Tropical Plant Pathology, vol. 38(5):387-397, 2013

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RESEARCH ARTICLE / *ARTIGO*

Increased enzymatic activity in rice leaf blast suppression by crude extract of *Epicoccum* sp.

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ABSTRACT

Epicoccum sp. showed *in vitro* antagonism to the rice pathogens *Magnaporthe oryzae*, *Rhizoctonia solani*, *Sarocladium oryzae*, *Monographella albescens* and *Cochliobolus miyabeanus* in dual culture. The colony growth of the rice blast fungus, *M. oryzae*, was reduced by 42.50%. The lethal doses (LD_{50}) determined based on probit-log dosage response curves at 3 and 6 hours after germination were 393.0 and 326.6 ppm, respectively. The crude extract (600 ppm) reduced appressorial formation by 95.68%. A greenhouse experiment comparing the relative efficiency of conidial suspension and crude extract of *Epicoccum* sp. in the suppression of leaf blast showed no statistical difference between both application methods. However, the crude extract of *Epicoccum* sp. (4000 ppm) 48 hours before the application of *M. oryzae* induced resistance and suppressed leaf blast by 97.6%. Scanning electron microscopy of rice leaves inoculated with crude extract of *Epicoccum* sp. and challenged with *M. oryzae* showed appressorial deformation on penetration phase. Peroxidase and β-1,3-glucanase activities increased in plants sprayed with crude extract of *Epicoccum* sp., 24 hours after the application of the challenger. PAL as well as chitinase activities increased 72 hours after challenge inoculation. *Epicoccum* sp. was shown to be a potential antagonist and inducer of resistance against *M. oryzae*.

Key words: *Epicoccum* sp., *Magnaporthe oryzae*, *Oryza sativa*, antagonism, ISR, PR proteins

INTRODUCTION

Brazil is the major upland rice producer in the world. This cereal is very important because more than half of the world population depends on rice as a staple food (FAO, 2011). Rice blast [*Magnaporthe oryzae* B. Couch, anamorph *Pyricularia oryzae* Cavara] is reported in all rice growing regions in the world and grain yield losses due to this disease may reach up to 100% (Prabhu et al., 2009; Fisher et al., 2012). Under tropical climatic conditions, combined with pathogen biology and limited durability of cultivar resistance, growers resort to intensive application of agricultural chemicals which result in environmental damage such as soil and water contamination, destruction of non-target microorganisms, human health hazards, selection of new pathotypes, besides increased production costs (Ramos, 2009).

Currently, plant disease biocontrol is considered economically viable because it reduces the negative impact on the environment and the risk of altering food integrity when compared with chemical control. Therefore, research on biological control is currently receiving greater attention as it is being considered as one of the main components to be included in integrated disease management for sustainable agriculture (Lahlali & Hijri, 2010). According to the model proposed by McSpadden-Gardener & Fravel (2002), the first line of defense in disease and pest management includes good agricultural practices, whereas the use of resistant cultivars developed by either traditional breeding or genetic engineering constitutes the second line of defense. However, these complementary measures for promoting economically sustainable agriculture must include biocontrol practices for diseases (Pal & McSpadden-Gardener, 2006).

Among the biological control agents, the fungus *Epicoccum* sp. of the order Moniliales and family Dematiaceae (Barnett & Hunter, 2006) is registered as an antagonist for *Monilinia* sp. in peach and nectarine (De Cal et al., 2009), *Sclerotinia sclerotiorum* in sunflower and dry beans (Huang et al., 2000; Pieckenstain et al., 2001), *Monilinia fructicola* in plums and apples (Wittig et al., 1997; Falconi & Mendgen, 1994), *Erysiphe cichoracearum* in lady's fingers (Derbalah et al., 2011), *Colletotrichum gloesporioides* in guava (Pandey et al., 2007), *Rhizoctonia solani* in tomato (Lahlali & Hijri, 2010), *Macrophomina phaseolina* and *R. solani* in soybean (Hashem, 2004), *R. solani* and *Fusarium sambuciunum* in potato (El-Kot, 2008). Also, its efficiency as antagonist to Oomycetes such as *Pythium* in *Vicia faba*, *Vicia unguiculata, Lupinus termis* (Koutb & Ali, 2010), *Gossypium hirsutum* (Hashem & Ali, 2004), *Plasmopora viticola* in *Vitis vinifera* (Kortekamp,

1997), and phytoplasm in *Catharanthus roseus* (Musetti et al., 2011) has been reported. Out of 189 fungal isolates obtained from rice phylloplane, 17 fungi were identified of which *Epicoccum* sp. (EP06) was the most efficient as an antagonist (Sena, 2012).

These biocontrol agents can act directly or indirectly by induction of defense mechanisms in plant diseases control (Doohan, 2005). The use of biotic and abiotic resistance inducers is one of the major strategies for increasing durability of disease resistance and to reduce toxic residues produced by the indiscriminate use of agricultural chemicals (Filippi et al., 2011). Studies on induction of acquired systemic resistance (SAR) and induced systemic resistance (ISR) in rice plants to *M. oryzae* were conducted after the application of avirulent isolates of *M. oryzae*, *Bipolaris sorokiniana* and rhizobacteria (Smith & Métraux 1991; Manandhar et al., 1998; Tsukamoto et al., 1999; Ashizawa et al., 2005; Filippi et al., 2007, 2011).

During SAR and ISR pathways key enzymes are expressed which are related to the metabolism of phenylpropanoids, such as phenylalanine ammonia lyase, peroxidases, polyphenoloxidases and pathogenesis-related (PR) proteins (Yoshikawa et al., 1983). Some examples of PR proteins related to SAR are β-1.3-glucanase and chitinase, beloning to the PR-2 and PR-3 families, respectively (Van Loon and Pieterse, 2006). However, the duration of protection by SAR is shorter compared to the protection mediated by agents of ISR (Wei et al., 1991).

