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Determining the presence of host specific toxin genes, *ToxA* and *ToxB*, in New Zealand *Pyrenophora tritici-repentis* isolates, and susceptibility of wheat cultivars

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Abstract Tan spot, caused by *Pyrenophora tritici-repentis* (Ptr), is an important disease of wheat worldwide, and an emerging issue in New Zealand. The pathogen produces host-specific toxins which interact with the wheat host sensitivity loci. Identification of the prevalence of the toxin encoding genes in the local population, and the susceptibility of commonly grown wheat cultivars to Ptr will aid selection of wheat cultivars to reduce disease risk. Twelve single spore isolates collected from wheat-growing areas of the South Island of New Zealand representing the *P. tritici-repentis* population were characterised for the Ptr ToxA and ToxB genes, *ToxA* and *ToxB*, respectively, using two gene specific primers. The susceptibility of 10 wheat cultivars to *P. tritici-repentis* was determined in a glasshouse experiment by inoculating young plants with a mixed-isolate spore inoculum. All 12 New Zealand *P. tritici-repentis* isolates were positive for the *ToxA* gene but none were positive for the *ToxB* gene. Tan spot lesions developed on all inoculated 10 wheat cultivars, with cultivars 'Empress' and 'Duchess' being the least susceptible and 'Discovery', 'Reliance' and 'Saracen' the most susceptible cultivars to infection by the mixed-isolate spore inoculum used. The results indicated that the cultivars 'Empress' and 'Duchess' may possess a level of tolerance to *P. tritici-repentis* and would, therefore, be recommended for cultivation in regions with high tan spot incidence.

Keywords *Drechslera tritici-repentis*, host-selective toxins, host susceptibility, race, tan spot, *Triticum aestivum*, virulence

INTRODUCTION

Tan spot caused by *Pyrenophora tritici-repentis* (Died.) Drechsler (syn. *Drechslera tritici-repentis* (Died.) Shoemaker), is an important foliar disease of wheat (*Triticum* spp.) worldwide, including Australia, Europe, USA, Canada and South America (Lamari & Strelkov 2010; Bertagnolli et al. 2019). In New Zealand, tan spot disease is considered a relatively new disease to affect wheat, with, until recently, only limited reports of *P. tritici-repentis* causing disease (Harvey et al. 2015). The first recorded identification in New Zealand was its isolation from infected wheat seed in 1976 (Hampton & Matthews 1978), with the first official report in the field being in 2013 (Harvey et al. 2015).

Tan spot infection can result in two distinct symptoms, necrosis (tan colour) and extensive chlorosis (yellow colour). On leaves, the lesions characteristically have small tan/brown centres, surrounded by a yellow circular border. The lesions initially appear as tan/brown flecks subsequently expanding into a diamond-shaped lesion. The lesions then develop into tan blotches on the leaf which later coalesce, often resulting in the death of the leaves (Kader 2010). As the plant matures, *P. tritici-repentis* infects the stem where it will begin to develop pseudothecia (Weise 1987).

The development of the different characteristic symptoms is highly specific and a result of an interaction between host-selective toxins (HST) secreted by the pathogen and the target receptors of a toxin-sensitive host wheat plant (Singh et al. 2010; Aboukhaddour et al. 2011). Three *P. tritici-repentis* HST, Ptr ToxA, Ptr ToxB and Ptr ToxC have been characterised to date. Both Ptr ToxA, which induces necrosis on susceptible wheat genotypes, and Ptr ToxB, which induces chlorosis on susceptible wheat cultivars, are proteinaceous in nature and are encoded, respectively by the genes *ToxA* (Ballance et al. 1989; Tomás et al. 1990; Tuori et al. 1995) and a number of multi-copy genes such as *ToxB* (Strelkov et al. 2002; Lamari et al. 2003; Martinez et al. 2004). At present, it is possible to screen for the presence of both *ToxA* and *ToxB* using specific molecular primers (Antoni et al. 2010). In contrast, although Ptr ToxC, which can induce chlorosis on specific wheat genotypes, has been

suggested to be a low molecular weight non-ionic polar molecule, its exact nature and the gene(s) encoding it have not been identified (Effertz et al. 2002). Additionally, there are also two other, uncharacterised HST, known as Ptr ToxD toxins, whose exact targets and functions have yet to be elucidated (Faris et al. 2013). Studies of *P. tritici-repentis* populations in the USA, Canada, South America, Australia, the Baltic States and Romania have shown that Ptr ToxA has been the predominant HST found in these populations, with Ptr ToxB almost completely absent (Strelkov & Lamari 2003; Antoni et al. 2010; Aboukhaddour et al. 2013, Abdullah et al. 2017). Based on the ability of isolates to produce the different HSTs (and thereby necrosis or chlorosis) on a set of differential wheat cultivars, currently eight races of *P. tritici-repentis* have been identified worldwide (Ali et al. 2010; Aboukhaddour et al. 2013). However, there is no information regarding the presence of Ptr ToxA and/or Ptr ToxB (or Ptr ToxC and/or Ptr ToxD) in the New Zealand *P. tritici-repentis* isolate populations.

