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RESEARCH ARTICLE

Biocontrol potential of *Waitea circinata*, an orchid mycorrhizal fungus, against the rice blast fungus

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Abstract Rice blast (*Magnaporthe oryzae*) occurs in rice producing areas worldwide. Microorganisms are capable of producing secondary metabolites that can control the pathogen. The present study aimed to identify the class of the secondary metabolites produced by the mycorrhizal fungus *Waitea circinata* obtained from a Cerrado orchid and to assess the in vitro and in vivo antagonism of the extracts against *M. oryzae*. Three mycorrhizal extracts (crude, mycelial and lyophilized) were obtained. In the in vitro antagonism assay, *W. circinata* produced the largest inhibition zone. The crude extract reduced the mycelial growth of the pathogen by 75 %, inhibited the formation of appressoria by 100 % and reduced the AUDPC by 25 % compared to the control. Analysis of the crude extract by electro spray ionization Fourier transform ion cyclotron resonance mass spectrometry revealed the presence of benzophenones. The results suggest that *W. circinata* extract has the potential for rice blast control.

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Introduction

Rice (*Oryza sativa* L.) is the most important grain crop in the world and is the staple food for over half of the world's population (FAO 2014). It is an excellent source of energy due to its high concentration of starch; it provides proteins, vitamins and minerals and contains a low amount of lipids (Naves 2007). Rice is a standout crop due to its productivity and large growing area and plays a strategic role in the economy and society, especially in developing countries (FAO 2014).

Rice blast, caused by the fungus *Magnaporthe oryzae* B. C. Couch (asexual morph *Pyricularia oryzae* Cavara), is one of the most widespread diseases (Prabhu et al. 2009; Fernandez and Wilson 2012; Fisher et al. 2012; Chen et al. 2013) and is widely regarded as the most damaging disease of rice. Under favorable conditions, the pathogen can cause severe epidemics that may lead to 100 % crop loss (Prabhu et al. 2009). For effective rice blast control, integrated disease management is necessary, which includes genetic resistance, cultural practices and the use of fungicides (Filippi et al. 2006).

Fungi are an essential part of all ecosystems and play major roles in their sustainability and biodiversity (Gunatilaka 2006). They are also potential sources of bioactive molecules with novel structures and innovative mechanisms of action. Biocontrol fungi (BCF) not only suppress plant diseases but are also able to induce resistance to abiotic stresses by improving the photosynthetic capacity (Shoresh et al. 2010). According to Pinto et al. (2002), fungal secondary metabolites may be a source of natural pesticides, such as in the case of the strobilurin fungicides.

Mycorrhizal fungi can increase crop yield and reduce the use of fertilizers, as well as facilitate the recovery of degraded soils, contributing to more sustainable agricultural practices. In addition, these fungi produce secondary metabolites during their interaction with plants that can control plant diseases (Li et al. 2000; Pinto et al. 2002; Siqueira et al. 2002).

The genus *Rhizoctonia* sp. includes plant pathogenic, saprophytic and mycorrhizal fungi (Roberts 1999; Agarwal 2010) and some orchid mycorrhizal species are potentially pathogenic, such as *Thanatephorus cucumeris* and *Waitea circinata*, teleomorph names of *Rhizoctonia solani* and *Rhizoctonia zeae*, respectively (Roberts 1999). Mycorrhizal *Rhizoctonia* spp., when interacting with orchids, increase the uptake of water and nutrients (Rasmussen and Rasmussen 2009) and can also act as antagonists to plant pathogens (Mosquera-Espinosa et al. 2013). Microorganisms which are capable of promoting plant growth and suppressing plant pathogens have been highlighted as an alternative for sustainable food production (Silva et al. 2012). For example, the number of seedlings of *Cyrtopodium saintlegerianum* increases when mycorrhizal *Rhizoctonia* sp. is mixed to the substrate (L.G. Araújo et al., unpublished results).

It is known that *Rhizoctonia* fungi produce bioactive secondary metabolites, especially alkaloids (Harris et al. 1987), phenolic compounds (Aliferes and Jabaji 2010) and steroids (Ma et al. 2004). It is also know that these metabolites decrease the conidial germination of some plant pathogens such as *Fusarium sporotrichioides*, *Heterosporium solani*, *Stachybotrys elegans* and the biological control agent *Trichoderma virens* (Aliferes and Jabaji 2010). Therefore, a study on the potential antagonistic interaction of selected orchid mycorrhizae and the rice blast fungus was conducted with the following objectives: (*i*) to screen a collection of orchid mycorrhizae isolates searching for potential antagonists against *M. oryzae*; (*ii*) to identify the class of secondary metabolites of the selected mycorrhizal fungi; (*iii*) to evaluate the supressive effects of the extracts from a selected fungal strain in vitro and in vivo against *M. oryzae*.

