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Characterization of *Sarocladium oryzae* and its reduction potential of rice leaf blast[1]

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ABSTRACT

The integrated management of rice leaf blast (*Magnaporthe oryzae*) is carried out mainly with the adoption of chemical control. However, the search for alternative practices has grown in recent years. Thus, the variability of 28 *Sarocladium oryzae* isolates was evaluated for the cerulenin production, as well as its potential for reducing the severity of rice leaf blast, quantifying the activity of enzymes linked to the plant defense mechanisms. More than 55 % of the *S. oryzae* isolates were antagonistic to the pathogens *M. oryzae*, *Cochliobolus miyabeanus*, *Thanatephorus cucumeris* and *Monographella albescens*, and 60 % of the isolates produced cerulenin at detectable levels. Both BRM 6461 (296.0 µg mL^{-1}) and BRM 6493 (undetectable cerulenin) inhibited the formation of *M. oryzae* appressoria in 89.5 % and 85 %, respectively. The BRM 6461 isolate, applied as conidial suspension and filtered, reduced the severity of rice leaf blast in 68.8 % and 75.5 %, respectively. The enzymatic activity in the presence of *M. oryzae* was higher for lipoxygenase at 5 h (filtered) and at 24 h and 72 h (conidial suspension) after the pathogen inoculation. For phenylalanine ammonia lyase, the highest expression was at 5 h (filtered) and 72 h (conidial suspension). The enzymes chitinase, β-1,3-glucanase and peroxidase and the salicylic acid phytohormone presented no differences, in relation to the controls (water and *M. oryzae*). The filtered from the BRM 6461 isolate, basically constituted by cerulenin, reduced the severity of rice leaf blast and possibly activated the defense mechanisms of the rice plants against *M. oryzae*.

KEYWORDS: *Magnaporthe oryzae*; cerulenin; biocontrol; enzyme activity.

RESUMO

Caracterização de *Sarocladium oryzae* e seu potencial na redução de brusone foliar em arroz

O manejo integrado de brusone (*Magnaporthe oryzae*) em arroz é realizado, principalmente, com a adoção de controle químico. No entanto, a busca por práticas alternativas tem crescido nos últimos anos. Assim, avaliou-se a variabilidade de 28 isolados de *Sarocladium oryzae*, quanto à produção de cerulenina, e o seu potencial na redução da severidade de brusone foliar em arroz, quantificando-se a atividade de enzimas ligadas ao mecanismo de defesa das plantas. Mais de 55 % dos isolados de *S. oryzae* foram antagônicos aos patógenos *M. oryzae*, *Cochliobolus miyabeanus*, *Thanatephorus cucumeris* e *Monographella albescens*, e 60 % dos isolados produziram cerulenina em níveis detectáveis. Tanto BRM 6461 (296,0 µg mL^{-1}) quanto BRM 6493 (cerulenina não detectável) inibiram a formação de apressórios de *M. oryzae* em 89,5 % e 85 %, respectivamente. O isolado BRM 6461, aplicado na forma de suspensão de conídios e filtrado, reduziu a severidade da brusone em 68,8 % e 75,5 %, respectivamente. A atividade enzimática, na presença de *M. oryzae*, foi maior para lipoxigenase após 5 h (filtrado) e 24 h e 72 h (suspensão de conídios) da inoculação do patógeno. Para fenilalanina-amônia liase, a maior expressão ocorreu após 5 h (filtrado) e 72 h (suspensão de conídios). As enzimas quitinase, β-1,3-glucanase e peroxidase e o fitohormônio ácido salicílico não apresentaram diferenças em relação aos controles (água e *M. oryzae*). O filtrado do isolado BRM 6461, constituído basicamente por cerulenina, reduziu a severidade da brusone e, possivelmente, ativou os mecanismos de defesa da planta de arroz contra *M. oryzae*.

PALAVRAS-CHAVE: *Magnaporthe oryzae*; cerulenina; biocontrole; atividade enzimática.

INTRODUCTION

Rice leaf blast, caused by *Magnaporthe oryzae* [B. Couch (anamorph: *Pyricularia oryzae* Cavara)] (Klaubauf et al. 2014), is one of the most destructive diseases in rice crops (Fisher et al. 2012) and may cause grain yield losses of up to 100 % (Prabhu et al. 2009) in all producing regions (Chen et al. 2013).

It is controlled using an integrated management system, involving strategies such as chemical control, crop management techniques and resistant cultivars. The application of chemical fungicides is the most

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widely used method, however, it has negative impacts on the environment (Meng et al. 2015), besides promoting the resistance of certain pathogen molecules, resulting in the effectiveness loss of some active ingredients (Oliveira et al. 2015). One of the most efficient rice leaf blast control methods is the use of resistant cultivars. However, this resistance is rapidly overcome in the field, due to the high variability and complexity of the disease-causing pathogen (Chen et al. 2013).

Recently, biological control agents have been suggested as an additional management strategy (Nie et al. 2014). These agents can act as direct antagonists, inhibiting the growth and/or development of plant pathogens, or resistance inducers, promoting a resistance to biotic and/or abiotic stresses by activating gene expression and metabolic pathways, thereby triggering defense mechanisms such as systemic acquired resistance and induced systemic resistance (Van Loon et al. 1998, Shoresh et al. 2010).