The susceptibility of cultivated wheat lines to tan spot has been extensively examined overseas (Lamari & Bernier 1989b; Tadesse et al. 2006; Jorgensen & Olsen 2007; Kollers et al. 2014; Tran et al. 2017; Liu et al. 2020). However, an issue that is often encountered when breeding tan spot resistance and tolerant cultivars is that the level of resistance, or tolerance, of one particular cultivar is often found to differ widely between different regions. For example, cultivars considered to be resistant in one study can often be found to be susceptible in another related study (Gilchrist et al. 1984; Lamari & Bernier 1989a). Possible reasons for these discrepancies in cultivar resistance include differences in the virulence and race of the *P. tritici-repentis* isolates selected and the methodologies employed by research workers to rate host reaction (Lamari & Bernier 1989a). Additionally, the fact that tan spot will often occur in association with other wheat foliar diseases also makes it more difficult to screen for tan spot resistance in the field. Therefore, the screening of wheat seedlings for their relative susceptibility to tan spot has been predominantly undertaken by artificial inoculation within a greenhouse environment. This method is known to permit the examination of resistance under uniform disease pressure (Singh et al. 2010). The utilisation of a lesion-type rating scale has been the preferred method of choice for tan spot researchers because it is regarded to be the most practical and convenient method for evaluating cultivar response to *P. tritici-repentis* (Singh et al. 2010). A study by Evans et al. (1999) reported that there is a positive correlation between assessment of tan spot resistance at seedling stage and rating of adult plants in the field.

Since tan spot disease is considered to be a relatively new disease to affect wheat in New Zealand, there have been no studies to determine which Ptr-HSTs the New Zealand populations of *P. tritici-repentis* possess. The aim of this study was to identify whether selected populations of *P. tritici-repentis* present in the South Island of New Zealand possessed the Ptr toxin genes *ToxA* and/or *ToxB* (but not the presence/absence of genes Ptr *ToxC* or Ptr *ToxD*). Furthermore, this study also examined the susceptibility of a cohort of commonly grown New Zealand feed and milling wheat cultivars to *P. tritici-repentis* under glasshouse conditions.

MATERIALS AND METHODS

Fungal isolates

Twelve single-spore *P. tritici-repentis* isolates were selected from the *P. tritici-repentis* isolate populations collected as part of a 2013–2014 survey (Weith 2015). All isolates were recovered from symptomatic wheat tissue and the isolates were selected so that they best represented the geographic distribution of *P. tritici-repentis* populations. The isolates were M13c, M14d (Methven, Mid-Canterbury), G14a (Greendale, North Canterbury), G22a (Geraldine, South Canterbury), F12d (Fairlie, South Canterbury), W12a, W12c, W15a (Waimate, South Canterbury), K16a (Kakanui, North Otago), S12a (Wedonside, Southland), S22a and S26a (Clinton, Southland). The isolates were stored as mycelium colonised agar discs in 20% glycerol at -80°C.

Detection of *Ptr-ToxA* and *Ptr-ToxB* using PCR

The 12 New Zealand *P. tritici-repentis* single-spore isolates were screened using PCR for the presence of the Ptr ToxA and Ptr ToxB genes using *ToxA* and *ToxB* specific primers, respectively. In addition, the genomic DNA of 7 international *P. tritici-repentis* isolates known to be either positive or negative for Ptr ToxA and Ptr ToxB, and from a range of different *P. tritici-repentis* races, were obtained to validate the PCR and for comparison against the local New Zealand *P. tritici-repentis* populations. Genomic DNA for the 7 international *P. tritici-repentis* isolates of varying geographic origin (ASC-1, AB47-10, Alg3-24, AB33-1, AB39-2, AB39-8 and TS93-71B) were provided by Dr Reem Aboukhaddour (University of Alberta, Canada) (Table 1).

The New Zealand *P. tritici-repentis* isolates were grown in potato dextrose broth (PDB; Difco) for 4 days at 20°C in continuous darkness. The mycelium was harvested onto sterile Miracloth™ (Cal–Biochem), squeezed between paper towels to remove excess moisture, wrapped with aluminium foil and snap frozen in liquid nitrogen and stored at -80°C until used for DNA extraction. Genomic DNA was extracted from the frozen and ground mycelium using the PUREGENE® genomic DNA isolation kit (Gentra systems, USA) following the manufacturer's instructions. DNA concentration was measured using a NanoDrop-ND-1000 spectrophotometer

Table 1 Details of the international *Pyrenophora tritici-repentis* isolates used in this study including the host-specific toxin (Tox) and pathogen race.

Isolate code[1]	Race	Tox	Region/Country collected
ASC1	R1	AC	Manitoba, Canada
AB47-10	R1	AC	Morley region, Alberta (Canada)
Alg3-24	R5	B	Guelma, Eastern Algeria
AB33-1	R1	AC	Wilson region, Alberta (Canada)
AB39-2	R3	C	Wilson region, Alberta (Canada)
AB39-8	R1	AC	Wilson region, Alberta (Canada)
TS93-71B	R8	ABC	Turkish–Syrian border

[1]International isolates provided by Dr Reem Aboukhaddour (University of Alberta, Canada).

