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Mapping QTL for Resistance against *Pyrenophora tritici-repentis* in Wheat

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Tan spot, caused by the fungus *Pyrenophora tritici-repentis* (Died.) Drechs is an important foliar disease of wheat (*Triticum aestivum* L.). From a set of phenotypically and molecularly characterized set of Argentinean isolates, two isolates H0019 and H0120 which do not correspond to known races of the pathogen were selected. Segregation for resistance among a set of recombinant inbred lines bred from the cross 'W7984' × cv. 'Opata 85' was used to identify the basis for resistance at the seedling stage, against those fungal isolates (H0019 and H0120), across three independent environments. On the basis of the mean performance across all three environments, a QTL against chlorosis located on the 6AS and linked to the RFLP locus *Xksuh4c* was significant for both isolates (with a LOD of 3.76 for isolate H0019 and 5.87 for H0120).

Keywords: tan spot, resistance, races, QTL

Abbreviations: PCR, polymerase chain reaction; RILS, recombinant inbreed lines; GS, growth stage; LOD, log likelihood ratio

Introduction

Tan spot of wheat (*Pyrenophora tritici-repentis* (Died.) Drechs. has been recognized as a major disease worldwide (Hosford 1982; Wiese 1987), responsible for losses in grain yield of 2–50% (Hosford 1971, 1974; Rees and Platz 1983; Simón 2011), or up to 75% in some situations (Rees et al. 1981). The disease is of growing importance in the Southern Cone region of South America (Kohli et al. 1992; Perelló et al. 2003; Simón et al. 2011).

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The race structure of populations of *P. tritici-repentis* has been concluded from the differential ability of isolates to induce necrosis or chlorosis on a standard cultivar panel (Lamari et al. 2003). The pathogen produces three host-specific toxins, namely Ptr ToxA, Ptr ToxB, and Ptr ToxC (Lamari and Bernier 1989a; Orolaza et al. 1995; Gamba et al. 1998; Effertz et al. 2002). Races can be characterized by the compatible interactions between an isolate and its corresponding susceptible differential cultivar plus the presence of the sequence of toxins (Andrie et al. 2007) (Table S1*). The *ToxA* gene is detectable in isolates belonging to races 1, 2, 7 and 8 (Lamari and Bernier 1991), and its product is the major cause of the necrosis shown by susceptible host cultivars. Ptr ToxB and Ptr ToxC are chlorosis-inducing host-specific toxins produced by races 5, 6, 7, 8 and 1, 3, 6 and 8 respectively (Orolaza et al. 1995). Tox B-like sequences have also been identified in race 3 and in race 4 (Strelkov and Lamari 2003), although neither produce Ptr ToxB.

Several recessive allele as *tsr1 (tsn1)* conferring resistance against Ptr ToxA have been located at the chromosome arm 5BL associated with resistance to necrosis caused by race 1 or 2 in both tetraploid and hexaploid wheat (Anderson et al. 1999; Faris et al. 1996), while *tsr2 (tsn2),* mapping to chromosome 3BL, confers resistance to race 3 which causes chlorosis in hexaploid wheat but necrosis in tetraploid wheat (Singh et al. 2006). In addition, *tsr3 (tsn3)* on chromosome 3D (Tadesse et al. 2006a, 2007), *tsr4 (tsn4)* on 3A (Tadesse et al. 2006b), *tsn5* on 3B (Singh et al. 2008) were located. Furthermore, the genes *tsn-syn1* and *Tsn-syn2* on chromosome 3D (Tadesse et al. 2006a) have also been mapped.

