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ARTIGO ORIGINAL

Induction of resistance to rice leaf blast by avirulent isolates of *Magnaporthe oryzae*

Resistência à brusone das folhas induzida por isolados avirulentos de Magnaporthe oryzae

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ABSTRACT: Rice blast (*Magnaporthe oryzae*) disease can cause losses of up to 100% in grain production. A sustainable management should consider alternative control measures. In the present work, biotic resistance induction to blast disease was studied by comparing disease severity and quantifying some pathogenesis-related proteins (PRPs). Rice plants of cultivars Metica-1 and Cica-8 were sprayed with biotic resistance inducers (incompatible isolates of *M. oryzae*) 48 h before inoculation with challenging isolates (compatible isolates of *M. oryzae*) under greenhouse conditions. For both cultivars, Metica-1 and Cica-8, the area under the disease progress curve was reduced by more than 80% when plants were induced with avirulent isolates of *Magnaporthe oryzae*. The biotic inducers also promoted an increase in the activity of peroxidase (POX), β-1.3-glucanase (GLU), chitinase (CHI), and phenylalanine ammonia-lyase (PAL). Systemic expression of resistance was also observed.

RESUMO: *A brusone nas folhas do arroz (Magnaporthe oryzae) pode causar perdas em até 100% da produção e o manejo sustentável deve considerar várias medidas de controle. No presente trabalho, foi estudada a indução biótica de resistência à brusone com o objetivo de comparar a severidade de brusone nas folhas e quantificar algumas proteínas relacionadas à patogênese (PRPs). Em condições de casa de vegetação, plantas de arroz das cultivares Metica-1 e Cica-8 foram pulverizadas com os indutores bióticos de resistência (isolados de M. oryzae incompatíveis) 48 h antes da inoculação feita com isolados desafiantes (isolados compatíveis de M. oryzae). Em ambas as cultivares, a área sob curva de progresso da doença foi reduzida em mais de 80% quando foram induzidas/inoculadas com isolado de M. oryzae aviruelento. Os indutores de resistência promoveram um aumento na atividade peroxidase (POX), seguida de β-1.3-glucanase (GLU), quitinase (CHI) e fenilalanina-amônio liase (PAL). Também foi observada a expressão sistêmica da resistência.*

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1 Introduction

Rice blast caused by *Magnaporthe oryzae* B.Couch [*Pyricularia grisea* (Cooke) Sacc.] is the most destructive rice disease causing yield losses, which are in staggering dimension. Yield losses up to 100% have been recorded in an outbreak of blast in Brazil, in an upland rice cultivar Colosso (Prabhu et al., 2009). In an ecologically sustainable agriculture, blast disease control requires integrated management of genetic resistance, cultural practices and chemical control. The durability of genetic resistance in improved rice cultivars is limited due to great variability of the pathogen. Consequently, the application of fungicides has been intensive in Brazil for reducing yield losses. Agnelli (2011) shows examples of using commercial resistance inducers, biotic/abiotic (Bion®, Ecolife®, Oryzemate®), as major strategies for increasing the durability of disease resistance and reducing toxic residues resulting from indiscriminate use of chemicals.

Avirulent isolates of *Pyricularia grisea* were known to suppress the infection by virulent isolates of the same fungus (Filippi et al., 2007). According to Manandhar et al. (1998), avirulent isolates of *P. grisea* substantially reduced rice blast both in greenhouse and field experiments. However, the knowledge on rice - *M. oryzae* interaction and the mechanisms of manifestation of resistance is limited. Ohata and Kozaka (1967) reported the production of two fluorescent compounds with antifungal properties in rice leaves when virulent and avirulent isolates were inoculated simultaneously. Kloepper et al. (1992) showed the accumulation of several defense–related transcripts in the rice leaves following inoculation with avirulent isolates of *P. oryzae*. Several pathogenesis related proteins (PRPs) accumulate in response to infection by pathogens or elicitors in rice plant (Eyal et al., 1992). Increased activities of enzymes β-1.3-glucanase (GLU) and chitinase (CHI), both responsible for degradation of pathogenic fungi cell wall, were observed after infection by *P. oryzae*. Phenylalanine ammonia-lyase (PAL) and peroxidase (POX) are some major enzymes in metabolic pathway leading to cell wall lignification (Bowels, 1990). Activities of PAL and POX were found to be significantly higher in response to infection by an avirulent isolate of *P. oryzae* than by a virulent *P. oryzae* (Ouyang et al., 1987). Although several PRPs show their activities in response to elicitors, their role in defense of plants with different genetic backgrounds is not known.

