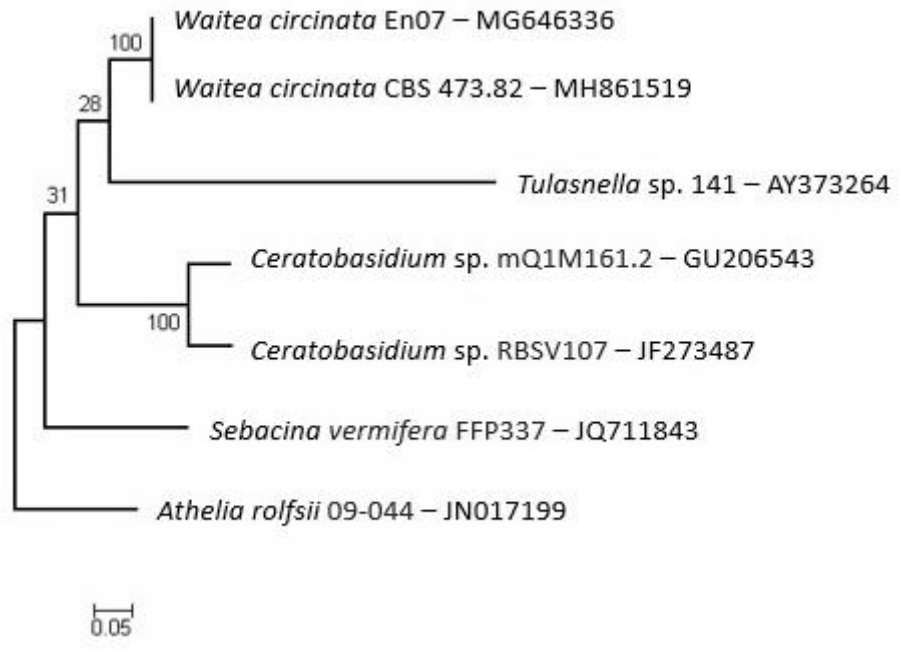
Statistical analyses

All assays described were repeated three times, and means were calculated for statistical analysis. Means for the dual-plate and volatile metabolite assays were compared using a t-test ($P < 0.05$). For other assays, analysis of variance was performed on the data collected, and means were compared according to Tukey's test ($P < 0.05$) using R version 3.0.1.