Studies on the interaction between *M. oryzae* in rice and *Epicoccum* sp., and the mechanisms of *in vivo* biological control are important for the adoption of adequate control measures for rice blast which are ecologically sustainable (Filippi et al., 2007). The knowledge of the mode of action and mechanisms involved in disease reduction by potential biocontrol agents such as *Epicoccum* sp. is limited. The present study reports the potential of crude extract of *Epicoccum* sp. in leaf blast suppression and elucidates the involved mechanisms on the basis of enzymatic activity.

MATERIAL AND METHODS

Antagonism of *Epicoccum* sp. against rice pathogens

Epicoccum sp. was isolated by Sena (2012) from the phylloplane of BRS Cateto rice cultivar from a comercial organic field, at Hidrolândia, GO. The following rice pathogens were used in antibiosis tests: *M. oryzae*; *R. solani* (sheath blight); *Sarocladium oryzae* (sheath rot); *Monographella albescens* (leaf scald) and *Cochliobolus miyabeanus* (brown spot), all obtained from the culture collection of Embrapa Arroz e Feijão. These isolates as well as the *Epicoccum* sp. isolate EP06 were transferred to Petri plates containing potato-dextrose-agar (PDA) and maintained at 25°C for four days under fluorescent light to obtain mycelial discs for antibiosis test. The experiment was conducted utilizing the dual culture technique (Romeiro, 2007). Mycelial discs 5 mm in size of each pathogen and *Epicoccum* sp. were placed in opposite sides of a plate containing PDA, maintaining a distance of 3.0 cm. The bioassay was run in a completely randomized design with three replications. Three plates for each pathogen without antagonist were used as control.

The evaluations were made 12 days after incubation under continuous fluorescent light at 25°C, measuring the colony diameter in horizontal and vertical directions of the pathogens and the isolate of *Epicoccum* sp. Also, the reduction in radial growth (the horizontal colony diameter times the vertical colony diameter) was calculated as (mean value of radial growth of the pathogen in the presence of the antagonist x 100/radial growth of control)-100.

Effect of crude extract of *Epicoccum* sp. on conidial germination and appressorial formation by *M. oryzae*

Crude extract preparation

The isolate of *Epicoccum* sp. was multiplied on PDA and after 18 days the colonies were cut and ground in a beaker with 100 mL of ethanol 95%. The suspension was filtered using a funnel containing filter paper (Whatman no. 1) for recovering part of the liquid in a Falcon tube. The liquid was centrifuged for 10 minutes at 500 rpm and the supernatant was concentrated by evaporation under low pressure utilizing a roto-vaporizer II at 40°C under vacuum, and later submitted to lyophilization for obtaining crystals of *Epicoccum* sp.

Preparation of conidial suspension of M. oryzae and Epicoccum sp.

The isolate of *M. oryzae* (Py 4075), showing compatible reaction to the cultivar Primavera, obtained from the Microorganism Multifunction Collection of Embrapa Arroz e Feijão, was multiplied in Petri dishes containing oat meal-dextrose-agar and incubated under continuous fluorescent light at 25-27°C for mycelial growth. After 14 days of incubation, the pathogen was subjected to physical stress by scraping the mycelium with a sterilized glass rod. The plates were transferred to a growth chamber at 25-27°C and were covered with a transparent plastic sheet under continuous fluorescent light for 48 to 72 hours. The inoculum was prepared by flooding the plates with sterile distilled water and removing the conidia with a paint brush. The conidial suspension was filtered through a sterilized cheesecloth and the concentration adjusted to 3 x 10^5 conidia mL^{-1} with a Newbauer haemocytometer.

The antagonist *Epicoccum* sp. was grown in Petri dishes containing PDA and incubated for 25 days at room temperature. The conidial suspension was prepared by flooding the plates with sterile distilled water and scraping mycelial fragments and conidia with a paint brush. The suspension obtained was filtered through cheesecloth and the final conidial suspension was adjusted to the desired concentrations.

Inhibition of conidial germination and appressorial formation

Germination of conidia and formation of appressoria were induced on the hydrophobic side of parafilm (Filippi, 2004). The parafilm, previously surface sterilized with sodium hypochloride followed by 70% ethanol, was placed on microscopic slides in each Petri dish containing two layers of moistend filter paper to maintain high humidity. A standard solution containing 80 mg of crude extract was diluted in sterile water to obtain six different concentrations (500, 2000, 4000, 6000 and 8000 ppm), in addition to a water control. Twenty microliters of conidia (1 x 10^5) and crude extract of *Epicoccum* sp. was dispensed on the hydrophobic surface of the parafilm. The bioassay was run as a completely randomized design with four replications. Conidial germination was assessed after three and six hours and appressorial formation after 12 and 20 hours of incubation at 25 ºC. The percentage of germinated conidia and formed appressorium were determined by microscopic examination of 100 conidia per replicate.

Relative efficiency of conidial suspension and crude extract of *Epicoccum* sp. in suppressing rice leaf blast

Planting

The experiment was conducted using cultivar Primavera in plastic trays (15 x 30 x 10 cm) containing 3 kg of soil fertilized with NPK (5 g of 5-30-15 NPK mix + 1 g of Zn and 3 g of ammonium sulfate at planting day). The seeds were sown in eight 10 cm-rows per tray and thinned to 10 to 12 plants per row after germination. Top dressing was done 20 days after seeding with 2 g of ammonium sulfate per tray. The bioassay was run as a completely randomized design with three replications.

Treatments

The experiment was designed to compare rice leaf blast suppression by conidial suspension and crude extract, applied 48 hours before spraying the plants with challenger or sprayed as a mixture of *Epicoccum* sp. (EP06) and *M. oryzae* (M.o.). The different concentrations were based on previous exploratory experiments which gave most promising results in the suppression of leaf blast. The following treatments were included: T1 = Crude extract of EP06 (2000 ppm) applied 48 hours before challenge inoculation; T2 = Crude extract of EP06 (4000 ppm) applied 48 hours before challenge inoculation; T3 = Conidial suspension of EP06 (5 x 10^5 con mL^{-1}) applied 48 hours before challenge inoculation; T4 = Conidial suspension of a mixture of EP06 (10 x 10^5 con mL^{-1}) and M.o. (3 x 10^5 con mL^{-1}); T5 = Conidial suspension of a mixture of EP06 (10 x 10^1 con mL^{-1}) and M.o. (3 x 10^5 con mL^{-1}); T6 = Crude extract of EP06 (2000 ppm) mixed with conidia of M.o. (3 x 10^5 con mL^{-1}); T7 = Crude extract of EP06 (4000 ppm) mixed with conidia of M.o. (3 x 10^5 con mL^{-1}); T8 = Control (M.o., 3 x 10^5 con mL^{-1}).

