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Virulence assessment of Australian *Pyrenophora tritici-repentis* isolates

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Abstract

The virulence of 57 Australian isolates of *Pyrenophora tritici-repentis* (Ptr), a necrotrophic fungal pathogen responsible for the major wheat disease tan spot, was assessed through plant infection assays. Isolates collected from the northern, southern, and western wheat-cropping regions of Australia were evaluated against 16 Australian bread wheat cultivars under controlled growth conditions. Following infection, the wheat panel displayed varying disease symptoms ranging from tiny necrotic specks to spreading chlorotic and necrotic lesions. Analysis of variance indicated that the wheat cultivar exhibited a greater effect on the disease response, explaining 62.7% of the variation, in comparison to the isolate (10.4%). The interaction between the cultivar and the isolate was statistically significant and was attributed to 9.8% of the total variation. All Ptr isolates examined were able to cause disease, but did not display a clear distinction in virulence on the wheat panel investigated, instead showing subtle differences in aggressiveness. Based on the disease responses, there was no obvious pattern between isolate aggressiveness and cropping region. Some cultivars, such as Hydra, exhibited an effective level of resistance in relation to the panel of isolates tested. All 57 Ptr isolates were found to possess the *ToxA* effector gene and lack the *ToxB* effector gene. The gene expression level of *ToxA* was up-regulated at 3 days postinfection in both ToxA-sensitive and -insensitive cultivars, independent of ToxA–*Tsn1* recognition.

KEYWORDS
necrotroph, tan spot, ToxA, virulence, wheat, yellow spot

1 | INTRODUCTION

Tan spot disease of wheat (also known as yellow spot) is caused by the necrotrophic fungal pathogen *Pyrenophora tritici-repentis* (Ptr). Disease symptoms are typified by tan-coloured foliar lesions surrounded by chlorotic halos (Ciuffetti & Tuori, 1999; Lamari et al., 1991). The extent of symptom development varies depending on the host genotype, with lesion types ranging from small black spots to extensive chlorosis or tan necrosis, which differentiate a resistant or susceptible reaction to the pathogen, respectively (Lamari & Bernier, 1989). Ptr is able to secrete necrotrophic effectors (e.g., host-selective toxins) that exert their effect on sensitive wheat

genotypes in an inverse gene-for-gene manner (Lamari et al., 2003; Oliver et al., 2016; Strelkov & Lamari, 2003; Tan et al., 2010). Like many parts of the world, in Australia the predominant Ptr effector is ToxA, which causes extensive necrosis on wheat lines possessing the sensitivity gene *Tsn1* and is generally correlated with tan spot disease susceptibility (Faris et al., 2010; Friesen et al., 2006; Kamel et al., 2019; See et al., 2018, 2019).

In Australia, tan spot was first documented in the 1950s (Valder & Shaw, 1952), and quickly spread via the introduction of susceptible dwarf wheat cultivars (Rees & Platz, 1979, 1983), such that tan spot has now become one of the major wheat diseases in Australia, with estimated annual yield losses of $212 million (Murray & Brennan, 2009). The wheat-growing areas in Australia are divided into three broad regions with distinct climate and soil characteristics. Although the disease was initially reported in the northern cropping region (New South Wales and Queensland; Valder & Shaw, 1952), the occurrence of tan spot is now widespread across the country.

Crop rotation, resistant cultivars, stubble management, and fungicide application are the primary disease management practices, forming an integrated control approach (Jorgensen & Olsen, 2007). The use of disease resistant cultivars is a very effective means of disease control, while reducing the need for repeated fungicide application and subsequent risk to the environment, as well as soil erosion as a result of tillage or stubble burning. New sources of disease resistant germplasm have been sought from international nurseries for use in Australia since the 1990s, particularly those from the International Maize and Wheat Improvement Centre (CIMMYT; Rees & Platz, 1990). Over the 30-year period of collaboration between the CIMMYT Australia ICARDA Germplasm Evaluation (CAIGE) breeding programmes and research institutes, Australian breeders have developed wheat cultivars with improved resistance to tan spot disease, such that the majority of new cultivars are rated as moderately resistant to moderately susceptible (MRMS) to tan spot. Yet cultivars rated as resistant to moderately resistant (MR) or resistant (R) are still lacking in Australia. In addition to efforts to identify wheat accessions with useful levels of disease resistance, a good understanding of the virulence of the current Ptr pathogen population in Australia is crucial for cultivar improvement.