The investigation of the mechanisms involved in the induction of resistance process could generate knowledge for the development of bioagents in controlling rice blast. The objective was to study the evolution of blast disease in both induced and noninduced plants of two rice cultivars and to quantify related proteins PRPs expressed in the host-plant interaction during the resistance induction process by avirulent isolates of *M. oryzae*.

2 Materials and Methods

The experiment was conducted in the greenhouse using two cultivars Metica-1 and Cica-8 exhibiting differential reaction to two distinct races of *M. oryzae*, IB-9 (Py-1050) and IB-45 (Py-435) respectively (Table 1).

Seeds were sown in plastic trays (30 x 15 x 10 cm) containing 3 kg soil fertilized with 5g of NPK (5-30-15 + Zn), and 3.0 g ammonium sulfate at planting. Ten to 12 seeds per row of each one of the cultivars Metica-1 and Cica-8 were sown in eight rows in separate trays. Top dressing was made18 days after planting with 2.0 g of ammonium sulfate.

The experimental lay-out was factorial 2 x 5 designs with 10 treatments. The induced resistance was measured by inoculating rice plants with inducer (avirulent isolates) on day 0 and challenging the plants 48 h later with virulent isolate, according to Filippi et al. (2007). The treatments include two cultivars and the following five spray inoculations: T1= control sprayed with water on day 0; T2= challenging plants with virulent isolates on day 0; T3= challenging plants with virulent isolates after 48 h; T4=sprayed with inducers on day 0; T5= sprayed with inducers on day 0 + challenging plants 48 h later.

Single conidial isolates were established from sporulating lesions of cultivars Metica-1(Py-1050) and Cica-8 (Py-435) and stock cultures were maintained on filter paper disks in sterilized butter paper bags at 4 + 1 °C. For sporulation, the isolates were grown on oat-meal agar medium in Petri dishes, during 10 days under fluorescent light at 25 °C. Eighteen day old plants, with three fully expanded leaves were inoculated by spraying with aqueous spore suspension on the leaves, until run-off, using an atomizer connected to an air compressor. The isolates utilized as inducers were sprayed 48 h prior to inoculation with virulent isolates using concentrations of 3.10^{-5} conidia.mL^{-1} according to method described by Filippi et al. (2007). The inoculated plants were kept in moist chamber for 24 h at 20 to 24 °C after which they were transferred to the greenhouse at temperatures ranging 25 to 28 °C.

Disease was assessed for five consecutive days soon after the first appearance of the symptom, based on percentage of leaf area affected on first fully opened leaf utilizing 10-grade scale according to Notteghem (1981).

The change in lesion type in response to disease resistance induction was studied on fully opened penultimate leaf. A modified score for lesion type (LT) was based on a scale for measuring leaf blast in greenhouse (IRRI, 1988). 0= no lesions; 1= small brown specks of pinpoint size hypersensitive lesions; 3=small round to oval necrotic brown lesion type; 5= typical elliptical susceptible sporulating lesions with brown margin; 7=broad spindle or irregular shaped lesions without distinct

Table 1. Reaction of rice cultivars Metica-1 and Cica-8 inoculated with two field isolates of *Magnaporthe oryzae* in the greenhouse.
Tabela 1. Reação de cultivares de arroz Metica-1 e Cica-8 inoculados com dois isolados de campo de *Magnaporthe oryzae* em estufa.