Results and discussion

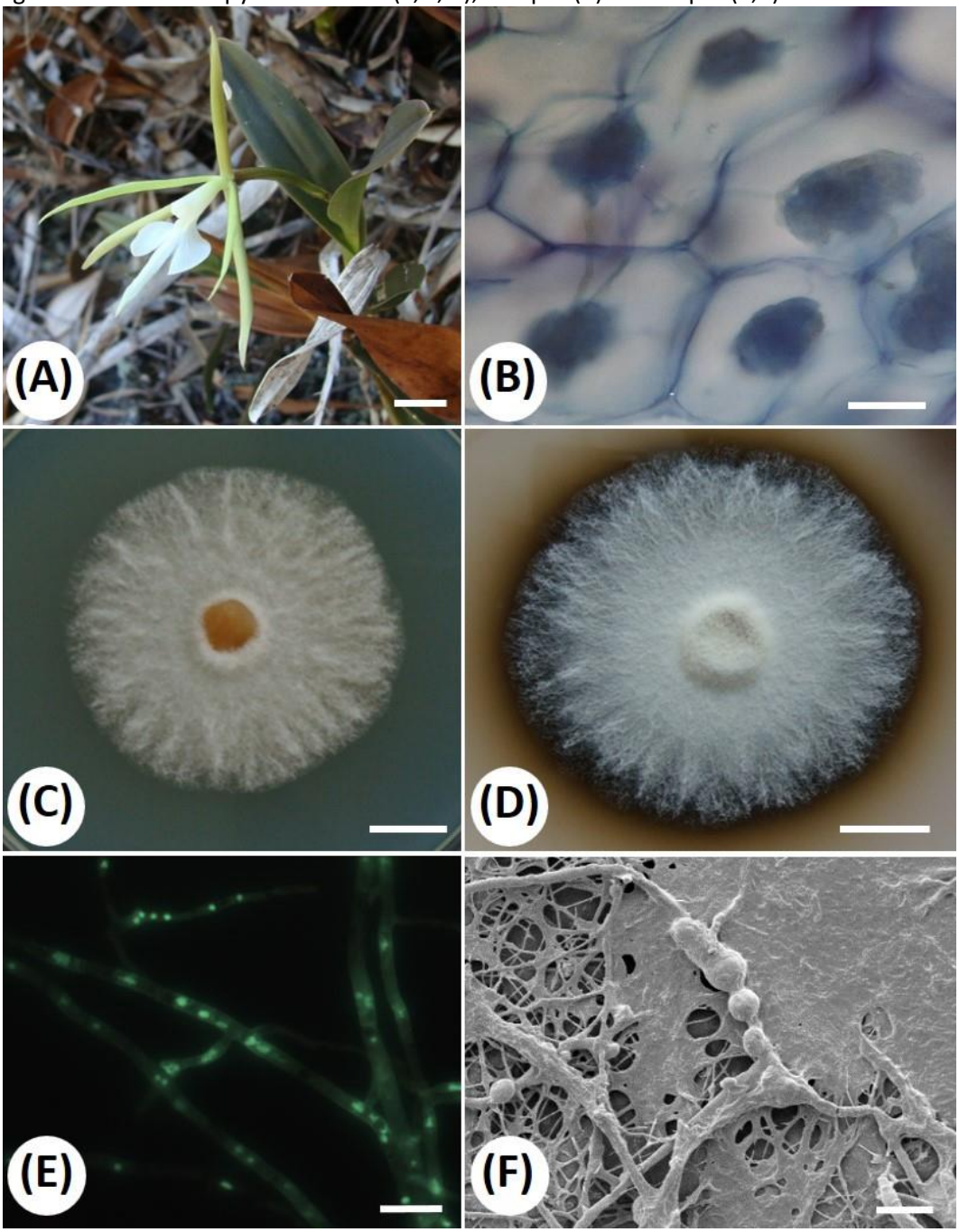
The ITS region was amplified, sequenced and compatibilized for analysis of En07; the results show that the isolate was *Waitea circinata* with NCBI code MG646336. The En07 isolate shows 99.52% identity with the *W*. *circinata* CBS 473.82 isolate (NCBI code: MH861519) (Figure 1).

Figure 1. The first of the 9 most parsimonious trees were obtained using the tree-bisection-regrafting (TBR) algorithm, which involved 7 nucleotide sequences. The most parsimonious tree was obtained using the ITS sequences of the En07 isolate of this work (MG646336) with *Waitea circinata* CBS 473.82 (MH861519), with higher identity and more accessions than other OMFs (*Ceratobasidium* sp. - GU206543 and JF273487, *Tulasnella* sp. - AY373264, and *Sebacina vermifera* - JQ711843). *Athelia rolfsii* (JN017199) was used as an outgroup. The values at the branches are percentages from 1000 bootstrap replications and are shown at the nodes.



Pelotons were observed in the cortex of the roots of *E*. *nocturnum* and were stained dark blue because of the presence of chitin in the hyphae (Figure 2A-B). Connective hyphae were also observed between pelotons (Figure 2B). The colony exhibited a submerged margin, mucous-like mycelia and a cream colour in PDA (Figure 2C). The isolate showed numerous nuclei per cell and the hyphae form a 90-degree angle (Figure 2E) and monilioid cells (Figure 2F). The bioagent produced IAA (2.3 µg mL^{-1}). It formed clear and amber (Figure 2D) halos, indicating qualitative production of cellulase and polyphenol oxidase.