Genes conditioning insensitivity to chlorosis have also been mapped. The gene *tsc1* (chromosome arm 1AS) protects against chlorosis caused by Ptr ToxC produced by race 1 and 3 (Effertz et al. 2001). Quantitative trait locus (QTL) based mapping using a population derived from crossing the synthetic hexaploid wheat 'W 7984' and 'Opata 85' has also shown that most of the resistance against chlorosis induced by race 1 isolates is controlled by a locus on chromosome arm 1AS (gene *tsc1*), with an additional contribution from a locus on chromosome arm 4AL, along with an interaction between the 1AS QTL and a locus mapping to chromosome arm 2DL (Faris et al. 1997). The major 1AS QTL was confirmed as also being effective in adult plants, but some minor QTL mapping to chromosome arms 1AL, 7DS, 5AL and 3BL were also identified. Furthermore the same QTL located in 1AS and 4AL conferred resistance to race 3 isolates in seedlings of ^W7984' × 'Trenton' population (Effertz et al. 2001). At the seedling stage, insensitivity to Prt ToxB due to the *tsc2* gene was mapped to a locus on chromosome arm 2BS, with a minor contribution from chromosome arms 2AS, 4AL and 2BL (Friesen and Faris 2004).

In Argentina and some other countries in the world new races of the pathogen seem to be present, as some isolates do not correspond to the known races (Ali et al. 2010; Patel and Adhikari 2013; Moreno et al. 2014) and location of the resistance has not been mapped.

*Further details about the Electronic Supplementary Material (ESM) can be found at the end of the article.

Our aim was to characterize two isolates from Argentina which apparently do not belong to known races and to map the location of the resistance to them in a wheat population of recombinant inbred lines (RILs) bred from the cross 'W7984' × cv. 'Opata 85'.

Materials and Methods

Isolates

Two isolates (H0019 and H0120, Faculty of Agriculture and Forestry Sciences, La Plata, Argentina) were selected and characterized from a wide collection of Argentinean isolates of *P. tritici-repentis*. For comparison, the following isolates were used as representatives of races 1 through 5: for race 1, isolate SD8; for race 2, isolate 86-124; for race 3, isolate D308; for race 4, isolate SD20; and race 5, isolate DW7. All these isolates were facilitated by Dra. Ciuffetti, Dep. of Botany and Plant Pathology, Oregon State University, OR, USA.

Pathogenicity test

The inoculum preparation and procedure of inoculation were made according to Odvody 1982. The inoculum was adjusted to contain 3×10^3 conidia per ml, and 0.5 ml of Tween 20 per litre were added to act as a surfactant made. To evaluate the reaction type, a differential set of six wheat cultivars/lines. Glenlea, Katepwa, 6B365, 6B662, Salamouni were selected based on Andrie et al. (2007). M3 (Synthetic wheat, also designated as W7976) was added to the standard set of differentials to increase the chance of finding more variants in the fungal population as suggested by Ali and Francl 2003. The seeds were proportionated by Dr. Shaukat of Dep. of Plant Pathology, North Dakota State University, USA. Three seeds of each differential line were sown in plastic cones (15 cm in diameter and 12 cm in length) and grown in a growth chamber at 20 ± 2 °C with 16 h photoperiod. The cones were arranged in a completely randomized design. All differential wheat cultivars were inoculated individually using both isolates. Wheat seedlings at the three leaf stage were sprayed until run-off using a small manual atomizer. After inoculation the plants were placed in the growth chamber with a plastic cover for 48 h to ensure high humidity. The plants were rated for symptoms development 7 days post-inoculation.

DNA extraction

Isolates were grown on PDA media. For each isolate, seven day old cultures were carefully scraped from the surfaces of two Petri dishes using a scalpel and DNA was extracted according to the technique of Ferreira et al. 1998. The DNA concentration was estimated by comparison against a 1 kb DNA ladder molecular marker of known concentration (Promega Biotech. Corp.) electrophoresed in 0.7% agarose.

Ptr ToxA and Ptr ToxB PCR analysis

Genotype characterization was carried out on the isolates of *P. tritici-repentis*. Primers corresponding to the coding region of Ptr ToxA, Ptr ToxB and Ptr toxb designed by Andrie et al. (2007) were selected. 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 detected 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. PCRs reactions were performed according to the protocol of Stenglein and Balatti (2006). 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 ml^{-1} of GelRed (Biotium, Hayward, USA) at 80 volts 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., Buenos Aires, Argentina). Gel images were photographed with a digital DOC 6490 system (Biodynamics S.R.L., Buenos Aires, Argentina).