Cultivar	Py-435 (IB-45)*	Py-1050 (IB-9)*
Cica-8	Compatible reaction (virulent)	Incompatible reaction (avirulent)
Metica-1	Incompatible reaction (avirulent)	Compatible reaction (virulent)

*Races were previously identified using eight standard international differentials.

margins; 9 = rapidly coalescing irregular white to bluish lesions without distinct margins. Blast severity assessment data made at random on 24 plants per treatment were used for variance analysis.

The area under disease progress curve (AUDPC) was used as another criterion for measuring the degree of resistance. The AUDPC was calculated for each treatment according to Shaner and Finney (1977). The data were analyzed using SPSS program, and the means were compared by test of Tukey ($p \leq 0.05$).

The quantification of enzymes was initially standardized by conducting exploratory trials in which the best results were obtained with samples taken 72 h after the inoculation with the challenger.

A sample of five leaves was macerated in liquid nitrogen with pistil until it became powder. The sample was collected and processed in micro tubes, to which a buffer solution was added at 1:4 (v/v) ratio. The buffer solution is composed of Tris-HCl 10 mM; NaCl 150 mM; EDTA 2 mM; DTT 2 mM; PMSF 1 mM; Leptin 10 µg mL^{-1} e Aprotinin 10 µg mL^{-1}. The suspension was agitated for 5 min and centrifuged under refrigeration for 30 min at 13000 x g. The supernatant was removed for protein quantification and enzymatic activity determination. A standard curve of protein was prepared with bovine serum albumin at concentration ranging 0-1 mg mL^{-1}. The readings were taken in spectrophotometer FENTO 600 Plus in wavelength of 595 nm (Côrtes et al., 2008).

Activity of β-1.3-glucanase (GLU) in rice leaf extracts from different treatments was assayed by measuring the rate of reducing sugar production using laminarin as the substrate (Pan et al., 1991). DNS reagent was used as the calorimetric agent. Activity was expressed in units U mg^{-1} protein. One unity (U) was defined as the enzyme activity catalyzing the formation of reducing sugar that increases the absorbency of one unit of abs per hour. The experiment was done in triplicate.

Peroxidase (POX) activity was assayed by measuring the rate of 2.2`-azino-bis-3-rthylbenzthiazoline-6-sulfonic acid (ABTS) oxidation, using its own calorimetric property. One unit was defined as the enzyme activity catalyzing the formation of 2.2`-azino-bis-3-rthylbenzthiazoline-6-sulfonic oxidized (ABTS*) that increases the absorbency of 1 unit of Abs per hour (Keesey, 1987).

Chitinase (CHI) activity in rice leaf extracts from different treatments was assayed by modified method of Pan et al. (1991). The rate of N-actyl glucosamine production was measured using coloidal chitin as the substrate. DNS reagent was used as the calorimetric agent. Activity was expressed in U mg^{-1} protein. One U was defined as the enzyme activity catalyzing the formation of reducing sugar that increases the absorbency of one unit of Abs per hour.

Phenylalanine-ammoniumlyase activity (PAL) was assayed by the method of Dickerson et al. (1984). It consists of measuring the rate of trans-cinamic acid production using L- phenylalanine as the substrate, using its own colorimetric property. Activity was expressed in units U.mg^{-1} protein. One unit was defined as the enzyme activity catalyzing the formation of trans-cinamic acid that increases the Abs per minute.

Another greenhouse experiment was conducted in plastic trays to study the systemic activity of resistance induction. Two susceptible irrigated rice cultivars Metica-1 and Cica-8 were used. Eight rows of the cultivars were sown separately in each tray as described in the above experiment. The treatments consisted of two cultivars sprayed and nonsprayed with spore suspension of avirulent isolates on plants with two fully expanded leaves followed by spraying with spore suspension of virulent isolate after the emergence of third leaf of the plants. A conidial concentration of 3.10^5 conidios mL^{-1} for both avirulent and virulent isolate was used. The multiplication of the fungus for spore production and inoculation procedure is as described above for induction of resistance Disease assessment was made only on third leaf, nine days after challenging with virulent isolate, using the scale developed based on percentage diseased leaf area (DLA) according to Notteghem (1981).

