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Profile of volatile compounds released by *Waitea circinata* against *Magnaporthe oryzae* under different periods and temperatures[1]

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ABSTRACT

Rice blast caused by the *Magnaporthe oryzae* pathogen is a major disease in this crop, and may cause devastating losses. This study aimed to investigate the profile of *Waitea circinata* mycorrhiza volatile compounds in antagonism to *M. oryzae*, under different growth periods and temperatures, using a completely randomized design. Volatile organic compounds were extracted by headspace solid phase microextraction and analyzed by gas chromatography mass spectrometry, while multidimensional scaling was used to compare the produced volatile organic compounds. The main compounds responsible by the antagonism to *M. oryzae* were longifolene, *trans*-*β*-farnesene, (*Z*)-*α*-bisabolene and *δ*-amorphene, which can be used as biofungicides and incorporated into rice blast management strategies.

KEYWORDS: Rice blast, mycorrhizal fungus, biofungicide.

RESUMO

Perfil de compostos voláteis liberados por *Waitea circinata* contra *Magnaporthe oryzae* sob diferentes períodos e temperaturas

A brusone causada pelo patógeno *Magnaporthe oryzae* é a principal doença do arroz, e pode causar perdas devastadoras. Objetivou-se investigar o perfil de compostos voláteis da micorriza *Waitea circinata* no antagonismo a *M. oryzae*, em diferentes períodos e temperaturas, utilizando-se delineamento inteiramente casualizado. Compostos orgânicos voláteis foram extraídos por micro extração em fase sólida, no modo *headspace*, e analisados por cromatografia gasosa acoplada a espectrometria de massas, enquanto o escalonamento multidimensional foi usado para comparar os compostos orgânicos voláteis produzidos. Os principais compostos responsáveis pelo antagonismo a *M. oryzae* foram longifoleno, *trans*-*β*-farneseno, (*Z*)-*α*-bisaboleno e *δ*-amorfeno, que podem ser usados como biofungicidas e incorporados às estratégias de manejo da brusone do arroz.

PALAVRAS-CHAVE: Brusone, fungo micorrízico, biofungicida.

INTRODUCTION

Rice is the most important cereal worldwide, being nutritious and providing a source of income and employment (Babaee et al. 2021). Rice growers provide food security for more than half of the world population, meaning that the production will need to double by 2050, in order to accompany its exponential growth (FAO 2019).

Rice blast is one of the greatest challenges in rice cultivation, causing up to 100 % of crop loss due to cultivar susceptibility and disease-friendly environmental conditions (Prabhu et al. 2009). Breeding to obtain disease-resistant cultivars and pesticide application are the main strategies used to control rice blast. Genetic resistance breeding is a lengthy process and physiological races of the pathogen mean that resistance is short-lived.

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Moreover, the application of fungicides causes toxic effects on other microorganisms, pathogen resistance to molecules, poisoning, environmental pollution, health risks and residual toxicity, and threatens food security (Nascimento et al. 2020). As such, the biological control of rice blast is a safe and environmentally friendly strategy that should be incorporated into disease management.

The use of volatile organic compounds produced by bioagents has become increasingly prominent among biological control mechanisms against plant pathogens, being responsible for a new generation of biocontrol. These metabolites exhibit low molecular mass (100-500 Da), high vapor pressure, low boiling point and are lipophilic, readily evaporating and diffusing through heterogeneous mixtures of solids, liquids and gases (Schmidt et al. 2015). Solid phase microextraction (SPME) coupled with gas chromatography mass spectroscopy (GC-MS) enables the extraction and characterization of volatile metabolites produced by microorganisms (Jeleń 2003). According to Jayakumar et al. (2021), different modes of action have been attributed to volatile organic compounds, such as inhibiting the growth and development of pathogens, activating plant defense mechanisms and promoting plant growth.

There are few examples in the literature of volatile organic compounds used against *Magnaporthe oryzae*, being the most noteworthy metabolite bacteria such as *Burkholderia cenocepacia*, *B. gladioli* and *Pseudomonas putida*, which inhibit mycelial growth and conidia germination (Chen et al. 2020, Lin et al. 2021, Patel et al. 2021).