Evaluation of mapping population for disease resistance

The mapping population comprised a subset of 49 recombinant inbred lines of the International Triticeae Mapping Initiative (ITMI) created by crossing 'W7984' × cv. 'Opata 85'. 'W7984' is a synthetic hexaploid line derived by amphiploidization of the durum wheat cv. 'Altar 84' × the D genome donor *Aegilops tauschii*, accession `GIGM86.940´ (van Deynze et al. 1995).

Phenotyping at the seedling stage was performed in three environments: two consecutive years at the Faculty of Agricultural and Forestry Sciences, La Plata, Argentina and in the field of the Experimental Station of Los Hornos, Argentina in one year. In each experiment, grains were pre-germinated in a petri dish and vernalized for four weeks at 4–8 °C. In the La Plata experiments, 8–10 vernalized seedlings per RIL were pot-grown in the field, and the pots were arranged in a split-plot randomized design with two replications. In the Los Hornos experiment, the seedlings were set out as rows (ten seedlings per row, spaced 2 cm apart) in the field. Each row was flanked by two rows of oat to decrease interplot interference. For all the experiments, soil was fertilized with 50 kg ha^{-1} N as urea and 50 kg ha^{-1} P as ammonium diphosphate at sowing and with 50 kg ha^{-1} N as urea at tillering. Each RIL was infected with either isolate H0019 or H0120. The conidial suspension was sprayed on to two leaf stage seedlings (GS 1.2, Zadoks et al. 1974) until run-off, and the plants were sprayed with water for 15 min every 2 h for the three days following

inoculation. The proportion of the leaf surface on the first and second leaves showing necrosis or chlorosis was assessed 28 days after inoculation visually. Evaluations in field conditions are usually delayed with respect to greenhouse or growth chamber assessments (7–14 days) due to suboptimum conditions for the development of the disease in the field. The reaction of each entry was deduced from the mean of these two measurements.

Statistical analysis of phenotypic data

The split-plot randomized block design consisted of three environments (main plots), two isolates (sub-plots), and the RILs and parental lines (sub-subplot) in two replications. The software package Genstat 12 (2008) was used to perform analysis of variance, following arcsine square root transformation of the mean leaf area measurements. Differences between entries were tested using the Fisher protected test, and comparisons between means by the LSD test at $P \leq 0.05$. The homogeneity of variance for both of the experiments was evaluated using Bartlett's test. Broad-sense heritability estimates were based on the partitioning of the phenotypic variance into its genetic and non-genetic components.

QTL mapping

For the analysis of the ITMI population, genotypic data previously gathered for 942 loci consisting of mainly SSR and some RFLP loci were used. The data were kindly provided by Dr. M. Röder, IPK Gatersleben, and discussed in detail by Ganal and Röder (2007). The subsequent QTL analysis was implemented within the software package QGene (Nelson 1997), first applying a single marker analysis to identify loci associated with the phenotypic variation, and then interval analysis to identify the genomic regions having a significant impact on the trait. A genome-wide LOD (log likelihood ratio) significance threshold was calculated for each set of phenotypic data using a permutation test with 1000 permutations.

Results

Isolates characterization

Based on the reaction on the differential lines, isolate H0019 could be assigned as a race 1 isolate, since it induces necrosis on the leaves of both cvs. 'Glenlea' and 'Katepwa, and chlorosis on those of cv. '6B365. The induction of chlorosis on cv. '6B365' should be a response to the presence of Ptr ToxC although this conclusion is not as yet verifiable using PCR. Based on the absence of symptoms on the differential set of cultivars to infection, isolate H0120 is thought to belong to race 4. However did not amplify for *Toxb* gene, according with the suggested primer by Andrie et al. (2007) (Table 1, Fig. S1).

Table 1. Phenotypic and genotypic characterization of two *Pyrenophora tritici-repentis* isolates and positive controls (C) for races 1 to 5