The systemic acquired resistance is normally triggered by localized infection and provides long-term systemic resistance. It involves the activation of pathogenesis-related genes with antimicrobial activity which encode proteins that use salicylic acid as a signaling molecule (Durrant & Dong 2004, Pieterse et al. 2009), with salicylic acid levels increasing when the pathogen is detected (Mishina & Zeier 2007, Tsuda et al. 2008). The induced systemic resistance occurs as a response to root colonization by non-pathogenic agents that can phenotypically induce this type of resistance (Van Loon et al. 1998, Pieterse et al. 2009), and requires additional signaling components not dependent on salicylic acid (Shoresh et al. 2010). Both systemic acquired resistance and induced systemic resistance have been employed successfully in rice, using different biotic and abiotic resistance inducers (Smith & Métraux 1991, Manandhar et al. 1998, Tsukamoto et al. 1999, Ashizawa et al. 2005, Filippi et al. 2007 and 2011, Sena et al. 2013).

A number of studies have been conducted in recent years using the rice blast pathosystem and different biocontrol agents, such as rhizobacteria, *Epicoccum* sp. and *Cladosporium* sp. (Fillipi et al. 2011, Sena et. al. 2013, Chaibub et al. 2016), which can act as resistance inducers. The *Sarocladium oryzae* [(Sawada) W. Gams & D. Hawksw] fungus is the causal agent of sheath rot, however, in rice, this disease only occurs in the stem during the reproductive phase and is not found in leaves.

S. oryzae is also known for its antagonistic potential against other disease-causing fungi in rice, including *M. oryzae* (Gnanamanickam & Mew 1991, Sakthivel et al. 2002, Silva et al. 2007). This fungus produces cerulenin as a secondary metabolite, which acts as a potent inhibitor of fatty acid and steroid metabolism (Omura 1976, Hittalmani et al. 2016), exhibiting *in vivo* and *in vitro* antagonistic behavior against certain fungal and bacterial rice pathogens (Sakthivel & Gnanamanickam 1986, Gnanamanickam & Mew 1991, Padmavathi et al. 1997, Prabhu et al. 2007, Côrtes et al. 2014).

This study aimed at evaluating the variability, in relation to cerulenin production, of 28 *S. oryzae* isolates, as well as the *S. oryzae* potential to reduce the severity of rice leaf blast, in addition to quantifying the activity of enzymes linked to plant defense mechanisms.

MATERIAL AND METHODS

The experiments were conducted at the Embrapa Arroz e Feijão, in Santo Antônio de Goiás, Goiás State, Brazil, from 2013 to 2014, using 32 isolates from its collection of multifunctional microorganisms and fungi. A total of 28 *S. oryzae* isolates, as well as one isolate of the pathogens *Magnaporthe oryzae*, *Cochliobolus miyabeanus*, *Thanatephorus cucumeris* and *Monographella albescens*, were used.

The antagonistic potential of the *S. oryzae* isolates against *M. oryzae*, *C. miyabeanus*, *T. cucumeris* and *M. albescens* fungi was assessed using the paired cultures method (Romeiro 2007). Mycelial discs with 5 mm in diameter were placed 45 mm apart on opposite sides of a Petri dish containing potato-dextrose-agar growth medium, with one disc consisting of the pathogen under study and the other a *S. oryzae* isolate. In the control treatment, pathogens were grown separately with a mycelial disc in the center of the dish. The inhibition zone was measured as soon as the control reached full growth. Analyses were carried out using a completely randomized design and the experiment was conducted in triplicate and means compared by the Scott-Knott test ($p \leq 0.05$), using the SASM-Agri software (Canteri et al. 2001). All pathogens were used in the asexual phase.

For the cerulenin quantification, the *S. oryzae* isolates were grown separately on potato-dextrose-agar medium and incubated at 25 ºC. After 10 days, a

5 mm-wide mycelial disc was transferred to 100 mL of a liquid medium (Omura 1976) containing 1.0 % of glucose, 3.0 % of glycerol, 0.5 % of peptone and 0.2 % of sodium chloride, and incubated for 8 days, at 150 rpm and 25 ºC, to obtain the crude extract. The extract was then submitted to a vacuum with chloroform at a ratio of 1:1 (v/v), crystallized and diluted in 2 mL of ethanol, resulting in the concentrated extract or cerulenin (Côrtes et al. 2014), which was quantified by high performance liquid chromatography using a Perkin Elmer Flexar™ system (Bills et al. 2004), in a reversed-phase C18 column measuring 150 mm x 4.6 mm x 5 µm, with a fixed temperature of 40 ºC, where the mobile phase B (acetonitrile) was diluted in water (A). A commercial cerulenin standard (Sigma) was used, obtaining a standard curve from concentrations of 0.00125-2 µg μL^{-1}. Readings were performed using the Chromera software (Perkin Elmer) at a wavelength of 210 nm. Analyses were carried out using a completely randomized design in triplicate and means compared by the Scott-Knott test ($p \leq 0.05$), using the SASM-Agri software (Canteri et al. 2001).

