Brazilian Journal of Microbiology (2022) 53:1091–1100
https://doi.org/10.1007/s42770-022-00749-7

BACTERIAL AND FUNGAL PATHOGENESIS - RESEARCH PAPER

Pre and post stage of infection of *Magnaporthe oryzae Oryza* in wheat leaves with different resistance levels

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Received: 8 November 2021 / Accepted: 30 March 2022 / Published online: 12 April 2022

Abstract

Blast fungus (*Magnaporthe oryzae* B.C. Couch) is an imminent threat to global food security because it causes serious yield losses in rice (*Oryza sativa* L.) and wheat (*Triticum aestivum* L.). The investigation of infection processes in resistant and susceptible varieties, as well as the cellular responses involved in resistance, can help us to better understand the process of interaction of the *M. oryzae*-Poaceae pathosystems. Thus, the objectives of this study were to evaluate the processes of pre- and post-infection of *M. oryzae* in leaves of wheat varieties with different levels of resistance. The percentage of germinated conidia, appressorium formed, tissue penetration and colonization, and the reaction of leaf tissue to infection were evaluated. A decrease was observed in the percentage of germinated conidia, appressorium formation, tissue penetration and colonization, especially in the tissues of resistant varieties, in addition to an increase in the plant's response to infection, with cell wall reinforcement, cell death, and autofluorescent cytoplasm aggregation. Nevertheless, our data produced a different temporal perspective regarding the expression of the known types of resistance. We found that, for a single genotype, recognition can start as early as 6 h after inoculation and continue to evolve until very late during the infection cycle, culminating in cell death. The combined and overlapping pre- and post-haustorial resistance mechanisms were sufficient to prevent disease symptoms, with a few punctual lesions observed in one of the resistant varieties (BR 18) and no visible symptoms in the other two (Ônix or BRS229) as opposed to susceptible variety.

Keywords Histopathology · Blast · *Triticum aestivum* · Cell death · Autofluorescence · Temporal pattern

Introduction

The fungus *Magnaporthe oryzae* B.C. Couch is an imminent threat to global food security. It causes serious disease that imposes yield losses on rice (*Oryza sativa* L.) and wheat (*Triticum aestivum* L.), the two more important crops in terms of sources of calories and protein for a major part of the world's population, thereby constituting a threat to global food security [14, 39]. In wheat, *M. oryzae* causes the disease called blast or "brusone" that first emerged in Brazil in 1985 [20] and spread during subsequent decades into neighboring countries in South America [5, 29, 41]. Depending on the host specificity, the pathogen has been classified in subpopulations within the *M. oryzae* species, with pathotypes named according to the preferential host [10].

Increasing concern for a worldwide dissemination of the pathogen has been underscored since the first outbreak outside South America was reported in Bangladesh [25]. The capacity for long-distance of genotype flow combined with a mixed reproductive system place *M. oryzae* in the category of pathogens with the highest evolutionary potential [24].

Most wheat varieties available in Brazil are highly susceptible to disease [12]. We believe that many wheat cultivars in various parts of the world are also susceptible to disease due to the aggressiveness of the pathogen. In addition

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to the scarcity of sources of resistance, breeding resistant varieties remains a challenge because *M. oryzae* also infects barley (*Hordeum vulgare* L.), rye (*Secale cereale* L.), triticale (×*Triticosecale* Wittmack), and a wide range of plants within the Poaceae family [24].

Because little is known about the cellular responses involved in resistance within the pathosystems of *M. oryzae*×wheat, its understanding may help wheat breeding programs to define microphenotypes to select more effective and durable forms of resistance.

The implantation of resistant material is the most efficient and viable method to control *M. oryzae* in wheat. Some varieties of wheat resistant to blast disease have been identified [12], but the mechanisms that confer resistance to fungal infection have not yet been elucidated in detail, nor the specificity of this interaction, if it follows Flor's gene-for-gene model or not. The infectious process of *M. oryzae* and host responses can be investigated through histopathological and histochemical analyses of infected plants of wheat varieties with different levels of resistance. The identification of structures and/or biochemical mechanisms that confer resistance can be important for obtaining new productive and long-term resistant varieties originated from breeding programs.

The Brazilian wheat varieties Ônix, BR 18, and BRS 229 were reported by Cunha et al. [12] to be among the few commercial cultivars showing moderate field resistance to *M. oryzae*, representing invaluable genetic resources for the solution of this puzzle. In this article, we investigated cytological events over time during host resistance in seedlings of these three cultivars infected with one isolate of a *M. oryzae Oryza* pathotype and compared them with responses observed in the susceptible variety Anahuac, which was previously tested for its response. Our findings help to understand some important mechanisms of resistance in wheat to its relatively new pathogen.

