DNA Fingerprinting and Sexual Characterization Revealed Two Distinct Populations of *Magnaporthe grisea* in Wheat Blast from Brazil

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Abstract: The wheat blast caused by *Magnaporthe grisea* (Hebert) Barr (anam. *Pyricularia grisea* Sacc.) is a disease reported only in Brazil and other countries of the Southern Cone of Latin America. The yield loss, lack of resistant varieties, absence of efficient fungicides to protect wheat spikes, and its geographical distribution have made the disease a major problem in wheat producing states of the country. The origin of the wheat blast generated much speculation until it was demonstrated that the causal agent was different from the rice blast pathogen. The present work showed that two distinct populations of *M. grisea* are causing wheat blast disease in Brazil based on the existence of isolates with different sexual characteristics and distinguished DNA fingerprinting. Sexual reproduction is suggested for one subpopulation of the wheat blast disease.

Keywords: *Pyricularia grisea*; distinct populations; host range; sex; DNA

Magnaporthe grisea (Hebert) Barr (anam. *Pyricularia grisea* Sacc.), the causal agent of blast disease, is a pathogen that attacks more than 50 grass species (Ou 1985). Rice (*Oryza sativa* L.) is the most important crop for which severe yield loss has been documented worldwide. In 1986 in Brazil, *M. grisea* was reported to be causing severe infection in wheat (*Triticum aestivum* L.) resulting in heavy yield constraint in some regions of Parana state, the Brazilian most important producer (Igarashi *et al*. 1986). This was the first report of occurrence of blast in wheat in nature in the world. In subsequent years, the disease spread to other states of the country and the yield loss it causes, its geographical distribution, the lack of efficient fungicides and resistant varieties make it one of the major diseases of wheat in Brazil today (Igarashi 1990; Urashima & Kato 1994; Goulart & Paiva 2000; Urashima *et al*. 2004b).

As soon as the importance of the disease was realized the relationship with the rice blast disease was examined. Igarashi *et al*. (1986) described positive pathogenicity of the wheat isolates towards rice and concluded that the rice blast fungus was the primary inoculum of this new disease in wheat. Further studies, however, were unable to find any wheat isolate pathogenic to rice and because of the impossibility of cross-inoculation it was demonstrated that the wheat blast pathogen was different from *M. grisea* from rice (Prabhu *et al*. 1992; Urashima *et al*. 1993; Urashima & Kato 1998; Urashima *et al*. 2004a).

Other parameter employed to examine relationship between these two organisms was the sexual characterization of *M. grisea*. Because the ability of the fungus to produce perithecia indicates a complex phenotype, mating ability is an excellent criterion to study relationship among isolates from different hosts (Viji & Gnanamanickam 1998). As in all other countries, Brazilian rice blast isolates had low fertility and inability to mate in sharp contrast to wheat blast isolates that showed high sexual fertility, presence of hermaphrodite isolates with both mating type in the same field at

the same time (Urashima *et al*. 1993; Bruno & Urashima 2001).

Studies on the relationship between *M. grisea* from rice and wheat employing molecular tools revealed quite different eletrophoretic kariotypes between rice and wheat isolates as well as in number of repetitive elements MGR583: 50 or more copies in rice blast and 20-40 in wheat isolates demonstrating that wheat blast is genetically different from the rice blast isolates (Hamer 1991; Orbach *et al*. 1996; Urashima *et al*. 1999).

The studies mentioned above indicated that the wheat blast disease did not originate from the rice blast fungus. Nevertheless, the discrepancy between data of the first study and following works suggests existence of two distinct populations of the wheat blast. The existence of different populations of *M. grisea* causing wheat blast in Brazil was first suggested by Urashima *et al*. (1993), one composed of isolates employed by Igarashi *et al*. (1986) which infected rice and another one used by other scientists which were non pathogenic to rice (Prabhu *et al*. 1992; Urashima *et al*. 1993; Urashima *et al*. 2004b). Thereafter, no further studies on this subject were documented.

