BLAST (*Magnaporthe oryzae*), A POTENTIAL THREAT TO WHEAT PRODUCTION IN SOUTH AMERICA

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SUMMARY

Magnaporthe oryzae has been reported infecting wheat spikes since 1985 in Brazil. The disease is called head blast or brusone. Few wheat cultivars were described as moderately resistant to the pathogen. Since 2009, Embrapa is coordinating a national initiative searching for wheat resistant genotypes and developing molecular genetics studies for assisting breeding programs. The research goals have been based on phenotyping under field and controlled conditions. From 2010 to 2014, 435 genotypes were characterized in blast nurseries, 308 varieties and 127 synthetic wheats. Under controlled conditions, 80 genotypes were inoculated, 10 of which were identified with contrasting responses to the disease (candidate-wheat genotypes, CWG). Molecular analyses including QTL mapping, association genetics, transcriptomics and proteomics have been carried on. Seedlings and adult plants from the ITMI population were phenotyped for their response to the blast disease. A doubled-haploid population was developed specifically for genetic studies of the resistance. For these parents analyses of differential transcriptional profiles have been carried on by suppression subtractive hybridization (SSH) and by Next-Generation Sequencing (NGS), 454 sequencing and Illumina, technologies.

Keywords: Brusone, *Pyricularia oryzae*, genetic resistance, gene prospection, phenotyping

INTRODUCTION

Wheat blast was first identified on wheat heads in 1985 in the state of Paraná, Brazil (Igarashi *et al.,* 1986). Until now, the disease was observed in several wheat growing regions in the country: São Paulo (Igarashi 1991), Mato Grosso do Sul (Goulart *et al.,* 1990), Rio Grande do Sul (Picinini and Fernandes 1990), Goiás (Prabhu *et al.,* 1992) and in the Cerrados region of Central Brazil (Anjos *et al.,,* 1996). In South America, wheat blast disease is endemic in north-eastern Argentina, lowlands of Bolivia and Paraguay (Kohli *et al.,* 2011).

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The causal agent (*Magnaporthe oryzae*, anamorph *Pyricularia oryzae*) may infect different parts of the wheat plant. The most serious symptoms are observed in wheat spikes which are frequently bleached. Infected rachis have a constriction region with a dark color. Above this region, grains are commonly small, wrinkled and have low test weight (Goulart, 2005).

In 1993, natural occurrence of blast on wheat was reported in the Cerrados region. Infected spikes showed a 10.4% reduction in the number of grains. The reduction in the grain weight per head varied from 8% to 29% in infected spikes depending on disease severity (Anjos *et al.,* 1996). In 2004, at Dourados county, early infections caused higher losses (63.4%) than later ones (46%). Furthermore, damages due to blast incidence depend on varieties and region (Goulart *et al.,* 2007). Grain yield losses caused by wheat blast have been reported from very low levels to almost 100% (Kohli *et al.,* 2011).

Wheat is cultivated in Brazil in several regions which are quite diverse and distributed along temperate, tropical and sub-tropical zones (Cunha *et al.,* 2011). Knowledge about environmental conditions that determine higher blast levels, under field conditions is scarce. The disease frequently occurs at high temperature and humidity (Torres *et al.,* 2009).

Until early 2000s, most wheat varieties showed heads with high levels of susceptibility to blast (Urashima and Kato, 1994, Goulart *et al.,* 1995, Arruda *et al.,* 2005). Differential reactions among plant varieties and fungal isolates are commonly observed (Urashima and Kato, 1994, Urashima *et al.,* 1993, Urashima *et al.,* 2004). Despite an intense search for sources of resistance to wheat blast, few cultivars are known or reported as moderately resistant to *M. oryzae* or presenting a good performance under disease pressure (Urashima *et al.,* 2004, Goulart *et al.,* 2007). Comparatively to rice blast, molecular studies about *Magnaporthe* x wheat interaction are quite few and concentrated in seedling responses to the pathogen infection (Tufan *et al.,* 2009, Tufan *et al.,* 2012). As far as we know, only two genes for resistance to *Triticum* isolates of *M. oryzae* were identified in a wheat cultivar (Zhan *et al.,* 2008). Consequently the infection process in wheat spikes and even the genetic basis for resistance are not yet well understood.