Materials and methods

In vitro pairing of fungal isolates

Eleven fungal isolates were used: ten mycorrhizal fungi of *Cyrtopodium saintlegerianum* Rchb. f. (Cs02, Cs10 and Cs21 - *Epulorhiza* sp.; Cs16 and Cs18 - *Xylaria* sp.) and *Epidendrum nocturnum* Jacq. (En02, En03 and En05 - *Epulorhiza* sp.; En07 - *Waitea circinata*; En24 - *Rhizoctonia* sp.), which were isolated by Sousa (2012); and one isolate of *Epicoccum* sp. (Ep06) obtained from rice (Sena et al. 2013). The mycorrhizal isolate En07 was identified as *W. circinata* based on ribosomal DNA analysis (data not shown). The other isolates were identified based on vegetative characteristics, and we adopted their anamorphic classification. The *M. oryzae* Py 10900 isolate used belongs to the Collection of Functional Microorganisms of Embrapa Arroz e Feijão (the *W. circinata* En07 isolate was deposited in the same collection under the code BRM 32644). All 12 isolates were grown in Petri dishes containing PDA. The fungi were incubated at 27 °C for 11 days with a photoperiod of 12 h in a growth chamber until the mycelia occupied the whole culture media surface.

The paired culture method described by Romeiro (2007) was used for evaluation of antagonistic effects. The assay was conducted in a completely randomized design with eight replications per isolate and 11 treatments. Five millimeter mycelial discs from the 11 mycorrhizal fungal isolates were transferred to the center of Petri dishes containing PDA and placed on opposite sides, approximately 3 cm apart. *Magnaporthe oryzae* was grown in the absence of antagonists as a negative control and in the presence of *Epicoccum* sp. as a positive control (Sena et al. 2013). The measurements were performed inside a laminar airflow chamber under constant white light. With the aid of a millimeter ruler, the lengths of the horizontal and vertical diameters of each treatment were evaluated daily until the negative control reached the plate edge, totaling five measurements. The inhibition zone was also measured and compared with the positive control. The reduction of the colony diameter of *M. oryzae* (Filippi et al 2011) was given by: [100 - [diam of *M. oryzae* in the presence of the probable antagonist × 100 / diam of *M. oryzae* alone (negative control)]. The average reduction of horizontal and vertical diameters were also calculated. The isolate presenting the greatest degree of in vitro antagonism against *M. oryzae* was selected for the identification of secondary metabolites. The data were subjected to analysis of variance and comparison of means by Tukey's test at 5 % probability using the software R version 2.11.0.

Pathogenicity test

The isolate preliminarily selected as the most promising antagonist, *W. circinata* En07, belongs to a genus known to include important plant pathogens, including strains of *Rhizoctonia solani* Kühn, causal agent of rice sheath blight (Prabhu et al. 2002), a highly important disease of rice. Therefore, it was necessary to demonstrate the non-virulence of *W. circinata* En07 against rice before it was further evaluated.

Seeds were surface-disinfected (soaking for five min in 70 % ethanol followed by five min in 3 % sodium hypochlorite) and dried before treatment with different concentrations of En07 mycelial suspension. En07 was grown in PDA for 11 days in a growth chamber at 27 °C under light. The mycelium was removed with a scalpel, lyophilized for 4 h and ground in liquid nitrogen with a mortar and pestle. After the disinfection process, 50 g of rice seeds (cv. BRS Primavera,

susceptible to *M. oryzae* isolate Py 10900) were placed in four different Erlenmeyer flasks containing 1 L of distilled water and 0, 0.1, 0.2 or 0.4 g of En07 ground mycelium. The flasks were kept in a shaker without heating during 24 h. The seeds were then sown in plastic trays (12x8) containing fertilized soil with 5 g of NPK (5–30–15), 3 g of ammonium sulfate and 1 g of zinc sulphate. The completely randomized design included four treatments and four replications. The trays were kept in a greenhouse with temperatures of 27–30 °C and the plants were examined daily for the presence of sheath blight symptoms.

Additionally, En07 was tested on 55-day old rice plants using three different inoculation methods: T1, colonized tooth pick; T2, culture discs; T3, foliar spray with mycelial suspension. In T1, 2-cm tooth picks were autoclaved and colonized with En07 mycelium for 10 days in Petri dishes with PDA. The tooth picks were placed in the base of the penultimate leaf of each plant. In T2, one 0.9-mm diameter disc obtained from the margin of an actively growing culture was placed on the soil surface next to the base of each plant. In T3, plants were sprayed with 50 mL of an aquous mycelial suspension (0.4 mg/L) until runoff. The plants were kept in a greenhouse at 95–100 % relative humidity, and temperatures ranging from 27–30 °C and 22–25 °C, day and night, respectively (Prabhu et al. 2002). The completely randomized design involved three replications, each consisting of one pot with four plants, and the control consisted of the application of distilled water.

Preparation of extracts

W. circinata En07 was grown on PDA for 11 days in a germination chamber at 27 °C in the presence of light. Twenty 25-mm culture discs obtained from the margin of actively growing colonies were transferred to flasks containing 1 L of liquid potato-dextrose (PD) medium. The flasks were kept under agitation for 15 days at 28 °C and 120 rpm. The content of the flasks was filtered through Whatman n° 4 filter paper under vacuum, to separate the mycelium from the culture medium.

