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RESEARCH ARTICLE

Potential for using crude extract of *Sarocladium oryzae* for suppression of rice blast

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ABSTRACT

Crude extract of an isolate of the fungus *Sarocladium oryzae* (CNPAF So 20G), containing the antimicrobial cerulenin, was produced and its antagonistic potential on the rice blast pathogen *Magnaporthe oryzae* was asessed. Cerulenin was detected in crude extract of *S. oryzae* through thin layer chromatography showing a R_f value of 0.63. The quantity of cerulenin in the crude extract was 237 $\mu g.mL^{-1}$. The *in vitro* inhibition of germination and appressorial formation of *M. oryzae* was assessed on an artificial hydrophobic surface, using eight different doses of cerulenin ranging from 0.05 to 30.0 $\mu g.mL^{-1}$. The LD_{50} values calculated based on the Probit-log analysis for germination and appressorial formation were 1.298 ± 0.123 $\mu g.mL^{-1}$ and 0.0705 ± 0.0062 $\mu g.mL^{-1}$ of cerulenin, respectively. The 30.0 $\mu g.mL^{-1}$ of cerulenin dose inhibited 98% and 99%, germination and appressorial formation, respectively. The mode of action of cerulenin was studied by staining conidia with Calcofluor White and fluorescent microscopy showing its effect on plasma membrane. Crude extract of *S. oryzae* suppressed 63% of rice blast disease in greenhouse conditions. The results indicate that a product based on cerulenin and/or *S. oryzae* has a great potential to be used in biological control of rice blast.

K**ey words:** *Magnaporthe oryzae*, *Oryza sativa, Sarocladium oryzae*, biological control, cerulenin.

INTRODUCTION

Magnaporthe oryzae B. Couch [anamorph *Pyricularia oryzae* (Cooke) Sacc.] is the causal agent of rice blast disease, one of the most devastating of all cereal disease worldwide. The major emphasis on blast disease control relies on genetic resistance, however the resistance durability is limited due to high genetic variability of the pathogen. Chemical control is another widely adopted measure to reduce grain yield losses in susceptible high yielding cultivars but it causes environmental damage and increases production cost (Khang & Valent, 2010). In addition, the knowledge on environment safe methods for disease control is limited.

The fungus *Sarocladium oryzae* (Sawada) W. Gams & D. Hawksw. is the causal agent of sheath rot disease and has been known to be antagonic against some microorganisms as a result of production of the antimicrobial metabolite cerulenin (Sakthivel & Gnanamanickam, 1986). Earlier studies on antagonism between *S. oryzae* and *M. oryzae* were based exclusively on inhibition of mycelial growth (Omura, 1986; Gnanamanickam & Mew, 1991; Prabhu et al., 2007), but it led to the recognition that antagonism against *M. oryzae* was less effective as compared to antagonism between *S. oryzae* and other rice pathogens (Gnanamanickam & Mew, 1991). On the other hand, information on lethal dosage (LD_{50}) of cerulenin on pre-infection stages of the pathogen such as germination and appressorial formation, its effects on structural integrity of *M. oryzae*, and the potential of using crude extract of *S. oryzae* as an alternative biocontrol agent to supress rice blast remains unknown.

The present investigation reports cerulenin activity from crude extract of *S. oryzae* on pre-infection stages of *M. oryzae*, and evaluation in greenhouse condition of the potential of using crude extract of *S. oryzae* as suppressor of rice blast..

MATERIAL AND METHODS

Microorganisms

The culture collection of Embrapa Arroz e Feijão, Santo Antônio de Goiás, Brazil, provided the strains of *Sarocladium oryzae* CNPAF So 20G and *Magnaporthe oryzae* CNPAF Py 435. *Candida albicans* (C.P. Robin) Berkhout ATCC 40006 strain was also used in this study.

Preparation of crude extract of *S. oryzae*

The isolate CNPAF So 20G of *S. oryzae* was grown in Petri dishes containing potato dextrose agar (PDA) and incubated at 25°C. After 10 days, a 5.0 mm diameter mycelium disc was transferred to a 500 mL flask containing 200 mL of the following sterile liquid culture medium (1.0% glucose, 3.0% glycerol, 0.5% peptone and 0.2% sodium chloride) and incubated for eight days under continuous shaking on an orbital rotary shaker (150 rpm) at 25ºC. The medium was then filtered through a layer of filter paper

to remove the mycelium resulting in a crude extract of *S. oryzae* (Omura, 1976).