(NanoDrop Technologies, United States of America) and DNA concentrations were diluted to 10–20 ng/µL for PCR.

The *P. tritici-repentis* isolates were screened for the presence of either the *ToxA* and *ToxB* genes using Ptr ToxA and Ptr ToxB gene-specific primers as described by Antoni et al. (2010). The forward (F) and reverse (R) primers for ToxA were: ToxAscreeningF ($^{5'}$CCTCGTACTTCTTTTCAGCG$^{3'}$) and ToxAscreeningR ($^{5'}$TCCTCTACCTTGAATTAAAGCG$^{3'}$); and the *ToxB* primers were TB10F ($^{5'}$TATGCGACCCTAACCTAGCC$^{3'}$) and TB12R ($^{5'}$GCCAGATAAAAAACCCCTATACC$^{3'}$). Each PCR reaction volume was made up to 25 µL containing 1xPCR buffer (Roche), 200 µM dNTPs, 10 µM primer, 1 U FastStart Taq polymerase (Roche) and 1 µL template DNA. A negative control with 1 µL sterile water instead of the template DNA was included. The cycling parameters for both primer sets were as follows: initial denaturation at 94°C for 2 min, followed by 35 cycles of 94°C for 30 sec, 57°C for 30 sec, and 72°C for 1 min, with a final extension at 72°C for 10 min. A 7–10 µL aliquot of each PCR product combined with 3 µL loading dye was separated by electrophoresis at 10V/cm for 50 min in a 1% agarose gel (Bioline USA Inc.) alongside the 1 Kb plus DNA Ladder (Invitrogen™, Thermo Fisher Scientific Inc., USA). Gels were stained in ethidium bromide solution and visualised on a UV transilluminator (UVItec Cambridge Imaging System, Total Lab Systems Ltd). The presence of a band with the expected size for each gene indicated the presence the *Tox* gene. To confirm primers amplified the correct target, the PCR products of three *P. tritici-repentis* isolates (G22a, M14d and S12a) were sequenced directly at the Lincoln University Sequencing Facility. The resulting sequences were submitted to a BLASTN search (http://www.ncbi.nlm.nih.gov/BLAST/) to confirm matching species in the GenBank database.

Determination of susceptibility of wheat cultivars

Selection of wheat cultivars

Ten New Zealand wheat cultivars were selected for screening to assess susceptibility and response to *P. tritici-repentis* infection based on results obtained from a regional survey conducted in 2013–2014 (Weith 2015) plus data and information supplied by Foundation for Arable Research (FAR). The survey examined 15 different wheat paddocks on the eastern side of the South Island, representing the main wheat growing region in New Zealand (https://figure.nz/), that were showing symptoms of tan spot disease. The wheat cultivars selected were: 'Empress', 'Saracen', 'Reliance', 'Wakanui', 'Duchess', 'Viceroy', 'Starfire' (KWW46), 'Inferno' (KWW47) and 'Torch'. Wheat seed was provided by Plant and Food Research, PGG Wrightsons Seeds Ltd and Seedforce NZ Ltd.

For each wheat cultivar, five seeds were planted at 1 cm deep in a 1 L pot (10 × 10 cm) containing general 3-month potting mix (80% composted pine bark, 20% pumice 1–7 mm, 3g/L Osmocote exact N:P:K: 16–3.5–10, 1 g/L horticultural lime and 1g/L hydraflo). For each wheat cultivar, 20 pots were prepared, with 10 replicate pots for both inoculated and uninoculated treatments. The pots were arranged in a complete randomised block design on a bench in a glasshouse at the Lincoln University nursery, with 10 blocks each containing one replicate for each treatment (cultivar and inoculated/uninoculated). At the Zadoks growth stage GS20 (Zadoks et al. 1974), the number of seedlings per pot was reduced to 3 by thinning.

Plants were grown at a temperature of 20–25°C during the day and ~15°C at night under artificial lighting (400 watt high-pressure sodium lamps) to extend the photoperiod to 12 hours light and darkness for 12 hours. Plants were watered once every day.

Preparation of fungal inoculum

Four *P. tritici-repentis* isolates (G14a, G22a, K16a and S12a) were randomly selected for inoculation of wheat plants based on the results of previous tox gene assessment and the genetic diversity studies of Weith (2015) that indicated limited genetic diversity. Further, a mixed isolate inoculum was used as the purpose of the experiment was to determine the overall susceptibility of the cultivars to the *P. tritici-repentis* population. Conidia of *P. tritici-repentis* were prepared using a modification of the method described by Lamari & Bernier (1989a). The isolates were initially cultured on potato dextrose agar (PDA, Oxoid Limited, Basingstoke, United Kingdom) for 4 days at 25°C in continuous darkness. The isolates were then plated onto V8-PDA (150 mL Campbell's V8 juice, 10 g Davis agar, 10 g PDA, 3 g $CaCO_3$ and 850 mL distilled water) and grown for 4 days at 25°C in continuous darkness. After 4 days incubation, the growing mycelium was flooded with 5 mL of sterile distilled water (SDW) and flattened using a flame-sterilised bent-glass rod. The water was then decanted and the plates were incubated in an Adaptis CMP6010 growth cabinet (Conviron) under white light for 12 hours at 22.5°C. After 12 hours incubation under white light, plates were incubated for 20 hours in continuous darkness at 16°C. Conidia were harvested by flooding the plate with 5 mL SDW and gently scrapping the conidia off the surface of the mycelium using a sterilised scalpel blade. The conidial concentration for each isolate was determined using a haemocytometer and adjusted to 3×10^3 conidia/mL. A mixed-isolate conidial suspension containing equivalent numbers of conidia of each isolate was prepared by mixing equal volumes of conidial suspension from each isolate. The conidial suspension were kept on ice during transit to the glasshouse and during the inoculation procedure.