The filtrates of the *S. oryzae* BRM 6461 and BRM 6493 isolates were assessed in terms of their effect on conidial germination and appressorium formation in *M. oryzae*. The filtrate was obtained based on the methodology proposed by Côrtes et al. (2014) and the *M. oryzae* conidial suspension according to Filippi & Prabhu (2001), and adjusted for a concentration of 1 x 10^4 conidia mL^{-1}. The suspensions were placed on a glass slide and covered with artificial superhydrophobic surface material. The treatments were: 10 µL of BRM 6461 + 10 µL of *M. oryzae* conidial suspension; 10 µL of BRM 6493 + 10 µL of *M. oryzae* conidial suspension; and 10 µL of *M. oryzae* conidial suspension + 10 µL of sterile distilled water. The experiment was performed in a completely randomized design at triplicate and analyzed after 1, 3, 6, 12 and 20 h of incubation (room temperature) and the data and means compared by the Tukey test ($p \leq 0.05$), using the SASM-Agri software (Canteri et al. 2001). Sixty *M. oryzae* conidia were observed using an epifluorescence microscope in 0.1 % calcofluor-white stain (Sigma) at 40X magnification. Conidia were considered germinated on formation of the germ tube and appressoria were deemed formed when there was a globular structure at the base of the germ tube (cell differentiation), responsible for penetrating the host.

In order to assess enzyme activity, a greenhouse experiment was carried out using the 'BRS Primavera' rice cultivar, which is susceptible to rice leaf blast. Seeds were planted in plastic trays (15 cm x 30 cm x 10 cm) containing 3 kg of soil fertilized with NPK (5-30-15), as well as the micronutrients zinc sulfate and iron sulfate. The soil used was collected from virgin Savannah and identified as Eutrophic Red Latosol (Embrapa 2013), with the following characteristics: pH (H_2O) = 5.4; clay = 589.0 g kg^{-1}; silt = 66.0 g kg^{-1}; sand = 144.0 g kg^{-1}; K^+ = 63.0 mg dm^{-3}; P = 4.0 mg dm^{-3}; Ca^{+2} = 0.4 mg dm^{-3}; Mg^{+2} = 0.2 $cmol_c$ dm^{-3}; Al^{+3} = 0.1 $cmol_c$ dm^{-3}; Si = 3.0 mg kg^{-1}.

Nitrogen in the form of ammonium sulfate [$(NH4)_2SO_4$ + Fe and Bo] was applied to the topsoil at 14 and 19 days after planting (DAP). The *S. oryzae* BRM 6461 isolate in the form of concentrated filtrate and conidial suspension (3 x 10^5 conidia mL^{-1}) was sprayed at 48 h prior to the inoculation with *M. oryza*e (BRM 31295 isolate), at a concentration of 3 x 10^5 conidia mL^{-1}, at 21 DAP. The *M. oryzae* inoculum was obtained as described by Filippi & Prabhu (2001). A randomized block design was used, with three replications, each consisting of one tray containing around 80 plants, and six treatments: T1 = control (water); T2 = spraying with *S. oryzae* filtrate; T3 = spraying with *S. oryzae* conidial suspension; T4 = spraying with *S. oryzae* filtrate at 48 h before the inoculation with *M. oryzae*; T5 = spraying with conidial suspension at 48 h before the inoculation with *M. oryzae*; and T6 = inoculation with *M. oryzae*.

Rice leaf blast severity was assessed in three treatments: plant inoculated with *M. oryzae* (control) and plants sprayed with *S. oryzae* conidial suspension and BRM 6461 filtrate, at 48 h before the inoculation with *M. oryzae*. Twenty plants per treatment were evaluated by using a grading scale with scores ranging from 0 to 9 (Leung et al. 1988). Four assessments were conducted, being the first at the onset of symptom emergence. The data were used to calculate the area under the disease-progress curve (Campbell & Madden 1990), and the reduction in disease severity was determined by using the McKinney index (Balardin et al. 1990). Analyses were carried out in a completely randomized design and the data submitted to analysis of variance and means compared by the Tukey test ($p \leq 0.05$), using the SASM-Agri software (Canteri et al. 2001).

The quantification of the enzymatic activity and salicylic acid content in the rice leaves was performed in six treatments (T1, T2, T3, T4, T5 and T6), using 10 to 15 leaves per treatment. The first leaf collection was done prior to the *M. oryzae* inoculation, while the second, third, fourth, fifth and sixth were carried out at 3, 5, 24, 48 and 72 h after the pathogen inoculation.

The samples were ground with liquid nitrogen and the protein extract obtained using a lysis buffer [(10 Mm of Tris-HCl; 150 mM of NaCl; 2 Mm of EDTA (ethylenediaminetetraacetic acid); 2 Mm of DTT (2-mercaptoethanol); 1 Mm of PMSF (phenylmethylsulfonyl fluoride), Leptin (10 mg mL^{-1}) and aprotinin (10 mg mL^{-1})]. Total protein was quantified by the colorimetric method (Bradford 1976) and readings were performed in a spectrophotometer, at a wavelength of 595 nm, using the Gen5 Data Analyses software (Biotek). The standard curve for total proteins with bovine serum albumin was obtained with different concentrations (0-1 mg mL^{-1}).