Purification, detection and quantification of cerulenin in crude extract of *S. oryzae*

Cerulenin from crude extract was extracted with an equal volume of chloroform (analytical grade). The chloroform extract was concentrated under vacuum until the production of light yellow crystals and diluted in 2 mL of ethanol (analytical grade), resulting in a concentrated extract of cerulenin.

Cerulenin was detected in the concentrated extract of *S. oryzae* through thin layer chromatography (TLC) on silica gel G60 20 cm x 20 cm plates, previously activated at 130ºC for 25 minutes and spotted with cerulenin standard. Plates were developed with solvent system of diethyl ester-acetic acid (100:0.5). The molecules spots were detected by iodine vapor as follows: a five liter glass chamber, with cap, was assembled with 1.0 g of iodine crystals on the base with a filter paper covering. After five minutes the chamber was saturated with iodine vapor and the TLC plate was inserted. After another five minutes the TLC plate developed a light brown color over the entire plate and the molecule spots were observed.

Quantification of cerulenin in the crude extract was performed by its specific biological activity against *C. albicans* (Sakthivel et al., 2002). Five mm diam sterile paper discs containing different quantities of standard cerulenin [Sigma-Aldrich, USA (1.0-60.0 µg)] and a 10 µL sample of concentrated extract of *S. oryzae* were placed at the center of the Petri dishes containing 25 mL of PDA embedded with $1.0x10^5$ CFU.mL^{-1} of *C. albicans*. The plates were incubated at 25ºC for three days and the inhibition zones of yeast growth were measured with an electronic digital caliper. A standard assay curve was developed based on the toxicity of cerulenin towards *C. albicans* measured by the inhibition zones. Means of three replicates were used to plot the standard curve.

Effect of cerulenin-containing crude extract of *S. oryzae* on mycelial growth of *M. oryzae*

Petri dishes containing PDA with six concentrations (0.0, 0.15, 0.3, 0.7, 1.5 and 2.0 µg.mL^{-1}) of cerulenin from crude extract of *S. oryzae* were seeded with a 5 mm diameter disc of *M. oryzae* mycelium and incubated at 25ºC for seven days. Colony diam (mm) of *M. oryzae* was measured with electronic caliper rule. A completely randomized design with three replicates was used and the data were subjected to linear regression analysis.

Effect of cerulenin-containing crude extract of *S. oryzae* on conidial germination and appressorial formation of *M. oryzae*

Magnaporthe oryzae was grown on oatmeal agar for eight days at 25ºC. Conidia were obtained by scraping the surface of colonized medium with a sterilized glass rod under aseptic conditions and keeping the plates under fluorescent light with lids open but covered with a plastic sheet, at 25ºC for three days. Conidia were harvested by flooding culture plates with distilled water and by scrapping the colony surface with a paintbrush. The conidial suspension was filtered through double layer of cheesecloth and adjusted to a concentration of 1×10^5 conidia.mL^{-1} with a Newbauer Chamber.

Conidial germination and appressorial formation were assessed on an inductive artificial hydrophobic polystyrene layer. Eight doses (0.05, 0.10, 0.20, 1.0, 2.0, 10.0, 20.0 and 30.0 µg.mL^{-1}) of cerulenin obtained from crude extract of *S. oryzae* CNPAF So 20G and standard cerulenin were tested. Control did not receive any cerulenin or extract. A 20 µL solution of the different cerulenin doses were placed on hydrophobic surface of the polystyrene on a microscopic glass slide and gently mixed with 20 µL of *M. oryzae* conidial suspension (1×10^5 conidia.mL^{-1}). The material was incubated in moist chamber in a Petri dish. After 24 h of incubation at room temperature the percentage of conidia which germinated and formed appressorium was determined by counting 100 conidia per replicate. The experiment was conducted using a completely randomized design with four replicates. A Probit-log analysis of Finney (1975) was used for determining the LD_{50} value (the concentration necessary for 50% inhibition of germination or appressorial formation). The method was based on linear regression by plotting values of Probit as dependent variable and concentration as independent variable. The data were subjected to linear regression analysis and the differences between means were analyzed by *t*- test at 5% probability.