Twenty one-day old plants were inoculated with conidial suspension of *M. oryzae*, according to the method of Filippi and Prabhu (2001). Following inoculation, the plants were incubated in plastic moist chambers for 24 hours and transferred to greenhouse benches at 27- 30ºC and high humidity until disease evaluation.

Rice leaf blast evaluations were made starting from the appearance of the first lesion using a percentage disease scale according to Notteghem (1981). The disease severity assessed at 2-day intervals was based on 24 rice plants per trial for AUDPC determination.

Induction of disease resistance by crude extract of *Epicoccum* sp. and enzymatic activity

Induction of disease resistance

Another greenhouse experiment was conducted to study the enzymatic activity and induced resistance by crude extract of *Epicoccum* sp. (4000 ppm) sprayed 48 hours before the application of challenger *M. oryzae*. The concentration and application timing were based on significant leaf blast suppression obtained in the previous experiment. Planting, preparation of inoculum of *M. oryzae* and crude extract of *Epicoccum* sp. were done as described above. The cultivar Primavera was planted in three plastic trays each of the following treatments: T1 = Crude extract of *Epicoccum* sp.; T2 = Crude extract of *Epicoccum* sp. sprayed 48 hours before *M. oryzae* and T3 = *M. oryzae*. The inoculation and evaluation was done as described above.

Enzymatic activity

Enzymatic activities of peroxidase, PAL and PR proteins (β-1,3-glucanase and chitinase) were quantified. The sample for analysis was based on the third leaf of 15 rice plants for each treatment. The first collection was done before the inoculation with challenger whereas the second, third, fourth, fifth, sixth and seventh were performed at 24, 48, 72, 96, 120 and 144 hours after inoculation of *M. oryzae*, respectively. The tests were performed in triplicates. One active enzymatic unit (1.0 U) was defined as variation of absorbance per hour at 25ºC and pH 9.0. The specific activity (U mg^{-1}) was calculated as the ratio between enzymatic activity previously defined in units (U) and protein content quantified in each sample (mg). Activity was expressed in units (U) mg^{-1} protein. For PR proteins, one U was defined as the enzyme activity catalyzing the formation of reducing sugar that increases the absorbancy by 1 unit per hour.

Protein extraction: A sample of five leaves was ground to a fine powder in liquid nitrogen. The buffer solution was composed of 10 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA, 2 mM DTT, 1 mM PMSF, leptin (10 µg mL^{-1}) and aprotinin (10 µg mL^{-1}). Protein determination was according to Bradford (1976).

β-1,3-glucanase activity (EC 3.2.1.6): Activity of β-1,3-glucanase was assayed by measuring the rate of reducing sugar production using laminarin (Sigma) as the

substrate (Pan et al., 1991). DNS reagent was used as the colorimetric agent.

Chitinase activity (EC 3.2.1.14): Chitinase activity was assayed by a modified method of Pan et al. (1991). The rate of N-acetyl glucosamine production was measured using colloidal chitin as the substrate. The 3,5-dinitrosalicylic acid (DNS) reagent was used as the colorimetric agent.

Peroxidase activity (EC 1.11.1.7): Peroxidase activity was assayed by measuring the rate of 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) oxidation, using its own colorimetric property. One unit was defined as the enzyme activity catalyzing the formation of 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) that increases the absorbancy by 1 unit per hour (Keesey, 1987).

Phenylalanine ammonia-lyase (EC 4.3.1.5): The determination of PAL activity was performed utilizing 2 mL of a 10 mM phenyalanine suspension in 0.1 M borate buffer pH 9.0, and 50 µL of plant protein extract. The mixture was homogenized and submitted for quantification of the product in spectrophotometer (Fento 600 Plus) at 290 nm.

Scanning electron microscopy

Leaves of rice plants of all three treatments were collected 24 hours after inoculation of challenger *M. oryzae* for scanning electron micrograph (SEM). The leaf bits were placed in microtubes containing Karnovsky fixative (glutaraldehyde and 2% isofar in combination with 2% formaldehyde), and later in cacodylate buffer (pH 7.2 plus sucrose 3%) for 24 hours at 23°C. Later, the samples were washed three times in 0.1M cacodylate buffer for 10 minutes each time, dehydrated in an ethanol series (70, 80, 90 and 99.1%), and submitted to critical point drying with liquid CO_2 (Autosamdri 815) during 40 minutes. The dry specimen was mounted on stubs using double-sided adhesive tape. The stubs were aluminum discs of 2.5 cm diameter and 0.8 cm thick which were previously sterilized with acetone and isopropylene alcohol. Later, the stubs were submitted for two minutes for the deposition of ultrathin films of gold using Denton Vaccum, Desc VA. After sputter-coated with gold, these stubs containing the samples were taken for observation in a Jeol JSM-6610 scanning electron microscope equipped with EDS.

Statistical analysis

The data on antagonism of *Epicoccum* sp. against rice pathogens, suppression of leaf blast by conidia of *Epicoccum* sp., conidial germination and formation of appressoria by crude extract of *Epicoccum* sp., induction of resistance by crude extract of *Epicoccum* sp. and enzymatic activity in plants challenged with the pathogen were submitted to analysis of variance using the Statistical Package for the Social Sciences (SPSS), version 18.0. Means were compared by Duncan's test ($P<0.05$). Leaf blast severity data in percentages were transformed to arc sine $\sqrt{x}+0.5$.