The activity of chitinase (EC 3.1.1.14) and β-1,3-glucanase (EC 3.2.1.6) were determined as described by Pan et al. (1991), with modifications, using colloidal chitin and soluble laminarin as substrate, respectively. The reducing sugars resulting from the reactions were quantified using the 3,5-dinitrosalicylic acid method. Readings performed were adjusted to 540 nm, with the Gen5 Data Analyses software. Lipoxygenase (EC 1.13.11.12) activity was assessed using the method described by Axelrod et al. (1981) and readings were performed on a spectrophotometer, at a wavelength of 234 nm. Peroxidase (EC 1.11.1.7) activity was established according to the method proposed by Keesey (1987) and modified by Côrtes et al. (2008), using a spectrophotometer adjusted to a wavelength of 405 nm and coupled with the WinSpec 2.3 software, in the kinetic mode. Phenylananine ammonia lyase (EC 4.3.1.5) activity was evaluated based on the method described by Alunni et al. (2003), through quantification of the trans-cinnamic acid generated by hydrolysis of the L-phenylalanine and readings on a spectrophotometer adjusted to 290 nm. The results were expressed as specific activity. Salicylic acid was analyzed in accordance with Yalpani et al. (1991) and quantified by high performance liquid chromatography with mobile phase composed of 23 % of methanol and 77 % 20 mM of acetate buffer (pH 5.0), in a C18 column maintained at a fixed temperature of 35 ºC. The ultraviolet detector was adjusted to 280 nm and coupled with the Chromera software (Perkin Elmer). The data were submitted to statistical analysis using the Statistical Package for the Social Sciences software and means compared by the Tukey test ($p \leq 0.05$).

RESULTS AND DISCUSSION

Of the 28 *S. oryzae* isolates analyzed, only six exhibited antagonistic activity against at least one of the pathogens studied (Table 1). BRM 6493 was the best antagonist, producing the largest zones of inhibition for the four pathogens tested.

In order to explain the rate of antagonism between the phytopathogens and the *S. Oryzae* fungus, isolates of the species were submitted to specific cultivation conditions to determine the production capacity of the secondary metabolite cerulenin. This molecule has been described as the main secondary metabolite responsible for the *in vivo* and *in vitro* antagonistic action of *S. oryzae* against rice pathogens (Sakthivel & Gnanamanickam 1986, Gnanamanickam & Mew 1991, Prabhu et al. 2007, Côrtes et al. 2014). The results of the present study indicated that 11 of the 28 isolates analyzed showed no detectable levels of this metabolite. BRM 6461 stood out from the remaining isolates, achieving a cerulenin production of 296 μg mL^{-1} (Table 2), more than twice as high as the second best cerulenin-producing isolate.

However, a comparison between data for *in vitro* antagonism and cerulenin production capacity found that isolates such as BRM 6493, which did not exhibit detectable cerulenin levels, were considered superior antagonists to the different pathogens. Thus, it is believed that *in vitro* antagonism was not only due to cerulenin production by some of the *S. oryzae* isolates, but also secondary metabolites such as helvolic acid, also often related to *in vitro* antagonism (Tschen & Wen 1980, Tschen et al. 1997). Another hypothesis is the production of other hitherto disregarded secondary metabolites, which may influence antagonistic behavior between species. Thus, it can be inferred that cerulenin was not the only molecule responsible for antagonistic action against pathogens in the *in vitro* mycelial development of *S. oryzae*. This information raises the possibility that molecules such as helvolic acid and others not described here, likely produced on a smaller scale,

Table 1. Inhibition of the mycelial growth of phytopathogenic rice fungi by *Sarocladium oryzae.*

Sarocladium oryzae	*Cochliobolus miyabeanus*	*Magnaporthe oryzae*	*Monographella albescens*	*Thanatephorus cucumeris*
	Inhibition zone (mm)*			
BRM 6458	7.16 c	3.55 d	7.20 b	0.28 c
BRM 6459	0.76 d	0.25 e	0.66 c	0.00 c
BRM 6461	9.27 b	3.87 d	2.31 c	2.29 c
BRM 6463	7.18 c	9.10 b	8.03 a	1.75 c
BRM 6464	0.64 d	3.61 d	0.52 c	0.00 c
BRM 6465	0.63 d	8.62 b	3.79 c	0.00 c
BRM 6466	8.25 b	0.31 e	1.50 c	0.00 c
BRM 6467	11.92 a	3.47 d	3.98 b	0.64 c
BRM 6469	9.76 b	9.60 b	9.12 a	0.00 c
BRM 6470	0.43 d	0.12 e	0.63 c	0.00 c
BRM 6472	10.77 a	6.61 c	8.07 a	5.47 b
BRM 6473	12.34 a	4.76 d	7.58 b	5.37 b
BRM 6475	11.96 a	4.20 d	2.70 c	0.00 c
BRM 6475	13.01 a	7.34 c	8.50 a	5.45 b
BRM 6476	9.01 b	0.87 e	5.63 b	0.47 c
BRM 6477	0.87 d	0.32 e	0.43 c	0.00 c
BRM 6481	7.51 c	1.13 e	9.00 a	1.25 c
BRM 6485	0.53 d	0.95 e	2.50 c	0.00 c
BRM 6486	8.98 b	9.30 b	9.02 a	0.46 c
BRM 6488	11.91 a	10.09 b	6.19 b	5.67 b
BRM 6489	8.28 b	7.87 c	8.58 a	2.87 c
BRM 6491	13.55 a	3.72 d	5.09 b	1.00 c
BRM 6493	13.76 a	13.28 a	10.29 a	8.69 a
BRM 6495	0.48 d	0.87 e	0.95 c	0.00 c
BRM 6497	5.58 c	1.72 e	1.62 c	1.15 c
So A	9.37 b	6.07 c	10.29 a	0.54 c
53B	8.10 b	2.33 d	6.58 b	0.35 c
Am 11	1.27 d	0.13 e	1.04 c	0.00 c
Control	0.00 d	0.00 e	0.00 c	0.00 c

* Means followed by the same letter do not differ statistically according to the Skott-Knott test ($p \leq 0.05$).

are acting as inhibitors of *M. oryzae* cell growth, *in vitro*, under the conditions described.