After evaluation of treatment containing 1.0 µg.mL^{-1} of cerulenin previously described, 50 µL of 0.01% (w/v) Calcofluor White was added to the inductive artificial hydrophobic polystyrene layer and after one hour of incubation at 25ºC, germination tube and appressorium formation was observed with a light microscope (Nikon Eclipse 80i) at 600× under fluorescent light.

Supression of rice blast using cerulenin-containing crude extract of *S. oryzae* in greenhouse conditions

An experiment was conducted in greenhouse conditions involving the rice cultivar Cica-1 grown in plastic trays (15 × 30 × 10 cm) containing 3 Kg of soil fertilized with NPK (5 g of 5-30-15) plus 1 g Zn and 3 g of ammonium sulfate immediately before planting day and 2 g of ammonium sulfate as top dressing 18 days after planting. The seeds were sown in eight 10 cm long rows per tray and thinned to 10 to 12 plants per row after germination. The bioassay was run as a completely randomized design with three replications.

Sarocladium oryzae crude extract was produced as described before. The content of cerulenin was adjusted to 100 µg.mL^{-1}. The solution was sprayed at twenty one-day old plants. One hour after spraying, the plants were inoculated with a *M. oryzae* conidial suspension (3×10^5 conidia.mL^{-1}) of isolate CNPAF Py 435 as described by

Filippi & Prabhu (2001). After inoculation, the plants were incubated in a humid chamber for 24 hours and transferred to greenhouse benches at temperatures varying from 27ºC to 30ºC and under high humidity until disease evaluation. The leaf blast evaluation was made seven days after inoculation using a diagrammatic disease scale (Notteghem, 1981). Data were submitted to analysis of variance using the program Statistical Package for the Social Sciences (SPSS), version 18.0.

RESULTS AND DISCUSSION

Detection and quantification of cerulenin in crude extract of *S. oryzae*

Cerulenin concentration in the crude extract was quantified as being 237 µg.mL^{-1}. The TLC analysis showed one or two spots for three samples studied indicating the presence of only two groups of molecules with similar polarity. The R_f (retention factor) of standard cerulenin and the sterile control culture media concentrated extract were 0.63 and 0.49, respectively. The concentrated extract from crude extract of *S. oryzae* showed two spots with R_f values equal to 0.49 and 0.63 which confirmed the specific production of cerulenin by the fungus *S. oryzae* (Figure 1A). Other almost imperceptible spots were also produced and probably represent the natural degradation of the target molecule under the presence of the organic solvent (Ghosh et al., 2002). It is important to note that the method selected for cerulenin production by *S. oryzae* that was described by Omura (1976) is acknowledged here as simple, cost-effective and safe.

Effect of cerulenin-containing crude extract of *S. oryzae* on mycelial growth of *M. oryzae*

Cerulenin produced by *S. oryzae* inhibitted the mycelia growth of *M. oryzae*. There was a decrease in radial mycelium growth of *M. oryzae* as the concentration of cerulenin increased ($F = 511.3$, $p < 0{,}001$, $R^2 = 0.9765$) (Figure 1B). The mycelia growth was inhibited at the concentration of 2.0 µg.mL^{-1} by 79% in relation to the control treatment. Growth inhibition at the concentrations 0.15, 0.3, 0.7, 1.5 µg.mL^{-1} was of approximately 10%, 21%, 50% and 68%, respectively. These results are in agreement with those obtained by Gnanamanickam & Mew (1991) in relation to growth inhibition of *M. oryzae* by *S. oryzae* involving pure cerulenin.

Effect of cerulenin-containing crude extract of *S. oryzae* on conidial germination and appressorial formation of *M. oryzae*

Different doses of cerulenin affected the germination and appressorial formation of *M. oryzae* differently (Figure 2). The highest dose (30.0 µg) of cerulenin inhibited germination and appressorial formation by 98% and 99%, and at the lowest dose (0.01 µg) inhibition was 3% and 33%, respectively, in relation to control without cerulenin.