Likewise, information on mycorrhizal volatile organic compounds is scarce, with the most prominent examples being the arbuscular mycorrhizal fungus *Scutellospora gilmorei*, which controls *Fusarium solani* in *Piper nigrum* L., and the ectomycorrhizal fungus *Schizophyllum commune*, which reduces the mortality of second-stage *Meloidogyne incognita* juveniles in tomato (Pimenta et al. 2017).

Waitea circinata is an orchid mycorrhizal fungus that controls rice pathogens by antibiosis, parasitism, lytic enzymes, volatile metabolites and defense mechanism activation (Carvalho et al. 2022, Sousa et al. 2022). As such, the chemical identification of volatile organic compounds for the formulation of bioproducts underlies this research. Thus, this study aimed to analyze the chemical profile of *W. circinata* volatile organic compounds during antagonism to *M. oryzae*.

MATERIAL AND METHODS

The assays were performed at the Universidade Federal de Goiás, in Goiânia, Goiás state, Brazil, from August 2017 to July 2018.

Waitea circinata (En 07) and *M. oryzae* (BRM 10900) were cultured on plates containing potato-dextrose-agar (PDA) medium consisting of 200 g of potato, 20 g of dextrose (Cromoline, SP, Brazil) and 20 g of agar (Cromoline, SP, Brazil) diluted in 1 L of water. After the incubation period, mycelial discs were transferred individually to the cultures in Petri dishes. The plates were incubated under continuous fluorescent light for 11 days (26 ± 2 ºC).

A completely randomized design, with four treatments and five replicates, was used. After the incubation period, discs (3 mm) of the fungal mycelia were transferred to polystyrene plates (90 × 15 mm with a division) previously prepared with a sterile PDA medium. The treatments were as it follows: T1: *W. circinata*; T2: *W. circinata* + pathogen; T3: pathogen only; T4: PDA plate as absolute control. The pathogen and mycorrhizal fungus were transferred individually to each site of the polystyrene plate. The plates were incubated at 27 ºC, for eight days, under continuous light. Colony diameter was measured using a digital pachymeter on the second, fourth, sixth and eighth day after incubation. The reduction was calculated according to Carvalho et al. (2015).

The extraction of volatile organic compounds was performed in triplicate on the fourth and eighth day after the onset of the experiment. A mixed polarity divinylbenzene/carboxen/polydimethylsiloxane (PDMS/CAR/DVB) SPME fiber (50/30 µm, Supelco Cat. Nº 57328-U) was used due to its greater versatility and ability to capture polar and nonpolar compounds. It was placed above (about 1 cm) the surface of the fungal culture and exposed for 20 min, in line with the headspace technique (HS-SPME), under two conditions (with and without water bath).

Without water bath, i.e., at 27 ºC, the plates were perforated, and the fiber was inserted and exposed for extraction. At 50 ºC (submitted to water bath), the plates were initially placed in ethylene glycol (Panreac) for 15 min and stabilized for 5 min, then perforated to insert the fiber, which was exposed for extraction [adapted from Oliveira et al. (2017)].

Next, the fibers from each experiment were analyzed by gas chromatography mass spectrometry. The chromatographic analysis was performed in a Shimadzu GCMS-QP2010 Plus gas chromatograph mass spectrometer (Shimadzu Corporation, Japan), equipped with a DB-5MS fused silica capillary column (30 m × 0.25 mm id × 0.25 µm film thickness), and the following temperature settings: 40 ºC for 2 min, 20 ºC min^{-1} to 195 ºC, 10 ºC min^{-1} to 220 ºC and 20 ºC min^{-1} to 280 ºC, totaling a chromatographic run of 15.25 min. Helium was used as carrier gas, at a flow rate of 1 mL min^{-1}, and the injector temperature maintained at 225 ºC. The following operational parameters were used: interface temperature of 240 ºC, electron impact ionization at 70 eV, mass range from 40 to 350 Da and sampling rate of 1 $scan^{-1}$.