This study aims to (a) determine the level of Ptr virulence in Australia across the three cropping regions, and (b) assess the effect of Ptr isolates and wheat cultivars on the tan spot disease response.

2 | MATERIALS AND METHODS

2.1 | Fungal isolates

Fifty-seven Australian Ptr isolates were used in this study. Of these, 56 were collected from the northern, southern, and western cropping regions in 2015 and 2016, while the control isolate (WAC13611) was collected from the western region in 2012 (Table S1). Isolate WAC13611 was selected as a control because it has previously shown good sporulation and consistent responses across a range of wheat lines (Dinglasan et al., 2019). Ptr isolates from the state of Victoria were kindly provided by Agriculture Victoria, while all other isolates were recovered from infected leaf material using a water agar single spore reisolation method described previously (Moffat et al., 2015). Ptr isolates were grown at room temperature on V8PDA plates (Campbell's V8 juice 150 ml/L, potato dextrose agar 10 g/L, $CaCO_3$ 3 g/L, and agar 15 g/L). Recovered isolates were confirmed as Ptr via morphological characteristics of the conidia, and all fungal isolates were screened for the presence of a Ptr unique genomic region via polymerase chain reaction (PCR) (Moffat et al., 2015) as described below.

2.2 | PCR assay for the identification of Ptr

Genomic DNA of individual fungal isolates was extracted using a Biosprint 15 DNA kit (Qiagen) according to the manufacturer's instructions. Colonies were confirmed as Ptr using the PtrUnique_F2 and PtrUnique_R2 primer set as described previously (Moffat et al., 2015). The PCR consisted of a 20 μl reaction of 1 × MyTaq Reaction Buffer (Bioline), 250 nM of each forward and reverse primer, 1 U MyTaq DNA polymerase (Bioline), and 100 ng DNA template, with the following cycling conditions: 94°C for 3 min; and 35 cycles of 94°C for 30 s, 57°C for 30 s, 72°C for 30 s. The PCR product was then analysed on a 1.2% agarose gel to detect the presence of the 490 bp amplification product specific to Ptr.

Individual Ptr isolates were also screened for the presence or absence of the necrotrophic effector genes *ToxA* and *ToxB* using the primer sets ToxAscreeningF (5′-CCTCGTACTTCTTTTCAGCG-3′) and ToxAscreeningR (5′-TGTAGAAGACAAGATTTTGA-3′) (Moolhuijzen et al., 2018) and TB10 (5′-TATGCGACCCTAACCTAGCC-3′) and TB12 (5′-GCCAGATAAAAAACCCCTATACC-3′) (Martinez et al., 2004), respectively. PCR conditions were the same as for the Ptr unique PCR assay.

2.3 | Plant infection assays

Sixteen Australian spring wheat (*Triticum aestivum*) cultivars with tan spot disease ratings ranging from MR to susceptible/very susceptible (SVS) were selected for this study (Table S2). Popular cultivars released from 1997 to 2014 were chosen, covering the full range of resistance responses, with more representation of the MR (two cultivars) and MRMS (eight cultivars) categories to help identify high virulence responses. For each wheat cultivar, four seeds were sown per pot (comprising one replicate), and each pot contained four different wheat cultivars arranged in a completely randomized design in triplicate. Plants were grown in 500 ml plastic pots in Richgro potting mix (Landsdale) and kept in controlled growth conditions of 20 ± 2°C with a 12 h photoperiod to avoid complexity in disease assessment caused by factors such as abiotic stress or coinfection by other pathogens. One week after planting, pots were fertilized with Thrive water-soluble fertilizer (Yates) according to the

manufacturer's recommended application rate. Fungal inoculum was prepared from cultures grown in V8PDA as described previously (Moffat et al., 2014). Two-week-old seedlings (a minimum of three seedlings per replicate) were spray-inoculated with 50 ml of conidial suspension at a concentration of approximately 2500 conidia/ml until run-off. Plants were incubated under high humidity (>80%) for 24 h. For assessment of virulence of the 57 Ptr isolates, experiments were conducted in batches of five to six isolates, including the control isolate WAC13611 in each assay to monitor for any variation between experiments.