Material and methods

Plant materials and growth

All experiments were conducted in the Department of Fitossanidade of the Federal University of Rio Grande do Sul, south Brazil. Four wheat varieties were used in the study: BR 18, Ônix, BRS 229, and Anahuac. Ten to 12 seeds of each variety were sown in 300-mL plastic pots containing field soil accordingly fertilized as recommended, totaling 10 pots per wheat variety. Plants were grown for 12 to 14 days in a growth chamber at 24 ± 2 °C, 80% relative humidity, and a light and dark cycle of 16 h and 8 h, respectively. When plants reached the five-to-six true leaves stage, a thinning was carried out, remaining four plants per pot.

Fungal inoculum

The isolate Py145 of *Magnaporthe oryzae Oryza* pathotype (MoO) used in this study was given by the Embrapa Rice and Bean unit, in Santo Antônio de Goiás city, Brazil, which was previously identified based on its morphological and molecular characteristics and stored in dry cellulose filter disc papers [18]. The isolate was originally collected from panicles of rice cultivar SCS 114 Andosan in Nova Veneza county (Santa Catarina State, Brazil—28°38′12″S,49°29′52″W). To grow it, dried cellulose filter disk papers were placed in Petri dishes containing oatmeal agar culture medium (40 g of oat flakes, 15 g agar, 15 g dextrose, and 1 L distilled water + 500 µg/mL of streptomycin) for 7 days in an incubator at 24 ± 1 °C with a photoperiod light and dark of 16 and 8 h, respectively. Hyphal tips were transferred to oatmeal agar and incubated again for 12 days under near-ultraviolet light at 22 °C to induce conidia production. For harvesting of conidia, sterile water containing 1% Tween 20 was added to the plates and the surface of the colony was scraped with a sterile Drigalski loop. The spore suspension was filtered through a double layer of gauze and adjusted to a concentration of 10^5 conidia mL^{-1}.

Inoculation protocol and incubation conditions

The ten pots containing four plants of each variety were transferred to a growth chamber (model CCP1200, Instalafrio®, Pinhais, PR) at 26 ± 2 °C with a photoperiod light and dark of 16 and 8 h, respectively. The adaxial surface of all the sixth fully expanded leaves was inoculated with 20 mL of a suspension of 10^5 conidia mL^{-1}. After the inoculation process, the plants were covered with transparent plastic bags for 24 h to maintain high humidity and incubated at 28 °C in the dark. After incubation, the plastic bags were removed, and the temperature in the growth chamber restored to 26 °C.

Symptom's evaluation

Five and twenty days after inoculation, the disease was scored according to the classification proposed by Valent et al. [40] as follows: type 0, no visible evidence of infection,type 1, uniform dark brown pinpoint lesions without visible centers; type 2, small lesions with distinct tan centers surrounded by a darker brown margin; type 3, small eyespot lesions approximately 2 mm in length with tan centers surrounded by dark brown margins; type 4, intermediate size eyespot lesions, approximately 3–4 mm in length; and type 5, large eyespot lesions that attain the maximum size seen for a particular cultivar. Types 0 and 1 were considered

incompatible interactions, because affected tissue cannot produce conidia under high humidity conditions. Types 2, 3, 4, and 5 were considered compatible, because conidia can be produced from such infected tissues under high humidity conditions.

Histological observation

Segments of the inoculated leaves were collected at 6, 12, 18, 24, 36, 48, and 72 h after inoculation (hai) for cytological analysis. From each time evaluated, four wheat leaves of each cultivar were collected. At least 10 segments of 2.5 cm in length were collected for each treatment (variety × collection time). Leaf samples were fixed and bleached, mounted on glass slides, and observed with a fluorescence microscope as described previously [17]. Briefly, samples were fixed for 24 h in 3:1 (v:v) ethanol:dichloromethane containing 0.15% (w/v) trichloroacetic acid, stained by boiling for 5 min in 1:2 (v:v) lactophenol:ethanol containing 0.05% (w/v) trypan blue, and cleared in a 5:2 (w:v) chloral hydrate:water mixture for 24 h. The stained samples were mounted on glass slides with 50% glycerol and analyzed under bright field or phase contrast microscopy using Olympus BX 41 microscope (Olympus Corporation, Tokyo, Japan). To detect autofluorescence, the same samples were dehydrated with ethanol (80% (v:v for 30 min; 90% for 30 min; and 100% twice × 30 min and stained for 5 min with a saturated solution of picric acid in methyl salicylate. Excess picric acid was removed by 15-min clearing in methyl salicylate [17]. Cell wall strengthening was assessed by the accumulation of stain beneath appressoria associated with cell wall appositions as previously reported [46]. The samples were mounted on glass slides containing methyl salicylate under cover slips sealed with nail polish and examined using blue-light epifluorescence microscopy (Olympus BX 41) fitted with a UMWB2 excitation filter set, consisting of a 460- to 490-nm dichroic beamsplitter 500-nm BA520 barrier filter. The percentages of germinated conidia, appressorium formed, tissue penetration, and colony formation were evaluated, as well as the occurrence of events associated with plant resistance including cell death, autofluorescent cytoplasmic aggregation, and cell wall strengthening.