The mycelial mass was lyophilized, ground with a mortar and pestle and then subjected to cold extraction with ethyl acetate. After 48 h, the solvent was removed by evaporation at reduced pressure on a rotary evaporator to obtain the mycelial extract (ME). The culture filtrate (liquid) was subjected to liquid-liquid partitioning with ethyl acetate (300 mL, 3×). The organic phases were combined and dried with anhydrous sodium sulfate and filtered. The solvent was removed on a rotary evaporator to obtain the crude extract (CE). The aqueous fraction was dried by lyophilization, followed by cold extraction in ethyl acetate in a rotary evaporator to remove the solvent and obtain the lyophilized extract (LE).

ESI FT-ICR MS analysis

Briefly, the samples were diluted to≈0.25 mg/mL in water:methanol (1:1) which contained 0.1 % NH_4OH for ESI in negative mode. The resulting solution was directly infused at a flow rate of 3 μL/min into the ESI source. The mass spectrometer (model 9.4 T Solarix, Bruker Daltonics) was set to operate over a mass range of *m/z* 150–2000. The ESI source conditions were as follows: nebulizer gas pressure of 3 bar, capillary voltage of 3.5 kV, transfer capillary temperature of 250 °C. The ions are accumulated in the hexapolar collision cell with time of 5.10^{-3} s followed by transport to the analyzer cell (ICR) through the multipole ion guide system (another hexapole). The time-of-flight in the hexapole was of 0.5 ms. Each spectrum was acquired by accumulating 200 scans of time-domain transient signals in four mega-point time-domain data sets. All mass spectra were externally calibrated using arginine (for ESI(+)) and NaTFA (for ESI(-)) solutions (*m/z* from 200 to 2000). A resolving power, $m/\Delta m_{50\%}$≈500 000, in which $\Delta m_{50\%}$ is the full peak width at half-maximum peak height, *m/z*≈400 and mass accuracy< 1 ppm provided the unambiguous molecular formula assignments for singly charged molecular ions. Mass spectra were acquired and processed using Data Analysis software (Bruker Daltonics). The MS data were processed and the elemental compositions of the compounds were determined by measuring the *m/z* values. The proposed structures for each formula were assigned using the chemspider (www.chemspider.com) database.

In vitro inhibition of mycelial growth of *Magnaporthe oryzae* by *W. circinata* En07 extracts

A completely randomized design was used comprising 16 treatments (including the control) and three replications. Five dilutions of each extract (CE, ME, LE) were prepared. First, the samples were weighed and diluted in 40 drops of ethanol. Next, Milli-Q water was added to obtain the final concentration. The treatments were: 1.040, 700, 520, 120 and 12 μg/mL (CE); 900, 800, 450, 130 and 13 μg/mL (LE); and 1.860, 930, 860, 280 and 28 μg/mL (ME). As a control, 40 drops of ethanol were added to 15 mL of PDA. Then, the samples were filtered through a syringe fitted with a Macherey-Nagel polytetrafluoroethylene (PTFE) membrane with a 20 μm pore diameter.

The target pathogen (*M. oryzae* Py 10900) was grown in PDA for 11 days at 25 °C. One milliliter of extract at each concentration to be tested and 15 mL of molten PDA were added to each of 48 Petri dishes, and the dishes were stirred so that the extract mixed with the culture medium. After solidification, one 5-mm disk of *M. oryzae* Py 10900 was transferred to the center of each Petri dish. These were placed in a growth chamber at 25 °C for 10 days. The control consisted

of PDA plates with a 5-mm disc of *M. oryzae* Py 10900 but without En07 extract. The horizontal and radial diameters of the colonies were measured with a digital caliper at the end of the experiment (day 10) (Côrtes et al. 2014) and the average reduction of radial and horizontal diameters were calculated. The experiment was repeated three times. The data (average results of the three experiments) were submitted to analysis of variance and means comparison of by Tukey's test at 5 % probability using the software R version 2.11.0.

Inhibition of conidial germination and appressorial formation in *Magnaporthe oryzae* by *W. circinata* En07 extracts

Magnaporthe oryzae Py 10900 was grown in PDA for 7 days and was subsequently transferred to Petri dishes containing oat-dextrose-agar (ODA; Filippi et al. 2006). The dishes were kept under continuous white light at 25 °C for 7 days for colony growth. Next, the colonies were induced to conidiation by surface scraping with a sterile glass rod and maintained for 2 days in a growth chamber under continuous white light at 25–27 °C. Conidia were harvested by flooding the plates with distilled water and scraping the surface of the colonies with a brush. The conidial suspension was then filtered through sterile fabric and the concentration was adjusted to $1x10^5$ conidia/mL (Filippi and Prabhu 2001).

The bioassay for conidial germination and apressorial inhibition was conducted in the hydrophobic side of parafilm, which was previously sterilized with a solution of 3 % sodium hypochlorite and 70 % ethanol and was organized into microscopic slides inside Petri dishes previously lined with paper towel moistened with sterile water to provide a high humidity environment (Sena et al. 2013). A 10-μL aliquot of each extract was separately placed on each hydrophobic side of parafilm and mixed with 10 μL of conidial suspension for a final concentration of $1x10^5$ conidia in a 20 μL drop. Evaluations for conidia germination and appressoria formation were performed after 3, 6 and 24 h by observing the slides under an light microscope (BelAnalyzer MicroImage software). The control consisted of a 20 μL drop of the conidial suspension placed on the same kind of support under the same conditions as described above, but without mixing with the En07 extract.