The existence of different populations causing one specific disease is a well-known phenomenon in plant pathosystems. Distinct subpopulations of *Puccinia recondita tritici* was identified in Canada based on phenotype characteristics (Kolmer 1991), two populations of *Phytophthora infestans* was detected in Mexico, one with sexual life cicle and another with clonal reproduction (Goodwin *et al*. 1992), *Cercospora zeae-maydis* has two distinct populations according to molecular data (Dunkle & Levy 2000). Distinct populations of *M. grisea* were also found in isolates causing gray leaf spot in *Lolium perenne* in Japan since isolates from west Japan had distinct DNA fingerprinting pattern when compared to east and north regions (Tosa *et al*. 2004).

The present work aims to demonstrate the existence of two different populations of *M. grisea* causing wheat blast disease in Brazil.

MATERIAL AND METHODS

Pathogen. Isolates of *M. grisea* were obtained from diseased plants randomly collected in one wheat field at Itaporã (MS) in Jul/1998 (designated MS01) and another at Londrina (PR) in Aug/1998 (designated PR01). The diseased wheat were collected from hot spot locations in two of the most blast affected regions in the country. Infected tissues were cut in small pieces, placed in petri dishes, and kept under moist condition for 24 h at 25°C. Single-conidial isolations were made on potato dextrose agar slants. For long-term storage, pure cultures were grown on sterilized wheat seeds in a vial, then dried completely at 25°C and stored in a plastic case with silica gel at 5°C. A total of 32 isolates from field MS01 and 27 from PR01 were employed.

Host. Seedlings employed in this study included rice, wheat, triticale (*X. triticosecale* Wittmack), barley (*Hordeum vulgare* L.), rye (*Secale cereale* L.), oat (*Avena sativa* L.), common millet (*Panicum mileaceum* L.), sorghum (*Sorghum vulgare* L.), and maize (*Zea mays* L.). Rice was composed by the Japanese differentials (Yamada *et al*. 1976), wheat by cultivars Embrapa10 and Br40, triticale by IAC3, barley by BRS180, rye by BR1, oat by IAC7, common millet by BRS1501, sorghum by BRS700, and maize by cv. P30K75. Four or five plants were grown in plastic pots (39 × 28 × 10 cm) with uniformly fertilized substrate (10 g of 4-14-8) + 5 g of ammonium sulfate and kept in a greenhouse at 23 ± 5°C until three- to four-leaf stage when they were inoculated.

Inoculation and disease evaluation. Inoculum was prepared from cultures grown on oatmeal agar medium. Transfers were made from potato dextrose agar slants onto oatmeal agar and incubated at 25°C under continuous fluorescent illumination. After 10–11 days, plates were flooded with sterilized distilled water, and aerial mycelia were removed by gently rubbing the colony with a paint brush. The suspension was then exposed to fluorescent light at 22°C for 3 days to induce sporulation. The conidial and mycelial suspension was filtered through two layers of cheesecloth and adjusted to 1×10^5 conidia per ml. Fifty ml of the conidial suspension with 500 µl of Tween20, was sprayed on the seedlings using a manual sprayer. The plants were placed in plastic bags to maintain a water-saturated atmosphere and placed in a greenhouse at 23 ± 5°C. After 18 h, bags were removed and the pots were randomized on the greenhouse bench with temperature ranging from 23–28°C until disease assessment. Four replications were used for each fungal isolate. Four to five seedlings were used per replication. Plants were examined for symptoms 7 days after inoculation. Six phenotypic reactions were recognized: 0 = no

visible reaction; 1 = minute, pinhead-sized spots; 2 = small brown to dark brown lesions with no distinguishable centers; 3 = small eyespot shaped lesions; with grey centers; 4 = typical blast lesions, elliptical with grey centers; 5 = complete blighting and leaf death. Lesion types 0, 1, and 2 were classified as non-compatible and lesion types 3, 4 and 5 as compatible. When different types of lesions were found on a single leaf, the largest lesions were considered.

Sexual characterization. Sexual characterization involved determination of mating type, and production of sexual organs (perithecia, asci, and ascospores). Crosses were performed by pairing the unknown isolate with fertile isolates of *MAT1-1* and *MAT1-2.* Two sets of isolates were employed as parentals: MS05-9 X PR02-5 and/or Br118.2d X Bp3a. Mycelial fragments of the unknown isolate and the parental isolates were placed about 4 cm apart at each point of the triangle on oatmeal agar in a Petri dish (Itoi *et al*. 1983). These crosses were kept in 22°C under continuous fluorescent illumination for about 4 weeks. The formation of perithecia occurred at the intersection of the mycelial growth if the cross was compatible. All crosses were performed at least twice.