MAJOR OBJECTIVES

In this context, in 2009, the Empresa Brasileira de Pesquisa Agropecuária (Embrapa) undertook the coordination of a Brazilian initiative to better characterize wheat resistance to blast disease. Institutions like Universidade Federal do Rio Grande do Sul (UFRGS) and Empresa de Pesquisa Agropecuária de Minas Gerais (EPAMIG) were involved, as well as several Embrapa centers. The national 'WheatBGIn project' (Wheat Blast Genes Interaction, from 2009 to 2012) aimed to find sources of resistance based on standardized phenotyping, specifically at the adult plant stage. Concurrently to the identification of contrasting genotypes and sources of resistance, molecular studies would be hold by QTL mapping, association genetics, transcriptomics and proteomics approaches (Figure 1). A second project (Wheat BGIn2, 2012-2016) is underway). It has the objective of characterizing strategies for the use of genetic resistance to combat blast disease (Figure 2).

Anahuac 75 is a wheat variety introduced from Mexico and largely used in Brazil in the early 1990s but withdrawn from cultivation because of blast outbreaks (Sousa, 1999). Even upon inoculation, Anahuac 75 is widely known as highly susceptible to blast disease (Urashima and Kato, 1994, Arruda *et al.,* 2005) and is used for our experiments as a susceptible check.

RESULTS

We have identified several wheat genotypes presenting lower disease levels either upon inoculation or in nursery conditions. Results of phenotyping activities and further molecular analyses will be presented in detail.
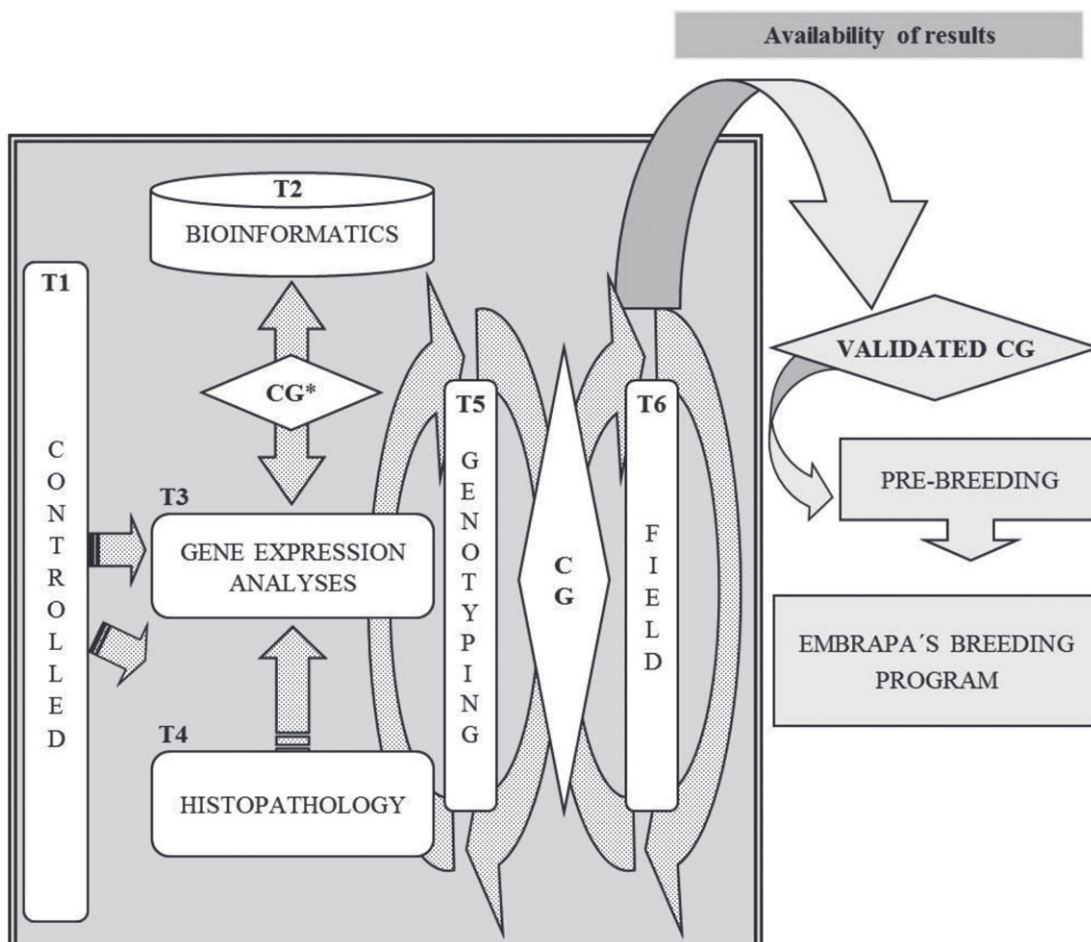
Figure 1. Research themes (T) of Wheat Blast Genes Interaction (Wheat BGIn) project. T1, wheat phenotyping under controlled conditions; T2, bioinformatics and information technology; T3, transcriptome and proteome analyses; T4, histopathology studies upon inoculation with *Magnaporthe oryzae* isolates; T5, wheat genotyping; T6, wheat phenotyping under field conditions. *The synergistic approach of the scientific themes of the WheatBGIn initiative has generated candidate-genes* (CG) *of wheat related to disease resistance.*