The assay for conidial germination inhibition was conducted twice and was comprised of three treatments (0.52 μg/mL CE; 0.45 μg/mL LE; 0.93 μg/mL ME) and the control. The assay for appressoria inhibition was comprised of three treatments represented by the best concentration for conidial germination inhibition of each tested extract, and the control. Assays were performed in triplicates in a completely randomized design. The data (average results of the two experiments) were submitted to analysis of variance and comparison of means by Tukey's test at 5 % probability using the software R version 2.11.0.

Suppression of rice leaf blast by *W. circinata* En07 extracts

Rice plants were grown as described above for the pathogenicity test and used in tests when 21 days old. Two assays were conducted under greenhouse conditions. Assays involved suspension of En07 extracts as follows: 1.040 μg/mL and 700 μg/mL CE; 1.860 μg/mL ME; 900 μg/mL LE. A volume of 30 mL of each solution was sprayed on each test plant. In assay 1 this was immediately followed by spraying a $3x10^5$ conidia/mL aqueous suspension of *M. oryzae* conidia. In assay 2, plants were sprayed simultaneously with the solutions of En07 extracts and the *M. oryzae* conidial suspension. The control consisted of spray inoculation of *M. oryzae* suspension ($3x10^5$ conidia/mL). The experimental design was completely randomized with five treatments and three replications. After inoculation, the plants were incubated in a moist chamber for 24 h at 24–26 °C and transferred to a greenhouse where temperatures ranged from 27 to 30 °C.

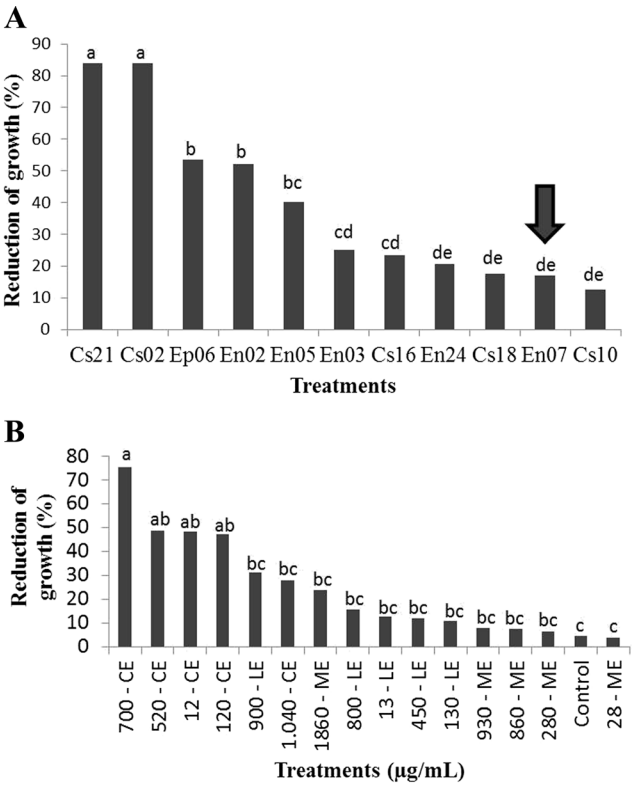
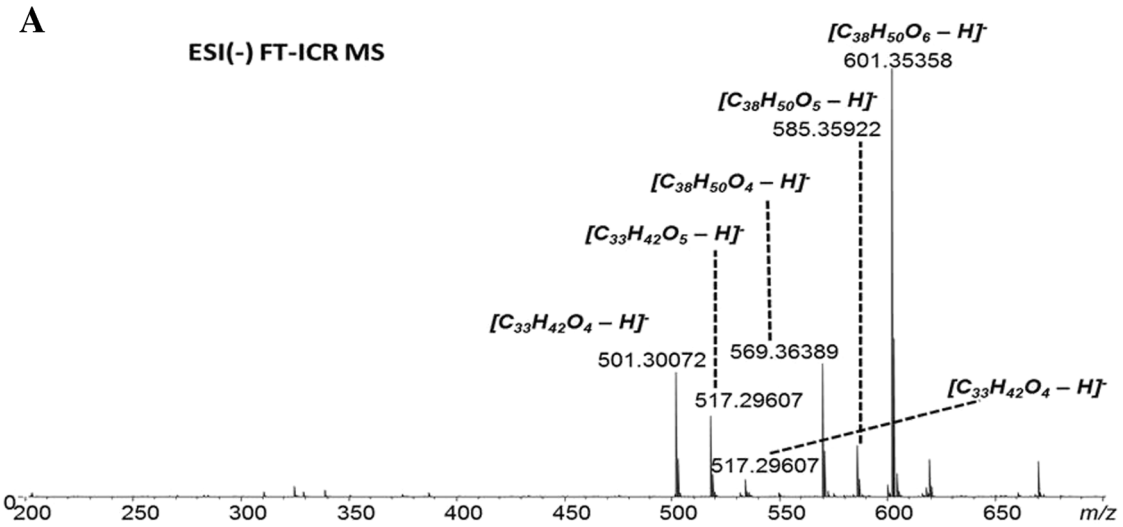