The lethal dose for conidial germination expressed in relation to the concentration of crude extract (ppm) that caused 50% inhibition (LD_{50}) was determined. The method of probit-log dosage analysis according to Finney (1975) was utilized for determining the LD_{50} assuming that the tolerance of conidia to concentrations of crude extract has a normal distribution. The method consists in estimating the linear regression of the probit value, corresponding to the percentage inhibition of conidia on log concentration of crude extract. The percentage conidial inhibition was obtained in relation to the germination of the control treatment.

RESULTS

Antagonism of *Epicoccum* sp. against rice pathogens

All rice pathogens tested using dual culture with *Epicoccum* sp. showed reduction in mycelial growth in relation to their respective controls. They also showed significant differences among the test pathogens in relation to inhibition percentage (Figure 1). The antagonism of *Epicoccum* sp. against *M. oryzae* was 42.50%.

Inhibition of conidial germination and appressorial formation of *M. oryzae* by crude extract of *Epicoccum* sp.

The percentage of germination and appressorial formation were significantly reduced in all six concentrations of crude extract in relation to control. The probit-log dosage curves for conidial germination are presented in Figure 2. The LD_{50} values were 393.0 and 326.6 ppm for 6 and 12 hours, respectively. The high percentage of conidial germination inhibition even after 6 hours shows that the crude extract contains a potential anti-fungal agent. The germinated conidia that formed appressoria at 12 and 20 hours after induction were reduced by 97.7% and 95.68%, respectively, with a crude extract concentration of 6000 ppm, compared with the control (Figure 3A, B).

Relative efficiency of conidial suspension and crude extract of *Epicoccum* sp. in the suppression of leaf blast

The results of the experiment are presented in Table 1. Treatment differences were significant in relation to leaf blast severity as well as AUDPC compared with the control. The crude extract was as efficient as conidial suspension in reducing leaf blast.

In relation to timing of application, conidial suspension or crude extract applied 48 hours before the application of challenger *M. oryzae* suppressed leaf blast. There was, however, no statistical difference between the application 48 hours before the challenger and simultaneous application as a mixture. The application of crude extract at 4000 ppm, 48 hours before the application of *M. oryzae* showed AUDPC of 1.1 compared to 45.0 in the control, which corresponds to 97.6% reduction in leaf blast.

Based on these results, the concentration of 4000 ppm of crude extract of *Epicoccum* sp. and the application

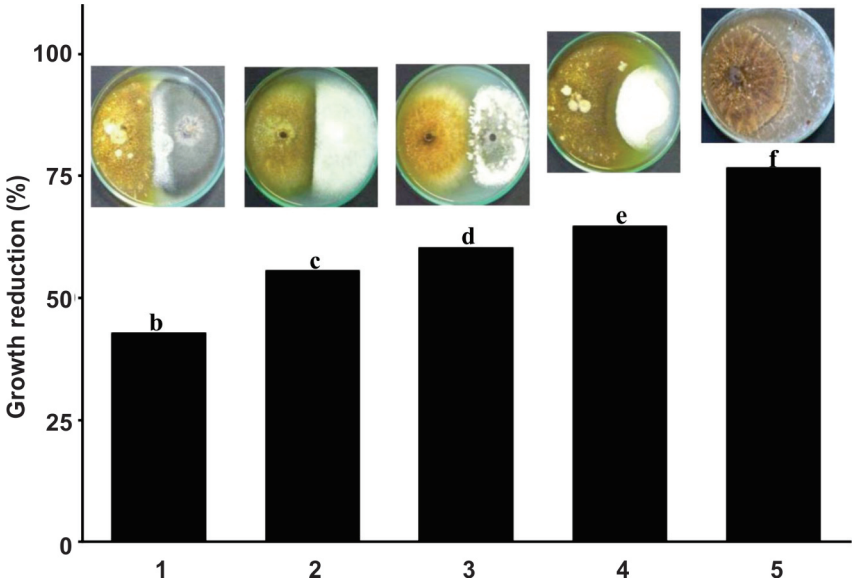

FIGURE 1 - Mycelial growth reduction (%) of plant pathogens of rice in dual culture with *Epicoccum* sp., after 12 days of growth on PDA. **1.** *Magnaporthe oryzae*; **2.** *Monographella albescens*; **3.** *Cochliobolus miyabeanus*; **4.** *Sarocladium oryzae*; **5**. *Rhizoctonia solani*. Columns followed by the same letter do not differ according to Duncan's test ($P<0.05$). The controls did not show any reduction in growth.

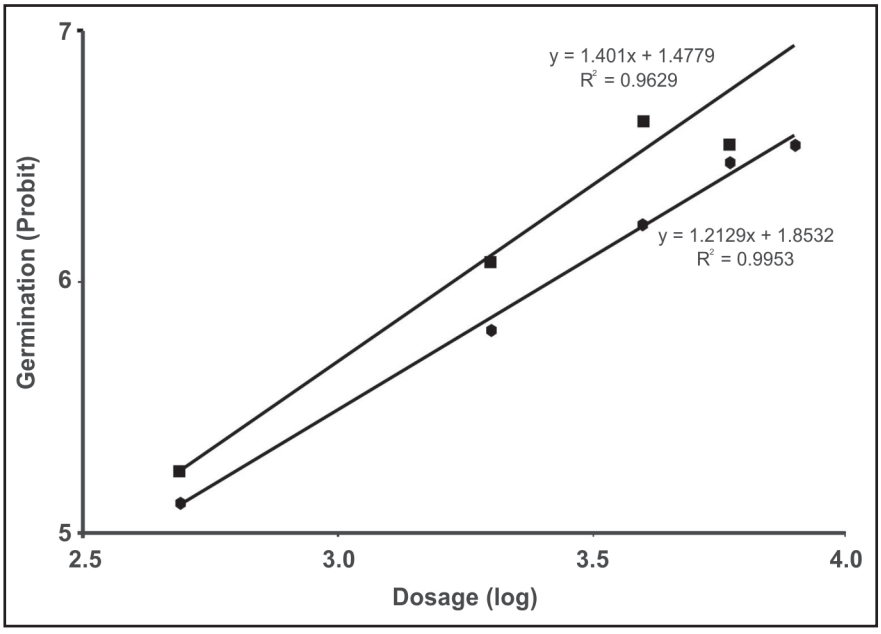

FIGURE 2 - Effect of crude extract of *Epicoccum* sp. on conidia germination of *Magnaporthe oryzae* after three (diamond; LD_{50}= 393.0 ppm) and six hours (square; LD_{50}= 326.6 ppm).

timing of 48 hours before spraying the challenger *M. oryzae* were utilized in further studies.