The LD_{50} values for inhibition of germination and appressorial formation of *M. oryzae*, calculated based on the Probit-log analysis were 1.298 ± 0.123 µg ($X^2 = 5.50$, g.l.= 6, $\alpha = 0.05$) and 0.0705 ± 0.0062 µg ($X^2 = 3.57$, g.l.= 6, $\alpha = 0.05$) of cerulenin at a concentration of $1x10^5$ conidia.mL^{-1} of *M. oryzae* under the experimental conditions, respectively. There was no difference between the doses mean responses for the two treatments (concentrated extract of cerulenin from *S. oryzae* versus standard cerulenin) according to *t*-test analysis, indicating that cerulenin present in the concentrated extract of *S. oryzae* is the unique responsible for the inhibition of *M. oryzae* (Figure 3A and Figure 3B).

The results in the present study showed that the sensitivity of conidial germination and appressorial formation to cerulenin was greater than the inhibition of mycelial growth . The great sensitivity of *M. oryzae* during

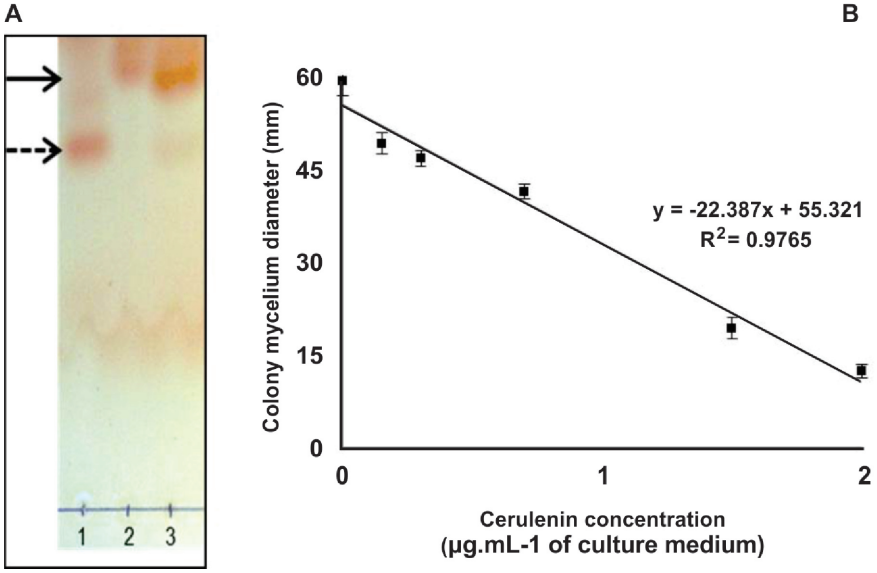


FIGURE 1 - A. Thin layer chromatography profiles. 1) control containing only sterile culture media extract, 2) standard cerulenin, 3) concentrated extract (purified fraction of cerulenin) from crude extract of *Sarocladium oryzae*. Continuous arrow indicates cerulenin molecule (R_f = 0.63) and dashed arrow indicates culture media extract (R_f = 0.49). **B**. *In vitro* inhibition of mycelial growth of *Magnaporthe oryzae* by cerulenin-containing crude extract of *S. oryzae*.

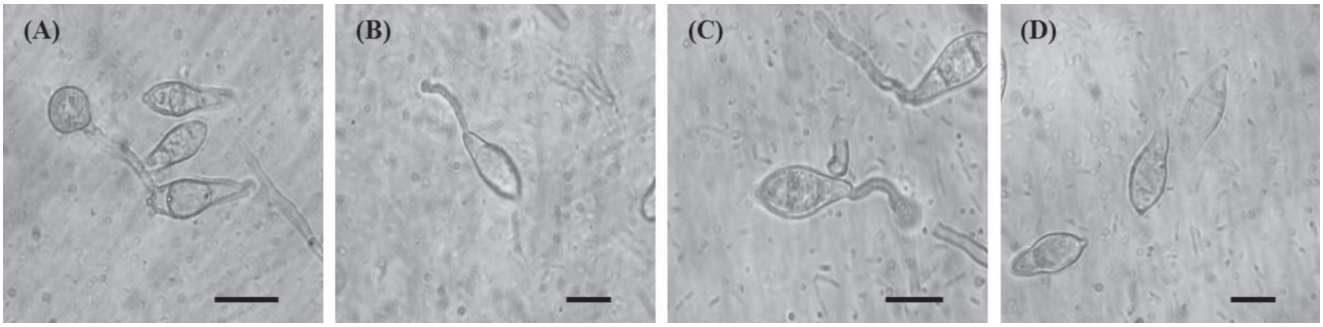
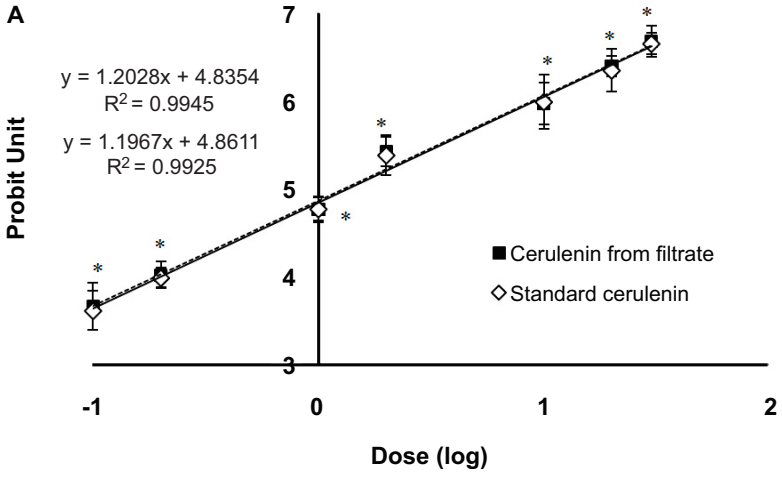