In addition to the antagonistic effect on mycelial growth, the inhibition of the conidial germination and appressorium formation of phytopathogens are of significant interest when the aim is to select biocontrol agents or molecules, particularly in terms of rice leaf blast management. In this respect, the *S. oryzae* BRM 6461 and BRM 6493 isolates were tested against *M. oryzae* conidia, exhibiting a delay in germ tube emergence in the first hour and 89.5 % and 85 % formation of *M. oryzae* appressoria, respectively (Table 3, Figure 1). The results shown by BRM 6461 were similar to those found by Côrtes et al. (2014) and Ohtake et al. (1999), who associated the reduced *M. oryzae* appressorium formation with increased cerulenin levels.

It is important to underscore that the metabolite cerulenin acts in the inhibition of lipid synthesis, one of the main components needed for the germ tube and appressorium formation in *M. oryzae* (Ebbole 2007). However, the BRM 6493 isolate displayed a marked inhibitory effect on appressorium formation: it did not produce cerulenin. The method used to produce the filtrate analyzed favors the cerulenin production and therefore hampers the helvolic acid production, which is generated in insignificant quantities (Omura 1976). This strengthens the hypothesis that secondary metabolites other than cerulenin and helvolic acid are produced and may be responsible for the inhibitory effect.

Based on the previous results, the *S. oryzae* BRM 6461 isolate was selected to assess the action of *S. oryzae* in reducing the severity of rice leaf blast. The experiment demonstrated that the concentrated filtrate or conidial suspension applied at 48 h before the inoculation with *M. oryzae* promoted reductions

of up to 75.5 % and 68.8 % in disease severity, respectively. Comparatively, the treatment with filtrate decreased rice leaf blast more effectively, in relation to the treatment with the *S. oryzae* conidial suspension (Figure 2). However, although this difference is statistically significant, it is not enough to be explained by the difference in the cerulenin concentration potentially present on the leaf surface at the moment of interaction with the pathogen. A far higher effect on disease reduction was expected in the presence of the concentrated filtrate, containing high cerulenin levels, than the treatment consisting of *S. oryzae* conidia isolated, together with their locally produced metabolites, possibly including cerulenin.

Table 2. Cerulenin production by *Sarocladium oryzae* isolates.

Sarocladium oryzae isolate	Amount of cerulenin (µg mL^{-1})
BRM 6461	296.0 a
BRM 6469	122.3 b
BRM 6475	64.5 c
BRM 6473	55.5 c
BRM 6472	53.8 c
BRM 6477	52.0 c
BRM 6476	48.9 c
BRM 6489	46.1 c
So A	38.3 c
BRM 6491	27.8 d
BRM 6488	26.5 d
BRM 6459	20.0 d
BRM 6463	14.6 d
BRM 6481	13.2 d
BRM 6465	7.5 e
BRM 6475	5.2 e
BRM 6497	5.2 e
BRM 6458	nd
BRM 6464	nd
BRM 6466	nd
BRM 6467	nd
BRM 6470	nd
BRM 6485	nd
BRM 6486	nd
BRM 6493	nd
BRM 6495	nd
53B	nd
Am 11	nd

* Means followed by the same letter do not differ statistically according to the Skott-Knott test ($p \leq 0.05$). nd: non-detectable levels.

In this respect, the hypothesis tested was whether both the *S. oryzae* filtrate and conidia, in addition to exhibiting antagonistic action against the pathogen, might be acting indirectly on the defense system of the rice plants through a process called induced resistance, promoting the expression of pathogenesis-related proteins. The pathogenesis-related protein expression was assessed by measuring the activity of five representative enzymes, in addition to the phytohormone salicylic acid. There are reports of increased expression of these enzymes and salicylic acid in potentially beneficial interactions among *M. oryzae*, rice and other microorganisms, confirming the induced resistance phenomenon and helping to suppress rice leaf blast (Filippi et al. 2014, Chaibub et al. 2016).

When applied in the absence of *M. oryzae*, the *S. oryzae* filtrate increased the chitinase activity (48 h after application), β-1,3-glucanase (5 h and 48 h after application) and peroxidase (48 h after application) in rice leaves, while for the conidial suspension the highest values were observed for β-1,3-glucanase (5 h, 24 h and 48 h after application) and peroxidase (5 h and 48 h after application). With respect to the enzyme activity in rice leaves sprayed with *S. oryzae* at 48 h before the inoculation with *M. oryzae*, the highest activity was recorded for lipoxygenase at 5 h when sprayed with the filtrate and at 24 h and 72 h with the conidial suspension. Phenylalanine ammonia lyase showed activity at 5 h after the treatment with the filtrate and after 72 h with the conidial suspension. No differences were found in the expression of chitinase, ß-1,3-glucanase, peroxidase and salicylic acid, in relation to the controls (Figure 3).