3 Results and Discussion

The percentage leaf area greatly increased with time in both cultivars in treatments challenged only with virulent isolate and decreased in treatments of prior inoculation with avirulent isolate (Figure 1).

The avirulent isolate of *M. oryzae* 48 h prior to inoculation with virulent isolate significantly reduced leaf blast in relation to the leaf area affected and lesion type in both cultivars Metica-1 and Cica-8 (Table 2). The cultivars, however, did not show significant differences in their response to biotic induction. The percentage leaf area affected in cv. Cica-8 was reduced from 0.39 to 0.068 and mean score for lesion type from 5.67 to 3.02, whereas, the leaf area in Metica-1 was reduced from 0.45 to 0.073 and score for lesion type from 4.98 to 2.78. The controls sprayed only with inducer but not the challenger did not show any blast lesion or necrotic spots.

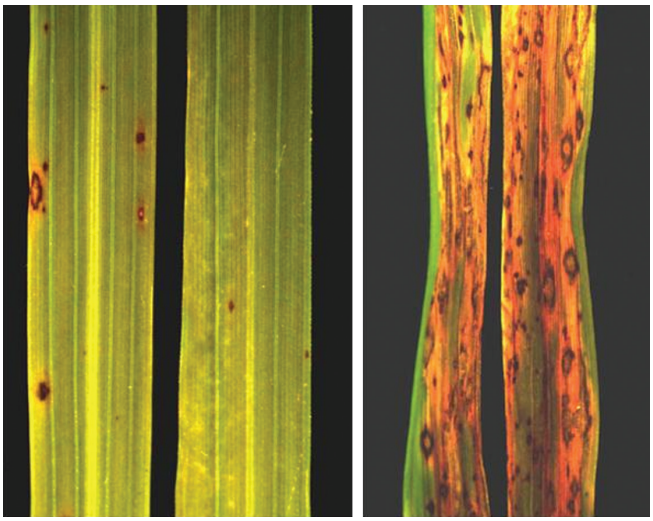

Figure 1. Rice leaf blast symptoms, 7 days after induction by prior inoculation with avirulent isolate (left) and control (right) challenged with only virulent isolate in greenhouse inoculation test (cv. Metica-1).

Figura 1. Sintomas da brusone no arroz sete dias após a indução por inoculação prévia com o isolado avirulento (à esquerda) e controle (à direita), desafiado apenas com o isolado virulento em teste de inoculação em estufa (cv. Metica-1).

Table 2. Mean score for lesion type (LT), percentage diseased leaf area (DLA) and area under disease progress curve (AUDPC) assessed 100 h after challenging with virulent isolate.

Tabela 2. Valores médios para o tipo de lesão (LT), porcentagem de área foliar lesionada (DLA) e área sob a curva de progresso da doença (AACPD) avaliadas 100 horas após desafio com isolado virulento.

Treatment[1]	LT (mean score)	DLA (%)	AUDPC
cv. Cica-8 inoculated with inducer and challenger	3.02 b[2]	0.068 a	0.09 a
cv. Metica-1 inoculated with inducer and challenger	2.78 a	0.073 a	0.13 a
cv. Cica-8 inoculated only with challenger	5.67 c	0.39 b	1.12 b
cv. Metica-1 inoculated only with challenger	4.98 c	0.45 b	0.92 b

[1]The cultivars Cica-8 and Metica-1 maintained as controls showed no leaf blast symptoms when inoculated with avirulent isolates Py 435 and Py 1050, respectively; [2]The means followed by the same letter in vertical columns do not differ significantly according to Tukey's test ($p \leq 0.05$).