The viability of the conidial inoculum was determined by spread plating 10 µL of conidial suspension of each *P. tritici-repentis* isolate onto 1.5% water agar plates. The percentage germination was assessed after 12 hours incubation at 16°C in continuous darkness. The conidial viability of all four *P. tritici-repentis* isolates was 100%.

Inoculation of plants

Wheat seedlings were inoculated at the two-tiller growth stage (GS20–29). To account for the time taken to inoculate the experiment, all seedlings in one block were inoculated at the same time, starting from block 1 to block 10. For all cultivars, all three seedlings within a pot were inoculated by placing a 5 µL drop of the mixed *P. tritici-repentis* isolate conidial suspension (3×10^3 conidia/ mL) on wheat leaves 2, 3 and 4. Inoculations were adjacent to points marked on the middle of each leaf using a marker pen, with one inoculation

on each leaf. Spore suspensions were shaken prior to inoculation to ensure conidia were in suspension. Control plants were left uninoculated.

A clean plastic bag sprayed with approximately 1 mL of sterile water was placed over the inoculated wheat plants to help generate a relative humidity of 90–100%. After 48 hours, the plastic bag was removed and the plants were tied up to a cane inserted into the pot. The plants were grown for 3 weeks under a 12 hour light/dark photoperiod and a day/night temperature of 20/15°C regime in the greenhouse.

Disease severity rating system

After 3 weeks' incubation, inoculated leaves were assessed for *P. tritici-repentis* symptom development. The largest lesion located closest to the inoculation point was assessed for leaves 2, 3 and 4 for two wheat plants per pot using the 0–5 lesion-rating scale developed by Lamari & Bernier (1989a) with modifications as outlined in Table 2. The length and width of the largest lesion present on leaf 3 of one wheat plant chosen at random per pot was also measured using a digital calliper (Mitutoyo, Kanagawa, Japan) and used to estimate lesion area.

Table 2 Disease score (0–5 scale) based on lesion size and appearance used to assess severity of *Pyrenophora tritici-repentis* lesions which developed on inoculated wheat cultivars (modified from Lamari & Bernier 1989a).

Disease Score (0–5 scale)	Lesion size and appearance	Example picture
0	No lesion present (indicates no infection or strong plant resistance) Note: black marker pen mark indicating inoculation point	
1	Small, dark brown to black singular spots (<0.5 mm diameter). Lack of any distinctive chlorotic (yellow) or necrotic zones (tan) (high tolerance level)	
2	Small dark brown to black spots (1–2 mm diameter) with very faint chlorotic borders. Some necrosis (tan to reddish brown) present (moderate level of tolerance)	
3	Small to medium (2–3 mm diameter) oval to diamond shaped lesion. Dark brown to black appearance which is completely or mostly surrounded with a chlorotic halo. Distinctive necrotic zone present (tan to reddish brown). Lesion may be coalescing with surrounding singular lesions (slight level of tolerance/ low susceptibility)	
4	Medium sized oval/ diamond shaped lesion (3–10 mm diameter). Central eyespot may be present. Lesion has distinctive necrotic zone and chlorotic halo. (Main lesion coalescing with surrounding singular lesions) (moderate to high level of susceptibility)	
5	Medium to large oval/ diamond shaped lesion (10–20 mm diameter) with distinctive central eye spot being indistinguishable. Main lesion coalescing with most surrounding singular lesions. Distinct necrotic zone with a clear surrounding chlorotic halo (high susceptibility)	

Isolations from symptomatic lesion tissue were carried out to confirm *P. tritici-repentis* as the causal agent, and complete Koch's postulates. One leaf from an inoculated seedling showing the characteristic tan spot symptoms (tan necrosis and yellow chlorosis) was selected from three different replicates chosen at random for each wheat cultivar. Three randomly selected leaves from three uninoculated plants were also taken for isolation. Lesioned tissue were surface sterilised in 2% sodium hypochlorite (25 g a.i./ 10 mL) for 1 min, and washed three times in SDW. The sterilised lesioned tissue were placed onto PDA and incubated at 25°C in continuous darkness. After 4 days' incubation, the plates were assessed for colonies characteristic of *P. tritici-repentis,* being colonies with a thick grey-green cottony mycelial surface and an olive-green underside, similar to the morphology of the isolates used to inoculate the plants.