Figure 2. Morphology of *W. circinata*. A. Flower (in the natural habitat), mycorrhizal colonization of *Epidendrum nocturnum*; B. parenchymatous cortex roots colonized by pelotons degraded with neighbouring cortical cells connected by hyphae; C. colony presenting a submerged margin, mucous mycelium and cream colour on PDA; D. amber halo from production of polyphenol oxidase; E. many nuclei per cell, as shown by an optical microscope with an epifluorescence accessory; F. monilioid cells revealed by scanning electron microscopy. Bars = 1 cm (a, c, d); 100 µm (b) and 10 µm (e, f).



W. *circinata* secreted GLU, CHI and PRO when cultured in TLE enriched with the cell walls of all pathogens and the co-culture. However, enzyme production kinetics varied when *W*. *circinata* was grown under different conditions (Figures 3 and 4).

M. *oryzae* cell wall: the greatest GLU and CHI activities were 4.8 and 5.5, respectively, at 48 h (Figure 3A and 3B). For PRO, the greatest production was 0.068 and 0.074 at 24 and 168 h, respectively (Figure 3C). *M*. *oryzae* co-culture: the highest GLU, CHI and PRO activities were 16.72 (Figure 4A), 2.97 (Figure 4E) and 0.094 (Figure 4I) at 72, 24 and 120 h, respectively.

S. *oryzae* cell wall: the greatest GLU activity was 12.59 at 168 h (Figure 3A). CHI values were 4.1 and 4.5 at 24 and 144 h, respectively (Figure 3B). The highest degree of PRO production was 0.085, 0.080 and 0.090 at 96, 120 and 168 h, respectively (Fig. 3C). *S*. *oryzae* co-culture: the greatest activities of GLU, CHI and PRO were 7.21 (Figure 4D), 3.1 (Figure 4H) and 0.075 (Figure 4I) at 48, 24 and 144 h, respectively.

C. miyabeanus cell wall: GLU activity peaked at 4.6 after 144 h (Figure 3A), CHI at 6.03 after 120 h (Figure 3B), and PRO at 0.067 and 0.066 at 96 and 120 h, respectively (Figure 3C). *C. miyabeanus* co-culture: the greatest GLU activity was 16.59 after 144 h (Figure 4B), with 4.48 CHI after 168 h (Figure 4F) and 0.082 PRO after 168 h (Figure 4J).

M. albescens cell wall: GLU activity was highest at 5.4 after 168 h (Figure 3A); CHI was highest at 4.1 after both 24 and 48 h (Figure 3B) and PRO at 0.072, 0.068 and 0.071 after 120, 144 and 168 h, respectively (Figure 3C). *M. albescens* co-culture: the greatest GLU, CHI, and PRO activities were 13.47 after 72 h (Figure 4C), 3.36 after 72 h (Figure 4G), and 0.094 after 120 h, respectively (Figure 4K).

Figure 3. Enzymatic activity of *W. circinata* grown in TLE with the cell wall (cw) of rice pathogens. **(**A) β-1,3-Glucanase, (B) Chitinase, and (C) Protease. Mo: *Magnaporthe oryzae*, So: *Sarocladium oryzae*, Cm: *Cochliobolus miyabeanus*, and Ma: *Monographella albescens*. Means with asterisks (*) were significantly different according to Tukey's test ($P < 0.05$). Bars indicate the mean standard error.