Fig. 1 Inhibition of *M. oryzae* Py 10900 mycelial growth by extracts of mycorrhizal fungi 10 days after growth on PDA. **a.** Mean reduction of the *M. oryzae* vertical and horizontal diam relative to the control. **En02, En05, En03**, *Epulorhiza* sp. from *Epidendrum nocturnum*; **En07, En24**, *Waitea circinata* and *Rhizoctonia* sp., respectively, from *E. nocturnum*; **Cs02, Cs10, Cs21**, *Epulorhiza* sp. from *Cyrtopodium saintlegerianum*; **Cs16, Cs18**, *Xylaria* sp. from *C. saintlegerianum*; **Ep06**, *Epicoccum* sp. **b.** Mean reduction growth of *M. oryzae* by extracts of *W. circinata* En07. **CE**, Crude extract; **LE**, Lyophilized extract; **ME**, Mycelium extract. Means followed by the same letter do not differ according to Tukey's test ($p<0.05$)



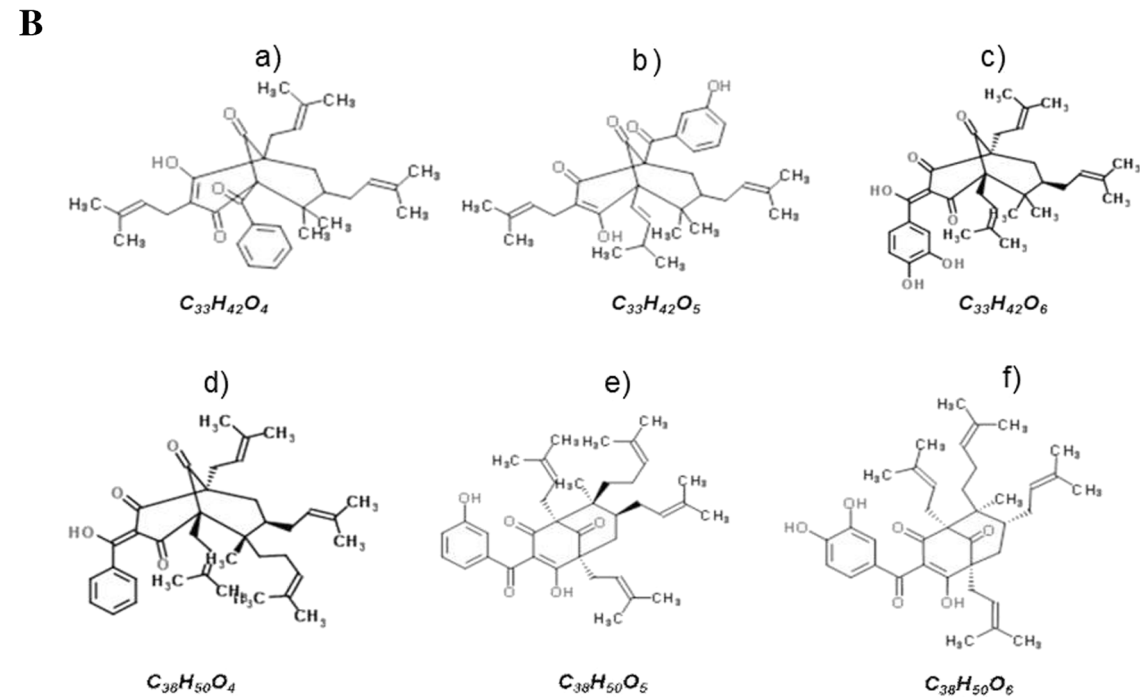


Fig. 2 **a.** ESI(-) FT-ICR mass spectrum of *Waitea circinata* crude extract. **b.** Proposed structures for the molecular formulas assigned from crude extracts. *a*) 1-Benzoyl-4-hydroxy-8,8-dimethyl-3,5,7-tris(3-methyl-2-buten-1-yl)bicyclo[3.3.1]non-3-ene-2,9-dione; *b*) 8-Benzoyl-4-(2-hydroxy-2-propanyl)-9,9-dimethyl-1,10-bis(3-methyl-2-buten-1-yl)-3-oxatricyclo[6.3.1.$0^{2,6}$]dodec-2(6)-ene-7,12-dione; *c*) 3-[(3,4-Dihydroxyphenyl)(hydroxy)methylene]-6,6-dimethyl-1,5,7-tris(3-methyl-2-buten-1-yl)bicyclo[3.3.1]nonane-2,4,9-trione; *d*) 3-[Hydroxy(phenyl)methylene]-6-methyl-1,5,7-tris(3-methyl-2-buten-1-yl)-6-(4-methyl-3-penten-1-yl)bicyclo[3.3.1]nonane-2,4,9-trione; *e*) 4-Hydroxy-3-(3-hydroxybenzoyl)-8-methyl-1,5,7-tris(3-methyl-2-buten-1-yl)-8-(4-methyl-3-penten-1-yl)bicyclo[3.3.1]non-3-ene-2,9-dione; *f*) 3-(3,4-Dihydroxybenzoyl)-4-hydroxy-5-[(2R)-2-isopropenyl-5-methyl-4-hexen-1-yl]-8,8-dimethyl-1,7-bis(3-methyl-2-buten-1-yl)bicyclo[3.3.1]non-3-ene-2,9-dione