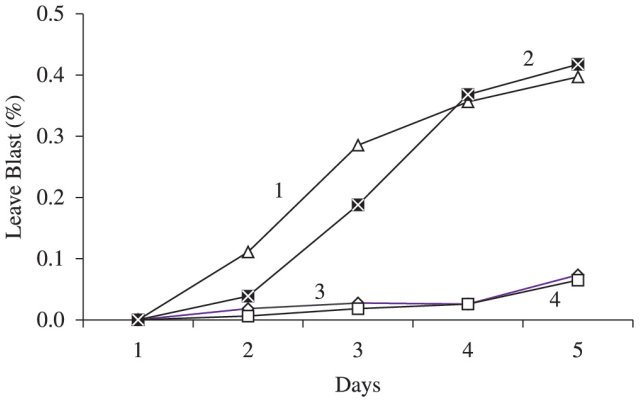

Figure 2. Blast disease progress in leaves after treatments: (1= cv. Metica-1, challenged with virulent isolate; 2= cv Cica 8, challenged with virulent isolate; 3= cv. Metica-1, induced with avirulent isolate prior to inoculation with virulent isolate; 4= cv. Cica-8, induced with avirulent isolate prior to inoculation with virulent isolate. The diseased leaf area in percentage was assessed for five consecutive days beginning first appearance of the symptom (Day 1= 4 days after challenging with virulent isolate).

Figura 2. Evolução do brusone nas folhas de arroz após os tratamentos: (1 = cv Metica-1, desafiado com o isolado virulento; 2 = cv. Cica 8, desafiado com o isolado virulento; 3 = cv. Metica-1, induzido com o isolado avirulento antes da inoculação com o isolado virulento; 4 = cv. Cica-8, induzido com o isolado avirulento antes da inoculação com o isolado virulento. A área foliar lesionada em porcentagem foi avaliada por cinco dias consecutivos, começando no primeiro aparecimento do sintoma (dia 1 = 4 dias após desafio com o isolado virulento)

Similar results were obtained with reference to AUDPC (Table 2, Figure 2). The AUDPC was reduced from 1.12 to 0.09 in cv. Cica-8. The corresponding reductions in cv. Metca-1 was from 0.92 to 0.13. The treatments sprayed with only challenger exhibited water soaked white to green irregular shaped lesions with grey center and often coalescing. The type 1 pinhead sized lesions and type 3 characterized by isolated round brown lesions and type 5 characterized by typical spindle shaped lesions with white to grey center were absent.

The enzymatic activity was greater for treatments induced with avirulent isolates 48 h prior to challenging with virulent isolates (T5) compared with controls (Figure 3). The control treatments T1 sprayed with water, T2 and T4 sprayed on the same day with virulent and avirulent isolates, respectively, showed less activity than in T3, another control sprayed with virulent isolate on the same day of treatment and T5 in which the resistance was induced 48 h prior to inoculation with challenger. The enzyme activity of peroxidase (POX) greatly increased, followed by β-1,3-glucanase (GLU), chitinase (CHI) and phenylalanine ammonia-lyase activity (PAL). However, there were differences between the cultivars in relation to the percentage increase of enzymatic activity. The enzymatic activity increased in cv. Cica-8 as expected in treatments induced with avirulent isolate 48 h prior to inoculation with challenger (T5). The treatment T1 sprayed with water indicated normal activity. The controls T2, T3 and T4 in which the activity was quantified 72 h after spray was greater than in T1.

The enzymatic activity in cultivar Metica-1 did not follow the same pattern as in cv. Cica-8. The control treatment T3 showed greater activity than T5 even though the isolate was highly virulent compared to the isolate used as challenger on Cica-8. The response in relation to enzymatic activity of PR3, PAL e POX, was greater than PR2 in relation to induced resistance. The activity of all enzymes was markedly higher in control T3 in which the lesions were still not developed and necrotic than in T2 where the lesions were well developed with grey centers after the spray was administered in both cultivars.

The plants that were sprayed with inducer on second penultimate leaf and challenged with virulent isolate on third topmost leaf showed systemic activity. Significant differences were obtained between induced and noninduced treatments in relation to the percentage leaf area affected. The differences were, however, greater in cv. Metica-1 than in cv. Cica-8 (Figure 4).