Statistical analysis of cultivar susceptibility scores and lesion area

The mean lesion rating scores and mean lesion area for each wheat cultivar were analysed with one-way analysis of variance (ANOVA) (with randomised blocks) using Genstat version 16. Means were separated using Tukey's 95% confidence interval test at $P \leq 0.05$.

RESULTS

Detection of Ptr ToxA and Ptr ToxB using PCR

The Ptr ToxA specific primers amplified a band of 510 bp from the genomic DNA of all 12 of the selected New Zealand *P. tritici-repentis* isolates (Figure 1A). A band of 510 bp was also amplified from 5 (ASC1, AB47–10, AB33–1, AB39–8 and TS93–71B) of the international *P. tritici-repentis* isolates (Figure 1) reported to be positive for *ToxA* gene (Table 1).

The sequences of the PCR products of three randomly selected isolates (G22a, M14d and S12a) were 99.75–100% similar to sequences on GenBank for Ptr ToxA encoding gene *ToxA* (MN062700.1 and MN052896.1).

No PCR products were obtained from the genomic DNA of the *P. tritici-repentis* isolates Alg3–24 and AB39–2 using the *ToxA* specific primers (Figure 1A).

The *ToxB* specific primers amplified a band of 646 bp from the genomic DNA of the *P. tritici-repentis* isolates Alg3–24 (Race 5) and TS93–71B (Race 8). Very faint bands were also observed in the reference ToxB negative strains (ASC1 and AB39-8; Table 1) and is likely to be a result of non-specific primer binding. No PCR product was amplified using the *ToxB* specific primers for any of the New Zealand *P. tritici-repentis* isolates tested (Figure 1B).

Visual observation of tan spot symptoms on cultivars

Symptoms of tan spot differed on each of the different wheat cultivars inoculated with *P. tritici-repentis* (Figure 2). No symptoms of tan spot were observed on any of the uninoculated wheat plants. The most characteristic diamond-shaped lesions with distinctive eyespots were observed on the wheat cultivars 'Discovery', 'Reliance' and 'Wakanui'. Less characteristic and irregular shaped tan spot lesions were observed on the other inoculated wheat cultivars. Very little chlorosis (yellowing) was observed on any of the wheat cultivars screened. The most distinctive and largest chlorotic zones were observed on the cultivars 'Discovery', 'Inferno' 'Reliance', and 'Wakanui' (Figure 2a, d, h and j respectively). Distinctive necrosis (tan) of varying degrees were observed on all cultivars inoculated with *P. tritici-repentis*.

Determination of cultivar susceptibility to *Pyrenophora tritici-repentis*

There was no significant effect ($P>0.05$) of block on the mean disease score. Cultivar had a highly significant effect on the mean disease score ($P \leq 0.001$) (Table 3). The wheat cultivar 'Empress' had a significantly lower ($P \leq 0.05$) mean score than 'Saracen', 'Reliance' and 'Discovery'. Of these cultivars, 'Discovery' was also significantly ($P \leq 0.05$) different from the cultivar 'Duchess'. None of the other cultivars differed significantly in mean disease score.

There was a significant effect ($P \leq 0.001$) of cultivar on the mean lesion size that developed on *P. tritici-repentis* inoculated plants (Table 3). The mean lesion area of 'Empress' was significantly smaller ($P \leq 0.05$) compared with that on cultivars 'Saracen', 'Reliance' and 'Discovery'. The mean lesion area that developed on 'Discovery' was also significantly larger ($P \leq 0.05$) compared with 'Duchess', 'Wakanui', 'Inferno', 'Viceroy' and 'Torch'. The cultivar susceptibility ranking using both mean disease score and mean lesion size was similar for all cultivars apart from 'Starfire', where the results of the lesion area score showed it was more susceptible compared with the disease score rating.

Figure 1 1% agarose gel of PCR products generated by: (A) *ToxA*-specific ToxAscreeningF/ToxAscreeningR primers; and (B) *ToxB*-specific TB10f and TB12r primers, using the genomic DNA of representative New Zealand and international *Pyrenophora tritici-repentis* single spore isolates. From left to right, lanes 1 to 13 are *P. tritici-repentis* isolates S12a, S26a, G14a, G22a, F12d, K16a, ASC1, AB47-10, Alg3-24, AB33-1, AB39-2, AB39-8 and TS93-71B. M = 1 kb plus DNA ladder (Invitrogen), -ve = negative control.