DNA manipulation. Isolates were grown in CM (3 g Casamino acids, 3 g Yeast extract, 5 g sucrose/l) media on a rotary shaker at 24°C for 7–9 days. Subsequently mycelia were harvested and stored at –20°C. DNA was extracted as previously described (Urashima *et al*. 1999).

Analyses of fingerprinting. The band pattern of DNA fingerprinting was scored visually by checking the presence (value 1) or absence (value 0) of bands in the 0.5–10 kb range. Bands with ambiguous interpretation were not evaluated in order to minimize unexpected errors. The data was analysed by NTSYS program. The genetic distance was calculated by similarity coefficient through Simple Matching analyses. A phenogram was constructed using the "unweighted pair group method using arithmetic means" (UPGMA). A bootstrap analysis was carried out with the WINBOOT program (Yap & Nelson 1996), and the robustness of each cluster was verified in 1000 replications.

RESULTS

Data on sexual characteristics of *M. grisea* from wheat field in Parana state (isolates PR01) and in Mato Grosso do Sul state (isolates MS01) are shown in Figure 1. Mating type was determined by the formation of perithecia when the unknown isolate was crossed with the fertile parentals (Figure 1A). The capacity of wheat isolates to cross varied according to the field as isolates PR01 had low mating ability (only 2 out of 27 isolates crossed with parental accounting for 7.4% of total) whereas isolates MS01 high mating capacity (27 out of 32 isolates crossed with one of the parentals, representing 84% of total). Both mating types were identified in both fields although frequency of *MAT1-1* isolates was higher in field MS01. In terms of production of asci and ascospores clear difference between fields was also detected (Figure 1B). Blast isolates of field MS01 was the only ones where perithecia, asci and ascospores were produced in great majority of crosses: 27 out of 32 isolates formed perithecia and from these 22 showed asci and ascospore formation (68.7%). In another wheat field (PR01) only two isolates produced perithecia, asci and ascospores totalling 7.4% of total. Overall, field MS01 stood out for high sexual fertility of its isolates.

DNA fingerprinting of wheat isolates were produced by digestion of genomic DNA with *Eco*R1,

