Genetic and molecular characterization of pathogenic isolates of *Pyricularia grisea* from wheat (*Triticum aestivum* Lam.) and triticale (*x Triticosecale* Wittmack) in the state of Paraná, Brazil

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Summary

Isolates of *Pyricularia grisea* from wheat *(Triticum aestivum* Lam.) and triticale *(x Triticosecale* Wittmack) spikes with blast symptoms were analyzed by classical (VCG) and molecular (RAPD) techniques. *P. grisea* mutants, unable to use sodium nitrate *(nit)* as nitrogen source, were obtained with potassium chlorate. For vegetative compatibility (VCG) tests, genetically complementary *nit* mutant pairs were inoculated in a medium with sodium nitrate as a single nitrogen source. *P. grisea* isolates were divided into two vegetative compatibility groups and two RAPD groups. Since vegetative compatible strains may mutually exchange genetic and cytoplasmatic material, the contribution of the parasexual cycle in the genetic variability of Brazilian *P. grisea* isolates is discussed.

Key words Genetic variability, Heterokaryosis, Parasexual recombination, RAPD

Caracterización molecular y genética de aislamientos patógenos de *Pyricularia grisea* del trigo (*Triticum aestivum* Lam.) y triticale (*x Triticosecale* Wittmack) en la Provincia de Paraná, Brasil

Resumen

Se analizaron por técnicas clásica (VCG) y molecular (RAPD) aislamientos de *Pyricularia grisea* de las espigas de trigo *(Triticum aestivum* Lam.) y triticale *(x Triticosecale* Wittmack) con los síntomas de piricularia o bruzone. Los mutantes de *P. grisea*, incapaces de utilizar nitratos como fuente de nitrógeno *(nit),* fueron obtenidos con clorato de potasio. Para las pruebas de compatibilidad vegetativa (VCG) se inocularon los pares complementarios de mutantes *nit* en un medio con nitrato de sodio como única fuente de nitrógeno. Los aislamientos de *P. grisea* fueron separados en dos grupos de compatibilidad vegetativa y dos de RAPD. Considerándose que los aislamientos compatibles de *P. grisea* pueden intercambiar materiales nucleares y citoplasmáticos entre sí, se discute la contribución del ciclo parasexual en la variabilidad genética de los aislamientos brasileños de *P. grisea.*

Palabras clave Variabilidad genética, Heterocarion, Recombinación parasexual, RAPD

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Blast, caused by the fungus *Pyricularia grisea* (Cav.) Sacc. [teleomorph *Magnaporthe grisea* (Herbert) Barr], is one of the most important rice *(Oriza sativa* L.) diseases. Isolates of the same fungus may infect other cereals as well, specially wheat *(Triticum aestivum* Lam.), triticale *(x Triticosecale* Wittmack), and other grasses, such as *Brachiaria plantaginea* (Link.) and *Setaria geniculata* (Lam.) [12,17].

Since there is no effective chemical control of blast disease, the development of resistant cultivars has become the chief aim of disease control programs. In spite of great efforts, resistant cultivars, especially in rice culture, have on the average, a useful life restricted to two or three years owing to the introduction of new pathogen strains which disrupt the acquired specific resistance [3]. Due to the high virulence of *P. grisea* in rice field populations, genetic variability of this fungus has been accounted as one of the chief causes of disruption in cultivar resistance [2,12,17]. Virulence diversity of *P. grisea* in rice has been mainly attributed to parasexual recombination and mutation processes [14,18,19].

The parasexual cycle starts with heterokaryosis or rather, the coexistence of nuclei from distinct origins in a common cytoplasm [15]. These nuclei may undergo fusion within the heterokaryotic mycelium and give rise to heterozygous diploid nuclei. Although diploid nuclei are relatively stable, random chromosome losses may occur in most species during the vegetative propagation of the nuclei until they reach the original haploid stage. The process is called haploidization. If prior to haploidization homologous chromosomes undergo mitotic exchanges, recombinant haploid nuclei may be obtained at the end of the process [9].

RAPD markers are used extensively for intraspecific characterization of several plant pathogens. When combined with VCG (Vegetative Compatibility Groups) data, these markers may elucidate the level of genetic diversity existing in plant pathogen populations [7,13]. The aim of this research was to characterize pathogenic isolates of *P. grisea* using genomic fingerprints generated via RAPD/PCR and to determine the vegetative compatibility among isolates.