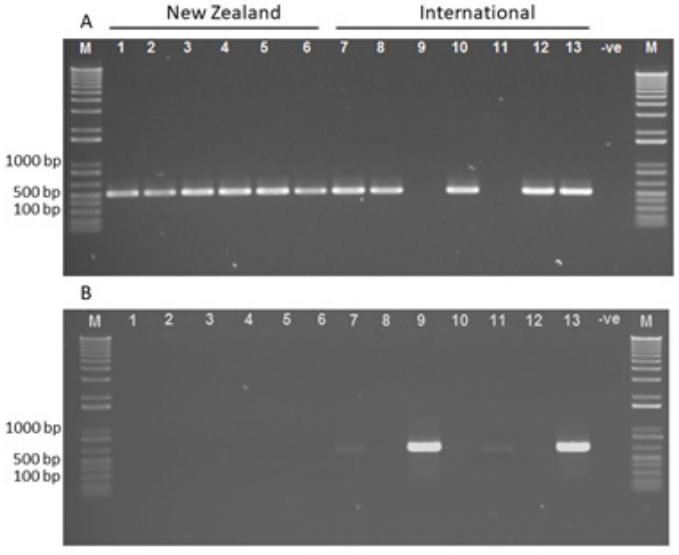
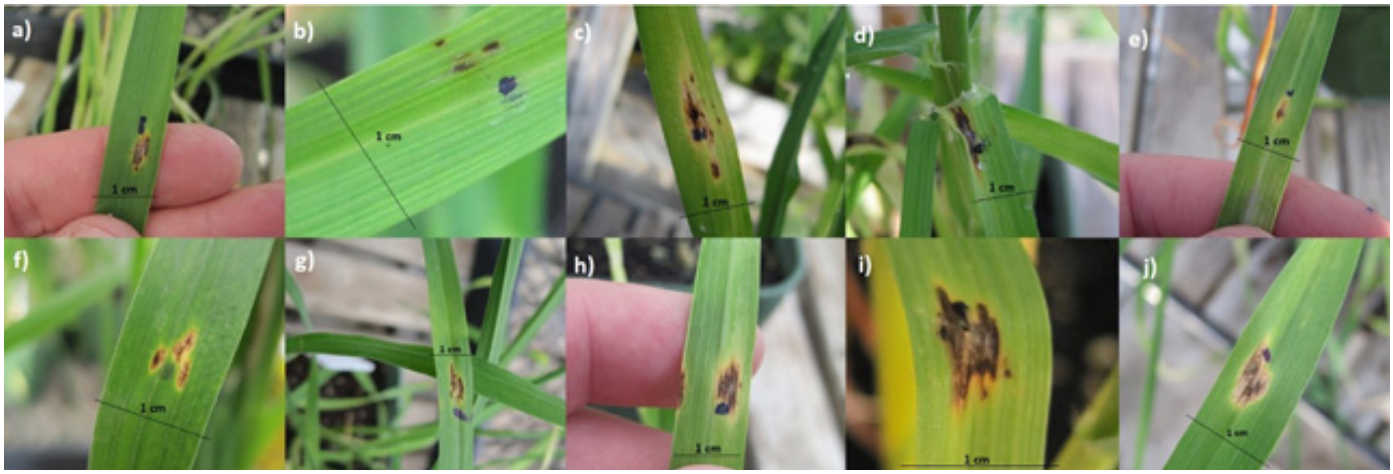


Figure 2 Representative tan spot symptoms observed on the different wheat cultivars 21 days after inoculation with *Pyrenophora tritici-repentis*: (a) 'Discovery', (b) 'Empress', (c) 'Duchess', (d) 'Inferno', (e) 'Saracen', (f) 'Starfire', (g) 'Torch', (h) 'Reliance', (i) 'Viceroy', and (j) 'Wakanui'.



Colonies characteristic of *P. tritici-repentis* were isolated from all inoculated wheat cultivar lesions plated onto PDA. No colonies characteristic of *P. tritici-repentis* or any other fungi were isolated from the tissue of un-inoculated plants on PDA.

DISCUSSION

This study is the first investigation of the race structure of *P. tritici-repentis* in New Zealand. All 12 New Zealand isolates, randomly selected to represent the South Island geographic distribution of the pathogen population, were positive for the *ToxA* gene but none had the *ToxB* gene. Aboukhaddour et al. (2013) related the presence of the *ToxA* gene to either virulence race 1 (if present with *ToxC*) or race 2 if occurring alone, while the presence of the *ToxB* gene was assigned to race 3. The absence of the *ToxB* gene in the New Zealand populations of *P. tritici-repentis* tested rules out race 3 but these populations could be classified as either virulence race 1 (Ptr ToxA + Ptr ToxC) or race 2 (Ptr ToxA only). More specific classification was not possible as the gene(s) that encode for the production of Ptr ToxC are currently unknown and therefore their presence could not be determined using PCR in the current study (Aboukhaddour et al. 2013). The New Zealand *P. tritici-repentis* population race structure is similar to that reported for Australian *P. tritici-repentis*, where all 119 isolates tested negative for the *ToxB* gene but were positive for *ToxA* so were classified as either race 1 or 2 as the presence of *ToxC* was not tested (Antoni et al. 2010). Further, races 1 and 2 are reported to be the predominant *P. tritici-repentis* races present in wheat growing areas in Canada (Lamari et al. 1998; Lamari et al. 2005; Aboukhaddour et al. 2013), USA (Friesen et al. 2005), Baltic States and Romania (Abdullah et al. 2017) and South America (Gamba et al. 2012; Bertagnolli et al. 2019). In contrast, in North Africa, *P. tritici-repentis* populations in Morocco and Tunisia were reported to be mostly positive for *ToxB* (Gamba et al. 2017; Kamel et al. 2019). Kamel et al. (2019) reported that whilst *ToxA* was only detected in 51% of isolates, *ToxB* was amplified in 97% of the population.