The model of efficiency of infection

To illustrate the differences observed along the infection process, we used a theoretical model, as proposed by Wesp-Guterres et al. [44], based on the assumption that 10^3 conidia were deposited on the leaves of susceptible and resistant plants. In this model, numbers in each stage were calculated with respect to the previous event and were based on the data collected, starting from spore germination, and following to appressorium differentiation, colony formation, cell death, and colonization.

Experimental design and statistical analysis

Experiments were conducted in a 4 × 7 factorial arrangement of treatments in a completely random design. The number of conidia found in the beginning of infectious process varied according with each treatment (Table 1). The response variables were measured as the percentage of spore germination, appressorium formation, penetration, and colony formation. The occurrence of events associated with plant resistance including cell death (recorded as percentages), cell wall strengthening, and autofluorescent cytoplasm aggregation was also observed. Treatments were compared using the 2 × 2 Fisher's exact test of independence for pairwise comparisons [22] with the Bonferroni correction for multiple tests [23].

Results

Symptom's evaluation

Five days after inoculation, Anahuac variety showed typical disease lesions type 4 or 5 and BR 18 showed few pinpoint lesions type 1; the varieties Ônix and BRS229 did not exhibit any visible symptom of the disease (Fig. 1). Disease symptoms evolved in Anahuac with coalescence of lesions recorded at 20 days after inoculations; however, the other three, resistant varieties, remained with their typical initial symptoms.

Table 1 Number of conidia of the *Magnaporthe oryzae Oryza* pathotype quantified on leaves of wheat (*Triticum aestivum* L.) varieties BR 18, Onix, BR 229, and Anahuac at 6, 12, 18, 24, 36, 48, and 72 h after inoculation and used to determine the defense mechanisms

Variety	Hours after inoculation						
	6	12	18	24	36	48	72
BR 18	213	360	616	795	482	767	263
Ônix	75	562	461	535	262	729	329
BRS 229	207	198	393	583	335	722	367
Anahuac	361	497	975	661	586	829	447

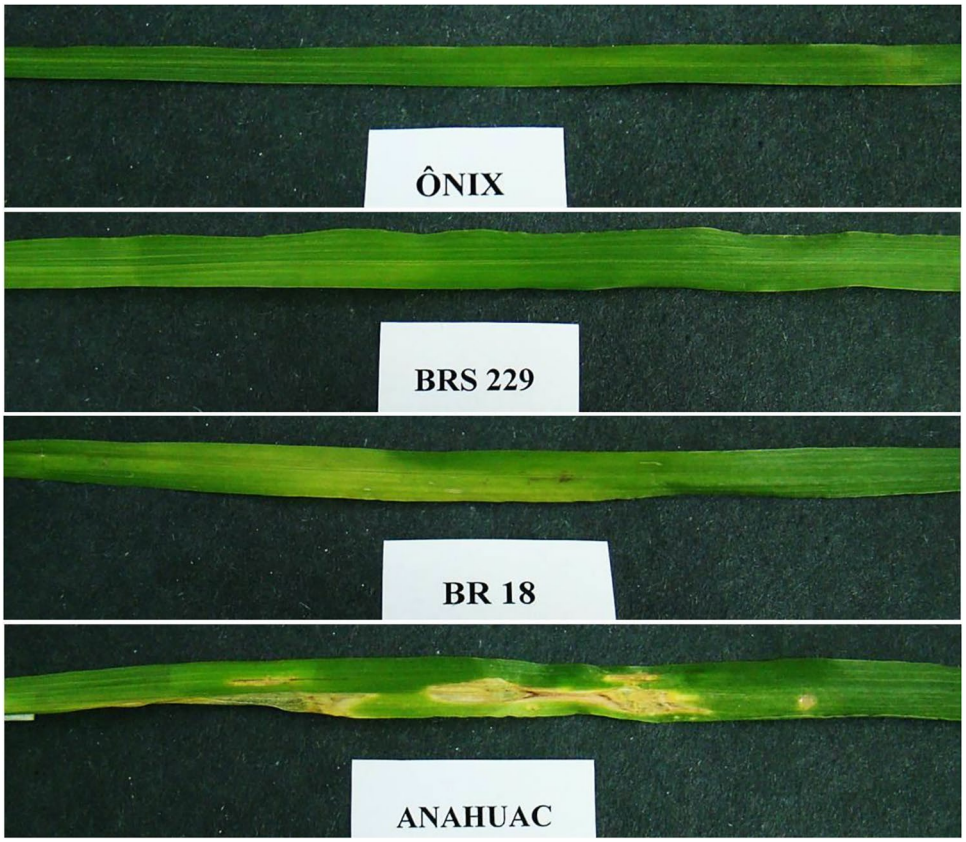

Fig. 1 Symptoms of blast disease caused by *Magnaporthe oryzae Oryza* isolated from rice (MoO pathotype) on leaves of resistant wheat (*Triticum aestivum* L.) varieties Ônix, BRS 229, and BR 18 compared to the susceptible cultivar Anahuac at 20 days after inoculation