Avirulent isolates of *M. oryzae* induced rice blast resistance in cultivars Cica-8 and Metica-1 under greenhouse inoculation tests confirming the earlier reports made using other cultivars in different countries (Fujita et al., 1990; Filippi et al., 2007). The induced resistance has also been reported in other important cereals such as wheat and oat. The rapid and efficient defense response is activated when plants receive initial biotic or abiotic stimulus (Zimmerli et al., 2000). Fujita et al. (1990) induced resistance in rice with avirulent isolate of *P. oryzae* and obtained more than 80% reduction in disease severity resulting in small lesions with brown margins. Our results demonstrated the same resistance induction, however, we were able to quantify CHI, GLU, PAL and POX, during plant defense.

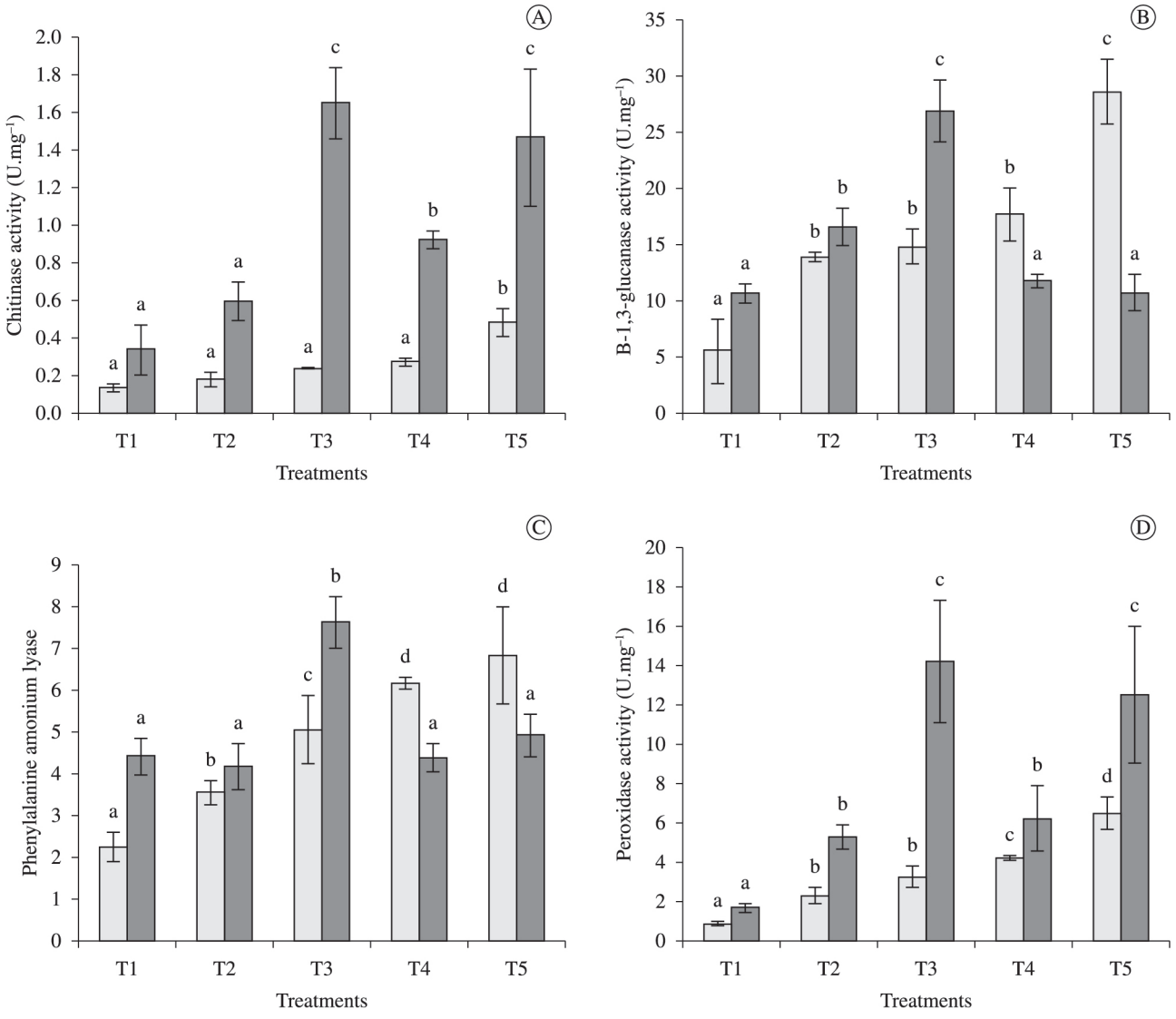

Figure 3. Quantification of enzymatic activity of chitinase (A), β-1,3-glucanase (B), phenylalanine ammonia-lyase (C) e peroxidase (D), in two test cultivars cv. Cica-8 and (white bars); cv. Metica-1 (shadowed bars). T1= non sprayed control; T2= 120 h after challenging with virulent isolate; T3= 72 after challenging with virulent isolate; T4= 120 h prior to induction with avirulent isolate; T5= 72 h prior to induction with virulent isolate. The means followed by the same letter in vertical columns do not differ significantly according to Tukey's test ($p \leq 0.05$).

Figura 3. Quantificação da atividade enzimática da quitinase (A), β-1,3-glucanase (B), fenilalanina amónia liase (C) e peroxidase (D) em dois cultivares de teste: cv. Cica-8 (barras brancas) e cv. Metica-1 (barras sombreadas). T1 = controle, não pulverizado; T2 = 120 h após desafio com o isolado virulento; T3 = 72 após desafio com o isolado virulento; T4 = 120 h antes da indução com o isolado avirulento; T5 = 72 h antes da indução com o isolado virulento. Médias seguidas pela mesma letra em colunas verticais não diferem significativamente pelo teste de Tukey ($p \leq 0,05$).