FIGURE 2 - Conidial germination and appressorial formation of *Magnaporthe oryzae* treated with cerulenin (1.0 µg.mL^{-1}) produced by *Sarocladium oryzae* under optical microscope (600x). **A**. Germinated conidia with appressorium on hydrophobic surface, free of cerulenin, after 24 hours. **B**. Conidia showing germination tube, without apressorium, treated with cerulenin, after 24 hours. **C**. Conidia with germination tube and defective appressorium, treated with cerulenin, after 24 hours. **D**. Conidia without germination tube treated with cerulenin, after 24 hours. Scale bar = 10 µm.



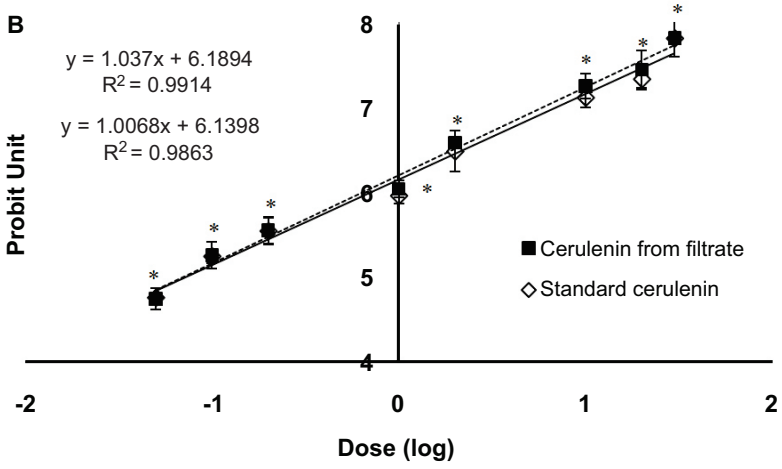


FIGURE 3 - Probit-log dosage curves showing the effect of cerulenin concentrations on **(A)** the germination and **(B)** the appressorial formation of *Magnaporthe oryzae.* Means followed by asterisks do not differ statistically according to *t*-test at 5% probability. The continuous lines represent standard cerulenin and the dashed ones represent the cerulenin from crude extract of *S. oryzae*.

appressorium formation to cerulenin, indicates the high potential of *S. oryzae* as a biological control agent since this is the key step for successful infection by *M. oryzae.*

Cerulenin is previously known to be a potent inhibitor of enzymes involved in fatty acid synthesis in a range of microorganisms (Omura, 1976). This group of enzymes is responsible for the production of lipids, which are vital components of cell plasma membrane (Campbell & Cronan, 2001; Oh et al., 2008). The damage caused by cerulenin to plasma membrane could be detected indirectly by staining the fungus cell wall structures using Calcofluor White reagent under fluorescent optical microscopy. Calcofluor White specifically stains the chitin of cell wall (Dolan & McNicol, 1986) generating fluorescence. Since chitin is synthesized by enzymes embedded in the plasma membrane (Chaffin et al., 1998), whatever alteration in plasma membrane will have an effect on cell wall chitin structure. Consequently, the cell wall damage will be evident by generating lower intensities of fluorescence compared to undamaged cell wall. The damaging effect of cerulenin molecule from *S. oryzae* on the formation of fungal infective apparatus structures, which are essential for infection and disease establishment, is shown for the first time using this technique (Figure 4).