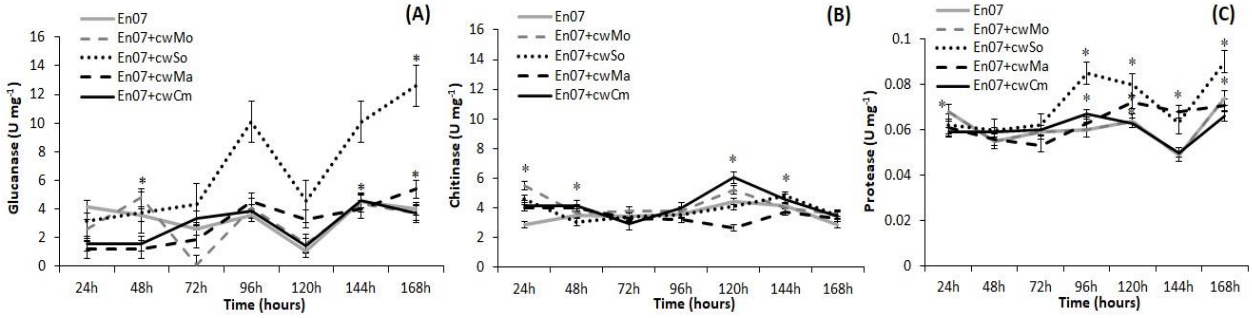
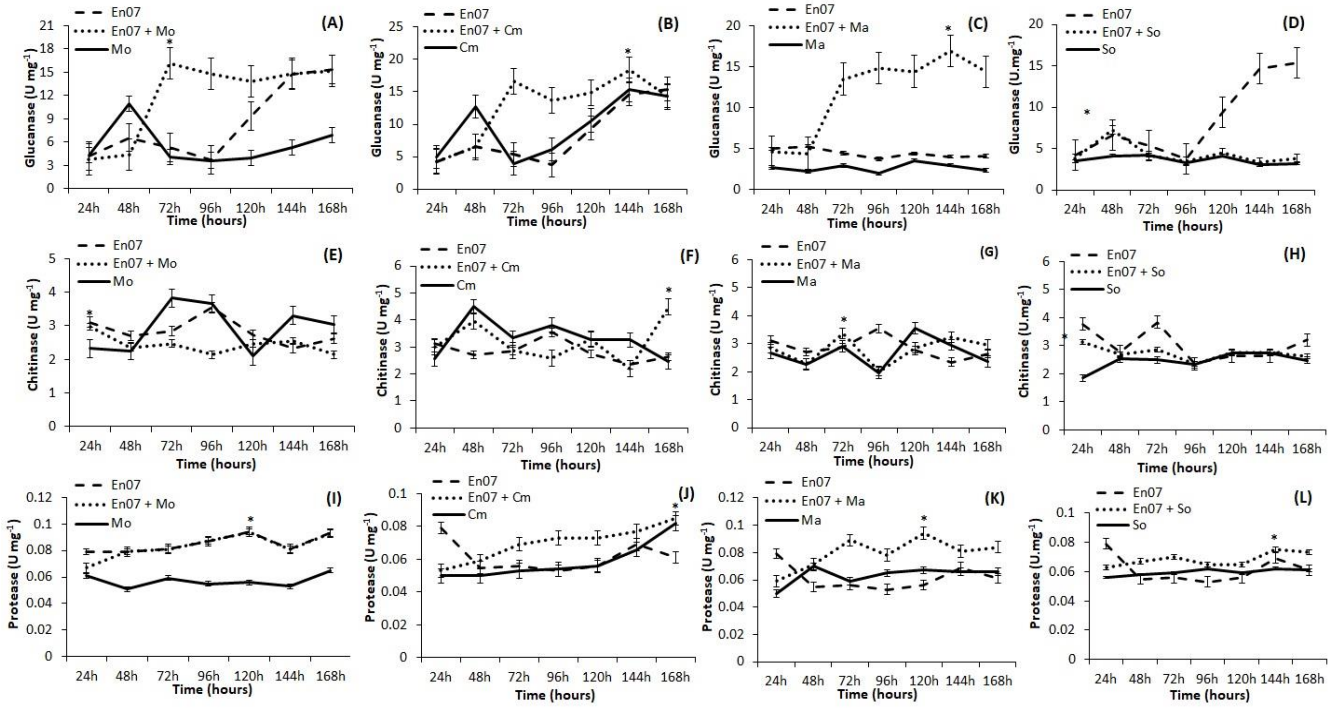


Figure 4. Enzymatic activity of *W. circinata* grown in TLE with coculture of rice pathogens. **(**A), (B), (C) β-1,3-Glucanase, (D), (E), (F). Chitinase, and (G), (H), (I). Protease. Mo: *Magnaporthe oryzae*, So: *Sarocladium oryzae*, Cm: *Cochliobolus miyabeanus*, and Ma: *Monographella albescens*. Means with asterisks (*) were significantly different according to Tukey's test (P < 0.05). Bars indicate the mean standard error.