Table 3. Number of conidia and appressoria formed during the exposure to *Sarocladium oryzae* filtrates of the BRM 6461 and BRM 6493 isolates.

Treatment	Time					
	GT/A	GT/A	GT/A	GT/A	GT/A	GT/A
	0 h	1 h	3 h	6 h	12 h	20 h
BRM 6461	$0^{ns}/0^{ns1}$	2.7 a*/0^{ns}	$47.3^{ns}/0.00^{ns}$	$51.0^{ns}/0.00^{ns}$	$51.3^{ns}/0.00^{ns}$	53.7^{ns}/0.00 a
BRM 6493	0/0	4.0 a/0	48.7/0.00	52.3/0.00	53.3/0.00	56.7/2.00 a
Control	0/0	32.3 b/0	50.3/0.00	52.7/0.00	52.7/0.00	52.3/42.7 b

GT = germ tube; A = appressoria. * Means followed by the same letter do not differ statistically according to the Tukey test ($p \leq 0.05$). ^{ns} non-significant.

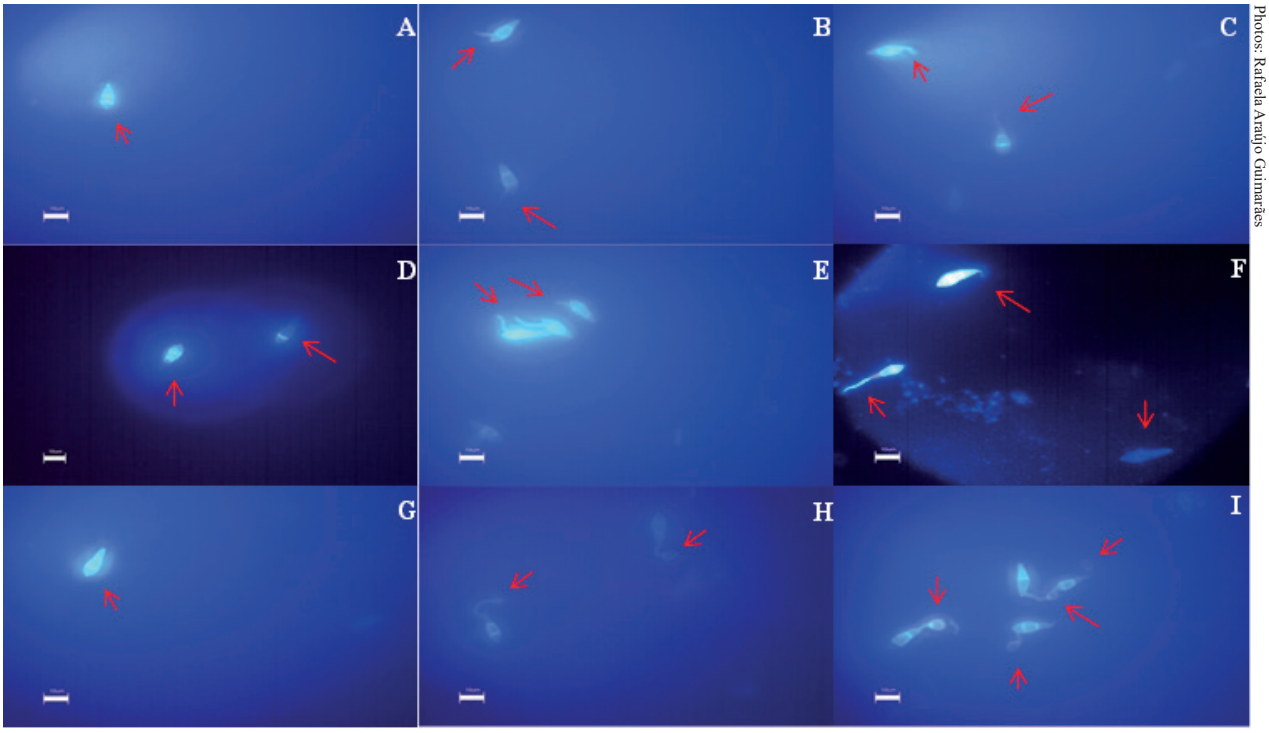

Figure 1. Effect of the *Sarocladium oryzae* filtrate on the germination and appressorium formation in *Magnaporthe oryzae*, where: A: 0 h/BRM 6461; B: 12 h/BRM 6461; C: 20 h/BRM 6461; D: 0 h/BRM 6493; E: 12 h/BRM 6493; F: 20 h/BRM 6493; G: 0 h/*M. oryzae*; H: 12 h/*M. oryzae*; I: 20 h/*M. oryzae*. Scale bar = 10 µm.