Phenotypic characterization						
Isolate /Differential line	Glenlea	Katepwa	6B662	6B635	Salamouni	Synthetic M3
H0019	Necrosis	Necrosis	R	Chlorosis	R	R
H0120	R	R	R	R	R	R
SD8 (C1)	Necrosis	Necrosis	R	Chlorosis	R	R
86-124 (C2)	Necrosis	Necrosis	R	R	R	R
3D308 (C3)	R	R	R	Chlorosis	R	R
4SD20 (C4)	R	R	R	R	R	R
DW7 (C5)	R	Chlorosis	Chlorosis	R	R	R
Genotypic characterization						
Isolate /Differential line	Tox A	ToxBTB71F /TB6R	ToxBTB71F /TB60R	toxbTB71F /TB58R		
H0019	+	–	–	–		
H00120	–	–	–	–		
SD8	+	–	–	–		
86-124	+	–	–	–		
3D308	–	+	+	–		
4SD20	–	+	+	+		
DW7	–	+	+	+		

Phenotypic analysis and broad-sense heritability

The RILs differed with respect to their seedling reaction to infection, and the genotype by environment interaction was also significant (Table S2). The reaction of 'W7984' seedlings varied from fully resistant to moderately resistant and that of cv. 'Opata 85' seedlings from moderately susceptible to fully susceptible. Most of the RILs displayed little development of leaf necrosis, but the level of chlorosis varied across a wide range. There was no evidence of a bimodal segregation among the RILs with respect to resistance, irrespective of the environment or the pathogen isolates involved (Figs. S2 and S3). The broad sense heritability of the disease reaction at the seedling stage was 0.56 for isolate H0019 and 0.61 for isolate H0120.

QTL analysis

The marker regression analysis for reaction to *P. tritici-repentis* identified a QTL (*QTs.fcayflp-6AS*) lying on the short arm of chromosome 6A, linked to the RFLP marker *Xksuh4c,* which was effective to both isolates (Tables S3 and S4). All markers with LOD values higher than 1.90 were indicated, however a LOD threshold of 3 was estimated to

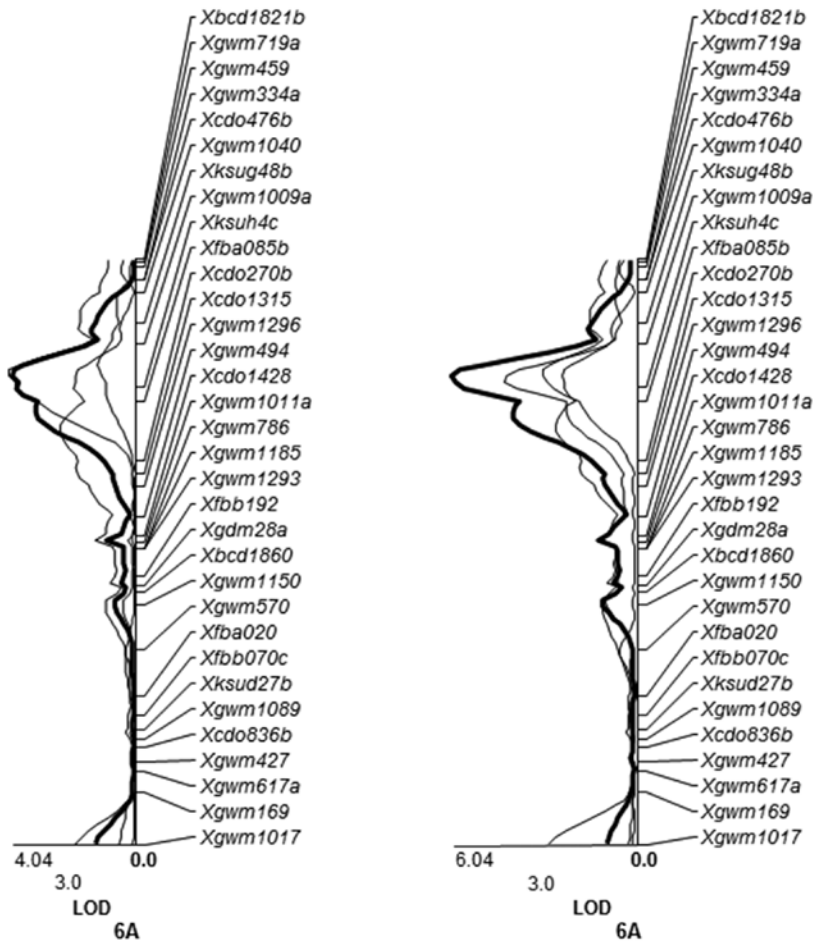

Figure 1. Interval QTL analysis along chromosome 6A, showing the location of a QTL *(QTsc3.fcayflp-6A)* determining seedling resistance to *Pyrenophora tritici-repentis* in three environments. Bold lines indicate the average of the three environments. A. to isolate H0019 B. to isolate H0120