Histological observation

Conidia germination, appressorium formation, and penetration varied across wheat varieties and evaluated times (Tables 2, 3, and 4; Fig. 3). A progressive increase in the germination of MoO pathotype conidia on the leaf tissues of the four evaluated wheat varieties was observed over 72 h (Table 2). In Anahuac and BR 18, spore germination significantly increased from 6 to 12 hai, and from 12 and 18 hai, when it reached a maximum. In Ônix and BRS229, spore germination was faster, significantly increasing from 6 to 12 hai, when maximum values were reached. However, the greatest difference in the percentage of conidia germination between the varieties was observed at 6 hai. The percentage of conidia germinated on Anahuac was 84%, significantly higher than that of BRS229 (73%) or BR 18 (64%), but it did not differ from the Ônix variety (77%).

At 6 hai, appressoria formation and melanization were low, varying from 15 to 30%, but not differing among the cultivars (Table 3). At 12 hai, appressorium formation increased significantly in all varieties, except for BRS 229 that had the lowest percentage of formed appressorium (17%). At 18 hai, the percentages of appressorium differentiation in Anahuac, BR 18, and Ônix reached 88%, 87%, and 80%, respectively, while in BRS 229, only 38% of germinated conidia showed appressorium formation. Over the evaluation periods, appressorium formation continued increasing on all varieties, peaking at 24 hai on Anahuac

Table 2 Percentage of conidia germination of *Magnaporthe oryzae Oryza* pathotype on leaf segments of wheat (*Triticum aestivum* L.) varieties sampled in seven time points after inoculation

Variety	Time points post-inoculation (hours)						
	6	12	18	24	36	48	72
BR 18	64.0 bC	97.7 aB	100 aA	95.4 cB	100 aA	100 aA	100 aA
Ônix	77.3 abB	98.4 aA	99.3 abA	99.2 abA	100 aA	100 aA	100 aA
BRS 229	72.9 bB	94.9 aA	96.5 cA	98.2 bA	99.7 aA	100 aA	100 aA
Anahuac	84.4 aC	95.9 aB	99.7 abA	99.8 aA	100 aA	100 aA	100 aA

Percentages followed by the same letter are not significantly different by Fisher's exact test of independence ($P < 0.05$) with Bonferroni correction for multiple tests. Lowercase letters indicate pairwise comparison between varieties. Uppercase letters indicate pairwise comparison between time points

Table 3 Percentage of appressorium formation of *Magnaporthe oryzae Oryza* pathotype on leaf segments of wheat (*Triticum aestivum* L.) varieties sampled in seven time points post-inoculation

Variety	Time points post-inoculation (hours)						
	6	12	18	24	36	48	72
BR 18	15.9 aG	64.4 bE	87.5 aC	92.9 aB	45.0 cF	96.3 aA	79.4 bD
Ônix	30.6 aE	87.3 aB	80.9 bC	77.2 cC	57.2 bD	93.0 bA	86.0 bB
BRS 229	24.1 aD	17.1 cE	38.1 cC	75.4 cB	82.1 aB	96.2 aA	95.6 aA
Anahuac	23.4 aC	82.4 aB	88.0 aB	92.1 bA	83.9 aB	93.9 abA	94.4 aA

Percentages followed by the same letter are not significantly different by Fisher's exact test of independence ($P < 0.05$) with Bonferroni correction for multiple tests. Lowercase letters indicate pairwise comparison between varieties. Uppercase letters indicate pairwise comparison between time points

Table 4 Percentage of appressorium-mediated penetration of *Magnaporthe oryzae Oryza* pathotype in leaf tissues of wheat (*Triticum aestivum* L.) varieties sampled in seven time points after inoculation

Variety	Time points post-inoculation (hours)						
	6	12	18	24	36	48	72
BR 18	0 aC	4.7 bC	2.2 aC	13.1 bB	3.2 bC	5.1 bC	27.8 aA
Ônix	0 aB	25.8 aA	3.7 aB	27.3 aA	9.3 bB	27.0 aA	26.2 aA
BRS 229	0 aB	2.9 bB	1.3 aB	20.5 dB	6.9 bA	8.4 bA	12.5 bA
Anahuac	0 aB	1.7 bB	3.7 aB	5.8 cB	30.4 aA	28.0 aA	6.4 cB

Percentages followed by the same letter are not significantly different by Fisher's exact test of independence ($P < 0.05$) with Bonferroni correction for multiple tests. Lowercase letters indicate pairwise comparison between varieties. Uppercase letters indicate pairwise comparison between time points

(92%) and at 48 hai for BR 18 (96%), Ônix (93%), and BRS 229 (93%).