Materials and methods

Strains. Five isolates of *P. grisea* (I-1 to I-5) from wheat and one from triticale (I-6) were collected from plants with blast symptoms in the region of Palotina PR Brazil. Isolate Bp3a from *Brachiaria plantaginea*, donated by Dr. Alfredo Urashima (Universidade Federal de São Carlos, São Carlos SP Brazil), was used for comparison in molecular analyses.

Culture media. Different culture media were used: Minimal Medium (MM), Czapek-Dox complemented with 1% (W/V) glucose. Complete medium (CM) and basal medium (BM) have previously been described by Pontecorvo et al. [15] and Correll et al. [5], respectively.

Auxotrophic mutants. *P. grisea* mutants which were unable to use sodium nitrate as a nitrogen source *(nit)* were obtained in BM + $NaNO_3$ (0.2%) + $KClO_3$ (3.0%). Plates were incubated at 24 ± 2 °C during four to six weeks. Chlorate resistant sectors were then identified, isolated and transferred to BM + $NaNO_3$ plates. Colonies with poor growth (without dense aerial mycelium) were considered mutants incapable of using nitrate *(nit)* [16].

Phenotype of nit *mutants*. *nit* mutants from *P. grisea* isolates (I-1 to I-6) were transferred to Petri plates with BM supplemented with different nitrogen sources: BM + sodium nitrate ($NaNO_3$) (0.2%), BM + sodium nitrite ($NaNO_2$) (0.085%), BM + hypoxanthine ($C_5H_4N_4O$) (0.01%) and BM + ammonium tartrate [$(NH_4)_2C_4H_4O_6$] (0.092%). Plates were incubated as above and mutants were characterized according to table 1.

Complementation between nit *mutants*. Mycelium plugs (5 mm) for each *nit* mutant were paired equidistantly apart (approximately 1.5 cm) in Petri dishes containing BM + $NaNO_3$ for vegetative complementation tests. Plates were incubated at a temperature of 24 ± 2 °C during 3 to 4 weeks and then analyzed for heterokaryon formation.

Genomic DNA extraction and RAPD/PCR amplification. Pure cultures of *P. grisea* were grown in CM for 5 days at 25 °C. Mycelia were harvested by filtration (filter paper Whatman No. 1) and ground to a fine powder in liquid nitrogen for DNA extraction, as described by Loudon et al. [11]. RAPD/PCR amplifications were carried out in a total volume of 25 µl containing 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM of each deoxy-nucleotide triphosphate, 10µM of a single primer, 1.5U Taq DNA polymerase (Invitrogen), and 10-30 ng of genomic DNA. All amplified DNA products were resolved by electrophoresis on agarose gel (2.0%) in TAE (Tris Acetate EDTA) buffer, stained with ethidium bromide and photographed under ultra-violet light in a Bio-Imaging System (Vilber Lourmat, France). All experiments were repeated at least twice.

RAPD product scoring and data analysis. Analysis included RAPD gel bands that could be scored unequivocally for presence or absence. Data were compiled as a binary 0/1 matrix by presence (1) or absence (0) of a band at a particular position. Dendrograms were produced by cluster analysis using the unweighted pair-grouped method by arithmetic average (UPGMA).

Results and discussion

The RAPD patterns of isolates of *P. grisea* were generated with primers PR1 (5'GGGTAACGCC3'), PR2 (5'TGCCGAGCTG3'), PR4 (5'CAGGCCCTTC3'), PR5

Table 1. Growth of *nit* mutants of *P. grisea* in different sources of nitrogen.

Mutation	Locus	Growth on nitrogen sources[a]			
		nitrate	nitrite	hypoxanthine	ammonium
Structural gene for nitrate reductase	*nit1*	–	+	+	+
Pathway-specific regulatory gene or structural gene for nitrite reductase	*Nit3*	–	–	+	+
Genes controlling production of a molybdenum-containing co-factor	*NitM*	–	+	–	+
Major nitrogen regulatory locus	n.d.[b]	–	–	–	+
Absent	wild-type	+	+	+	+

[a]: modified from Beever and Parkes [1].
[b]: not determined.

