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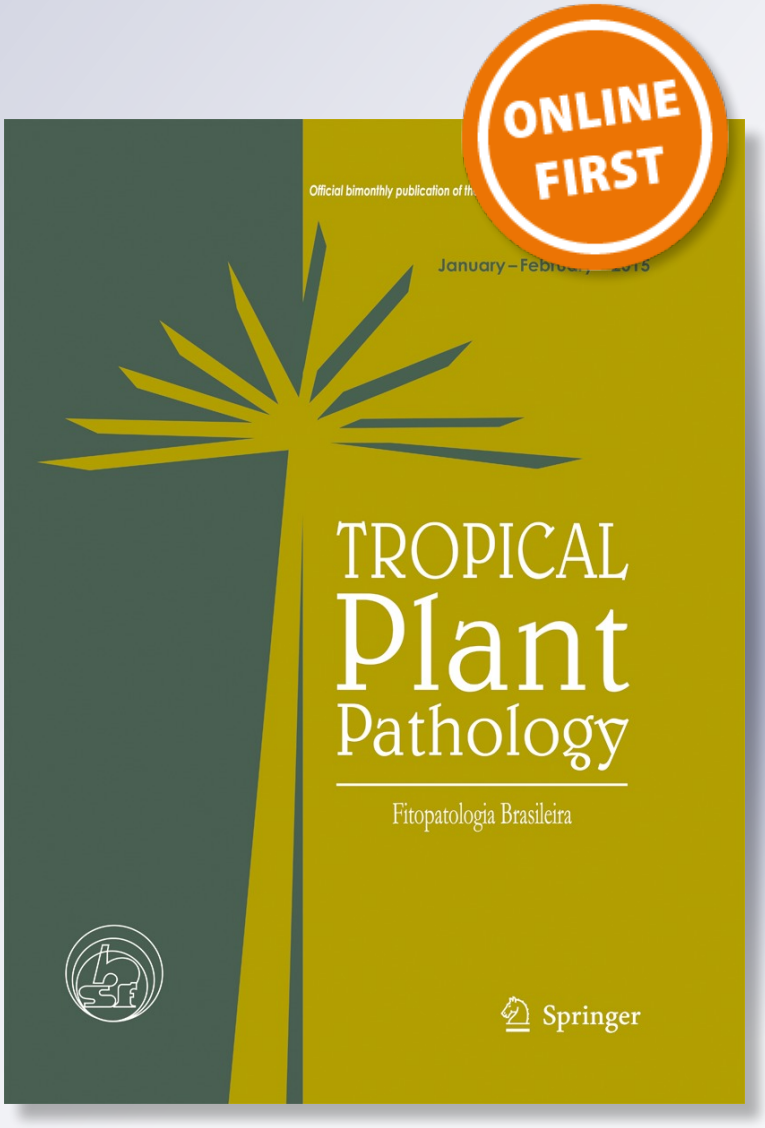

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Distribution of races and *Tox* genes in *Pyrenophora tritici-repentis* isolates from wheat in Argentina

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Abstract Tan spot, caused by *Pyrenophora tritici-repentis*, is a common disease in wheat-growing regions of Argentina. In this study 65 isolates of *P. tritici-repentis* obtained from different cultivars and wheat regions of Argentina were assessed for their virulence on six wheat cultivars/lines (Glenlea, Salomouni, Katepwa, M-3, 6B365 and 6B662) and for the presence/absence of the *Tox* genes based on a PCR approach. Thirty-six isolates were assigned to races, of which races 4 and 8 were dominant. Results for molecular analysis of *ToxA*, *ToxB*, *ToxB*-like and *toxb* genes showed that 57 isolates possessed the *ToxA* gene whereas only one isolate possessed *ToxA* and *ToxB* genes. There was no correlation between races and the toxin genotypes. It is suggested that *P. tritici-repentis* exhibits a complex race structure in Argentina.