Induction of resistance by crude extract of *Epicoccum* sp., enzymatic activity and scanning electron microscopy

The application of crude extract of *Epicoccum* (4000 ppm) in rice plants 48 hours before spraying the challenger (M.o.) reduced leaf blast severity assessed at 2-day interval up to 9 days, in relation to plants sprayed only with *M. oryzae*. No disease symptoms were evident in plants sprayed with *Epicoccum* sp. (Figure 4).

Spraying rice plants with crude extract of *Epicoccum* sp. significantly increased the activity of PAL and chitinase 96 hours after the application of challenger. PAL activity increased in plants challenged as well as induced and challenged 48 and 72 hours after inoculation with M.o.. Chitinase activity increased only 72 hours after challenge inoculation. Chitinase activity in plants sprayed with *M. oryzae*, *Epicoccum* sp. and resistance induced plants did not differ but differed significantly compared to the control sprayed with water (Figure 5A, C).

The activities of peroxidase and β-1,3-glucanase increased in plants sprayed with crude extract of *Epicoccum* sp., 24 hours after the application of challenger (Figure

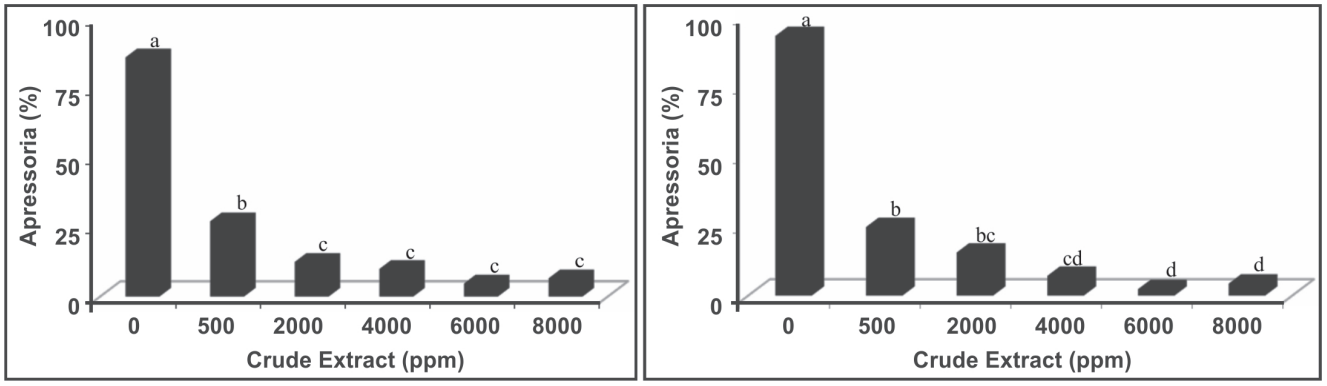

FIGURE 3 - Effect of crude extract concentration of *Epicoccum* sp. (500, 2000, 4000, 6000 and 8000 ppm) on the formation of appressoria of *Magnaporthe oryzae*. **A.** After12 hours; **B.** After 20 hours. Columns followed by the same letter do not differ significantly according to Duncan's test (P<0.05).

TABLE 1 - Relative efficiency of conidial suspension and crude extract of *Epicoccum* sp. on rice leaf blast suppression

Treatments	LBS (%)[1]	AUDPC[2]
T1: 2000 ppm of crude extract applied 48 BCI[3]	1.46 a[4]	3.30 a
T2: 4000 ppm of crude extract applied 48 BCI	0.36 a	1.10 a
T3: EP06 5 x 10^5 con mL^{-1} applied 48 BCI	0.91 a	3.63 a
T4: mixture of EP06 (10 x 10^5 con mL^{-1}) and M.o.	4.53 b	9.09 bc
T5: mixture of EP06 (10 x 10^1 con mL^{-1}) and M.o.	6.31 c	9.81 bc
T6: mixture of 2000 ppm of crude extract EP06 and M.o.	6.37 c	12.43 c
T7: mixture of 4000 ppm of crude extract EP06 and M.o.	4.70 bc	7.93 b
T8: Control (M.o. 3 x 10^5 con mL^{-1})	34.32 d	45.01 d

[1]Percentage leaf area affected using a 10 grade scale (0-9) according to Notteghem (1981); [2]AUPDC: area under disease progress curve; [3]BCI: before challenge inoculation; [4]Means followed by same letters in column do not differ statistically according to the Duncan (P < 0.05).

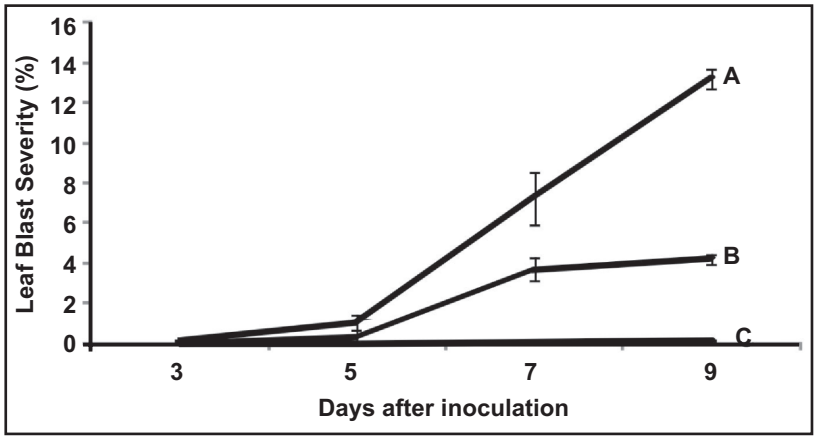

FIGURE 4 - Induced resistance to rice leaf blast in plants sprayed with crude extract of *Epicoccum* sp. 48 hours before challenge inoculation with *M. oryzae*. **A.** Control; **B.** Crude extract of *Epicoccum* sp. (4000 ppm) followed by *M. oryzae* (3 x 10^5 con mL^{-1}). **C.** Crude extract of *Epicoccum* sp. The bars represent standard error of the mean.