PHENOTYPING UNDER FIELD CONDITIONS

Since 2010, we have evaluated a collection of wheat germplasm in a blast nursery (or hotspots) conditions. Wheat genotypes are sown in three locations where the occurrence of blast is frequently observed in Brazil: Londrina-PR (Paraná state), Dourados-MS (Mato Grosso do Sul state) and Planaltina-DF (Distrito Federal).

We are focused on characterizing molecular responses of wheat under high pathogen pressure and identifying genes/QTLs/markers

associated to resistance. Studies and reports about disease evaluations for wheat blast are quite diverse. This variability makes comparison of phenotypic data difficult. Standard disease evaluations are crucial for phenotyping is and for efficient molecular analyses (Postman *et al.,* 2010, Rafalski, 2010).

We established a standardized methodology for disease evaluation (data not published). Our experimental design aims at characterizing plant responses to the blast disease in nurseries rather than screening germplasm.
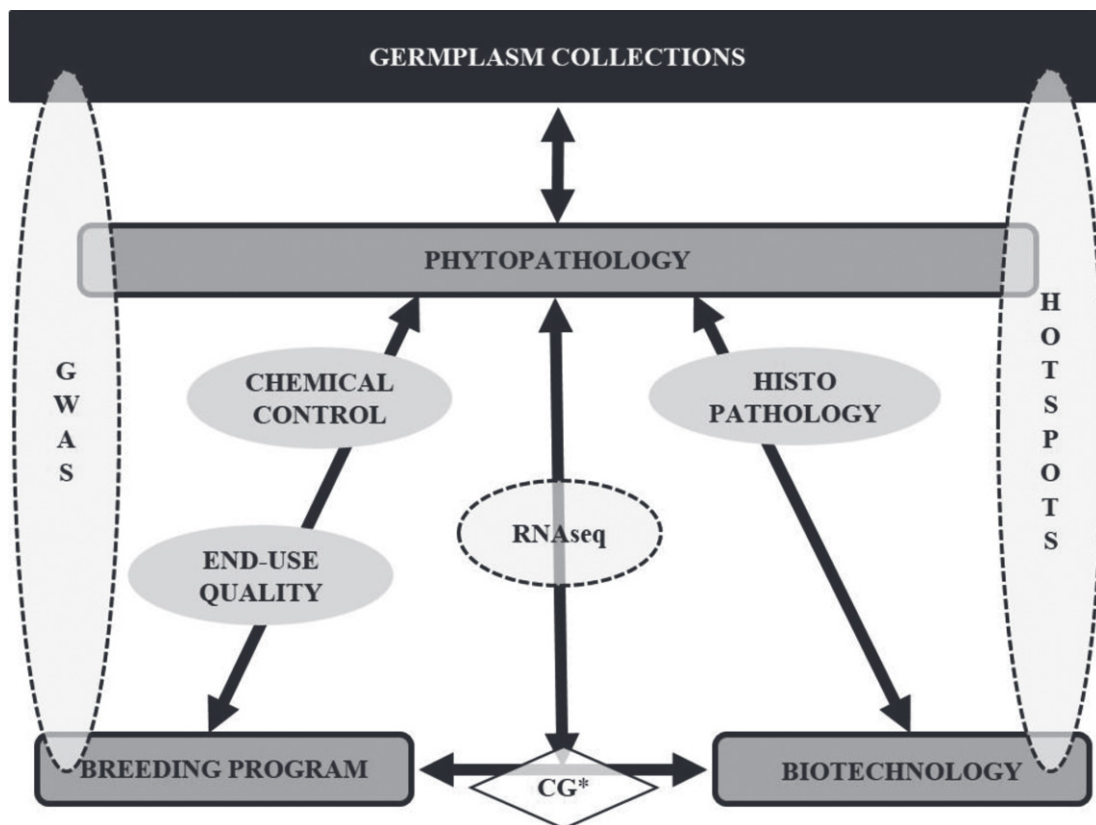
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Figure 2. Research themes (T) of 'Wheat BGIn2' project. Integrated strategies to characterize wheat resistance to blast disease. CG means candidate-genes. *Fine phenotyping of germplasm collections have been sustained by integrative approaches of phytopathology, breeding and biotechnology.*