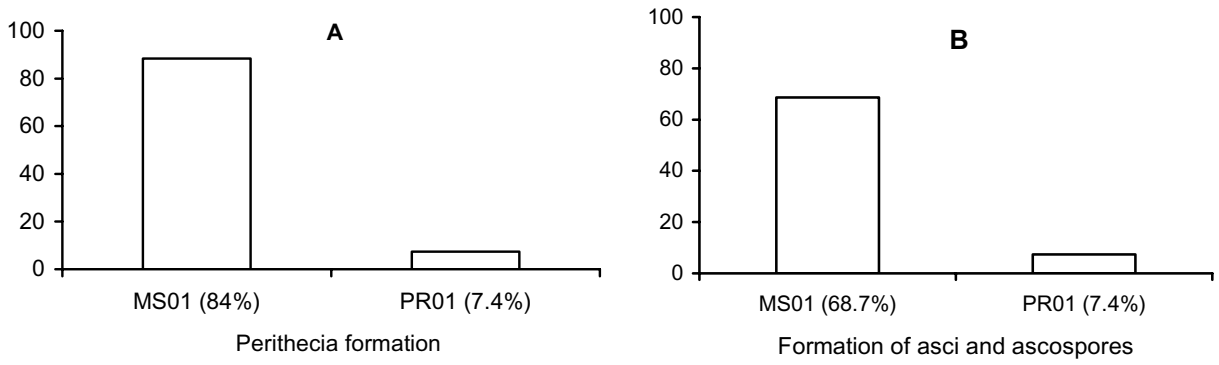

Figure 1. Sexual characterization of *Magnaporthe grisea* from two wheat fields

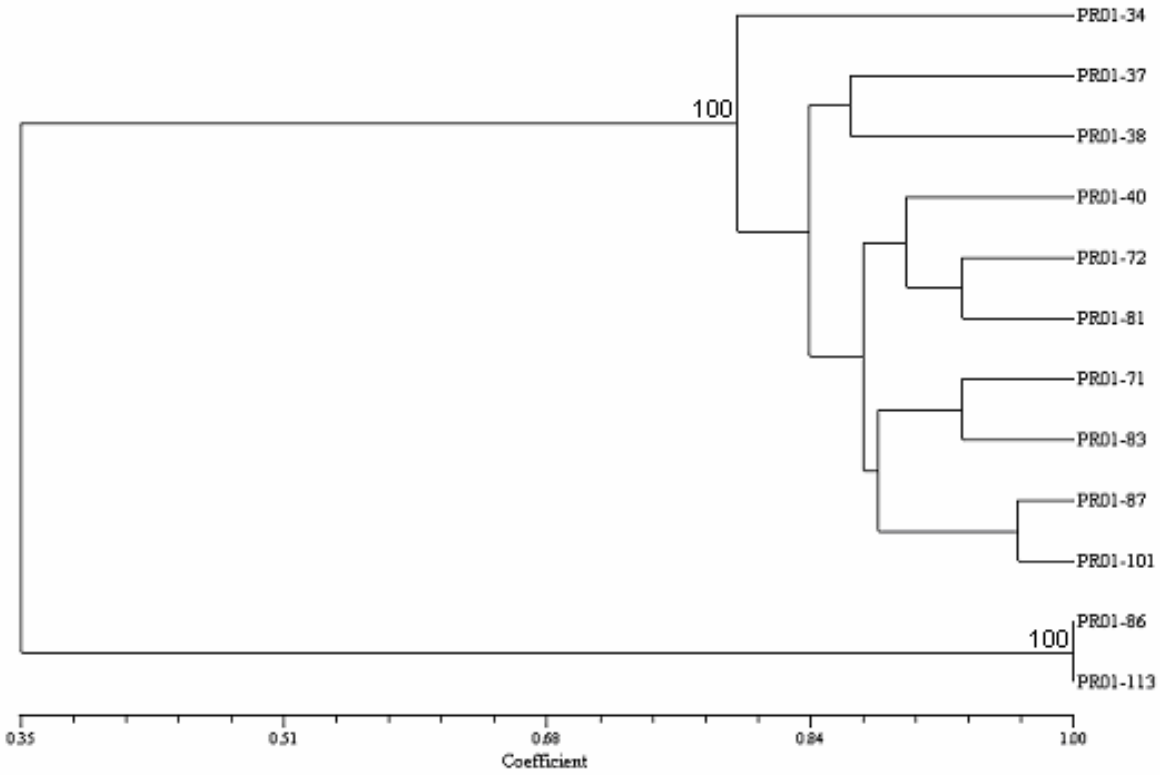

Figure 2. UPGMA dendrogram of *Magnaporthe grisea* isolates from field PR01 based on fingerprints with MGR583. Values at cluster branches indicate results of the boostrap analysis

Table 1. Reaction of gramineous plants to Magnaporthe grisea from two different wheat fields

Isolate	Sorghum	Barley	Rye	Triticale	Millet	Wheat	Oat	Maize
PR01-37	5*	5	5	5	5	5	5	5
PR01-87	5	5	5	5	5	5	5	5
PR01-30	1	4	4	4	N.T.**	4	4	0
PR01-39	1	4	4	4	3	4	4	0
PR01-83	2	4	4	4	0	4	4	0
P01-85	2	4	4	4	0	4	4	2
MS01-12	2	4	4	4	0	4	4	4
MS01-50	3	5	5	5	0	4	4	5
MS01-55	1	4	5	4	0	4	4	4
MS01-59	3	5	5	5	0	5	4	5
MS01-66	3	5	5	5	0	5	4	5
MS01-82	2	5	5	5	0	5	4	5

*Infection score: 0 = no visible reaction; 1 = minute, pinhead-sized spots; 2 = small brown to dark brown lesions with no distinguishable centers; 3 = small eyespot shaped lesions; with grey centers; 4 = typical blast lesions, elliptical with grey centers; 5 = complete blighting and leaf death
**Not tested