W. circinata produced and secreted lytic enzymes when cultured in TLE enriched with pathogen cell walls and live pathogens (co-culture); however, the enzyme kinetics varied according to the type of culture and pathogen. Overall, enzymatic activity was always higher when the bioagent was cultured with pathogen cell walls or in co-culture than when it was cultured alone. These results indicate parasitism as the first likely mechanism used by *W. circinata* against the rice pathogens tested. Under both assay conditions, enrichment with cell walls and in co-culture, *W. circinata* displayed rapid responses, and the fact that the greatest enzyme activities were detected in the first 24 h revealed the ability of *W. circinata* to recognize the pathogens. This rapid recognition may be responsible for interrupting important phases of the pathogen cycle, such as adhesion, germination, and consequently, penetration.

Dual-plate assays: *W. circinata* in co-culture significantly decreased the colony areas of *C*. *miyabeanus*, *M*. *albescens* and *S*. *oryzae* by 76, 81 and 69%, respectively, with 0.012 cm inhibition halos for *M*. *albescens*. In terms of direct antagonism, the reduction in mycelial growth was statistically similar for all pathogens analyzed (Table 1).

Volatile metabolites: *W. circinata* volatile metabolites inhibited the colony growth of *C*. *miyabeanus*, *M*. *albescens* and *S*. *oryzae* by 58.5, 74 and 64.7%, respectively (Table 1).

Thermostability: treatments involving 5 and 10 g L^{-1} suspensions of autoclaved mycelia significantly inhibited colony growth of *M. oryzae*, *M*. *albescens* and *C*. *miyabeanus* compared with the control (Table 2).

These results explain the inhibition of *C. miyabeanus*, *M. albescens,* and *S. oryzae* colony growth by 76, 81 and 69%, respectively, in the dual-plate assay. In addition, the release of volatile metabolites also inhibited *C. miyabeanus*, *M. albescens,* and *S. oryzae* colony areas by 58.5, 74 and 64.7%, respectively. In another work, the mycelial mass extract of *W*. *circinata* also inhibited all the pathogens evaluated in present study. Chaibub *et al*. (2019a) also showed that *Cladosporium* sp. reduce rice pathogens mycelial growth by producing hydrolytic enzymes in dual plates, corroborating our results. Therefore, this shows that this other studied bioagent was also able to exert parasitism detected for same method used in this study.

Moreover, autoclaved mycelial suspensions (5 and 10 g L^{-1}) inhibited the colony growth of all pathogens that may facilitate the

use of *W. circinata* under tropical conditions and for industrial-scale production by using its metabolites.

Table 1. Mycelial growth (mm^2) and inhibition of mycelial growth (%) of *Magnaporthe oryzae* (Mo), *Cochliobolus miyabeanus* (Cm), *Monographella albescens* (Ma) and *Sarocladium oryzae* (So) when in dual plate and cultured in the presence of *W. circinata* volatile metabolites.