Keywords *Triticum aestivum* · *Pyrenophora tritici-repentis* · Tan spot · Races

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Introduction

Tan spot, caused by *Pyrenophora tritici-repentis* (Died.) Drechs., is an important disease of wheat (*Triticum aestivum* L.) worldwide and a serious concern for wheat producers in the Southern Cone region of South America (Kohli 1995). In Argentina, the disease has gained importance in most of the wheat field areas particularly under no-tillage (Moreno and Perelló 2010). Tan spot epidemics potentially reduce kernel weight, number of grains per spike and total biomass (Simón et al. 2011), thus leading to reduced yield, but also grain quality may be affected (Fernandez et al. 1994). Knowledge of pathogen variability is an important component for developing resistant cultivars (Araya 2003). Variability for virulence in *P. tritici-repentis* population have been reported in several countries (Lamari and Bernier 1989a, b; Ali and Francl 2002; Ali et al. 2002; Strelkov et al. 2002). Currently, eight races of *P. tritici-repentis* have been identified based on virulence patterns on a standard differential set of wheat cultivars worldwide (Lamari et al. 1995, 2003; Ali et al. 2002; Manning et al. 2002; Strelkov et al. 2002; Ali and Francl 2002, 2003; Andrie et al. 2007; Gamba et al. 2012). The ability of *P. tritici-repentis* to induce necrosis and chlorosis in wheat is correlated with the production of host selective toxins (HSTs) including Ptr ToxA, Ptr ToxB and Ptr ToxC (Ballance et al. 1996; Ciuffetti et al. 1997; Effertz et al. 2002; Strelkov and Lamari 2003). Several molecular methods have been used to analyze diversity of the pathogen at the genome level (Moreno et al. 2012). For instance, Andrie et al. (2007) proposed the molecular identification of races based on the presence/absence of genes associated with the necrosis and chlorosis symptoms. The genetic structure of Argentinean population of *P. tritici-repentis* is unknown with respect to the presence of genes that encode for toxins responsible for producing symptoms and the races of pathogen based on a differential set. In this context, the aim of this study was two-fold: 1) determine

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Table 1 Information for the collection of *Pyrenophora tritici repentis* isolates obtained from wheat in Argentina