The penetration rate of MoO hyphae on the leaf tissues varied between the varieties and in relation to the evaluated times (Table 4). At 6 hai, no hyphae penetration was observed in any wheat variety. In the susceptible variety Anahuac, rates of germinated conidia that had differentiated appressoria and succeeded in penetrating leaf tissue were low until 24 hai. The highest percentage of penetration occurred at 36 hai, when they abruptly reached a peak of 30%. In Ônix, the maximum penetration rate (around 26%) occurred at 12, 24, 48, and 72 hai. At 18 and 36 hai, a low percentage of hyphae penetration was observed. In BRS 229 and BR 18, the maximum values of differentiated appressoria whose hyphae successfully penetrated leaf tissues were observed at 24 hai (20%) and 72 hai (27%), respectively.

Colonization, defined as invasion of hyphae into various neighboring cells from the point of penetration below the appressorium, was analyzed only at 48 hai, because this was the only time point at which the event could be most clearly observed. After this time, the necrotrophic growth of the fungus over dead cells turned critical the microscopic observation. The four wheat varieties significantly differed from one another (Table 5). The highest percentage of colony formation was observed in the susceptible variety Anahuac (71%), followed by Ônix (43%), BRS 229 (18%), and BR18 (2%).

A reaction in the leaf tissues to MoO infection was also observed (Table 6 and Figs. 2 and 3). A hypersensitivity

Table 5 Percentage of colony formation from the hyphae penetration of the *Magnaporthe oryzae Oryza* pathotype in leaf tissues of four wheat (*Triticum aestivum* L.) varieties at 48 h after inoculation (hai)

Variety	Leaf colonization at 48 hai (%)
BR 18	2.6 c
Ônix	43.6 b
BRS 229	19.0 c
Anahuac	71.0 a

Averages followed by the same letter in a column did not differ according to Fisher's exact test of independence ($P < 0.05$) with Bonferroni correction for multiple tests

Table 6 Percentage of cell death following infection of *Magnaporthe oryzae Oryza* pathotype in leaf tissues of four wheat (*Triticum aestivum* L.) varieties in four time points after inoculation

Variety	Hours after inoculation			
	24	36	48	72
BR 18	70.8 aA	70.5 aA	91.3 aA	93.3 aA
Ônix	5.3 bC	9.3 bC	61.7 bA	23.3 bB
BRS 229	3.6 bD	10.9 bC	84.5 aA	29.1 bB
Anahuac	4.7 bC	11.6 bB	39.9 cA	14.5 cB

Averages followed by the same lowercase letter in a column and capital letters in a line did not differ according to Fisher's exact test of independence ($P < 0.05$) with Bonferroni correction for multiple tests