declare a QTL significant. The positive allele was inherited from 'W7984', as it was indicated by the additive effect. Data were analysed separately for each environment and combined through environments considering that variances were homogeneous through them. The QTL explained on average of the three environments 29.80% of the variation for resistance to chlorosis to isolate H0019 and 42.41% of the resistance to chlorosis to isolate H0120.

The interval analysis indicated that the locus was present in two environments and associated with a LOD score of between 1.90 and 3.76 with respect to isolate H0019, and in the three environments between 2.25 and 4.29 with respect to isolate H0120. On the

basis of the mean performance across all three environments, the QTL was significant for both isolates (with a LOD of 3.76 for isolate H0019 and 5.87 for H0120); in addition it was significant for environment 1 for isolate H0019 and for environment 2 for isolate H00120. Some other QTL associated by single marker regression analysis did not reach the significant LOD threshold and were not consistent through the different isolates and environments (Tables S3 and S4; Fig. 1).

Discussion

Both parents were resistant to tan spot necrosis, for that reason the lines did not segregate for necrosis, but only for chlorosis (Faris et al. 1997). Two common rating scales have been used for tan spot evaluation: a 1-to-5 lesion-type rating scale (Lamari and Bernier 1989b; Faris et al. 1997) and another scale of using an average percent leaf area diseased (necrosis + chlorosis) (Faris et al. 1997; Bockus et al. 2007, Sun et al. 2010, Noriel et al. 2011). A high correlation was observed between the two rating scales (Faris et al. 1997) and they both provide accurate rating of tan spot damage in wheat leaves. However, estimation of necrosis + chlorosis percentage is easy to estimate and showed high repeatability among different plants of each accession (Bockus et al. 2007). In this study, we used average of chlorosis and necrosis percentage across two leaves in seedlings.

The population of *P. tritici-repentis* has been shown to possess a distinct race structure, which implies that novel races can readily arise. Genes (or QTL) effective against races 1, 2, 3 and 5 have already been identified in the literature and races 7 and 8 contain combinations of virulence from other races (Faris et al. 2013). An earlier analysis of the identical 'W7984' / cv. 'Opata 85' RIL population has shown that seedling resistance to extensive chlorosis against race 1 isolates is quantitative in nature, with a major proportion of the variation under the control of a QTL mapping to chromosome arm 1AS (Faris et al. 1997). This same locus is also responsible for resistance at the adult stage (Effertz et al. 2001). In both cases, a number of minor QTL were also identified. The 1AS QTL also confers seedling resistance against infection by race 1 isolates among segregants from the cross 'W7984' × cv. 'Trenton' (Effertz et al. 2001), while an assay based on seedling sensitivity to Prt ToxB was able to locate a gene on chromosome arm 2BS, along with several other minor QTL (Friesen and Faris 2004).

In this work, as it was indicated H0019 induces necrosis on the leaves of both cvs. 'Glenlea' and 'Katepwa', and chlorosis on those of cv. '6B365. However, as the presence of Pr ToxC inducing that chlorosis is not as yet verifiable using PCR (Effertz et al. 2002), it is not possible to assure that the isolate belong to race 1. The segregation among the RILs for resistance to isolate H0019 was inconsistent with the presence of the well established resistance QTL on chromosome arm 1AS (Faris et al. 1997; Effertz et al. 2001), suggesting also, that it may after all not be a genuine race 1 isolate. Although isolate H0019 carries Ptr ToxA, the gene in 5BL effective to this toxin (Faris et al. 1996; Anderson et al. 1999) was not identified in this population because it did not segregate for tan necrosis.