The volatile organic compounds were identified using mass spectral data (NIST 2022) obtained from the equipment by calculating the experimental retention index (ERI) at a non-linear programmed temperature (Van Den Dool & Kratz 1963), relatively to the retention time in a series of n-alkanes (C_7-C_{30}, Sigma-Aldrich). Next, the data were compared to the retention index described in the literature (LRI) (Adams 2017), and the obtained spectra were compared against those found in spectral libraries, organic compound databases and described in articles. The treatment containing only the PDA medium (T4) was also analyzed as a blank sample, and the detected compounds were excluded from the analysis.

The means for the antagonism volatile organic compounds assays were compared by the t-test ($p < 0.05$), using the SPSS software (version 20.0). The chromatographic data were processed by converting the presence or absence of the volatile organic compounds chromatographic peak into binary data: '1' for presence and '0' for absence. Next, multidimensional scaling (Gower 1966) was carried out considering the matrix of binary (asymmetric) distances from chromatographic data. Two-dimensional multidimensional scaling plots were represented by the main axes Dim1 and Dim2, and distances between the samples were then projected onto this new coordinate space. This made it possible to visualize the similarities between the samples after reducing the data dimensionality. The multidimensional scaling was calculated using the R software, version 4.0.4 (R Core Team 2020).

RESULTS AND DISCUSSION

The average diameters of the pathogen colony in the presence of *Waitea circinata* were 1.4, 2.5, 3.5 and 3.7 cm, respectively at 2, 4, 6 and 8 days after culturing, being significantly lower than the pathogen cultured alone, which was 1.7, 4.5, 6.0 and 7.9 cm over the same period. Another important observation is that *W. circinata* exhibited the same growth under antagonism and when cultured alone on all the assessment days (Figure 1A). The inhibition of the pathogen colony was greater at four (55.6 %) and eight (62.5 %) days after culturing (Figure 1B), and, as such, these days were selected to assess the chemical profile of volatile organic compounds. A previous study demonstrated that volatile organic compounds released by *W. circinata* inhibited the colony growth in *Cochliobolus miyabeanus*, *Monographella albescens* and *Sarocladium oryzae* by 90, 58.5, 74 and 64.7 %, respectively, and changed the coloring of the pathogen's colony due to the inhibition of melanin production (Carvalho et al. 2022). The investigation was only qualitative, and no study of the chemical profile was carried

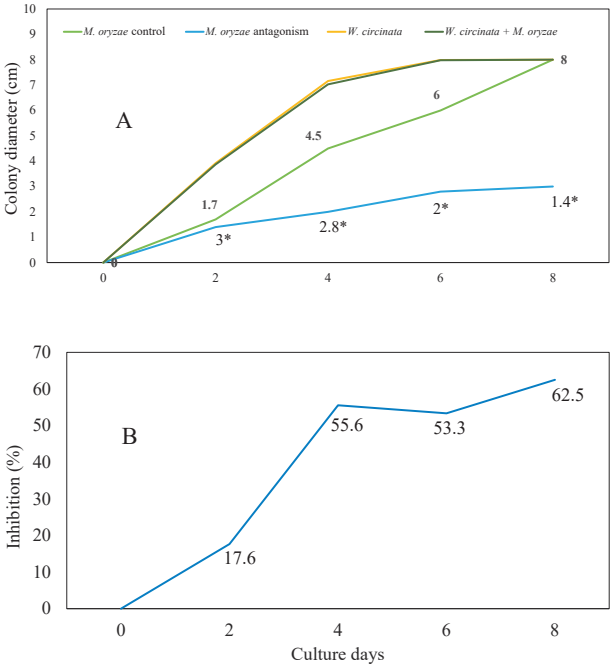

Figure 1. Mycelial growth (cm): A) inhibition of mycelial growth (%); B) *Magnaporthe oryzae* (Mo), when cultured in the presence of *Waitea circinata* volatile metabolites. * Significantly different from the control according to the T-test ($p < 0.05$).

out to identify the compounds responsible for this observation.

Sousa et al. (2022) reported that *W. circinata* was efficient at suppressing sheath blight by *in vitro* parasitism and induced resistance by activating biochemical mechanisms. In another study, *W. circinata* also produced secondary metabolites (benzophenones) that suppressed leaf blast (Carvalho et al. 2015).