The New Zealand populations of *P. tritici-repentis* screened in this study were isolated from symptomatic wheat leaves, with both necrosis and extensive chlorosis observed in the field indicating the isolates were capable of inducing chlorosis on all wheat cultivars sampled (Weith 2015). However, whether this observation was indeed the result of a Ptr ToxC-sensitive wheat reaction, or just an indirect cultivar specific reaction to *P. tritici-repentis* requires further investigation. The development of Ptr ToxC-specific PCR primers would facilitate the identification of the race(s) present in the New Zealand *P. tritici-repentis* populations. As a result, further work is required in order to determine whether the populations of *P. tritici-repentis* possess the ability to produce Ptr ToxC. However, it has also been

Table 3 The mean disease score (0–5 scale; Table 2) and mean lesion area (mm^2) of ten different New Zealand wheat cultivars assessed 3 weeks after inoculation with *Pyrenophora tritici-repentis*.

Cultivar	Mean disease score	Mean lesion area (mm^2)
'Empress'	1.3 a	1.6 A
'Duchess'	1.6 ab	2.2 AB
'Wakanui'	1.9 abc	2.1 AB
'Inferno'	1.9 abc	2.1 AB
'Starfire'	2.0 abc	2.7 ABC
'Viceroy'	2.0 abc	2.5 AB
'Torch'	2.3 abc	2.1 AB
'Saracen'	2.6 bc	3.6 BC
'Reliance'	2.6 bc	3.6 BC
'Discovery'	3.0 c	4.6 C

Values within columns followed by the same letter are not significantly different according to Tukey's test at $P \leq 0.05$. Susceptible mean score (a–c) was significant ($P \leq 0.001$; F = 4.01). Lesion area (A–C) was also significant ($P \leq 0.001$; F = 4.56).

suggested by Andrie et al. (2007) that it may not be possible to accurately identify the race structure of a *P. tritici-repentis* population with just a molecular-based system as genes encoding for toxins C and D have not been identified. These authors suggested screening isolates using an established wheat differential system, which are currently not available in New Zealand.

The current study confirmed the pathogenicity of New Zealand *P. tritici-repentis* isolate mixtures, and is the first study to investigate the susceptibility of New Zealand wheat cultivars to inoculation with *P. tritici-repentis*. The results showed that all of the screened cultivars possess varying degrees of susceptibility to tan spot disease. Of the ten wheat cultivars assessed, the cultivars 'Empress' and 'Duchess' had the lowest levels of tan spot infection indicating they may possess a level of resistance or high tolerance to *P. tritici-repentis*. In contrast, 'Discovery', 'Reliance' and 'Saracen' were the most susceptible cultivars to infection by *P. tritici-repentis*. These results strongly indicate that, at present, the majority of the most commonly grown wheat cultivars commercially available in New Zealand should be considered as either susceptible, or at most, moderately resistant to tan spot. However, based on these results, the cultivars 'Empress', 'Duchess' and potentially 'Wakanui' and 'Inferno' would be the best cultivars to recommend to a farmer growing in a high tan-spot incidence region.

The sensitivity of a wheat line to Ptr ToxA HST is conferred and determined by a dominant allele located on the *Tsn1* locus of the wheat *Tsn1* gene, whereas sensitivity to Ptr ToxB HST is conferred by a dominant allele located on the *Tsc2* locus of the wheat *Tsn2* gene (Haen et al. 2004; Strelkov & Lamari 2003; Faris et al. 2013; Antoni et al. 2010). The results of this study therefore indicate that the *ToxA–Tsn1* gene interaction is an important factor in the pathogenicity relationship between *P. tritici-repentis* and wheat in New Zealand. The observation of necrosis of varying levels on all of the wheat cultivars inoculated with *P. tritici-repentis* suggests that all the wheat cultivars evaluated in this study very likely possess the gene *Tsn1*, which confers susceptibility to the HST Ptr ToxA. Combined with the knowledge that all of the inoculated isolates were characterised as possessing the Ptr ToxA-inducing gene *ToxA,* the necrosis observed in this study can be presumed to be the likely result of a *ToxA–Tsn1* interaction (Faris et al. 2013). Furthermore, since the isolates screened in the current study were recovered from plants which were expressing necrosis in the field (Weith 2015), these results possibly suggest that the majority of the New Zealand wheat cultivars sampled in this study also possessed this dominant *Tsn1* gene. In contrast, the results of the study imply that the *ToxB–Tsc2* gene interaction is an insignificant factor determining the pathogenicity of *P. tritici-repentis* towards wheat in New Zealand. This result is similar to that of Antoni et al. (2010) where the *ToxA–Tsn1* interaction was the dominating factor in Australian tan spot, whereas the *ToxB–Tsc2* was, at the time, reported to be an insignificant factor.