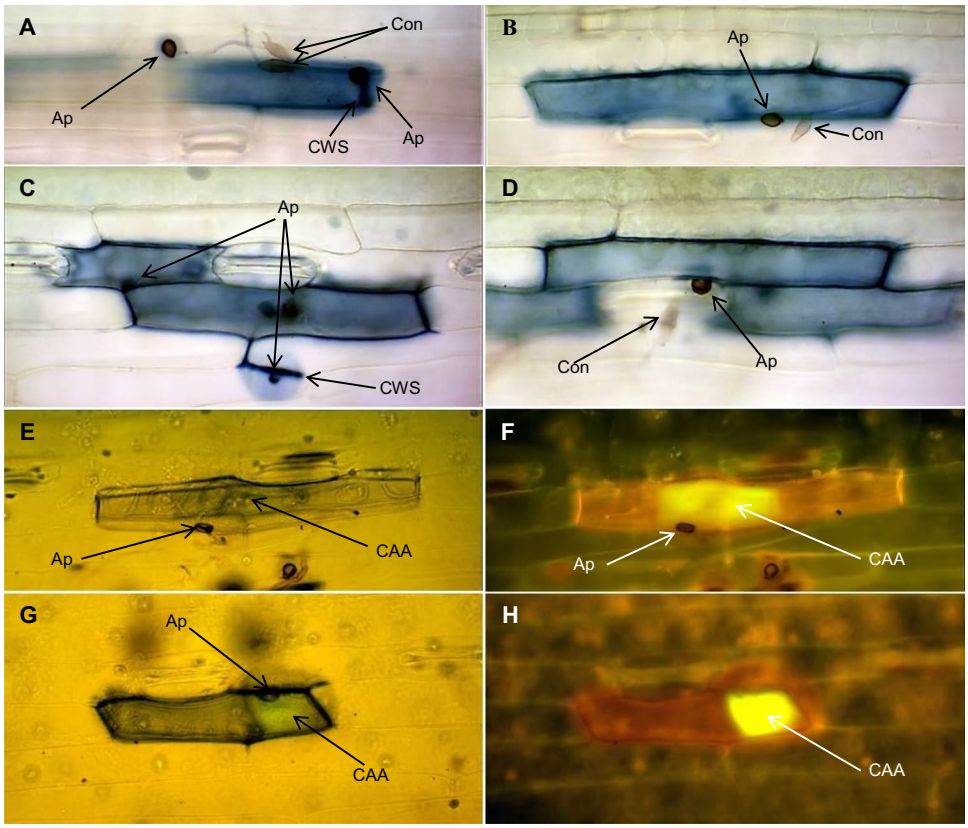

Fig. 2 Hypersensitive cell death in four wheat varieties in response to infection of *Magnaporthe oryzae Oryza* pathotype. Images obtained by phase contrast light microscopy (**A**–**D**), brightfield microscopy (**E** and **G**), and fluorescence light microscopy (**F** and **H**). Variety BR 18 cell death shown at 24 h after inoculation (hai) (**A** and **B**); variety BRS 229 with cell death peaked at 48 hai (**C** and **D**); and variety Ônix with cell death peaked at 48 hai (**E** and **G**) with cytoplasm aggregation visible at 48 and 72 hai (**F** and **H**). Images captured using an Olympus BX-41 microscope, 200 × magnification. Con—Conidium; Ap—appressorium; CWS—cell wall strengthening; CAA—cytoplasm aggregation, autofluorescent under UMWB2 (blue excitation filter set), consisting of a 460- to 490-nm dichroic beamsplitter 500-nm BA520 barrier filter

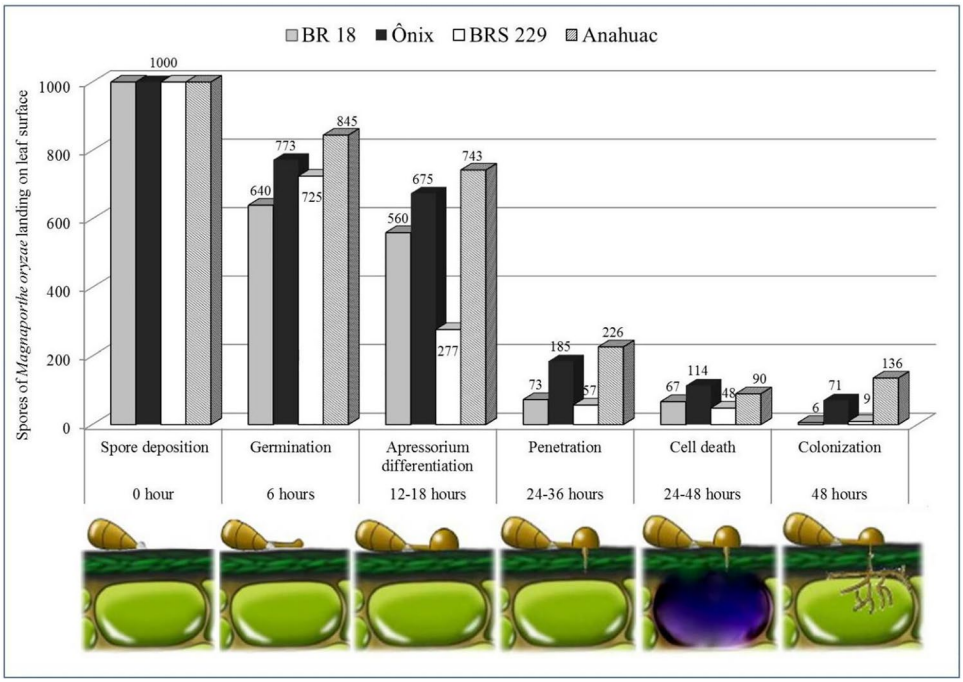

Fig. 3 Theoretical model adapted from Wesp-Guterrez et al. [44]of a pre- and post-infection cycle of the Magnaporthe oryzae Oryza pathotype in leaf tissues of resistant wheat varieties BR 18, Ônix, BRS 229 compared to the susceptible variety Anahuac. Based on the results obtained, the bars indicate the subsequent efficiency in the events of infection, starting from an equal number of 1000 conidia landed on leaf surface. Pictures of the infection cycle were adapted from Ribot et al. [31]

(HR) response due to epidermal cell death under appressoria was observed with greater intensity in resistant varieties (Onyx, BRS 229, and BR18) compared to the susceptible (Anahuac). Cell death could be visualized as soon as 24 hai in all four cultivars; however, this response was significantly higher in BR 18 (70%) of cell death associated with melanized appressoria (Table 6). In Ônix (5%) and BRS229 (3%), it was not significantly different from that observed in

Anahuac (4%). However, in Ônix and BRS229, this response reached a peak at 48 hai, with 61% and 84% of cell death associated with melanized appressoria, respectively, while in the susceptible variety, it was only 39%. In all four wheat varieties, the observed cell death clearly could be categorized in two types: single that consisted of collapse restricted to only one cell (Fig. 2A and B) and multiple cell death that consisted of cell death spanning several adjacent cells (Fig. 2C and D). In addition to cell death, strengthening of cell wall in the penetration sites of the fungus was observed in resistant varieties BR18, Ônix, and BRS229 (Fig. 2A and C; Fig. 3). In Ônix, in addition to the hypersensitive cell death and cell wall strengthening, infection attempts were also blocked by an additional resistance response that could only be observed at 72 hai. At this time point, 55% of cells undergoing HR also showed dense cytoplasm aggregation (Fig. 2E and G) with intense lemon-yellow coloration under fluorescence light (Fig. 2F and H).