Based on the GC-MS analysis, considering the days four and eight, 21 compounds were detected and are summarized in Table 1. To support their identification, the spectra were compared against the data of compounds described in the literature with an approximate retention index. Seventeen volatile organic compounds were identified, and four [retention times and identification numbers of 7.14 (**1**), 9.49 (**7**), 10.02 (**13**) and 12.46 min (**21**)] could not be characterized.

Among these 17 compounds, fifteen hydrocarbons and two alcohols were identified as longifolene (**2**), α-cedrene (**3**), α-gurjunene (**4**), β-cedrene (**5**), 8-α-cedrene (**6**), α-humulene (**8**), *trans*-β-farnesene (**9**), α-macrocarpene (**10**), β-acoradiene (**11**), cumacrene (**12**), *cis*-1,4-candinadiene (**14**), (*Z*)-α-bisabolene (**15**), δ-amorphene (**16**), (*E*)-γ-bisabolene (**17**), α-cadinene (**18**), epiglobulol (**19**) and cubenol (**20**), respectively (Figure 2). Sesquiterpenes were the main class of volatile organic compounds obtained (71.4 %).

Few studies have investigated the chemical profile of bioagents against *M. oryzae*. Volatile organic compounds in *Burkholderia cenocepacia* ETR-B22 (dimethyl trisulfide, methyl salicylate, indole and methyl anthranilate) inhibited 100 % of the *M. oryzae* mycelial growth (Chen et al. 2020). *B. gladioli* (BBB-01) produced 2,5-dimethylfuran, which decreased the *M. oryzae* growth (Lin et al. 2021). The volatile organic compounds 2-methylpyrazine and 2-ethyl-3,6-dimethylpyrazin, released by *Pseudomonas putida* BP25, reduced the mycelial growth by up to 100 %, conidial germination and sporulation in *M. oryzae* (Patel et al. 2021). In this study, other classes of compounds were identified, revealing application possibilities from natural sources, as well as inspiration for structures to be synthesized in the laboratory, aiming at fungicidal potential.

Trindade et al. (2021) observed the presence of *δ*-cadinene, cubenol and *δ*-amorphene during

Table 1. Volatile organic compounds released by *Waitea circinata* and *Magnaporthe oryzae* in antagonism, detected by solid phase microextraction coupled with gas chromatography mass spectroscopy.

Number	RT[(a)] (min)	Name	ERI[(b)]	ALRI[(c)]	LLRI[(d)]	Reference
1	7.14	NI[(e)]	1188	-	-	-
2	8.86	longifolene	1405	1407	1402	Mitić et al. 2021
3	8.95	*α*-cedrene	1410	1410	1409	Pino et al. 2005
4	9.00	*α*-gurjunene	1414	1409	1409	Szafranek et al. 2005
5	9.08	*β*-cedrene	1420	1420	1422	Al-Jaber et al. 2020
6	9.43	8-*α*-cedrene	1445	1447	1449	Ghazouani et al. 2017
7	9.49	NI	1450	-	-	-
8	9.52	*α*-humulene	1452	1452	1453	Reidel et al. 2017
9	9.58	*trans*-β-farnesene	1456	1456	1460	Torky et al. 2021
10	9.68	α-macrocarpene	1463	1470	1472	Costa et al. 2021
11	9.76	β-acoradiene	1469	1469	1471	Giuliani et al. 2020
12	9.79	cumacrene	1471	1470	1474	Joffard et al. 2017
13	10.02	NI	1488	-	-	-
14	10.13	*cis*-1,4-candinadiene	1495	1495	1498	Courtois et al. 2009
15	10.22	(*Z*)-α-bisabolene	1505	1506	1503	Courtois et al. 2009
16	10.26	δ-amorphene	1511	1511	1511	Joffard et al. 2017
17	10.39	(*E*)-*y*-bisabolene	1530	1530	1535	Costa et al. 2021
18	10.44	α-cadinene	1538	1538	1538	Joffard et al. 2017
19	10.52	epiglobulol	1549	-	1532	Zhang et al. 2017
20	11.20	cubenol	1645	1645	1648	Jiang et al. 2020
21	12.66	NI	1845	-	-	-

[(a)] Retention time; [(b)] experimental retention index; [(c)] Adams linear retention index (Adams 2017); [(d)] literature linear retention index; [(e)] not identified.