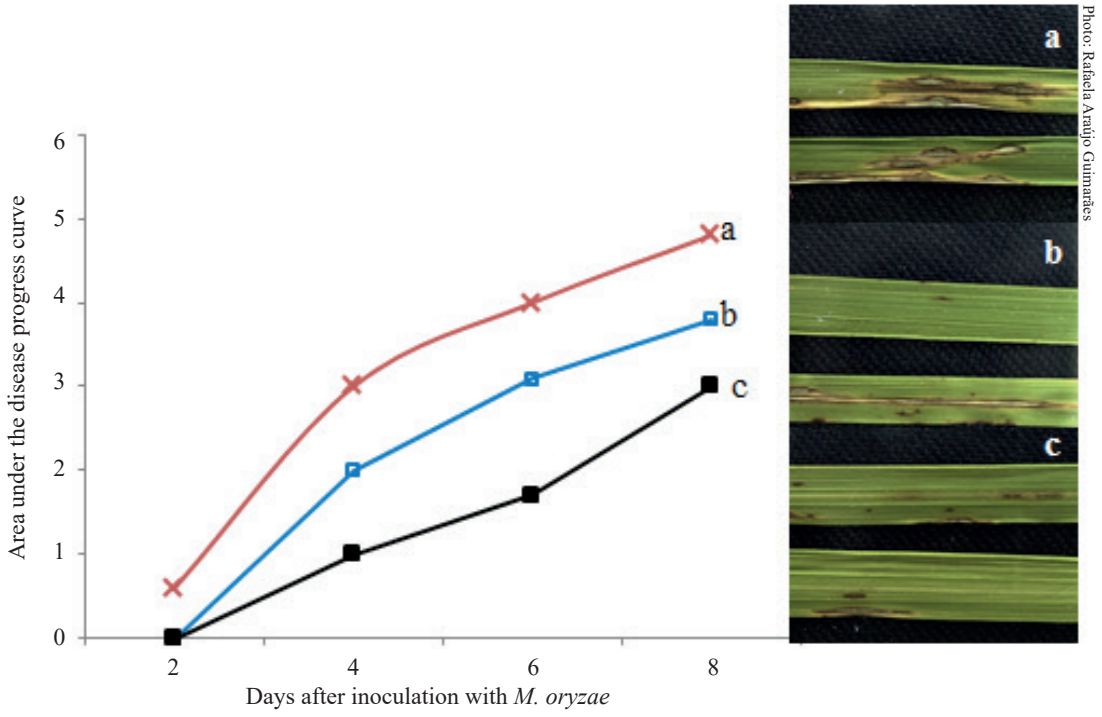

Figure 2. Area under the disease progress curve, where: *a* is the plant inoculated with *Magnaporthe oryzae* alone and *b* and *c* the plants sprayed with *Sarocladium oryzae* conidial suspension and BRM 6461 filtrate, at 48 h before the inoculation with *M. oryzae*, respectively. * Means differ according to the Tukey test ($p \leq 0.05$).