The projected numbers of infective events taking place in the studied varieties, starting with a theoretical 1000 conidia landing on a leaf surface, are shown in Fig. 3. Numbers in each stage were calculated with respect to the previous event and were based on the data collected, as displayed in Tables 2 through 6. At each stage of the infection cycle, the efficiency of infection decreased, with BR 18, Ônix, and BRS 229 showing fewer established infection events than those of Anahuac. Although for all varieties the infection process started to be delayed as early as 6 hai, during the germination stage, for BRS 229, most pronounced reductions in infection efficiency were seen during both the appressorium formation and penetration stages, where of the 725 germinated conidia, only 277 succeeded to differentiate appressoria; and of these, only 57 successfully penetrated the leaf epidermal cells. For BR 18 and Ônix, infection was more efficiently delayed during the penetration stage: where of the 560 and 675 melanized appressoria, only 73 and 185 successfully penetrated, respectively.

Discussion

In the present study, we investigated the cytological events occurring in seedling leaves of Brazilian field-resistant wheat varieties in response to one isolate of *M. oryzae Oryza* pathotype from 6 to 72 hai. The data presented in this paper provide evidence that resistant wheat plants possess a few defense mechanisms against *M. oryzae Oryza* pathotype, such as inhibitors of germination, infection structure growth, haustorium formation and functioning, physical and chemical barriers. To date, only few studies have demonstrated the pre- and post-infection processes of *M. oryzae* in wheat and the plant's reaction mechanisms to its infection.

Among the four varieties studied, the susceptible variety Anahuac also showed a reduction in infection efficiency, especially in the penetration stage; of the 743 conidia that differentiated appressoria, only 226 successfully penetrated; and of these, 136 were able to colonize leaf tissues. This was also observed in wheat leaves inoculated with 1000 urediniospores of *Puccinia triticina* Erikss. & Henn., in which only 20 urediniospores managed to colonize the susceptible variety, reaching a ratio of only 1000:1 for the partially resistant variety [44].

The type of cellular responses observed in the Brazilian wheat varieties BR 18, Ônix, and BRS229 was similar to those reported previously for many *Magnaporthe* × Poaceae interactions. Tufan et al. [37] reported that wheat variety Renan exhibited autofluorescence in several cells in the epidermis and mesophyll at 72 hai with a *Triticum* isolate of *M. oryzae*, occasionally accompanied of the formation of denser papilla-like structures. Resistance to *Digitaria* isolates produced no visible macroscopic symptoms, and most infection sites were arrested from 24 hai, with formation of cell wall appositions (autofluorescent halo). Hyphae in first invaded epidermal cell were associated with epidermal and mesophyll fluorescence by 72 hai, occasionally accompanied of the formation of denser papilla-like structures. Our findings regarding the occurrence of single or multiple epidermal cell autofluorescence were similar to those observed in both host and non-host resistance of rice and wheat against *M. oryzae* isolated from *Digitaria*, *Triticum*, and *Oryza* [3, 13]. So far, the role of autofluorescence response concerning cell resistance, however, has been mainly associated with cell death [21].

Despite that our work has observed resistance expressed on leaves of young plants, it may be of epidemiological significance to reduce epidemics. In another interaction, it has been reported that with the *M. oryzae Triticum* pathotype (MoT) only 57% of the head reaction could be explained by the seedling reaction [8], and that the reduction of inocula on lower leaves could be a factor contributing to disease management [9]. Mechanisms of cellular resistance responses in the *M. oryzae* × Poaceae pathosystems, once well characterized, can be an efficient breeding tool for achieving durable, effective forms of disease management. Knowing the mechanisms of resistance of wheat varieties to this pathogen is of significant scientific importance, particularly the pre- and post-infection mechanisms in different genetic materials with different levels of resistance.

Our results suggest that recognition can start in the very early hours after inoculation with a sequence of events occurring in order to prevent infection that continues evolving until very late, culminating in cell death, which would involve combined and overlapping mechanisms of resistance, such as pathogen-triggered immune (PTI), by pathogen or microbe-associated molecular patterns (PAMP or

MAMP) that are conserved between species of a microbial group, and that triggered by isolate-specific pathogen effectors (ETI) [32]. The current understanding is that PTI is an important factor in non-host resistance and may contribute to quantitative resistance,ETI forms the basis of qualitative resistance [16, 27].