The current study also showed that the degree of chlorosis observed on the wheat cultivars inoculated with *P. tritici-repentis* appeared to be very low and not as pronounced as the levels reported on some wheat cultivars grown in other countries (Singh et al. 2010). This result may indicate that there is either a low prevalence of the chlorosis inducing *Tsn1* or *Tsc2* genes in the assessed wheat cultivars, or absence of *ToxB*- and/or *ToxC*-inducing *P. tritici-repentis* isolates in New Zealand (Faris et al. 2013). The chlorosis observed in this study was most likely not the result of a *ToxB–Tsc2* interaction due to the inoculated *P. tritici-repentis* isolates being found to lack the *ToxB* gene. Whether this result could indicate that the *P. tritici-repentis* isolates used to inoculate the wheat cultivars possess the HST Ptr ToxC was outside the scope of this study. However, as with the *ToxA–Tsn1* gene interaction, the presence of HST Ptr ToxC could be further investigated by evaluating for the presence of *Tsn1* and more specifically *Tsc2* in the current range of wheat cultivars in New Zealand. In addition, New Zealand isolates of *P. tritici-repentis* should also be screened for the *ToxC* gene(s) should a PCR-based method be developed in future. Together this knowledge would provide valuable information for New Zealand wheat breeders to enable the selective breeding of new wheat lines that lack HST-susceptible genes and lead to cultivars with reduced susceptibility to *P. tritici-repentis*.

Tan spot is typically expressed on a resistant wheat plant as small, dark brown lesions that do not increase in size (Singh et al. 2010). In contrast, a susceptible wheat plant will express dark brown spots surrounded by extensive yellow chlorosis and/or tan necrosis (Lamari & Bernier 1989b; Singh et al. 2010). In this study, eight of the ten inoculated cultivars expressed characteristic symptoms of tan spot which indicated they were susceptible or tolerant to varying degrees to *P. tritici-repentis*. Based on relevant literature (Singh et al. 2010; Faris et al. 2013), cultivars 'Empress' and 'Duchess' displayed symptoms that indicated they may possess a level resistance to the mixture of 4 *P. tritici-repentis* isolates tested here. However, application of a relatively high concentration of conidia at a single specific inoculation point on a leaf may have prevented characteristic resistance symptoms on inoculated cultivars by overloading the resistance threshold of the evaluated cultivar. The number of conidia inoculated in the current study, although sufficient to cause infection on the evaluated wheat cultivars, was relatively low (approximately 15 conidia / 5 µL inoculation drop). But this concentration may have still been high enough to induce the expression of large necrotic lesions which were interpreted as indicating a degree of susceptibility or tolerance. For example, the cultivars 'Empress', 'Inferno', 'Starfire' and 'Viceroy' expressed irregularly shaped very dark black lesions with little chlorosis three weeks after being inoculated with *P. tritici-repentis*. It is possible that they may have expressed a degree of resistance to tan spot had the whole surface of these cultivars been uniformly inoculated by a spray-based method using an atomiser. Further greenhouse or field-based work would need to be conducted in order to confirm this.

The stages which the wheat cultivars were inoculated could also affect the cultivar response to *P. tritici-repentis* infection. In the current study, wheat cultivars were inoculated at GS-20-29 (Zadoks et al. 1974) mainly because the leaf surfaces were large enough to make it easy to inoculate with *P. tritici-repentis* conidia. In contrast, a

number of studies have inoculated wheat plants at the seedling stage e.g. two to five leaf stages (GS 13-15) (Lamari & Bernier 1989a; Lamari & Bernier 1989b; Hosford et al. 1990; Tadesse et al. 2006). However, it is unlikely that this had a significant effect on the cultivar responses observed in this study since Shabeer & Bockus (1988) reported that plants were most physiologically susceptible when inoculated in the field at booting (GS 40–49) and flowering (GS 60–69) growth stages. Furthermore, evaluation of wheat cultivars in the current study was carried out under greenhouse conditions and the results should be confirmed under New Zealand field conditions. However, other studies have reported that results from glasshouse studies provide a good correlation with relative susceptibility of cultivars under field conditions (Evans et al. 1999).

The drop-based inoculation method and modified rating system used in the current study was found to provide sufficient information on the degree of each cultivars response to *P. tritici-repentis.* For this study, a mean disease score and mean lesion area were measured for each inoculated wheat cultivar. The system was effective in allowing the responses of the cultivars to be rated and compared against each other to determine their overall susceptibility or tolerance. The disease score scale was designed to help quantify, to some extent, the large range of known responses that can be induced by *P. tritici-repentis* when inoculated onto different wheat genotypes. In general, similar ratings of susceptibility were recorded with both measuring methods in the current study. However, with the cultivar 'Starfire', the results showed that it was more susceptible with the lesion area score compared with the disease score rating. This outcome may have been due to the disease score taking into account the chlorosis whereas the lesion area score did not.

The results of the current study could have important practical implications on the way the breeding of wheat in New Zealand is approached in the future whereby wheat lines are selectively bred to lack the *Tsn1* gene in order to confer resistance to *ToxA*-possessing isolates of *P. tritici-repentis* (Antoni et al. 2010). However, the results also indicate that the cultivars 'Empress' and 'Duchess' would be most suitable for cultivation in regions of New Zealand with a high incidence of tan spot. However, all of the evaluated cultivars could potentially be grown in areas of high tan spot incidence provided their cultivation includes a robust and appropriate fungicide programme.

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