Based on the absence of symptoms on the differential set of cultivars to infection, isolate H0120 is thought to belong to race 4. However, it did not amplify for *Toxb* gene, ac-

cording with the suggested primer by Andrie et al. (2007). It is known that the races 3 and 4 have present in their genomes single copies of distinct *ToxB* homolog (Martínez et al. 2004; Strelkov et al. 2006). If a criterion for assigning an isolate to race 4 is the presence of *Toxb* gene, then H0120 may also belong to an as yet undescribed *P. tritici-repentis* race. The race structure of *P. tritici-repentis* is rather complex, and discrepancies have frequently been observed between the race assignments derived from phenotype (host reaction) and genotype (presence of Ptr ToxA and Ptr ToxB) (Andrie et al. 2007; Ali et al. 2010; Lepoint et al. 2010). In addition, the presence of frequent sexual reproduction increases the potential for the development of new races. In Argentina, race characterization of isolates of the pathogen is still in progress. From 65 isolates, 29 do not correspond to any new race according to phenotypic reaction and genotypic characteristics. Fifteen of them were similar to H0120 based on the absence of symptoms on the differential set of cultivars, although 12 of them amplified for *ToxA* gene and none of them amplified for *Toxb* gene (Moreno et al. 2015). In addition, in that set only H0120 corresponds phenotypically to race 1.

In our work, the analysis of seedling reaction to *P. tritici-repentis* infection resulted in the detection of one QTL determining resistance to chlorosis, mapping to chromosome arm 6AS. This QTL was effective to both isolates, indicating that is effective to ToxA gene (isolate H0019) and to the unidentified virulent factors present in the H0120 isolate. The reasonable level of heritability associated with the resistance trait suggests that the level of plant resistance against this pathogen can be improved by directed selection.

Using association mapping, QTL involved in resistance to an isolate that do not fit in the current race classification and with absence of *ToxA* gene were identified, which are located in chromosomes 1A, 1D, 2B, 2D, 6A, and 7A (Patel et al. 2013) but in that study, the QTL in chromosome 6A are located on the long arm of the chromosome. Furthermore Arseniuk et al. (2004) also found a region associated with resistance to *Stagonospora nodorum* blotch in seedlings to a mixed isolate in chromosome 6AL. However, in a study using 567 landraces, markers associated with resistance to race 1 and 5 isolates were found in a similar region as the QTL in this work, on the short arm of chromosome 6A (Gurung et al. 2011).

The present data suggest that both isolates (H0019 and H0120) belong to as yet unclassified race of the pathogen, which could have a toxin still not identified and that the locus resistant to chlorosis detected on chromosome 6A would be effective against that toxin.

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Electronic Supplementary Material (ESM)

Electronic Supplementary Material (ESM) associated with this article can be found at the website of CRC at http://www.akademiai.com/content/120427/

Electronic Supplementary *Table S1*. *Pyrenophora tritici-repentis* pathotype/race classification

Electronic Supplementary *Table S2*. Analysis of variance among a set of 'W7984' / cv. 'Opata 85' RILs for tan spot occurrence at the seedling stage

Electronic Supplementary *Table S3*. Statistical parameters associated with the segregation for tan spot occurrence among a set of 'W7984'/cv 'Opata 85' RIL seedlings following inoculation with isolate H0019 in three environments

Table S4. Statistical parameters associated with the segregation for tan spot occurrence among a set of 'W7984'/cv 'Opata 85' RIL seedlings following inoculation with isolate H0120 in three environments

Electronic Supplementary *Figure S1*. DNA fragments of the *Pyrenophora tritici-repentis* isolates H0120 and H0019 compared to positive control isolates for races 1 to 5 with the primers ToxA (TA51F/TA52R), Tox b (TB71F/TB58R), Tox B (TB71F/TB60R) and Tox B (TB71F/TB6R). C1; C2; C3; C4; C5 positive controls for races 1 to 5

Electronic Supplementary *Figure S2*. Frequency distribution for disease occurrence among a set of 'W7984' / cv. 'Opata 85' RIL seedlings following inoculation with isolate H0019. Average disease scores in a) environment 1, b) environment 2, c) environment 3, d) the mean of the three environments

Electronic Supplementary *Figure S3*. Frequency distribution for disease occurrence among a set of 'W7984' / cv. 'Opata 85' RIL seedlings following inoculation with isolate H0120. Average disease scores in a) environment 1, b) environment 2, c) environment 3, d) the mean of the three environments