Although the concepts of qualitative and quantitative resistance often have been presented as a dichotomy, a continuum of scenarios can exist [27, 30] and overlapping mechanisms mediating both non-host resistance and quantitative resistance have been hypothesized [16, 27]. More recently, Yuan et al. [45] propose a revised model in which potentiation of PTI is an indispensable component of ETI during bacterial infection. This model conceptually unites the two major signaling cascades in plants and mechanistically explains some of the long-observed similarities in downstream defense outputs between PTI and ETI. The differences found among the levels of resistance among the varieties BR 18, Ônix, and BRS 229 and the susceptible variety Anahuac occurring sequentially and in all stages of the infection cycle (Fig. 3) may suggest a combined and overlapping PAMP-triggered immunity (PTI) and effector-triggered immunity (ETI). This hypothesis is also in accordance with the results reported by Casassola et al. [6] who analyzed the differential expression of genes involved in adult plant resistance for wheat variety Toropi-*P. triticina* pathosystem. They found that classical defense genes, including peroxidases, β-1,3-glucanases, and an endochitinases, were expressed both early (pre-haustorial) and late (post-haustorial) over the 72-h infection time course. The response seen in Toropi indicated a possible PTI resistance response at 24 hai, associated with an ETI resistance response, leading to the hypersensitive cell death at 120 hai. The authors pointed out that pre-haustorial leaf rust resistance in adult plants of Toropi is unusual. Phenotypically, it resembles non-host resistance in *Arabidopsis* to barley powdery mildew (*Blumeria graminis* DC. Speer f.sp. *hordei* Marchal) [4, 7] and in barley to non-adapted rust species [48],in these cases, infection is suppressed early by pre-haustorial mechanisms without cell necrosis, with the few haustoria that may elicit a post-haustorial hypersensitivity response.

The combined events related to plant resistance, including cell death (which was faster in BR 18), cell wall strengthening, and autofluorescent cytoplasm aggregation (Fig. 2) contributed to prevent symptoms of infection in the studied varieties. The few pinpoint lesions type 1 were the only symptoms observed in BR 18; however, cultivars Ônix and BRS229 did not exhibit any visible symptom of the disease (Fig. 1). Sherwood and Vance [33] reported that grasses have constitutive and inducible resistance mechanisms associated with the epidermis. The first mechanism restricts the frequency of penetration and the second is correlated with appositional cell wall formation (papillae), a general mechanism of resistance to fungal penetration in the Gramineae. Deposition of papillae at sites of pathogen detection is thought to act as a physical barrier to limit access of pathogens to the underlying protoplast and chemical due to the accumulation of antimicrobial compounds [38]. In addition to two avirulence loci, *Pwt1* and *Pwt2*, conditioning HR and papilla formation, respectively, were postulated in wheat Norin 4 inoculated with hybrid isolates of *M. grisea* from *Setaria* and *Triticum* [26] and inoculated with hybrid *Oryza*×*Triticum* isolates of *M. oryzae* [36].

To date, 10 *Rmg* blast R genes have been identified in wheat [11, 28, 34, 35, 42, 43, 47]. More recently, Anh et al. [2] suggested that *Rmg7* and *Rmg8* appear to participate in gene-for-genes (plural) interactions [15], in which one avirulence gene corresponds to more than one resistance gene, and these two resistance genes would be equivalent to a single gene from the viewpoint of resistance breeding. Some varieties were found to carry more than one *Rmg* gene; for example, Norin 4 and Shin-chunaga carry *Rmg1*, *Rmg4*, and *Rmg6* [19, 28, 35, 42],Norin 26 carries *Rmg1* and *Rmg4* [28, 35],and Thatcher carries *Rmg2* and *Rmg3* [47]. Since both Ônix and BRS229 have Norin 26 and Thatcher in their genetic background (http://www.wheatpedigree.net), it can be hypothesized that one or more *Rmg* genes identified in these genotypes could be present in the varieties of wheat plants in Brazil. Alternatively, these varieties may carry new R genes, since none of the *Rmg* genes previously identified in wheat has been found against an avirulent isolate of *M. oryzae* from rice (MoO pathotype), but from *Digitaria sanguinalis* (L.) Scop. [28], *Lolium perenne* L. [42], *Avena sativa* L. [11, 19, 35], and *Triticum aestivum* L. [1, 34, 43, 47]. Nevertheless, the resistance response in each variety results from combined mechanisms. Further studies will determine whether Ônix, BR 18, and BRS 229 carry new or already known resistance genes to *M. oryzae*.

The work presented in this paper, by testing the interaction of MoO on young leaves of wheat varying in their levels of resistance, offers a suitable and helpful model to understand some mechanisms by which plants can reduce infection and, therefore, the epidemic impact of wheat blast. The two most remarkable effects were seen on the rate of successful penetration and on the colonization, although not alone but along with other mechanisms they can be of significance.

Acknowledgements Thanks are given to Dr. John H. McDonald (University of Delaware, USA) for the assistance in the statistical analysis.

Author contribution Conceptualization: Chaves, M.S.; Martinelli, J.A.; Barbieri, M. Data acquisition: Barbieri, M. Data analysis: Barbieri, M.; Chaves, M.S.; Silva, G.B.P. Design of methodology: Chaves, M.S.; Graichen, F.A.S. Writing and editing: Barbieri, M.; Chaves, M.S.; Martinelli, J.A.; Silva, G.B.P.; Graichen, F.A.S.; Torres., G.A.M.

Funding The authors wish to thank the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Empresa Brasileira de Pesquisa Agropecuária (Embrapa- (Project SEG 02.11.08.004.00.00)) for financial support.

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