The results obtained were submitted to publication elsewhere (Torres *et al.,* submitted).

PHENOTYPING UNDER CONTROLLED CONDITIONS

A collection of eighty genotypes was inoculated at heading stage, under controlled conditions. Ten genotypes (12.5%) had contrasting responses to the disease development (data not published). These genotypes, which had a susceptibility or a resistance response to fungal infection, were named 'candidate wheat genotypes' (CWG) and they were further characterized. Here, we present results for a susceptible and a resistant CWG.

Genotypes were sown in three pots with four seeds per pot. Plants were cultivated under greenhouse conditions until heading and then inoculated. Other diseases were controlled by fungicides application of until booting. Growth stages of wheat plants were assessed three up to five times per week. The development stage of each spike selected for inoculation was assessed using the Zadoks *et al.* (1974) scale.

Some isolates of *M. oryzae* (from Plant Pathology Laboratory, Embrapa Wheat) were used in preliminary tests with seedling and adult plants. Isolate Py6025 was selected as it caused the most severe symptoms and was employed in further experiments.

Later studies (A. Nhani Jr., data not published) corroborated these results. Using 263,987 SNPs, Py6025 isolate was grouped with the most aggressive isolates of *M. grisea* from wheat (Maciel *et al.,* 2014).

Py6025 was cultivated on oat agar medium for 10 to 12 days, under 24 to 25 ° C (± 2 ° C), with 12 hours of light. For the preparation of inoculum suspension, dishes were washed with distilled water and Tween 80 (two drops per liter) was added. Concentration was adjusted to 2 x 10^5 conidia/ml.

One spike composed a replicate per treatment per genotype and four replicates were used per experiment. Inoculation experiment was repeated ten times. Twenty-four hours before inoculation, pots were transferred to an inoculation chamber with temperatures fitted from 26 to 28 ° C. Non inoculated spikes were also sprayed with distilled water with Tween 80 and protected with polyethylene bags to prevent contamination with spores. Fifteen minutes after spraying inoculum, plants were submitted to 12 hours of dark, 24 hours of humidity saturation at 28 ° C, to optimize infection conditions. After the first twenty-four hours (24 hours post inoculation, hpi), plants were maintained for seven days in the same chambers programmed to temperature of 26 ° C +- 2 ° C, 12 hours of light and nebulization of water every 2 minutes during 30 minutes. At 8 days post inoculation (dpi), pots were transferred to greenhouse conditions where plants remained until their physiological maturity and harvest of grains.

Since 3 dpi, wheat heads were inspected for the appearance of symptoms and for the type of blast lesions (Urashima et al.,, 1993). Blast symptoms on spikes were daily evaluated until the maturation of plants. Spikelets with lesions and bleached spikes were counted. Disease severity, considering the presence of lesions and bleaching severity was estimated based on the total of spikelets per spike.

Results of disease evaluations, for 52 spikes on average, are presented in Table 1. At 6 dpi, while Anahuac 75 presented 49.6% of spikelets with lesions, S-CWG presented 13.6% and R-CWG, 28.6%. However, if we consider disease severity expressed as percentage of bleaching, disease quantitation was quite different. At 9 dpi, S-CWG presented the highest bleaching (74.7%), followed by Anahuac 75 (66.5%) and the R-CWG (1.8%). It took 20 days to the resistant-candidate to attain 19.4% of bleached spikelets.