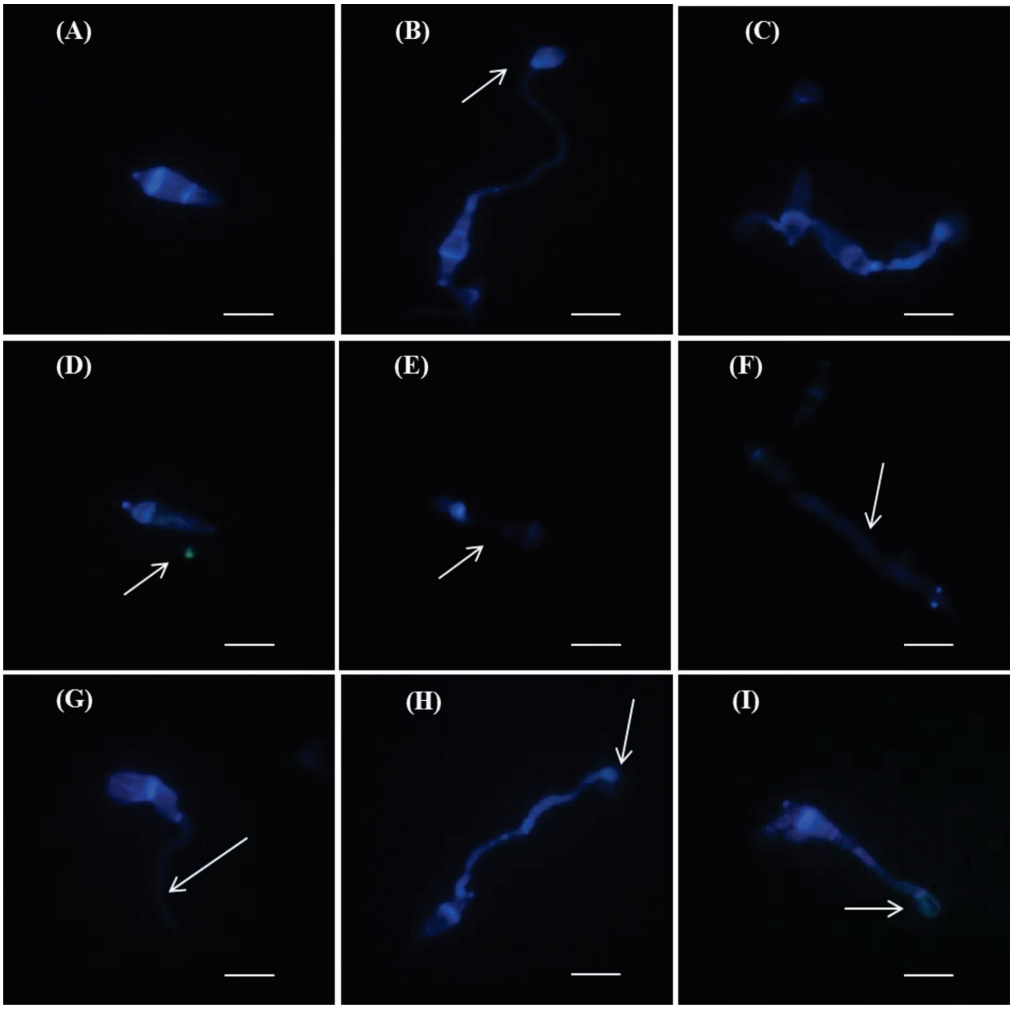


FIGURE 4 - Conidial germination and appressorial formation of *Magnaporthe oryzae* treated with cerulenin (1.0 µg.mL^{-1}) produced by *Sarocladium oryzae* under fluorescent light optical microscope (600x). Conidia were stained with 0.01% (w/v) Calcofluor White (Sigma). **A**. Non-germinated conidia on hydrophobic surface free of cerulenin, at time zero. **B, C**. Germinated conidia showing induction of appressorial formation on hydrophobic surface, free of cerulenin, after 24 hours. **D, F**. Conidia showing abnormal germination tube treated with cerulenin, arrows point towards fragments of germination tube and defective conidia, after 24 hours. **G**. Defective germination tube submitted to cerulenin treatment, after 24 hours. **H, I**. Germinating conidia with germination tube apparently without damage, but with deformed appressorium treated with cerulenin, the arrows points towards defective appressorium, after 24 hours. Scale bar = 10 µm.