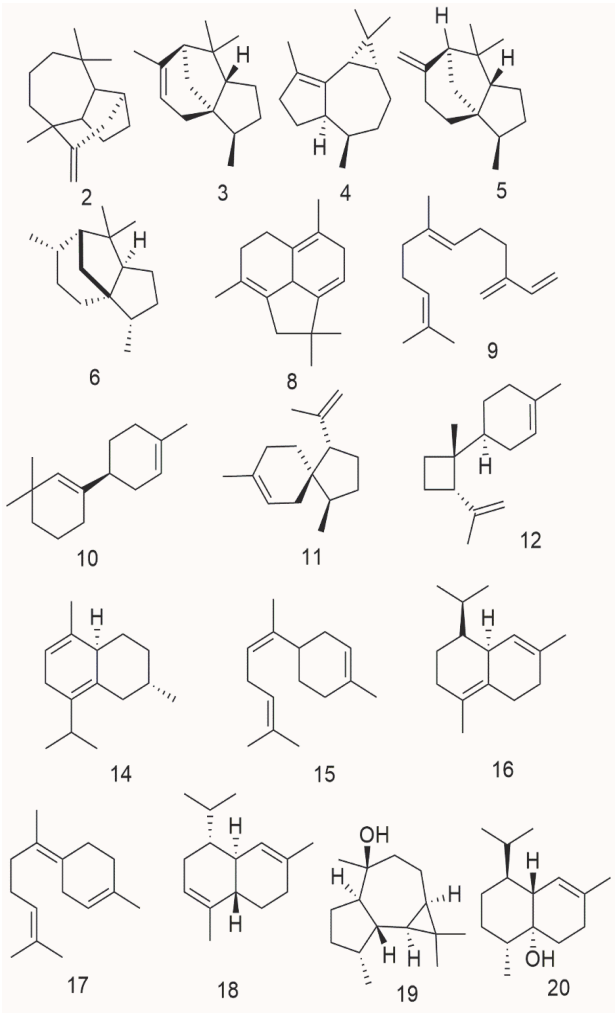

Figure 2. Chemical structures of the seventeen volatile organic compounds identified in the solid phase microextraction coupled with gas chromatography mass spectroscopy analysis. The numbers assigned to the volatile organic compounds are described in Table 1.

the antagonism of the arbuscular mycorrhizal *Scutellospora gilmorei* and *Fusarium solani* in *Piper nigrum*. The ectomycorrhizal *Schizophyllum commune* (basidiomycota) produced γ-bisabolene, which immobilized the exposed *Meloidogyne incognita* J2 in tomato, when compared to the control. The present study demonstrates that *W. circinata* also produces volatile compounds in a non-obligate symbiont that can be easily multiplied in PDA, what does not occur with other mycorrhizal fungi.

The volatile organic compounds extraction was carried out during two incubation periods (days four and eight), because the pathogen growth inhibition was greater on those days, and under two conditions (with and without water bath). Venn diagrams were constructed with 21 and 10 volatile organic compounds detected for samples submitted or not to water bath, respectively (Figures 3 and 4).

Without water bath, *W. circinata* released nine volatile organic compounds, i.e., five (**1**, **3**, **7**, **8** and **12**) only on the day eight and four on both days: longifolene (**2**), (*Z*)-α-bisabolene (**15**), δ-amorphene (**16**) and cubenol (**20**). These last four were also observed in antagonism, whereas *M. oryzae* only released one (**21**) and only on the day eight (Figure 3).