Isolate code	Wheat region	Locality	Cultivar	Year
A029	II Sur	Alberti	Klein Escudo	2002
B024	II Sur	Bragado	Klein Escorpion	2002
B028	II Sur	Bragado	Klein Escorpion	2002
B033	II Sur	Bragado	Klein Escorpion	2003
CH004	IV	Chillar	Klein Don Enrique	2000
CH005	IV	Chillar	Klein Don Enrique	2000
CH006	IV	Chillar	Klein Don Enrique	2000
CH007	IV	Chillar	Klein Don Enrique	2000
CH009	IV	Chillar	Klein Don Enrique	2000
CH0010	IV	Chillar	Klein Don Enrique	2000
CP021	II Norte	Comodoro Py	Baguette 10	2002
CR0819	IV	Azul	Cronox	2008
CR0810a	IV	Azul	Cronox	2008
CR0822a	IV	Azul	Cronox	2008
CR0823	IV	Azul	Cronox	2008
G0300	III	Gualeguaychu	Baguette 10u	2003
G032	III	Gualeguaychu	Baguette 10	2003
G033	III	Gualeguaychu	Klein Zorzal	2003
G036	III	Gualeguaychu	Buck Bigua	2003
G037	III	Gualeguaychu	Buck Bigua	2003
G0311	III	Gualeguaychu	Buck Mataco	2003
G0313	III	Gualeguaychu	Buck Mataco	2003
G0315	III	Gualeguaychu	Buck Guapo	2003
G0316	III	Gualeguaychu	Buck Guapo	2003
G0318	III	Gualeguaychu	Buck Guapo	2003
G0321	III	Gualeguaychu	Klein Chaja	2003
GO322	III	Gualeguaychu	Klein Chaja	2003
G0323	III	Gualeguaychu	Klein Chaja	2003
G0324	III	Gualeguaychu	Klein Chaja	2003
G0328	III	Gualeguaychu	Klein Zorzal	2003
G0331	III	Gualeguaychu	INTA Tijereta	2003
G0332	III	Gualeguaychu	INTA Tijereta	2003
G0333	III	Gualeguaychu	Klein Churrinche	2003
G0334	III	Gualeguaychu	Klein Churrinche	2003
G0336	III	Gualeguaychu	Klein Zorzal	2003
H001	II Sur	Los Hornos	Buck Brasil	2000
H003	II Sur	Los Hornos	Buck Brasil	2000
H004	II Sur	Los Hornos	Buck Brasil	2000
H006	II Sur	Los Hornos	Buck Brasil	2000
H0011	II Sur	Los Hornos	Buck Brasil	2000
H0014	II Sur	Los Hornos	Buck Brasil	2000
H0019	II Sur	Los Hornos	Buck Brasil	2000
HO15	II Sur	Los Hornos	Buck Brasil	2001
H016	II Sur	Los Hornos	Buck Brasil	2001
9J031	II Sur	9 de Julio	Klein Escorpion	2003
9J032	II Sur	9 de Julio	Klein Escorpion	2003
25M031	II Sur	25 de Mayo	Buck Mataco	2003
25M033	II Sur	25 de Mayo	Buck Mataco	2003
25M035	II Sur	25 de Mayo	Buck Mataco	2003
25M036	II Sur	25 de Mayo	Buck Mataco	2003
O001	IV	Orense	Buck Sureño	2000
O0014	IV	Orense	Buck Sureño	2000
O0015	IV	Orense	Buck Sureño	2000
O0018	IV	Orense	Buck Sureño	2000
O0019	IV	Orense	Buck Sureño	2000
O0020	IV	Orense	Buck Sureño	2000
P021	II Norte	Pergamino	Klein Don Enrique	2002
P022	II Norte	Pergamino	Klein Don Enrique	2002
P026	II Norte	Pergamino	Klein Don Enrique	2002
P028	II Norte	Pergamino	Klein Don Enrique	2002
P0313	II Norte	Pergamino	Klein Don Enrique	2003
TA022	IV	Tandil	Baguette 10	2002
V021	III	Victoria	Baguette 10	2002
V0212	III	Victoria	Baguette 10	2002
V0214	III	Victoria	Baguette 10	2002

the reaction type of *P. tritici-repentis* isolates on a differential wheat cultivars/lines, and 2) detect the *ToxA*, *ToxB*, *ToxB*-like and *toxb* genes for the same isolates using molecular assays.

The isolates were obtained from leaf samples collected in various cultivars growing under no-till in different regions of Argentina during 2000, 2001, 2002 and 2008 year. The isolates were obtained following Moreno et al. (2008). Sixty-five *P. tritici-repentis* isolates which were purified through monosporic culturing were identified to species based on morphology (Ellis 1976) (Table 1). For comparison, five *P. tritici-repentis* isolates (SD8, 86–124, D308, SD20, DW7) were kindly provided by Dra. Lynda M. Ciuffetti (Department of Botany and Plant Pathology, Oregon State University, USA).

To determine the reaction types, a differential set of six wheat cultivars/lines (Glenlea, Katepwa, 6B365, 6B662, Salomouni and M3) were selected based on Andrie et al. (2007) and Ali et al. (2010). Three seeds of each differential were sown in plastic cones (15 cm diameter × 12 cm length) and grown in a chamber with environmental control (20±2 °C, 16 h photoperiod - 180 μmol/m^2/s -, and 60 % relative humidity). The cones were arranged in a completely randomized design. All differential wheat cultivars were inoculated individually with each isolate. The procedures of inoculum preparation and inoculation were as described elsewhere (Moreno et al. 2008). After inoculation the plants were covered with a plastic bag for 48 h to ensure high humidity. The plants were rated for symptom development 9 days after inoculation.