Additionally, infection assays were repeated for a subset of 10 Ptr isolates against a subset of three cultivars (Mace, Westonia, and GBA Sapphire) in a single experiment to validate the initial results. This was designed as a split-plot design with isolate as the main plot and cultivar as the subplot in three replicates. For the split-plot design with a nested structural component, the highest level of structure corresponded to complete replicates of the set of treatments (i.e., block). Each block was then divided into 10 main plots, with the 10 levels of isolate randomized to the main plots separately within each block. Finally, each main plot was divided into three subplots, and the three levels of cultivar were randomized onto subplots within each main plot. The experimental design was generated using the experimental design tool DiGGer in R (Coombes, 2018).

2.4 | Disease assessment

Plants were examined for disease symptoms at 7 days postinoculation (dpi) by scoring the two newest leaves that were fully elongated at the time of inoculation. Disease phenotypes were recorded based on visual assessment of leaf lesions, with 1 = presence of tiny necrotic specks; 2 = lesions with little necrosis and chlorosis; 3 = lesions with distinct necrosis and chlorosis; 4 = coalescing type 3 lesions; and 5 = extensive necrosis and chlorosis and the absence of well-defined borders between lesions (Lamari & Bernier, 1989). Disease reactions of type 1 and 2 were considered as resistant, while 3–5 were susceptible. If multiple types of lesions were observed on the seedlings, the highest score was recorded. For both experiments (57 isolates against 16 cultivars, and 10 isolates against 3 cultivars) three replicates per cultivar were assessed for each fungal isolate. Each replicate consisted of three to four seedlings depending on germination. An example of observed disease symptoms is shown in Figure S1.

2.5 | ToxA sensitivity assay of wheat cultivars

To determine if each wheat cultivar harboured the *Tsn1* sensitivity locus to the Ptr effector ToxA, plant bioassays were performed on each cultivar as described previously (See et al., 2019). Briefly, the ToxA protein was heterologously expressed in *Escherichia coli* SHuffle using the expression vector pET28(+) (Novagen) and purified using HisPur Ni-NTA purification spin columns (Thermo Fisher Scientific) by gravity flow according to the manufacturer's instructions. Purified ToxA protein (10 ng/μl) was infiltrated into the first leaves of 2-week-old seedlings using a needleless syringe and scored at 7 days postinfiltration (Figure S2). Cultivars that harboured the *Tsn1* sensitivity locus produced a necrotic symptom while insensitive cultivars had no symptoms (Figure S2). A minimum of three independent seedlings were infiltrated for each cultivar.

2.6 | Detection and quantitative gene expression of *ToxA*

The expression of the *ToxA* gene during infection was evaluated from two isolates that differed in aggressiveness (S6561 and Q4034). One seedling from each replicate of the disease assessment was snap-frozen at 0, 3, 5, and 7 dpi from the experiment of 10 Ptr isolates against three cultivars. RNA was extracted from the leaf tissue using TRIzol reagent (Thermo Fisher Scientific). First-strand complementary DNA was synthesized from RNA using the iScript cDNA synthesis kit (Bio-Rad). Briefly, RNA was treated with DNase (Ambion DNA-free kit; Thermo Fisher Scientific) to remove any residual genomic DNA according to the manufacturer's instructions. This was followed by the synthesis of complementary DNA in a 20 μl reaction consisting of 1 μg of RNA, 1× iScript reaction mix, 1 U iScript reverse transcriptase, and nuclease-free water. The reaction was incubated for 5 min at 25°C, 30 min at 42°C, and 5 min at 85°C to terminate the reaction. Quantitative expression analysis of *ToxA* was performed using the QuantiTect SYBR green PCR kit (Qiagen) in a CFX384 Touch Real-Time PCR Detection System (Bio-Rad). The primer sets ToxAF (5′-GACGACCTGGTGCTATAGGC-3′) and ToxAR (5′-CGATTAGTCCTGCCGGTGTT-3′), as well as Act1F2 (5′-AGACCTTCAACGCTCCCGCC-3′) and Act1R2 (5′-TGGCGTGGGGAAGAGCGAAAC-3′) were used to amplify the *ToxA* and actin (*Act1*) genes, respectively (Rybak et al., 2017). The PCR consisted of a 10 μl reaction of 1× QuantiTect SYBR Green PCR Master mix, 300 nM of each forward and reverse primer, and 4 μl of cDNA template, with the following cycling conditions: 95°C for 15 min, and 39 cycles of 95°C for 15 s, 62°C for 30 s, 72°C for 30 s. The housekeeping gene *Act1* was used to normalize the gene expression of *ToxA*. Five biological replicates and two technical replicates were assayed for each wheat cultivar.