Pathogens	Pairing	Inhibition by pairing (%)	Volatiles	Inhibition by volatiles (%)
Mo			6323.6 ± 20.6	-
Mo x En07			599.5 ± 30.6*	90.5 ± 0.5 a
Cm	4764.2 ± 407.8	-	2468.6 ± 118.1	-
Cm x En07	1000.6 ± 130.3*	75.5 ± 3.8 a	952.2 ± 67.6*	58.5 ± 4.4 c
Ma	3361.5 ± 397.5	-	982.3 ± 43.3	-
Ma x En07	501.9 ± 53*	80. 9 ± 2.9 a	250.4 ± 6.5*	74.0 ± 0.9 b
So	1806.5 ± 138.3	-	667.8 ± 65.2	-
So x En07	520.8 ± 46.1*	68.9 ± 3.7 a	214.9 ± 12.9*	64.7 ± 3.5 bc

Means ± SEs (standard errors) followed by the asterisk (*) indicate significant differences between treatments according to the T-test ($P \leq 0.05$). Means ± SEs followed by the same letters in columns do not differ from each other according to Tukey's test ($P < 0.05$)

Table 2. Thermostability effects of *W. circinata* mycelial suspensions on the colony areas (mm^2) of rice pathogens. Suspensions: 2, 5 and 10 g of *W. circinata* mycelium per litre of PDA. Some of these suspensions were autoclaved, forming treatments A2g, A5g, and A10g; non-autoclaved suspensions constituted treatments S2g, S5g, and S10g. The control consisted of the pathogens cultured in PDA without the presence of mycorrhizal fungi.

Treatment	Pathogen			
	Magnaporthe oryzae	***Cochliobolus miyabeanus***	***Monographella albescens***	***Sarocladium oryzae***
Control	4841.4 ± 436.4 a	6358.5 ± 0.0 a	6358.5 ± 0.0 a	4065.9 ± 984.8 ab
A2g	5376.2 ± 59.5 a	6358.5 ± 0.0 a	6358.5 ± 0.0 a	5491.2 ± 542.1 a
A5g	3006.7 ± 466.6 bc	4607.1 ± 445.5 bc	2952.8 ± 686.8 cd	5111.5 ± 511.2 ab
A10g	1000.6 ± 184.2 d	2411.2 ± 396.1 d	1443.4 ± 205.5 d	4692.3 ± 688.0 ab
S2g	4838.4 ± 655.8 ab	5321.4 ± 472.2 ab	4904.0 ± 434.2 ab	6358.5 ± 0.0 a
S5g	2393.9 ± 544.5 cd	4820.5 ± 587.4 abc	3099.4 ± 506.7 cd	2375.4 ± 148.5 b
S10g	2403.0 ± 260.0 cd	3684.4 ± 61.6 cd	3531.7 ± 282.2 bc	4592.9 ± 878.5 ab

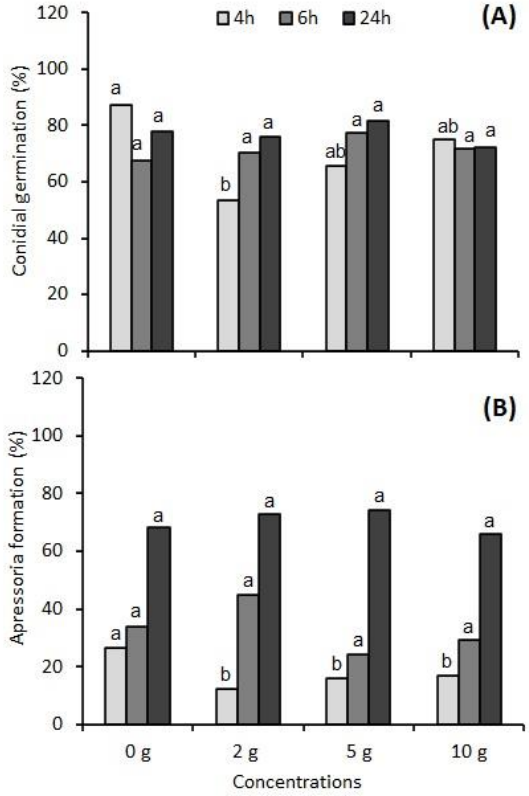
Means ± SEs (standard errors) followed by the same letters in columns do not differ from each other according to Tukey's test ($P < 0.05$)