Mature plants were harvested and tagged spikes were individually threshed. Production evaluation was based on counting total grains and the weight of grains produced per spike. Estimations were further made for mean weight of grain for each sample. Significant differences were found among the means of grain production data using t test (GeneStat, VSN International 2013) for control and inoculated treatments with probability < 0.05 for all cases but number of grains per spike of R-CWG (prob < 0.255).

In inoculation experiments (Figure 3), control treatments (white histograms), statistically significant differences among the three genotypes were found for both grain production parameters: number of grains per spike (Figure 3-A), and mean grain weight, in milligrams (Figure 3-B). R-CWG produced more grains per spike than Anahuac 75 and this one produced more than S-CWG (Figure 3-A). For mean grain weight (Figure 3-B), R-CWG had the highest value followed by Anahuac 75 and finally by S-CWG. In inoculated spikes (gray histograms), the R-CWG presented both parameters significantly higher than the other genotypes. While S-CWG produced more grains per spike than

Table 1. Disease severity, expressed as percentage of spikelets with lesions (%) at 6 dpi and as percentage of bleached spikelets (%) at 9 dpi, of Anahuac 75, a susceptible-candidate wheat genotype and a resistant-candidate wheat genotype submitted to inoculation with *P. oryzae*.

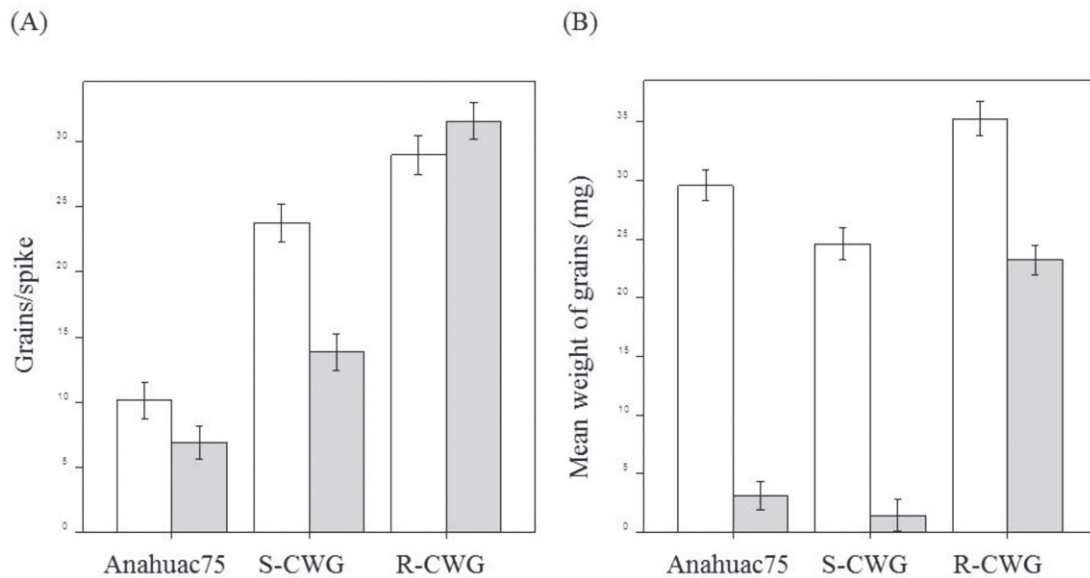|	Anahuac 75	Susceptible-CWG	Resistant-CWG
% spikelets with lesions at 6dpi	49.6	13.6	28.6
% bleached spikelets at 9dpi	66.5	74.7	1.8

Figure 3. Number of grains produced per spike (A) and mean weight of grains (B) of Anahuac 75, a susceptible-candidate (S-CWG) and a resistant-candidate (R-CWG) wheat genotypes. White histograms represent control spikes and the gray ones represent spikes inoculated with *P. oryzae* suspension.

Anahuac 75 (Figure 3-A), there was no significant difference for mean weight of grains between both susceptible genotypes (Figure 3-B).

Comparing mock and inoculated treatments within each genotype, significant differences were observed for both variables (number of grains per spike and mean grain weight) in susceptible genotypes. When inoculated, Anahuac 75 presented a reduction of 32% and 89% and S-CWG presented a reduction of 42% and 94%, respectively for number of grains and mean weight of grains. For R-CWG there was a statistical difference in mean grain weight between control and inoculated spikes (reduction of 34% upon inoculation), but not for the number of grains produced per spike.