Rice leaf blast ratings (RLB %) were performed using a 0–10 scale according to Notteghem (1981), 8 days after *M. oryzae* inoculation for assay 1. Both assays (1 and 2) were evaluated with the same scale. However, for assay 2, rice leaf blast severity was evaluated at 2-day intervals to determine the area under the disease progress curve (AUDPC). Twenty four plants per tray were random marked for AUDPC evaluation. The reduction in rice leaf blast severity as compared to control was calculated for both assays and for

the AUDPC using the following formula: 100 - (Severity of the treatment × 100 / Severity of the control). The suppression data was transformed using $\sqrt{x+0.5}$ and submitted to analysis of variance and comparison of means by Tukey's test at 5 % probability using the software R version 2.11.0.

Results

In vitro pairing of fungal isolates and pathogenicity of selected isolate

The greatest level of suppression of *M. oryzae* (reduction of colony diameter) was 84 % (Cs02 and Cs21, *Epulorhiza* sp.; Fig. 1a). A correlation between vertical and horizontal diameters (r=0.895, $p \leq 0.01$) was detected, indicating data consistency and uniformity.

Regarding the formation of inhibition zone, two isolates, En07 (*W. circinata*) and En03 (*Epulorhiza* sp.), produced significant results. *W. circinata* isolate En07 presented the largest inhibition zone and, consequently, was selected for further evaluations.

No sheath blight symptoms were observed on rice, either on plants originating from seeds exposed to En07 or on plants inoculated directly with this isolate by three different methods. When culture discs of En07 were used as inoculum, mycelium grew from the discs and was observed surrounding plant culms but no lesions resulted. Sheath blight symptoms were also not observed in the control plants.

ESI FT-ICR MS analysis

ESI(±) Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR MS) (Cooper and Marshall 2001) was used to characterize the composition of En07 extracts without prior extraction or separation. FT-ICR MS routinely provides ultra-high mass resolving power, $m/\Delta m_{50\%}$>400.000, and mass accuracy better than 1 ppm. These high specifications mean that FT-ICR is ideal for analyzing complex mixtures (Colati 2013). Moreover, it becomes possible to assign molecular formulas ($C_cH_hN_nO_oS_s$) unambiguously by mass measurement from singly charged ions such as $[M+H]^+$, $[M+Na]^+$or $[M - H]^-$, where M corresponds to a neutral molecule.

The ESI(-)FT-ICR mass spectrum of *Rhizoctonia* isolate En07 extracts (Fig. 2a) shows that ions of *m/z* 501.30072, 517.29607, 569.36389, 585.35922 and 601.35922 are the most abundant, detected as deprotonated ions: $[C_{33}H_{42}O_4 - H]^-$, $[C_{33}H_{42}O_5 - H]^-$, $[C_{38}H_{50}O_4 - H]^-$, $[C_{38}H_{50}O_5 - H]^-$ and $[C_{38}H_{50}O_6 - H]^-$. The proposed structures for these compounds as benzophenones are presented in Fig. 2b.

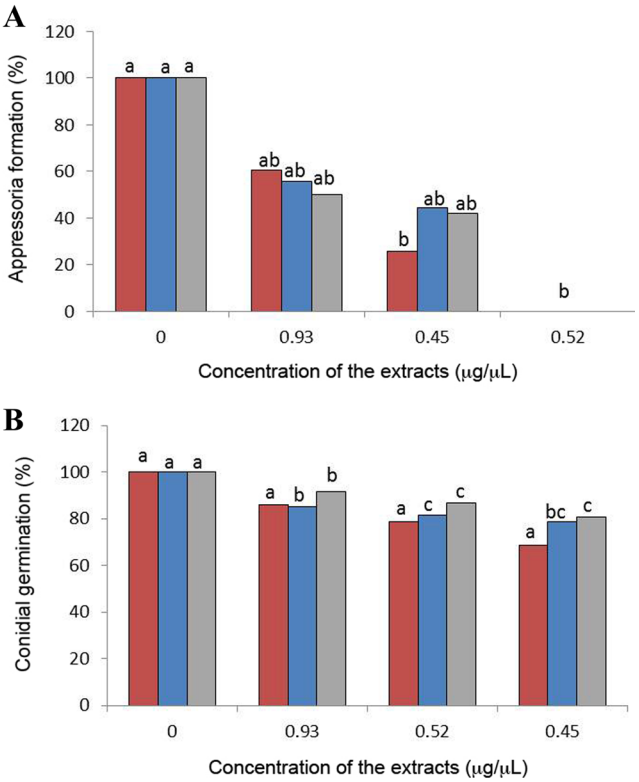


Fig. 3 Effect of *Waitea circinata* En07 extracts on *Magnaporthe oryzae* appressoria formation (**a**) and conidial germination (**b**). Red, 3 h after conidial deposition; Blue, 6 h after conidial deposition; Gray, 24 h after conidial deposition. Control (no extract), 0; Mycelial extract, 0.93 µg/µL; Lyophilized extract, 0.45 µg/µL; Crude extract, 0.52 µg/µL. Means followed by the same letter do not differ according to Tukey's test (p<0.05)

In vitro inhibition of *Magnaporthe oryzae* by *W. circinata* En07 extracts

Inhibition of *M. oryzae* mycelial growth by the extract produced by En07 was observed, confirming the result of the paring test. Some treatments reduced the mycelial growth of *M. oryzae* compared to the control (*M. oryzae*). The treatment consisting of 700 µg/mL CE had the greatest inhibitory effect (75 %; Fig. 1b).