The area under disease progress (AUDPC) was significantly reduced in both cultivars in response to induction by avirulent isolates compared to controls sprayed with virulent isolate. Activation of genes related to defense leads to a faster and/or higher level of expression after inoculation with the challenger, and it is one common characteristic of different types of induced resistance (Pieterse; Van Loon, 2004). These intracellular biochemical alterations delimit the infected region avoiding disease spread. In the expression of hypersensitive reaction (HR), a chain reaction occurs in activated cells, resulting in several responses against the pathogen, such as cell wall fortification, phytoalexin production and activation of proteins related to pathogenesis (PRPs) (Daugrois et al., 1990).

In addition to the reduction in disease severity, the present study also showed systemic activity in response to the application of avirulent isolate on lower leaf. The earlier investigators (Manandhar et al., 1998) failed to observe any such systemic activity. According to Park and Kim (1983) the lesion size was reduced up to 3.0 cm above the site of inoculation with avirulent isolate of *P. oryzae*. The only report on systemic activity was made by Smith and Metraux (1991) using *Pseudomonas syringae* pv. *syringae* as resistance inducer. Later studies did not confirm this report (Reimmann et al., 1992). The reported systemic induced resistance (SAR) in the whole rice plant to M.oryzae utilizing avirulent isolates of the same species could be attributed to interaction between the