Table 2 Reaction type of the infection by *Pyrenophora tritici repentis* isolates in a set of six differentials and the respective race and the presence/absence (+/−) of toxin genes by molecular analysis

Isolate	Reaction type[1]						Race[2]	PCR assay[3]	
	Glenlea	Katepwa	6B662	6B365	Salomouni	M3		*ToxA*	*ToxB TB6R*
A029	NecCl	−	−	−	−	−	NC	+	−
B024	NecCl	−	−	−	−	−	NC	+	−
B028	−	−	−	−	−	−	NC	+	−
B033	Nec	NecCl	Cl	Cl	−	−	8	+	−
CH004	−	−	−	−	−	−	4	+	−
CH005	−	−	−	−	−	−	4	+	−
CH006	−	−	−	−	−	−	4	+	−
CH007	Cl	Nec	−	−	−	−	NC	+	−
CH009	−	−	−	−	Nec	−	NC	−	−
CH0010	−	−	−	−	−	−	4	−	−
CP021	NecCl	Nec	−	−	−	−	NC	+	+
CR0819	−	NecCl	−	Cl	−	−	NC	+	−
CR0810a	−	−	−	−	−	−	4	+	−
CR0822a	Nec	NecCl	Cl	Cl	−	−	8	+	−
CR0823	Nec	NecCl	Cl	Cl	−	−	8	+	−
G0300	Nec	NecCl	Cl	Cl	−	−	8	+	−
G032	Nec	−	−	−	−	−	NC	−	−
G033	−	−	−	−	−	−	4	+	−
G036	Nec	NecCl	Cl	Cl	−	−	8	+	−
G037	−	−	−	Cl	−	−	3	+	−
G0311	−	−	−	−	−	−	4	+	−
G0313	−	−	−	−	−	−	4	+	−
G0315	−	Cl	Cl	Cl	−	−	6	+	−
G0316	Nec	−	−	Cl	−	−	NC	+	−
G0318	−	−	−	−	−	−	4	−	−
G0321	−	−	−	Cl	Cl	−	NC	−	−
GO322	Nec	−	−	−	−	−	NC	+	−
G0323	Nec	NecCl	Cl	Cl	−	−	8	+	−
G0324	Nec	NecCl	Cl	Cl	−	−	8	+	−
G0328	Nec	−	Cl	Cl	−	−	NC	+	−
G0331	Nec	−	−	−	−	−	NC	+	−
G0332	Nec	Nec	−	−	−	−	2	+	−
G0333	−	Nec	−	−	−	−	NC	+	−
G0334	Nec	NecCl	Cl	Cl	−	−	8	+	−
G0336	Nec	NecCl	Cl	Cl	−	−	8	+	−
H001	Nec	NecCl	Cl	Cl	Nec	−	NC	+	−
H003	−	−	−	−	Nec	−	NC	+	−
H004	Nec	−	−	Cl	−	−	NC	+	−
H006	Nec	NecCl	Cl	Cl	−	−	8	+	−
H0011	−	Nec	−	−	−	−	NC	+	−
H0014	Cl	−	−	Cl	−	−	NC	+	−
H0019	Nec	Nec	−	Cl	−	−	1	+	−
HO15	−	−	−	−	−	−	4	+	−
H016	Nec	−	Cl	Cl	Cl	−	NC	+	−
9J031	Nec	NecCl	Cl	Cl	−	−	8	+	−
9J032	NecCl	NecCl	Cl	Cl	−	−	8	+	−

Table 2 (continued)

Isolate	Reaction type[1]						Race[2]	PCR assay[3]	
	Glenlea	Katepwa	6B662	6B365	Salomouni	M3		*ToxA*	*ToxB TB6R*
25M031	–	NecCl	–	–	–	–	NC	+	–
25M033	–	–	–	–	–	–	4	–	–
25M035	Nec	Nec	–	–	–	–	2	+	–
25M036	–	Nec	–	Cl	–	–	NC	+	–
O001	NecCl	Nec	–	–	–	–	NC	+	–
O0014	–	Nec	Cl	–	–	–	5	+	–
O0015	Nec	Nec	–	–	–	–	2	+	–
O0018	–	–	–	–	–	–	NC	–	–
O0019	Nec	NecCl	Cl	–	Cl	–	NC	+	–
O0020	Nec	Nec	–	–	–	–	2	+	–
P021	–	–	–	–	–	–	4	+	–
P022	–	–	–	–	–	–	4	–	–
P026	–	–	–	–	–	–	4	+	–
P028	Nec	–	–	Cl	–	–	NC	+	–
P0313	Nec	NecCl	Cl	Cl	–	–	8	+	–
TA022	NecCl	–	Cl	Cl	–	–	NC	+	–
V021	–	Nec	–	–	–	–	NC	+	–
V0212	–	–	–	–	–	–	4	+	–
V0214	–	Nec	–	–	–	–	NC	+	–