5B, D), the critical period in the infection process of M.o Peroxidase activity increased 48 and 72 hours after induced resistance followed by challenge. After 72 and 96 hours of inoculation with M.o. all treatments differed from the control in relation to peroxidase activity (Figure 5B). β-1,3-glucanase activity significantly increased in plants that received only crude extract of *Epicoccum* sp., 24 after inoculation of the challenger (Figure 5D).

In leaves treated with only crude extract of *Epicoccum* sp. the presence of crystal fragments of *Epicoccum* sp. can be observed without the presence of M.o. (Figure 6A) whereas in leaves inoculated with crude

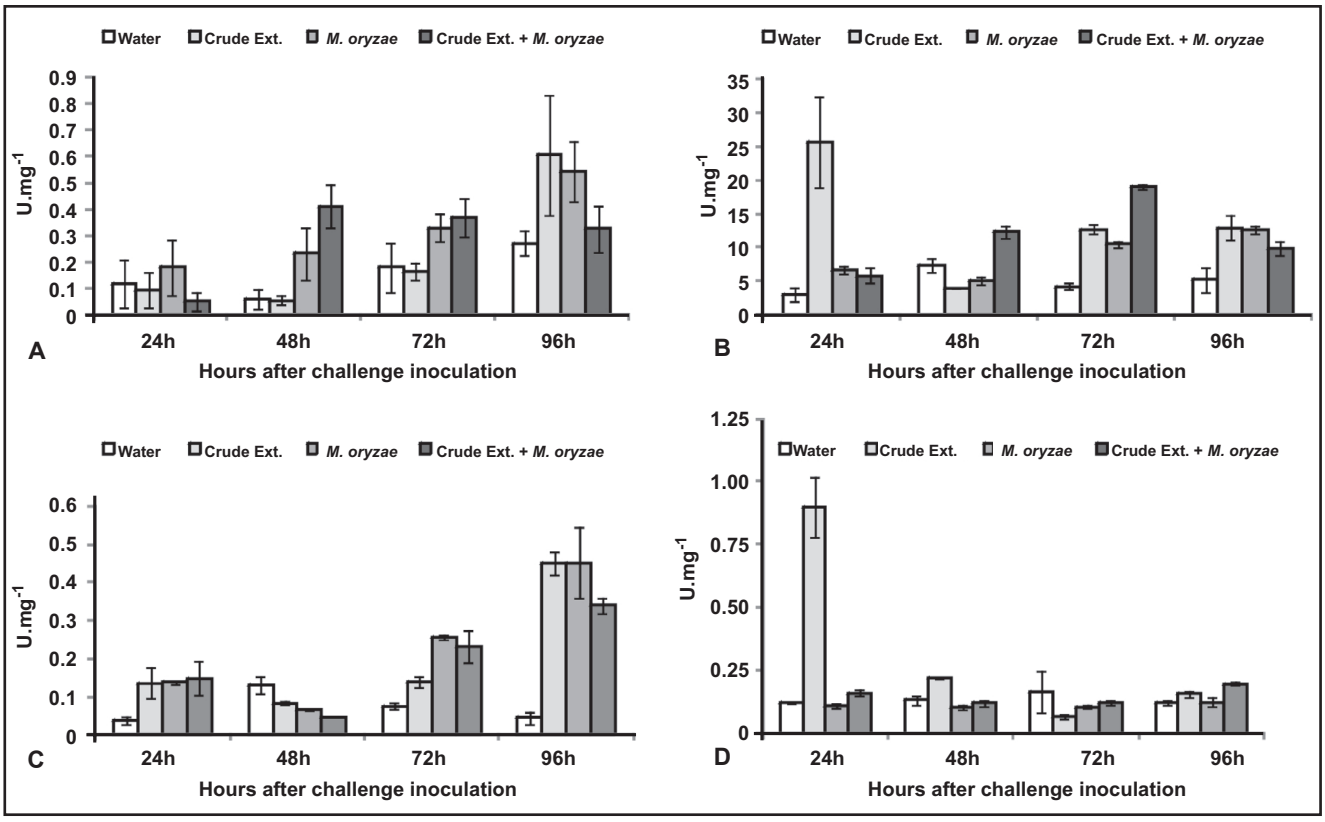

FIGURE 5 - Enzymatic activity of rice plants treated with crude extract of *Epicoccum* sp. (4000 ppm) hours after challenge inoculation with *M. oryzae*. **A.** Phenylalanine ammonia-lyase; **B.** Peroxidase; **C.** Chitinase; **D.** β-1,3-Glucanase. The bars represent standard error of the mean.

extract of *Epicoccum* sp. followed by *M. oryzae* the conidia of *M. oryzae* with deformed appressoria were evident (Figure 6B). In control inoculated only with the pathogen, the conidia and appressoria were formed normally without any deformation (Figure 6C).

DISCUSSION

Epicoccum spp. is considered a saprophytic fungus and phylloplane inhabitant in rice (Araújo et al., 2010). In the present study, the reduced mycelial growth and significant colony inhibition of *M. oryzae*, *R. solani*, *S. oryzae*, *M. albescens* and *C. miyabeanus* indicate its potential to control several rice diseases. These results are in agreement with the earlier reports of mycelial growth inhibition by *Epicoccum* sp. of various pathogens such as *M. oryzae* (Araújo et al., 2010), *Colletotrichum gloesporioides* (Pandey et al., 2007), *Rhizoctonia solani* (Lahlali & Hijri, 2010), *Macrophomina phaseolina* (Hashem, 2004) and *Fusarium sambuciunum* (El-Kot, 2008).