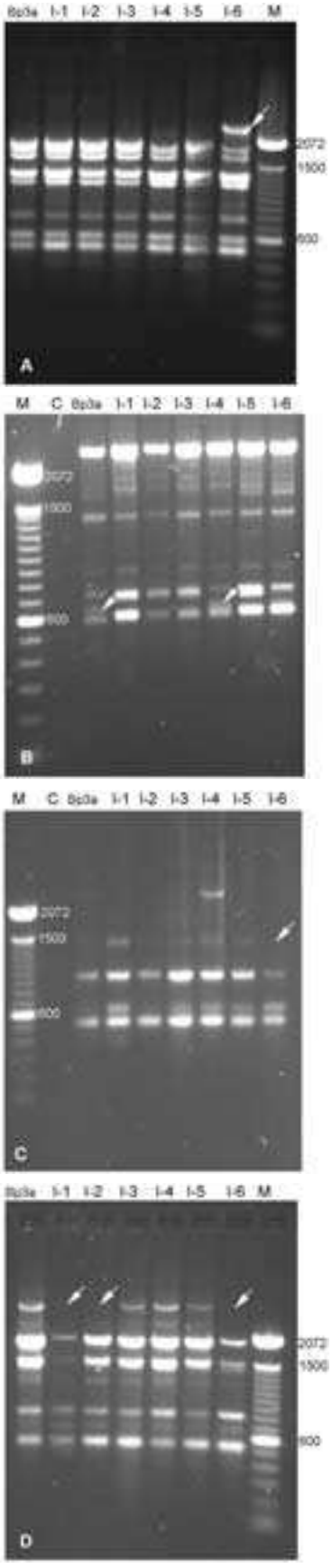

Figure 1. Agarose gel analysis of RAPD. Amplified products of seven isolates (Bp3a, I-1 to I-6) of *P. grisea* with primers: (A) OPA-18, (B) PR-5, (C) PR-1 and (D) PR-2. M, molecular marker; C, negative control.

(5'ACATGCCGTG3'), OPA-13, OPA-18, OPA-19, OPW-2, OPW-3 and OPW-7 (Operon Technologies) and produced 67 distinct reproducible polymorphic bands ranging from 0,4 to 3,0 Kb. Discrete genetic variation was detected among isolates I-1 to I-6 of *P. grisea*. Polymorphic variations among some isolates were identified in spite of their high genetic similarity. Primer OPA-18 identified an exclusive polymorphic band with more than 2000 bp in I-6 of triticale (Figure 1A). Two polymorphic bands of approximately 650 bp have been located in isolates Bp3a and in I-4 (Figure 1B) when primer PR5 was employed. The lack of two bands, one with approximately 1500 bp (primer PR1, Figure 1C) and the other with over 2000 bp (primer PR2), has been reported in I-6 isolate (Figure 1D). The latter was also absent in isolates I-1 and I-2 (Figure 1D). UPGMA-elaborated dendrogram (Figure 2) separated isolates *P. grisea* in two RAPD groups. Group A included isolates Bp3a and I-4 with a 91% genetic similarity index and isolate I-6 with an 83.6% genetic similarity index with regard to former isolates. Group B, which included isolates I-1, I-2, I-3 and I-5, had an 83.6% similarity index when compared to group A.

Vegetative compatibility among *nit* mutants of *P. grisea* has been currently identified through the development of dense aerial mycelium only in the contact region between two mutant colonies when cultivated in selective conditions (Figure 3), or rather, in the presence of sodium nitrate as the only nitrogen source. Under these conditions, *nit* mutants individually develop thin hyphae without the formation of aerial mycelium. However, genetically complementary *nit* mutants, such as *nit1* x *Nit3*, *nit1* x *NitM* or *NitM* x *Nit3*, may establish heterokaryons when they are vegetatively compatible [4,5,16].