[1] *Cl* Chlorosis, *Nec* Necrosis, *NecCl*, Necrosis with chlorosis

[2] Known race number; *NC* Non-correspond to any known race

[3] Tox A: PCR using TA51F/TA52R primer set; Tox B TB 6R PCR using the TB71F/TB6R primer set; "+" and "–" means the presence and absence of the respective fragment, respectively

DNA of the 65 isolates of *P. tritici-repentis* was extracted following Stenglein and Balatti (2006). Primers corresponding to the coding region of Ptr ToxA, Ptr ToxB, Ptr ToxB-like and Ptr toxb designed by Andrie et al. (2007) were used to genotype the isolates. *ToxA* primers (TA51F/TA52R) amplify a≈600 bp- fragment only in races 1 and 2, whereas races 3, 4 and 5 yield a≈250-bp amplicon specific to *ToxB* using primer pair TB71F/TB6R. Reverse primer TB60R paired with TB71F is specific to *ToxB-like* sequences that are characteristic of races 3 and 5, whereas reverse primer TB58R is specific to *toxb* in race 4 (Table 1). PCRs were performed in a 25 μl final volume containing 12 ng of genomic DNA, 10X reaction buffer (2 mM Tris–HCl pH 8.0+10 mM KCl + 0.01 mM EDTA + 1 mM DTT + 50 % glycerol + 0.5 % Tween®20+ 0.5 % Nonidet® P40.), 0.7 μM of primer, 200 μM of each dNTP (Promega Biotech) 2.5 mM $MgCl_2$, 1.25 units of *Taq* DNA polymerase (Higway Molecular Biology-InBio-UNICEN-Tandil). DNA amplifications were performed in a XP thermal cycler (Bioer Technology Co, Hangzhou, China) using the following cycling protocol: an initial denaturation step of 95 °C for 2 min, followed by 29 cycles at 95 °C for 30 s, 50 °C for 35 s (TA51F/TA52R and TB71F/TB58R), and 72 °C for 45 s, and a final extension cycle at 72 °C for 2 min. Annealing temperatures were 52 °C for TB71F/TB6R and for TB71F/TB60R. Each reaction was performed at least twice. PCR products were electrophoresed on 1.5 % (wt/vol) agarose gels containing 3 μl of GelRed (Biotium) at 80 V in 5X Tris-borate-EDTA buffer for 3 h at room temperature. Fragments were visualized under UV light. The size of the DNA fragments was estimated by comparing the DNA bands with a positive control of *P. tritici-repentis* and a 100 bp DNA ladder (Genbiotech S.R.L.). Gel images were photographed with a digital DOC 6490 system (Biodynamics S.R.L.).

The reaction types observed were categorized as necrosis (Nec), chlorosis (Cl) and necrosis with chlorosis halo (NecCl) (Moreno et al. 2008). The Nec reaction was observed in 38 isolates inoculated in the three wheat cultivars (Glenlea, Katepwa and Salomouni). The most frequent interaction was observed on Glenlea cv. (22 isolates); eight isolates produced Nec reaction on Katepwa cv. and three isolates produced the same reaction on Salamouni. Five isolates produced Nec reaction in both Katepwa and Glenlea. The Cl reaction was found for 30 isolates inoculated on the three cultivars (Glenlea, Katepwa and Salomouni) and two lines (6B365 and 6B662). Sixteen isolates produced Cl reaction in both 6B365 and 6B662. The isolates CR0819, G037, G0316,