The normal structures of conidia, germ tube and appressorium of *M. oryzae* in the absence of cerulenin (control) can be observed in Figures 4A, B and C. Also, two types of deformities caused by the action of cerulenin on tube germination can be seen in Figures 4D, F and G. Additionally, Figure 4F shows an abnormally thick and wide germ tube formed probably due to defects in the cellular plasma membrane caused by the action of cerulenin. The appressorium was ,however, normally formed.

Less intensity of fluorescence emission was observed in the cell wall structure of the germ tube , due to the deformation of cellular plasma membrane, but not on appressorial formation (Figures 4D and G). The superficial cell structure of conidia was also affected in the presence of cerulenin (Figures 4D, E and F). In the presence of cerulenin, the structural modification resulted in deformation of the conidial primordium. It is also evident from Figures 4H and 4I that the formation of the appressorium is incomplete due to the action of cerulenin.

Supression of rice blast using cerulenin-containing crude extract from *S. oryzae* in greenhouse conditions

The difference between leaf blast severity in *S. oryzae* extract-treated plants and control was significant (Table 1 and Figures 5A and B). The cerulenin-containing crude extract of *S. oryzae* (100 µg.mL^{-1}) reduced blast severity at approximately 63%. Figure 5C also shows images of rice leaves sprayed only with crude extract of *S. oryzae* (cerulenin 100 µg.mL^{-1}). No phytotoxic effect of cerulenin was observed on rice leaves (chlorotic lesions) under the experimental conditions used in this work, differently to observed by Sakthivel (2002).

In conclusion, the present study demonstrated that cerulenin present in crude extract, *S. oryzae*, affected the pre-infection structures of *M. oryzae* such as germination tubes and appressoria. The inhibition of germination and the reduction in number of conidia with normal appressoria, besides the formation of conidia with defective appressoria, can result in less percentage of viable conidia and successful penetration of the leaf surface resulting in suppression of rice blast. Therefore, cerulenin-producing *S. oryzae* has a great potential to be used as a bio-fungicide for blast control. The ease with which it can be produced by fermentation in liquid phase at low cost justifies further studies on scale-up production, formulation development and field for rice blast control.

TABLE 1 - Efficiency of crude extract (cerulenin 100 µg.mL^{-1}) from *Sarocladium oryzae* on leaf blast suppression.

Treatments	*LBS (%)* [1]	*LBS Standard Error*
Control	27.03	± 2.39
Crude extract (cerulenin 100 µg.mL^{-1})	9.80	± 1.08

[1]Percentage leaf area affected using a diagrammatic scale, according to Notteghem (1981). The analysis of variance differenced statistically control versus crude extract ($p < 0.001$).

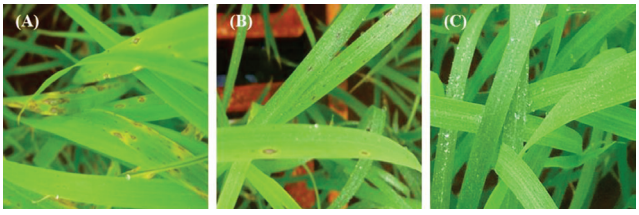


FIGURE 5 - Suppression effect of the application of crude extract of *Sarocladium oryzae* (containing cerulenin 100 µg.mL^{-1}) on rice blast suppression on leaves, seven days after inoculation. **A.** Control - plants inoculated with conidial suspension (3 x 10^5 conidia.mL^{-1}) of isolate CNPAF Py 435 of *M. oryzae*. **B.** Plants treated with crude extract (cerulenin 100 µg.mL^{-1}) and inoculated with a 3 x 10^5 conidia.mL^{-1}conidial suspension (isolate CNPAF Py 435 of *M. oryzae*) and **C.** Plants sprayed with the crude extract (cerulenin 100 µg.mL^{-1}) only.

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