Conidial germination and appressorium formation: conidial germination percentages with a 2 g L^{-1} *W. circinata* suspension and the control were 53.1 and 87.4%, respectively, 4 h after inoculation (Figure 5A). Appressorium formation percentages were 12.4, 16.2 and 17.3% for 2, 5 and 10 g L^{-1} suspensions 4, 6 and 24 h after evaluation, respectively, compared to the control with 26% (Figure 5B). Carvalho et al., (2021) also demonstrated that the mycelial mass extract inhibited the *M. oryzae* conidial germination and appressorium formation by 80 %. This demonstrates that both, the extract obtained by these authors and the fungus studied here exert a mechanism of inhibition of pathogens from the production of toxic compounds to the pathogens studied.

Another important observation is that a mycelial suspension (2 g L^{-1}) of *W. circinata* reduced *M. oryzae* conidial germination by 61% 4 h after germination induction. Furthermore, all mycelial suspensions (2, 5 and 10 g L^{-1}) of *W. circinata* reduced *M. oryzae* appressorium formation by 87.6, 83.8, and 82% 4, 6, and 24 h

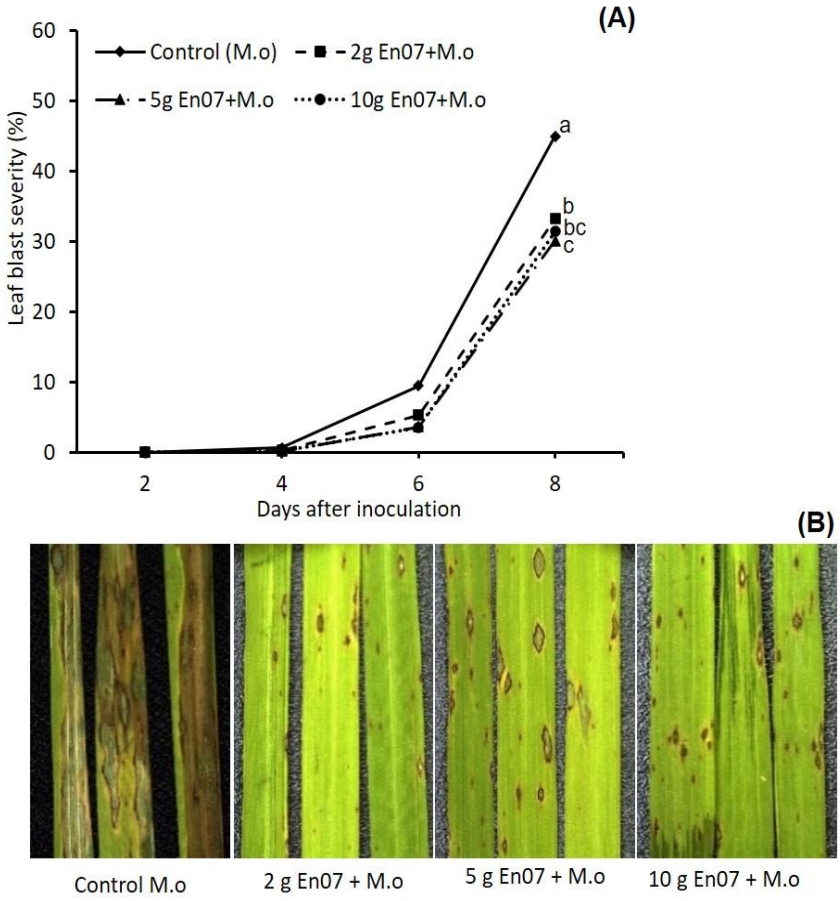
after induction, respectively. According to Howard e Valent (1996), *M*. *oryzae* conidia secrete mucilage after they are deposited onto surfaces. Adherence to an inductive surface and the initiation of germination followed by appressorium formation are necessary. It is likely that the *W. circinata* mycelial suspension prevented contact between the conidia and the inductive surface, thereby interfering with adhesion and recognition, as well as *M. oryzae* germination and appressorium formation processes on the inductive surface.