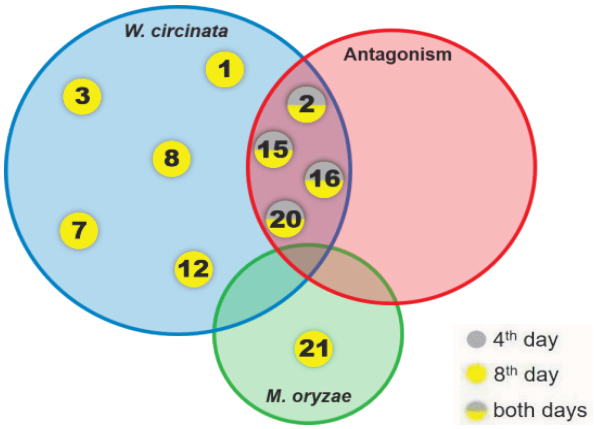

Figure 3. Venn diagram describing the volatile organic compounds profiles of *Waitea circinata* and *Magnaporthe oryzae* (isolated and in antagonism) not submitted to water bath. The numbers assigned to the volatile organic compounds are described in Table 1.

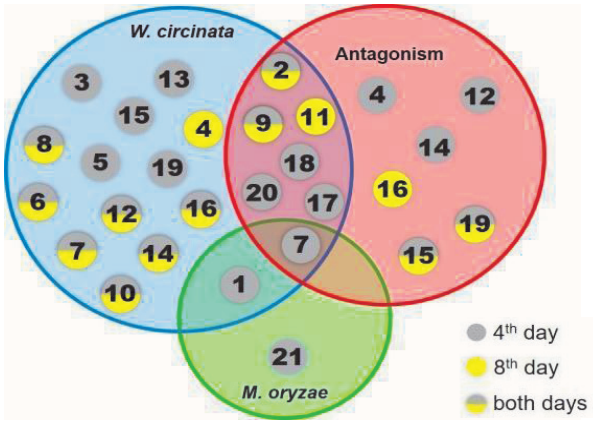

Figure 4. Venn diagram describing the volatile organic compounds profiles of *Waitea circinata* and *Magnaporthe oryzae* (isolated and in antagonism) submitted to water bath. The numbers assigned to the volatile organic compounds are described in Table 1.

The temperature variation promoted an efficient volatile organic compounds extraction for all the treatments. For samples submitted to water bath (Figure 4), the number and variety of volatile compounds identified on the day four were greater in all the treatments. *W. circinata* was the largest producer of volatile organic compounds, totaling 19, being eight exclusively on the day four, nine on both days and two on the day eight. According to Oliveira et al. (2017), temperature is a variable that influences volatile organic compounds extraction, what was also observed in the present study, reflected in the larger number of volatile organic compounds collected under water bath conditions.

In the antagonism treatment (day four, with water bath), the compounds *α*-gurjenene (**4**), cumacrene (**12**), *cis*-1,4-candinadiene (**14**), (*E*)-*γ*-bisabolene (**17**), *α*-cadinene (**18**) and cubenol (**20**) were observed, and *β*-acoradiene (**11**) and *δ*-amorphene (**16**) on the day eight. The volatile organic compounds (**2**) and (**9**) are exclusive to *W. circinata* and showed antagonism on both days.

In addition, the volatile organic compounds types differed according to the incubation period. In antagonism (day four) and for *W. circinata* (days four and eight) submitted to water bath, cumacrene (**12**) and *cis*-1,4-candinadiene (**14**) were detected (Figure 4). Longifolene (**2**) and δ-amorphene (**16**) were also detected in antagonism (with no water bath) on both days; with bath, (**2**) was observed on both days and (**16**) only on the day eight.

Longifolene, cedrene, *γ*-acoradiene and *β*-gurjunene were also released by *Trichoderma longibrachiatum* and controlled *Sclerotium rolfsii*, *Macrophomina faseolina* and *Fusarium oxysporum* (Sridharan et al. 2020, Rajani et al. 2021). Additionally, *T. harzianum* produced *β*-bisabolene and inhibited the growth of *F. oxysporum* f. sp. cubense, while cadinene and *α*-gurjunene released by *T. virens* inhibited *Rhizoctonia solani* growth (Zhang et al. 2014, Inayati et al. 2019), showing that *W. circinata* produces the same compounds as the main bioagent used in the world.

It is also worth mentioning that synthetics volatiles based on longifolene, bisabolene and farnesene have successfully controlled plant pathogens, performing better than commercial fungicides (Zhang et al. 2021, Ricciardi et al. 2021), demonstrating that *W. circinata* volatile organic compounds can be synthesized or commercially acquired for rice disease biocontrol.