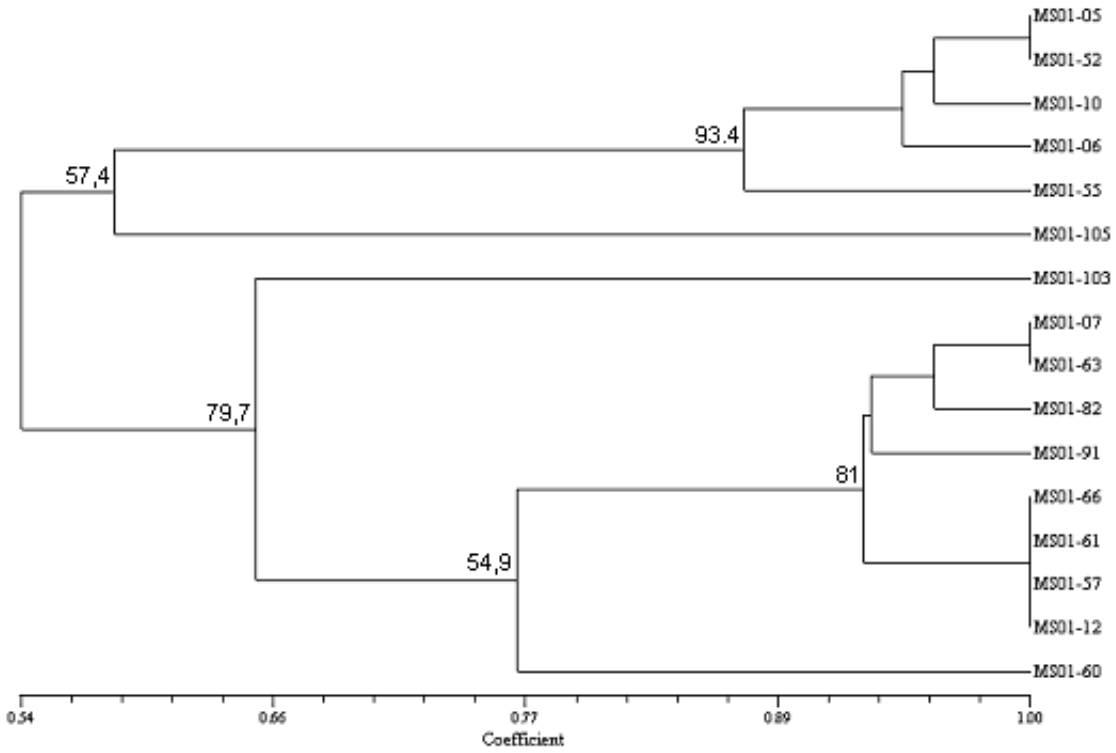

Figure 3. UPGMA dendrogram of *Magnaporthe grisea* isolates from field MS01 based on fingerprints with MGR583. Values at cluster branches indicate results of the boostrap analysis

eletrophoresed, and hybridized with probe MGR583. All isolates from wheat carried this repetitive element in a moderate copy number (30–40 copies). For a better understanding of the generated DNA fingerprinting, hybridization profiles were examined through phenogram of genetic similarity. Two clearly distinguished groups were detected among isolates from field PR01 (Figure 2). A group formed by isolates PR01-86 and PR01-113 showed 35% of genetic similarity to another group composed by the remaining 10 isolates. Similarity among isolates within each group was 100% for PR01-86 and PR01-113 and more than 70% for the group of 10 isolates. Each population formed a robust cluster, represented by the boostrap value of 100%, suggesting that each represents a distinct genetic group.

Isolates of field MS01 could be examined through phenogram in Figure 3. Various clones were detected among the 16 isolates examined: MS01-05 with MS01-52, MS01-07 with MS01-63, and MS01-12, MS01-57, MS01-61, MS01-66. Genetic similarity among isolates was 54% but lineages could not be observed because boostrap values revealed that confidence of clusters within isolates was low, indication that isolates from this field had high variation and could not be separated into lineages.

Data on pathogenic reaction of different gramineous species to *M. grisea* from MS01 and PR01 wheat fields are shown in Table 1. Although some isolates PR01 caused immune reaction on maize, no clear difference in host range was identified between field isolates MS01 and PR01 as blast symptoms with abundant sporulation was observed on aerial parts of sorghum, barley, rye, triticale, wheat, oat, and maize when inoculated with isolates from both field (lesions 3, 4, 5). Common millet was the sole host that had different reaction according to the field isolate employed, all isolates from field MS01 caused non-compatible reaction (immune type) contrasting with a compatible reaction by some isolates PR01, a few even caused the most susceptible reaction with death of plants (isolates PR01-37, PR01-87).