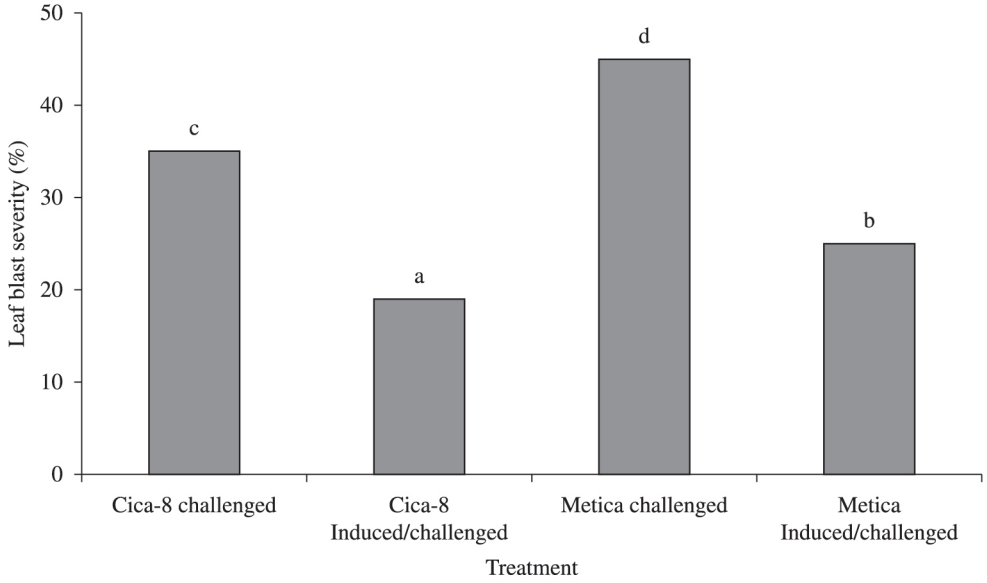

Figure 4. Systemic activity on third top most leaf in response to inoculation with avirulent isolate on second penultimate leaf. The mean leaf blast severity's represented by letters differed significantly by Tukey's test ($p \leq 0.05$).

Figura 4. Atividade sistêmica na terceira folha mais alta em resposta à inoculação com o isolado avirulento na antepenúltima folha. Severidade média da brusone representada por letras diferiram significativamente pelo teste de Tukey ($p \leq 0,05$).

avirulence gene and resistance. Wang et al. (1999) identified and isolated *Pib*, gene that confer resistance to most of the *M. oryzae* races in Japan. The gene *Pib* is a member of class of genes NBS-LRR that confer resistance to blast and the characteristic of its structure suggests that the product of this gene interact with other proteins for defense manifestation and its expression is influenced by temperature, light and water and treatments with chemical products such as jasmonic acid, salicylic acid, ethanol and probendazole.

Several biochemical changes elicited in incompatible rice blast fungus interactions have been identified. Salicylic acid is considered a signal responsible for the induction of SAR and acts via local systemic induction of PR proteins (Silverman et al., 1995). The importance of PR proteins in rice SAR is not as clear as in tobacco or cucumber. Increase in chitinase and glucanase activities were detected only in *P. syrigae* inoculated leaves but not systemically. However, the trancription of chitinase and glucanse activities were induced in rice by pathogen derived elicitors and salicylic acid (Nishizawa; Hibi, 1991). Phenylproponoid pathway plays an important role in plant defense system. The activity of enzyme PAL was enhanced in response to the inoculation with avirulent isolate in the present study. The PAL is an enzyme that utilizes L-phenylalanine as substrate and can be induced by external supplementation of elicitors such as salicylic acid and jasmonic acid (Yao; Tian, 2005) resulting in increase of phenolic compounds (Taiz; Zeiger, 2006). Rice converts *trans*-cinnamic acid to salicylic acid via benzoic acid. This reaction is catalized by a BA- inducible BA2H, which functions as a Cyt P450 monooxygenase as in tobacco (Silverman et al., 1995).

The inducers of resistance utilized in the present study promoted increased activity of enzymes related to pathogenesis such as β-1.3-glucanase and chitinase. Both these enzymes are known to be involved in the degradation of glycose polymer formed by linkages of type β-1.3 and chitin present in some fungal cell walls exhibiting direct action against aggressive pathogen.

Eventhough the cultivars Cica-8 and Metica-1 did not exhibit significant differences in disease severity in response to biotic induction there were differences in enzymatic activities (Figure 4). Rice is known to have the highest level of endogenous salicylic acid. The clear demonstration of SAR in the present study and the induction of rice PR proteins by salicylic acid show its possible role in SAR.

4 Conclusions

For Both cultivars, Cica and Metica, the area under curve of disease progress was reduced by more than 80%, when plants were spray inoculated with Magnaporthe oryzae aviruelnto isolate. The inducer, Magnaporthe oryzae aviruelnto isolate, increased peroxidase (POX), β-1,3-glucanase (GLU), chitinase (CHI) and phenylalanine ammonia-lyase (PAL) activity, and a systemic expression of resistance was also observed.

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