Figure 5. Effect of *W. circinata* mycelial suspensions (2, 5, and 10 g L^{-1}) on (A) *M. oryzae* conidial germination and (B) appressorium formation at 4, 6, and 24 h after conidial deposition on the surface. Means followed by the same letters were not significantly different from each other according to Tukey's test ($P < 0.05$).



Compared to the control, three mycelial suspensions (2, 5, and 10 g L^{-1}) significantly reduced LBS 2, 4, 6, and 8 days after inoculation with *M. oryzae*. The AUDPCs for treatments with the suspensions (2, 5, and 10 g L^{-1}) were 44.5, 37.6 and 39, respectively, which differed from that of the control treatment (65.2) (Figure 6A). The reduction in AUDPC was illustrated by the slow disease progression in these treatments with *W*. *circinata* suspensions 8 days after inoculation (Figure 6B).

Figure 6. A. Area under the disease progress curve (AUDPC) for rice plants treated with *W. circinata* mycelial suspensions (2, 5, and 10 g L^{-1}). B. Symptoms of blast on rice leaves treated or not treated with En07 mycelial suspensions. Means followed by the same letters were not significantly different according to Tukey's test ($P < 0.05$).



It is important to underscore that a *W. circinata* mycelial suspension is alive, with mycelia and other structures that probably prevent contact, acting as a physical and chemical barrier. We believe that the enzymes secreted by *W. circinata* diffuse into the culture medium, inhibiting pathogen growth, and that this would also occur under greenhouse conditions. Ours results showed that three suspensions of *W. circinata* (2, 5, and 10 g L^{-1}) suppressed LBS by 77.7, 75 and 84% 8 days after inoculation with *M. oryzae*. The effects of the *W. circinata* suspensions were shown by suppression of the infected leaf area and lesion type. All treatments sprayed together with mycorrhizal suspensions showed few lesions, with brown edges and small non-sporulating aspects. However, the lesions did not coalesce over time, retaining more green leaf area. Conversely, in the positive control, we observed typical lesions: a greyish center and brown edges that coalesced over time, causing leaf death. It is noteworthy that the extract of the mycelial mass of *W. Circinata* also suppressed leaf blast by 94%, and therefore this work demonstrates that the bioproduct can also be formulated from this live fungus.

The suppression efficiencies based on the AUDPC for 2, 5 and 10 g L^{-1} suspensions were 31.7, 42.3 and 40.2%, respectively, in relation to the control. *T. asperellum*, *Cladosporium* spp. and *C. cladosporioides* also reduced blast severity (SOUSA *et al*., 2018; CHAIBUB *et al*., 2016, 2019b, 2020). This corroborates that the fungus studied here is capable of controlling blast as well as other bioagents already explored.

Lytic enzyme secretion after 24 h is one of the mechanisms that may explain the efficiency of reducing severity 48 h after inoculation. Corroborating our results, the mycorrhiza fungi *Glomus margarita* inoculated on coffee roots simultaneously with *Meloidogyne paranaensis* was efficient in suppressing the pathogen compared to the control, through the production of hydrolytic enzymes (RAMOS *et al*., 2005).

Based on the results obtained, the *W. circinata* antagonism mechanisms against rice pathogens are lytic enzymes, antibiosis and volatile thermostable toxic compounds. These actions also guaranteed the efficiency of leaf blast suppression.

Thus, our results could contribute to developing new formulations containing this fungus for applications in rice blast management.

Acknowledgements

We thank the Federal University of Goiás, Embrapa Rice and Beans and the Goiás Research Support Foundation (FAPEG) for supporting this work and the Brazilian National Council for Scientific and Technological Development (CNPq) for financial support to the first author.

Conflict of interest

No content confict of interest.

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