The overlapping antagonism (Ant) and *W. circinata* (Wc) in Figure 5A indicates a high similarity between the two volatile organic compounds profiles. Under these experimental conditions, the four compounds in the Ant chromatogram are the same as those identified for Wc: (**2**), (**15**), (**16**) and (**20**). These compounds are not present in the PDA medium or *Magnaporthe oryzae* (Mo), which are far from each other and from Ant and Wc.

The two-dimensional multidimensional scaling also indicates a greater similarity between the Ant and Wc volatile organic compounds profiles in relation to those of PDA and Mo (Figure 5). The longer time period, in relation to the result in Figure 5A, led to a larger number of compounds produced by Wc (from 4 to 9), while the number released by Ant remained unchanged (4), with these Ant compounds also detected in the Wc chromatogram.

A total of 11 peaks were observed in the chromatogram for Ant and 18 for Wc. This similarity is due to the presence of 10 common volatile organic compounds [(**2**), (**7**), (**9**), (**12**), (**14**), (**15**), (**17**), (**18**), (**19**) and (**20**)]. In other words, of the 11 compounds detected for Ant, 10 are common to Wc. Of these 10,

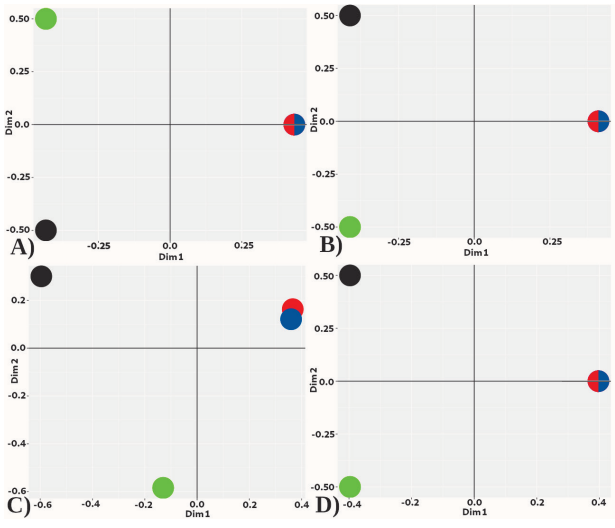

Figure 5. Two-dimensional multidimensional scaling plots of the volatile organic compounds profiles in four experimental treatments: A) day four, no water bath; B) day eight, no water bath; C) day four, with water bath; D) day eight, with water bath. Ant: antagonism (red circle); Wc: *Waitea circinata* (blue circle); Mo: *Magnaporthe oryzae* (green circle); PDA: potato-dextrose-agar (black circle).

three (**2**, **15** and **20**) were also observed under the conditions of Figures 5A and 5B. With the increase in temperature after 4 days, the number of compounds rised from 4 to 11 for Ant and from 4 to 18 for Wc (Figure 5C).

Ant and Wc exhibited a considerable similarity, with 4 compounds in common (**2**, **9**, **11** and **16**) (Figure 5D). In this case, the longer time period, in relation to the Figure 5C, reduced the number of compounds produced by Ant (from 11 to 6) and Wc (from 18 to 11). A comparison of the effect of temperature (higher when compared to Figure 5B) indicates an increase in the number of compounds released by Ant (from 4 to 6) and Wc (from 4 to11). That is, while a longer time period (from 4 to 8 days) decreased the number of compounds for both Ant and Wc, higher temperatures (from no water bath to water bath conditions) increased the number of Ant and Wc compounds. As such, the larger number of compounds produced by Ant and Wc is favored by the increase in temperature and not the longer experiment duration (number of days).

Biocontrol agents can be used to protect crops based on the knowledge of substances produced during interactions between these agents and plant pathogens. These results support the important role of the volatile organic compounds from *W. circinata* in antagonism to *M. oryzae* and suggest its potential as a natural and ecological alternative in the biocontrol of this pathogen in rice crops.

CONCLUSION

Longifolene, *trans*-β-farnesene, (Z)-α-bisabolene and δ-amorphene produced by *Waitea circinata* are responsible for the antagonism to *Magnaporthe oryzae*.

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