The crude extract is being widely utilized with the objective of identifying potential biological agents. Li et al. (2011) showed that the crude extract of *Streptomyces globisporus* inhibited mycelial growth, conidial germination and appressorial formation of *M. oryzae* in histological observations of cut leaves. According to Madrigal et al. (1991) the secondary metabolite flavipine poduced by *Epicoccum* sp. inhibited conidial germination of several plant pathogens. In the present study, the partially purified crude extract of *Epicoccum* sp. affected conidial germination and appressorial formation in all six concentrations tested. The reduction in appressorial formation was as high as 97.7% and is greater than the inhibition of conidial germination. Further studies are underway with reference to components of crude extract that are responsible for antagonistic effect against *M. oryzae*.

In this study, the antagonistic effect of *Epicoccum* sp. detected *in vitro* was also confirmed *in vivo* in greenhouse inoculation tests. An experiment was conducted to determine the relative efficiency of crude extract and conidial suspension in suppressing leaf balst. The results showed that crude extract of *Epicoccum* sp. is as efficient as spraying conidial suspension of the same (Table 1). The application of crude extract of *Epicoccum* sp. (2000 and 4000 ppm) sprayed 48 hours before the inoculation with challenger *M. oryzae* reduced leaf blast severity by 97.56% in relation to control. The crude extract applied as a mixture with the conidial suspension of *M. oryzae* or as a mixture of conidia of both fungi also controlled leaf blast compared with control. The efficiency of conidial suspension of

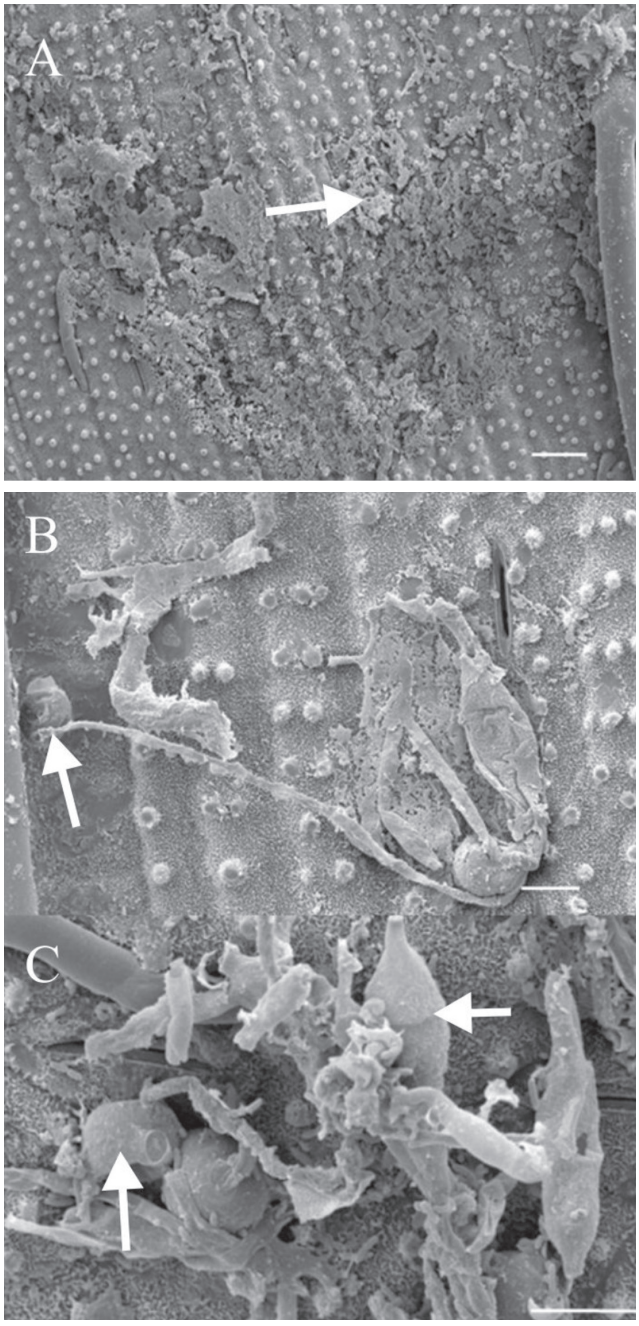

FIGURE 6 - Scanning eletron micrographs of rice leaves treated with crude extract of *Epicoccum* sp. (4000 ppm) 24 hours after inoculation with challenger *Magnaporthe oryzae* (3 x 10^5 con mL^{-1}). **A**. Cristals (arrow) of crude extract of *Epicoccum* sp. Bar = 20 µM; **B.** Crude extract of *Epicoccum* sp. followed by *M. oryzae*. Bar = 10 µM. Note *M. oryzae* conidia and deformed apressoria (arrow); **C.** *M. oryzae* alone. Bar = 10 µM. Note normal conidia and apressorium (arrow).

Epicoccum sp. in the suppression of other plant diseases and its potential as biological agent were reported by several investigators (Madrigal et al., 1991; Wittig et al., 1997; Pieckenstain et al., 2001; Hashem, 2004; Pandey et al., 2007; El-Kot, 2008, Koutb & Ali, 2010; Lahlali & Hijri, 2010; Musetti et al., 2011).

The *Epicoccm* sp. isolate utilized in the present study (EP06) was obtained from the rice phylloplane. Kawamata et al. (2004) verified that 7% of 967 rice phylloplane fungal isolates reduced leaf blast severity, including *Epicoccum* sp. The chances of success of phylloplane constituent as biological agent are considered greater, because they already developed the potential to colonize their host plants.