Conidial germination began 3 h after deposition on parafilm. After six and 24 h of conidial deposition, there were significantly lower percentages of germinated conidia for the three extract solutions as compared to the control (Fig. 3b). As for the appressorial formation, in both assays, the crude extract (0.52 µg/µL) led to complete inhibition, whereas abundant appressoria were formed for other treatments and the control (Fig. 3a).

Table 1 Rice blast progress at 4, 6 and 8 days after inoculation (dai) with *M. oryzae* and three different extracts of *W. circinata* isolate En07

Treatments	4 dai	6 dai	8 dai	AUDPC*
3×10^5 conidia/mL M.o.	0.49a	3.42a	14.56a	7.46b
900 μg/mL LE+3×10^5 conidia/mL M.o.	0.46a	2.56a	6.79b	7.47b
700 μg/mL CE+3×10^5 conidia/mL M.o.	0.44ab	3.74a	6.37b	9.20a
1040 μg/mL CE+3×10^5 conidia/mL M.o.	0.43ab	1.41a	3.25b	5.60c
1860 μg/mL ME+3×10^5 conidia/mL M.o.	0.30b	2.70a	5.15b	7.96ab

Means followed by the same letter in the column do not differ according to Tukey's test ($P<0.05$)

*Area under disease progress curve

Suppression of rice leaf blast by *W. circinata* En07 extracts

In the first assay it was observed that all treatments had significantly lower blast severity compared to the control. However, there were no significant differences in severity among different extract concentrations. In assay 2, at 4 and 6 days after inoculation, there was no statistical difference among treatments for blast severity. However, 8 days after inoculation all treatments presented significantly smaller blast severity compared to the control (Table 1 and Fig. 4). The treatment consisting of 1.040 μg/mL CE+3×10^5 conidia/mL M.o. resulted in the smallest AUDPC (5.6), whereas the value for the control reached 7.46. This represented a 25 % increase in disease suppression as compared to the control (Table 1).

Discussion

Based on the results from the assay conducted with different inoculation methods, the mycorrhizal isolate *W. circinata* En07 was not pathogenic to rice, and thus it is assumed to be safe to use it as an antagonist to the rice blast fungus. Similar results were observed for a *Ceratobasidium* sp. (another teleomorphic phase of mycorrhizal *Rhizoctonia*) isolated from orchids (Mosquera-Espinosa et al. 2013). Furthermore, we observed, in vitro, the antagonism between *W. circinata* En07 and *M. oryzae*. Three different mycorrhizal extracts (crude, mycelial and lyophilized) were tested and the most effective was the crude extract, which reduced *M. oryzae* mycelial growth by 75 %, inhibited the formation of appressoria by 100 % and reduced the AUDPC by 25 % compared to the control. The analysis of the crude extract by electro spray ionization Fourier transform ion cyclotron resonance mass spectrometry (ESI (-) FT-ICR MS) revealed the presence of benzophenones. This is the first time that a mycorrhizal