DISCUSSION

The hypothesis that two distinct populations of *M. grisea* are causing wheat blast in Brazil was confirmed by the data of the present study. One of the

populations has high mating ability and the other low (Figure 1). Isolates with high mating ability is a common feature in wheat blast (Urashima *et al*. 1993; Bruno & Urashima 2001) but the discovery of a subpopulation with low sexual fertility was surprising. Sexual fertility was one characteristics used for distinguishing the wheat blast fungus from the rice blast because the majority of *M. grisea* pathogenic to rice is female sterile (Itoi *et al*. 1983; Notteghem & Silué 1992; Zeigler 1998). This led some scientists to suggest that wheat blast disease had arisen from a fertile subpopulation of *M. grisea* and not from rice blast pathogen (Orbach *et al*. 1996). More recent data demonstrated that blast isolates from *Digitaria insularis* were the only ones among various weed isolates that showed positive cross-infectivity, high mating ability and sexual compatibility, and same genetic cluster of wheat isolates by DNA fingerprinting suggesting that this was the weed from which the disease originated (Urashima *et al*. 2003). Isolates from field PR01 were not included in that work.

The existence of two different populations causing wheat blast is supported by isolates from field PR01 that showed sexual characteristics more similar to rice blast because only 7.4% of its isolates had its mating type determined. Sexual traits of isolates from field MS01 showed to be similar to wheat isolates examined in previous studies (Urashima *et al*. 1993; Bruno & Urashima 2001). Other sexual parameter that demonstrated existence of two distinct populations was the ability of isolates to form perithecia, asci and ascospores (Figure 1B). Because the formation of these organs involves various genes (Valent *et al*. 1986) difference in degree of asci and ascospore production suggest that isolates of field PR01 have suffered many different single mutations that resulted in female sterility (Zeigler 1998).

The discovery of sexually distinct subpopulation led us to speculate on other aspects to further differentiate both field isolates. Comparison of the phenogram of genetic similarity constructed from the DNA fingerprinting with MGR583 revealed crystal clear difference between subpopulations. The population structure of isolates from field PR01 is very much similar with the population structure of the rice blast isolates from around the world in the sense that they can be grouped in different lineages due to high boostrap values (Chen *et al*. 1995; Don *et al*. 1999; Kumar *et al*. 1999). Robust clusters from phylogenetic analysis of DNA fingerprinting of *M. grisea* have been interpreted as evidence of population structuring into genetic lineages (Levy *et al*. 1993). On the other hand, isolates MS01 showed a population structure characteristic of wheat isolates where no lineages could be identified because of their high degree of variation reflected in the low value of boostrap (Urashima *et al*. 1999). Low boostrap value indicates that clusters in the phenograms were not robust and that population structure into lineages is not well defined (Kumar *et al*. 1999). These features combined with the high fertility and the existence of both mating type in the field at the same type (Figure 1) suggest that sexual reproduction played an important role in population structure of blast isolates from field MS01.

The next stage of this work was to investigate the phenotypic consequences of the existence of two blast populations discovered by sexual and DNA studies. This was done examining the host range of isolates. Difference in the host range was not clear although some isolates from field PR01 completely killed common millet because former work had already observed that reaction of this grass varied greatly according to the isolate (Urashima *et al*. 1993). The fact that many gramineous plants were susceptible to wheat isolates is a matter of extreme concern because many of these hosts are cultivated in the same geographical area or during the same growing season, it is not unusual to find wheat fields side by side to triticale or in fields where rice was grown as summer crop. Moreover, the high susceptibility of maize demonstrate that wheat blast poses as potential threat to this crop that is cultivated in large areas of the country. All together, the broad host range of isolates PR01 and MS01 showed that crop rotation as one strategy for the control of the wheat blast disease does not seem to be an effective means of disease management.

The existence of two different populations in the wheat blast disease that was first suggested by Urashima *et al*. (1993) was demonstrated for the first time by the present work. One practical consequence of this finding is that strategy of control should result different according to the subpopulation of the area. It is speculated that in subpopulation with high mating ability and DNA fingerprinting with low boostrap values (represented in this work by isolates MS01) sexual reproduction could be responsible for the generation of the variability resulting in a much larger number of lineages. This poses an extra task for wheat blast control strategies as resistance breed-

ing lines or chemical-based disease management strategies may be less effective.

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