The results in the present study demonstrated the efficiency of crude extract in controlling leaf blast under greenhouse conditions. The hypothesis that crude extract of *Epicoccum* sp. activating the plant defense mechanisms is supported by our results. It is possible that PAL and chitinase may not have participated in the initial defense which is important for successful infection. PAL is a precursor and regulator of salicylic acid, the plant hormone required for induction of SAR (Chen et al., 2009; Dempsey et al., 2011). The results indicate that crude extract of *Epicoccum* sp. did not induce SAR. However, the antagonistic effect of crude extract with *M. oryzae* may have promoted the liberation of molecules that had effect on PAL and chitinase (PR-3, PR-4, PR-8 and PR-11), which was observed 96 hours after challenge inoculation with the pathogen. It is known that the *Pal 1* gene that codifies PAL is activated by jasmonic acid and ethylene during the process of plant defense (Shoresh et al., 2010).

On the other hand, the crude extract of *Epicoccum* sp. induced increased activity of peroxidase and β-1,3-glucanase (PR-2). In earlier *in vitro* tests the production of chitinase and β-1,3-glucanase were not detected by the isolate of *Epicoccum* sp. (data not shown). However, we need further studies concerning enzyme production by *Epicoccum* sp. and composition of its crude extract. The increased enzymatic activity refers to its proper plant product and known to be related to the activation of ISR. Increased activity of β-1,3-glucanase and peroxidase during leaf blast suppression by rhizobacteria and acibenzolar-S-metyl was reported by Filippi et al. (2011) and Côrtes et al. (2008). Peroxidase is related to diverse responses to plant stress. In the present study, as the plants were not subjected to abiotic stress, we may conclude that the crude extract is responsible for increased enzymatic activity, mainly after 24 hours, the critical period for the suppression of infection in which the fungus initiates the penetration process. These results are further supported by our SEM analysis, confirming the validity of data on suppression of rice leaf blast severity compared with the control.

According to Walters et al. (2007) and Pieterse et al. (2009), three types of systemic resistance ae characterized which are effective against biotrophic and necrotrophic plant pathogens: SAR, ISR, and resistance induced by β-aminobutíric acid (ABA-IR). The metabolic pathways that break or control these systems and the associated defense responses were well characterized in *Arabidopsis thaliana* (Van der Ent et al., 2008; Pieterse et al., 2009).

SAR, depending on the presence of salicylic acid, can be induced by necrotizing pathogens, and by specific elicitors which are associated to the related pathogenesis proteins and regulated by proteins and by NPR1. ISR can be induced by beneficial microorganisms such as rhizobacteria and the fungus *Trichoderma* spp. which do not require the presence of salicylic acid and not PR dependent, but dependent on jasmonic acid, ethylene and of NPR1. On the other hand, the ABA-IR metabolic pathway depends on the presence of salicylic acid and abscisic acid. The results herein presented allow us to conclude that *Epicoccum* sp. as well as its crude extract elicited plant defense mechanisms in rice, but what is still lacking is the knowledge on the metabolic pathway that is activated. For this it will be necessary to quantify plant hormones, jasmonic acid, ethylene and salicylic acid to explain when and which pathways are elicited by its product. Also, further studies are necessary to elucidate the fraction of crude extract that has the capacity to elicit the plant defense and establish the antibiosis with the rice pathogen. In addition, through molecular techniques it is possible to clone genes related to defense pathways of host plants such as elicitors by crude extract of *Epicoccum* sp., as well as fractions of crude extract responsible for antibiosis against rice pathogens. Various investigators provided evidence regarding the induction of SAR in rice after the application of microorganisms utilizing avirulent isolates of *M. oryzae* and *Bipolaris sorokiniana* (Smith & Métraux 1991; Manandhar et al., 1998; Tsukamoto et al., 1999; Ashizawa et al., 2005; Filippi et al., 2007) as well as ISR in rice with *Pseudomonas fluorescens* for the control of sheath blight (Nandakumar et al., 2000; Radjacommare et al., 2004), with rhizobacteria and with *Bacillus* sp. for leaf blast (Nascimento, 2009; Filippi et al., 2011). Umamaheswari et al. (2009) showed that seed treatment and foliar application of biocontrol agents induced resistance to *Alternaria alternata* in watermelon. Besides, the six pre-treatment agents increased protection of the plant to the pathogen as shown by the increased activity of PAL, peroxidase and β-1,3-glucanase.

Our SEM results indicate that the increased activity of these products is capable of inhibiting germination and appressorial formation. The crude extract did not impair the initial phases of infection because the conidia of *M. oryzae* are visible, germinated, and formed appressoria, however, without success for penetration.

There are still some limitations in adopting resistance induction (RI) in commercial crops, including instability of the efficiency of some inducers, growers' demands in relation to quality and performance of the elicitor. Under field conditions, the expression of RI is influenced by climatic conditions, genotype and nutritional status. Elicitors such as crude extract of *Epicoccum* spp., which possess both characteristics of antagonism and resistance induction, are highly promising for integrated management of cultivated plants.

The results of this study showed that *Epicoccum* sp., obtained from the phylloplane of rice plants in commercial fields, showed antagonistic potential against *M. oryzae, R. solani, S. oryzae, M. albescens* and *C. miyabeanus*. Also, the partially purified crude extract of *Epicoccum* sp. as well as conidial suspension reduced conidial germination, appressorial formation, rice leaf blast severity and induced plant defense responses by increasing activity of peroxidase and β-1,3-glucanase, 24 hours after the application of challenger *M. oryzae*. These results further confirm that this fungus is both antagonist and systemic resistance inducer against *M. oryzae*. Further studies are necessary with crude extract of *Epicoccum* sp. for identifying the elicitor molecule(s) during the initial stages of the rice-*M. oryzae* interaction responsible for induction of ISR, as well as studies on transduction of signals used for activating local and systemic defense.

ACKNOWLEDGEMENTS

We thank Fundação de Amparo à Pesquisa do Estado de Goiás - FAPEG and Universidade Federal de Goiás - UFG for providing financial support for this research, Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - CAPES for financial support to the first author, and the Multiuse Microscopy Laboratory (LabMic) of UFG for scanning electron microscopic analysis.

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TPP 2013-0044 - Received 28 March 2013 - Accepted 23 April 2013
Section Editor: Bernardo A. Halfeld-Vieira