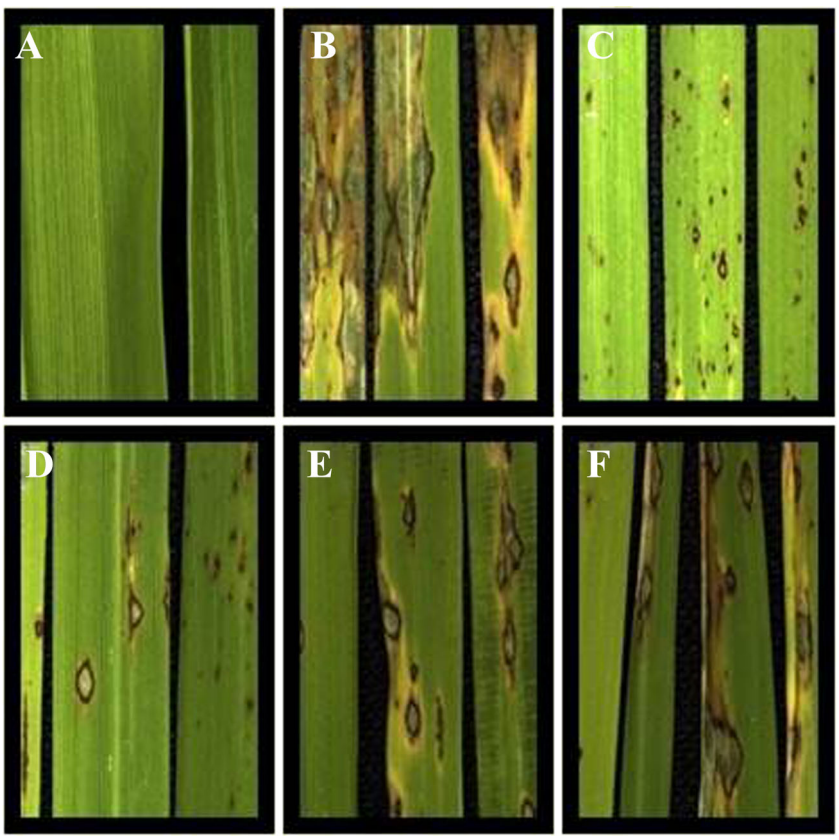


Fig. 4 Rice blast suppression by *Waitea circinata* En07 extracts in vivo. **a**, Water control; **b**, 3×10^5 conidia/mL M.o.; **c**, 1.040 μg/mL CE+3×10^5 conidia/mL M.o.; **d**, 900 μg/mL LE+3×10^5 conidia/mL M.o.; **e**, 1.860 μg/mL ME+3×10^5 conidia/mL M.o.; **f**, 700 μg/mL CE+3×10^5 conidia/mL M.o

fungus and its crude extract are identified as rice blast suppressors.

Based on the results from our in vitro assays, we identified an antagonistic relationship between orchid mycorrhizae (*Epulorhiza* and *Waitea* - isolates Cs02, Cs21 and En07) and the rice blast fungus *M. oryzae*. En07 promoted a pronounced inhibition zone when paired with *M. oryzae*. The formed inhibition zone led us to propose that En07 may be secreting metabolites in the medium which may have adverse effects on *M. oryzae*, similarly to the results reported by Sena et al. (2013) for an isolate of *Epicoccum* sp., which reduced the mycelial growth of *M. oryzae* by 42.50 % in vitro. The same *Epicoccum* sp. isolate was used as a positive control in this study, reducing *M. oryzae* mycelial growth by 53 % but forming a smaller inhibition zone than En07.

The crude extract showed to be the most efficient for in vitro tests in terms of mycelial growth, conidial germination and appressoria formation. A concentration of 700 μg/mL of crude extract inhibited the pathogen's mycelial growth by 75 %, and 0.52 μg/mL of the same extract inhibited 100 % of appressoria formation at 3, 6 and 24 h. The other extract concentrations delayed conidial germination and appressoria formation. Other endophytes producing these secondary metabolites have been reported, such as *Daldinia concentrica* (Hashimoto et al. 1994) and *Emericella nidulans* (Eidam) Vuill. (Kawahara et al. 1994). Another example of a fungal metabolite with in vitro suppressive activity against *M. oryzae* is cerulenin, an extracellular metabolite produced by *Sarocladium oryzae* (Sawada) W. Gams & D. Hawksw at similar levels to those found in this study (Côrtes et al. 2014). Sena et al. (2013) have reported that *Epicoccum* sp. crude extract at concentrations of 6 and 8 mg/mL applied at 12 and 20 h after deposition of conidia reduced appressoria formation in *M. oryzae* by 98 and 96 %, respectively. Therefore, the concentrations used in this assay were selected for the in vivo leaf blast suppression test.

The *W. circinata* En07 crude extract was also efficient in the in vivo assay, where 1.040 μg/mL CE+3x10^5 conidia/mL M.o. suppressed rice leaf blast severity by 25 % 8 days after *M. oryzae* inoculation, and also presented the lowest AUDPC (5.6, while the control treatment presented 7.46). In similar studies, crude extract of *Epicoccum* sp. and cerulenin from *Sarocladium oryzae* reduced leaf blast severity by 27 and 63 %, respectively (Sena et al. 2013; Côrtes et al. 2014). The effect of EN07 extracts can be observed not only in suppressing the infected leaf area, but also in lesion type. In the treatment consisting of 1.040 μg/mL CE+3x10^5 conidia/mL M.o., the formed lesions were non-sporulating, contrary to the few small sporulating lesions dispersed on the leaf surface of the other treatments, and the many large ones in the control.

The efficiency of crude extracts showed in this study may be related to their composition. The presence of phenolic compounds (most likely benzophenones) was detected in the crude extract of En07. Thus far, we can only assume that the activity is due, at least in part, to the benzophenones, since the MS analysis suggests that they are the main compounds in the crude extract. Benzophenones comprise a large class of phenolic compounds that show a wide range of biological properties, including antifungal, anti-HIV, antimicrobial, antioxidant, antiviral and cytotoxic activities (Wu et al. 2014). The inhibitory activity could be due to a synergistic effect, and further studies and bioguided fractionation are needed to corroborate this hypothesis. Therefore, these extracts should be studied and purified until the molecules that show activity are identified.

Resistance induction may also play a role in the interaction between *W. circinata* mycorrhiza and the rice plant. However, further studies are necessary to elucidate if *W. circinata* signals the plant defense system, and which mechanisms and compounds are involved in the antagonism between *M. oryzae* and *W. circinata* En07.

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