H004, H0019, 25 M036 and P028 produced Cl reaction only in 6B365. The remaining seven isolates produced Cl in different combinations on the named cultivars and lines (Table 2). The isolates CH007 and H0014 produced Cl on Glenlea; G0321, H016 and O0019 produced Cl on Salomouni. Twenty-two isolates caused NecCl on Glenlea and Katepwa. Sixteen isolates produced these symptoms on Katepwa, five isolates on Glenlea and one isolate on both Katepwa and Glenlea (Table 2). Based on these reactions, 35 isolates of the fungus were assigned to eight races: race 1 (n=1e); race 2 (n=4); race 3 (n=1); race 4 (n=14); race 5 (n=1), race 6 (n=1), and race 8 (n=13). The remaining 30 isolates of *P. tritici-repentis* were not assigned to any known races, because they did present the known reactions for certain races (Table 2). Results of the genotypic analyses showed that 57 in 65 isolates amplified for the *ToxA* gene. Only one isolate (CP021) amplified for *ToxB* gene, but also amplified to *ToxA* gene.

We found a discrepancy between the phenotypic characterization of *P. tritici-repentis* and the molecular analysis for the *ToxA*, *ToxB, ToxB*-like, and *toxb* genes. Within the group of isolates that produced Nec on Glenlea, Katepwa and Salomouni, the isolate G032 did not amplify for the *ToxA* gene. Similar results were reported for isolates obtained from Arkansas, USA (Ali et al. 2010). These isolates may contain different toxin compounds that could induce similar phenotype to Ptr ToxA on Glenlea. The wheat cultivar Salomouni was resistant to all of the characterized HSTs of *P. tritici-repentis*, which is in agreement with a previous report (Strelkov and Lamari 2003). However, these isolates may contain different toxin compounds and belong to a potential new race. The isolates that produced only Nec on Glenlea or Katepwa cvs., and amplified for the *ToxA* gene, were not assigned to any known race. Manning et al. (2004) suggested that a reduction in Ptr ToxA activity may occur by competition with mutant proteins and that this Tox required multiple motifs for complete activity on susceptible wheat cultivars/lines. Further studies on the *ToxA* gene of these isolates should check whether there is any mutation within the coding region. In addition to races 1 and 2, races 7 and 8 may also synthesize Ptr ToxA (Lamari et al. 2003).

We found isolates of race 8 and all of them amplified the product for the *ToxA* gene, similar to another study (Andrie et al. 2007). Chlorosis in 6B662 and Katepwa is now known to be due to the production of Ptr ToxB (Ciuffetti et al. 1998), encoded by the *ToxB* gene (Orolaza et al. 1995). Ptr ToxC is associated with Cl on 6B365 (Effertz et al. 2002), but to the best of author's knowledge, there are no set of primers to amplify the *ToxC* gene. Similary to Andrie et al. (2007), *P. tritici-repentis* isolates assigned to race 8 possesses the *ToxA* gene, but lacked the *ToxB* gene. Glenlea and Salomouni were considered resistant to Cl (Lamari et al. 2003). However, Cl on Glenlea was detected previously (Moreno et al. 2008). Ptr ToxA was amplified for the race 3 isolate, supporting the statement by Manning et al. (2004). The race 4 isolates did not amplify for the *toxb* gene and more than ten of them amplified for the *ToxA* gene. Following Andrie et al. (2007) we could assigned any isolate to race 4. More studies are needed with these isolates to understand and correlate phenotypic with genotypic data. Races 5, 6, 7 and 8 have the *ToxB* gene, which is know to produce Ptr ToxB (Lamari et al. 2003; Strelkov and Lamari 2003). However, in our study the isolates assigned to these races did not amplify for the *ToxB* gene (Table 2), similarly to previous reports for race 8 (Andrie et al. 2007). Ali and Francl (2002) determined the presence of races 1 and 7 in Argentina by using only two isolates in their study. Similarly Gamba et al. (2012) analyzed isolates sampled only in the wheat region III of Argentina (province of Entre Ríos, which contributes with only 3 % of the national wheat production) and reported the presence of races 1 and 2. Therefore, our study constitutes the first comprehensive analysis for a collection of *P. tritici-repentis* isolates obtained from a large wheat growing-region using both virulence and molecular data, thus providing valuable information on the races and *Tox* genes present in the Argentinean population.

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