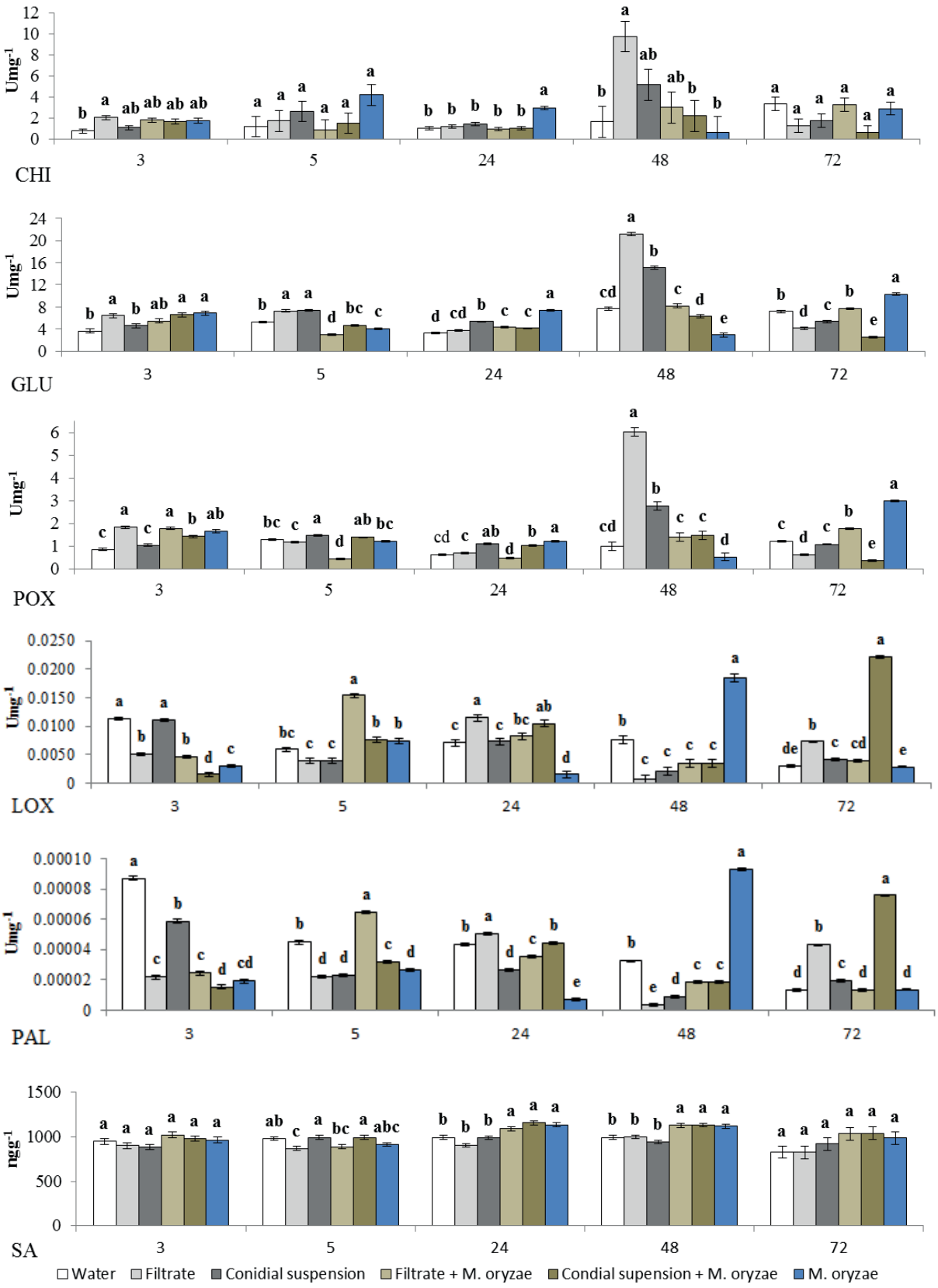

Figure 3. Enzyme activity and salicylic acid content at rice leaf collection times (3, 5, 24, 48 and 72 h after the inoculation with *M. oryzae*), where: CHI: chitinase; GLU: β-1,3-glucanase; POX: peroxidase; LOX: lipoxygenase; PAL: phenylalanine ammonia lyase; SA: salicylic acid. * Means followed by the same letter do not differ according to the Tukey test ($p \leq 0.05$).

Defense mechanisms are associated with metabolic changes such as alterations in key enzymes of the primary and secondary metabolism (Araújo & Menezes 2009), including: chitinase and β-1,3-glucanase, well known pathogenesis-related proteins (Van Loon et al. 2006); lipoxygenase, which inhibits pathogen growth, inducing the phytoalexin production (Wang 2004); phenylalanine ammonia lyase, which is involved in the phenylpropanoid synthesis (Hyodo et al. 1978), resulting in the synthesis of compounds such as phytoalexins and primary lignin, making the cell walls of plants more pathogen-resistant (Nakazawa et al. 2001); and peroxidase, which acts in the oxidation of phenolic compounds that are toxic to pathogens (Sutic & Sinclair 1991).

Hydroperoxide lyase is a reaction product of lipoxygenase that produces aldehydes responsible for inhibiting the growth of fungi, insects and protozoa on plant tissue. Hydroperoxide cyclase is also a lipoxygenase reaction product formed by lipid degradation that, after reduction and oxidation, produces jasmonic acid, which regulates growth and is also involved in the development of plant response to lesions and pathogens (Croft et al. 1993). Thus, spraying with cerulenin, a secondary metabolite composed primarily of fatty acids and steroids (Hittalmani et al. 2016), increased lipoxygenase levels and activated the defense of rice plants against *M. oryzae*, evidenced by the reduced disease severity.

Another enzyme responsible for the reduction in the rice leaf blast severity by *S. oryzae* was phenylalanine ammonia lyase, which produces phenolic compounds with antimicrobial potential, as well as some phytoalexins (Schuster & Rétey 1995). The phenylalanine ammonia lyase production is regulated during plant growth, but is also induced in cells adjacent to the infection site by different environmental stimuli, such as infection, injury, contamination by heavy metals, light and growth regulators (Rahman & Punja 2005). According to Baysal et al. (2003) and Macagnan et al. (2008), changes in the activity of key enzymes such as lipoxygenase and phenylalanine ammonia lyase make it possible to monitor induced resistance in plants, when exposed to pathogens.

Some studies have already demonstrated the activation of defense mechanisms in rice plants against blight by antagonists using bacteria and fungi. Sha et al. (2016) reported that *Bacillus subtilis* can activate these defense mechanisms by acting on the growth hyphae of *M. oryzae*, consequently reducing the length of the germinative tube, reflecting on the germination of conidia. These morphological changes compromise the *M. oryzae* infection in plants and increase the activity of some enzymes, such as peroxidase. Law et al. (2017) evaluated different species of *Streptomyces* in a greenhouse and also obtained success, reducing in up to 88.3 % the severity of the disease, a fact linked to the production of bioactive compounds by these strains. Some rhizobacteria, such as the *Pseudomonas fluorescens* genus, have been described by De Vleesschauwer et al. (2008), promoting the induction of systemic resistance due to the production of siderophores of the pseudobactin type. The same authors concluded that this interaction of *M. oryzae* with rice presents not only one, but multiple resistance pathways. Other studies using fungi as antagonists reported reduction in the rice leaf blast severity, such as those by Chaibub et al. (2016) and Sena et al. (2013), using *Cladosporium* sp. and *Epicoccum* sp., respectively. Both antagonists, when applied before the presence of *M. orzyae*, reduced the rice leaf blast severity by more than 70 % and increased levels of defense-related enzymes.

These results should be tested in the field for possible inclusion in integrated disease management, in order to reduce the use of fungicides in the control of rice leaf blast. But it is still necessary a better understanding of the defense mechanisms, as well as the metabolic pathways and the genes involved in the defense system of the rice plant, where *S. oryzae* can act in the protection against *M. oryzae*.

CONCLUSION

The *Sarocladium oryzae* BRM 6461 isolate, in filtrate form, composed primarily of the secondary metabolite cerulenin, delays the germination and inhibits the formation of *Magnaporthe oryzae* appressoria, besides reducing the rice leaf blast severity.

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