According to our results, whereas five isolates (I-1 to I-5) were included in a single vegetative compatibility group (VCG1), the triticale isolate I-6 was included in an other distinct one (VCG2). Vegetative incompatibility barriers among isolates of *P. grisea*, detected in our analysis, corroborate results by Correll et al. [4] who separated 538 isolates of *P. grisea* from rice in four VCGs, namely US-01, US-02, US-03 and US-04, by complementary tests among *nit* mutants. Contrasting results, however, were previously reported by UV-induced auxotrophic mutants of *P. grisea* where interstrain barriers were not detected [6]. Correll et al. [4] explained the difference between their results and those by Crawford et al. (1986) [6] stating that UV light-induced mutagenesis might have changed the loci that affected the pathogen's vegetative incompatibility.

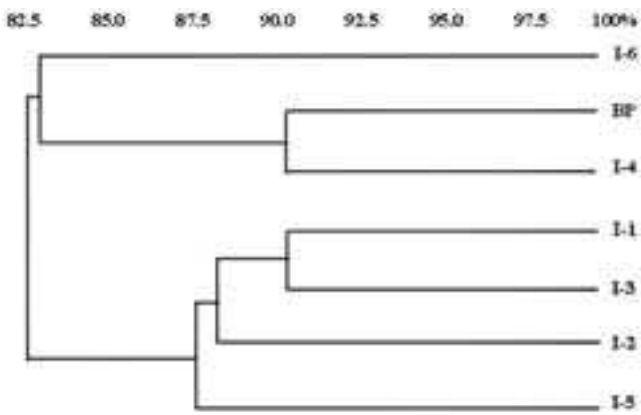

Figure 2. Dendrogram obtained from seven isolates of *P. grisea* with UPGMA, based on Simple Matching Coefficient. Isolates are indicated at the terminal branches. The line above the dendrogram represents the similarity index.

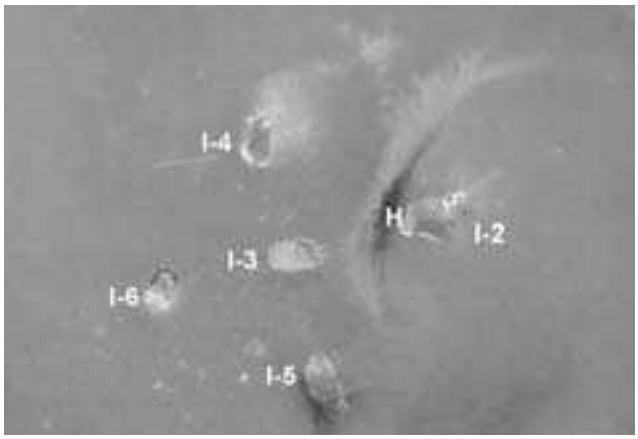

Figure 3. Pairing of nitrate non-utilizing mutants of *P. grisea* isolates. Heterokaryons formation (H) was observed in the following pairings: I-2 x I-4; I-3 x I-2; I-5 x I-2.

Vegetative incompatibility systems in fungi normally impair genetic exchanges among strains and are under strict control of *het* (heterokaryon incompatibility) and *vic* (vegetative incompatibility) loci. Natural populations of fungi are generally polymorphic for vegetative incompatibility systems [10]. Whereas members of the same VCG undergo anastomosis and form stable heterokaryons among themselves, these strains have a higher genetic similarity among themselves than with isolates from different VCGs [8,10].

With the exception of isolates I-4 and I-6, included in the same RAPD group with an 83.6% genetic similarity index, albeit separated in different VCGs, RAPD analyses in this research were compatible to the vegetative relationship degree of isolates of *P. grisea* (Figures 1 and 2). Distinct results were obtained in *Colletotrichum acutatum* isolates, which showed indistinguishable by RAPD analysis, but could be differentiated when the VCG method was employed [7].

Since it is highly possible that compatible strains of *P. grisea* establish contact among themselves, in field conditions the formation of heterozygous diploid nuclei, followed by mitotic recombination and haploidization processes, may justify the rise of other pathogen strains with greater or lesser virulence. Such a process may occur in greater proportions if incompatible strains undergo mutations in the genes that control the vegetative incompatibility systems and widen the possibilities of genetic exchange among pathogenic isolates.

Although the number of isolates analyzed in this research is too small and their geographic origin is too limited, our results demonstrate the potential of the VCG analysis to differentiate even closed related isolates of *P. grisea* (I-4 and I-6 isolates) and suggest the participation of the parasexual cycle in the generation of genetic variation observed in the pathogen.

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