Several genotypes whose reaction to blast was phenotyped had not been studied before. CWG whose results were presented are BRS209 and CBFusariumENT014. BRS209 is a variety from Embrapa that had already been reported as susceptible to blast (Prestes *et al.,* 2007). CBFusariumENT014 is a synthetic wheat belonging to a crossing block (CB) from the International Maize and Wheat Improvement Center (CIMMYT, Van Ginkel *et al.,* 2002). The CIMMYT CB, are collections of elite breeding parental stocks and source materials arranged by mega-environment (ME). Genotypes from each section of the CB carry specific genes valuable for their defined ME. In this case the ME is *Fusarium* head blight. As far as we know, CBFusariumENT014 had not been characterized for blast resistance. Interestingly, it showed low levels of blast incidence when characterized in Planaltina´s nursery conditions.

The scope of use of a particular germplasm is directly related to the availability and quality of its characterization. Costs for molecular analyses have sharply declined. The challenge now is linking their fine characterization of gene(s) related to defence to blast disease.

BRS209 and CBFusariumENT014 were used as parents of a mapping population developed for genetic studies of wheat resistance to blast (Webber *et al.,* in preparation). Nowadays, we have developed 118 doubled-haploid lines at Embrapa Wheat (Scagliusi *et al.,* in preparation).

EXPRESSION ANALYSES

Tissues of plants inoculated with *M. oryzae* were collected at 2 dpi. Total RNA was extracted combining TRIzol (Invitrogen, Carlsbad, California) and RNeasy methods (Qiagen Biosciences, Maryland). PCR-based suppression subtractive hybridization (SSH) was conducted to search for defense-related genes from a wheat genotype which is possibly resistant to *M. oryzae*. Two subtraction libraries, named forward and reverse, were generated by using cDNA synthesized with RNA from inoculated spikes and RNA derived from mock-inoculated wheat spikes as the tester, respectively.1728 clones were sequenced which corresponded to 420 contigs. Forty expressed sequences were selected for validation by qRT-PCR.

The application of next-generation sequencing (NGS) technologies can accelerate wheat crop improvement (Berkman *et al.*, 2012). Aiming to understand the biological basis of wheat response to *Pyricularia* sp. infection we have undertaken transcriptome analyses using NGS technologies. Roche 454 sequencer uses pyrosequencing technology (Margulies *et al.*, 2005) and HiSeq2000 from Illumina adopts the technology of sequencing by synthesis (SBS) (http://www.illumina.com). While the former current systems can produce around 400 Mbp of sequence data with reads of up to 400 bp, Illumina generates the greatest data volume of any NGS platform and equal to more than 35 hexaploid wheat genomes. Assembly and analysis of NGS data are underway.

CONCLUDING REMARKS AND FURTHER EXPECTED OUTCOMES

- Under favourable climatic conditions wheat blast disease can be devastating. In spite of representing a potential threat to wheat cultivation, the exploration of genetic resources of *Triticum* germplasm is an important strategy to improve plant resistance to blast.
- Coordinated by Embrapa, a Brazilian research initiative on wheat resistance to blast started in 2009. From 2010 to 2014, 435 wheat genotypes, including *T. aestivum* (306), *T. durum* (2) and synthetic wheats (127), have been phenotyped in wheat blast nurseries.
- There are currently four evaluation sites of the disease under natural field conditions: Planaltina-DF, Dourados-MS, Londrina-PR, Passo Fundo-RS. Since 2013, we have had a new site of disease evaluation at Passo Fundo-RS, where the disease occurs under experimental conditions during summer time. We intend to analyse local environmental variables and pathogen spore density that could be restrictive to disease development.
- Genotypes presenting contrasting responses to pathogen infection were identified under controlled conditions and in blast nurseries. A set of wheat genotypes identified as resistant-candidates have been evaluated under different planting dates in Minas Gerais state.
- Currently, novel research topics have been developed as chemical control strategies and the evaluation on the effects of blast on wheat end-use quality
- Wheat blast is a big challenge because plant-pathogen interactions still need to be better understood. Five years after the structuring and coordination of an interdisciplinary research group in Brazil, the first results of research started to be published. These integrative approaches will shed new light on the *Magnaporthe* x wheat interaction.

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