2.7 | Statistical analysis of the tan spot disease response

The disease response data was analysed using linear mixed models (LMMs) fitted with ASReml-R 4.1.0 (Butler et al., 2018) to examine the spatial variations including local autocorrelation, global trends, and extraneous variations, and to produce predicted values. Variance parameters in the mixed models were estimated using the residual maximum likelihood (REML) procedure (Patterson & Thompson, 1971). Residual diagnostics were performed to examine the validity

of the model assumption. All data were analysed with ASReml-R using R platform v. 3.6.3 (R Project for Statistical Computing; R Core Team, 2018). As the main objective of the experiments was to determine the difference between specific pairs of treatments, the cultivar, isolate, and the interaction term between cultivar and isolate were fitted as fixed effects (Smith et al., 2005). Wald tests for fixed effects in the fitted LMM were applied to evaluate the significance of effects. For each of the fitted models, the empirical best linear unbiased estimates (eBLUEs) were produced. The R package asreml-Plus (Brien, 2020) was used to compute the least significant difference (with $\alpha = 0.05$) values for multiple comparison.

2.8 | Hierarchical cluster analysis

The hierarchical cluster analysis was performed for cultivars and isolates based on the eBLUEs of tan spot scores using the R package cluster (Maechler et al., 2019) with the ward.D2 method (Murtagh & Legendre, 2014). The optimal number of clusters was identified using the successive K-means approach (Hartigan & Wong, 1979) with an increasing number of clusters by optimizing the variance between groups while minimizing the variance within groups (Figure S3). Principal component analysis (PCA) was conducted using the functionality of prcomp in Base R platform (R Core Team, 2018). A heat map with dendrograms was created with the functionality heatmap.2 in the R package gplots (Warnes et al., 2016) along with R package dendextend (Galili, 2015).

3 | RESULTS

3.1 | Evaluation of Australian Ptr isolates for variation in virulence

A total of 57 Ptr isolates were obtained across the three wheat-cropping regions of Australia comprising 24 isolates from the northern region, 14 from the southern region, and 19 from the western region (Figure 1; Table S1). All 57 isolates were found to possess the ToxA effector gene (*ToxA*) and lack the ToxB effector gene (*ToxB*) (Table S1). To assess isolate virulence, plant infection assays were performed in a controlled growth environment on a panel of 16 Australian bread wheat cultivars with tan spot disease ratings that ranged from MR to SVS (Table S2). All three groups of isolates displayed comparable virulence patterns across the 16 wheat cultivars (Figure 2). The overall mean disease scores of isolates ranged from 1.21 ± 0.05 (standard error of mean [*SEM*]) to 4.21 ± 0.07 (*SEM*), with the lowest disease score on the MRMS cultivar Hydra, and the highest disease score observed on the SVS cultivar Yitpi (Figure 2).

Hierarchical clustering of the 57 isolates and 16 wheat cultivars was performed based on the tan spot disease response scores (Figure 3). As illustrated in the dendrogram, the Ptr isolates were grouped into two broad clusters; one comprised 36 isolates with the highest disease scores, while the 21 remaining isolates formed the other cluster. The clade of the more aggressive isolates could be subdivided into a further four clusters, of which the most aggressive comprised only four isolates (W0152U, N1084, S2084, and S2259). In comparison, the cluster of the remaining 21 isolates comprised a further two clusters, of which the least aggressive clade consisted of seven isolates (WAI2300, N6857, V-15-109, W0160HY, Q7650, S6561, and N0141). Overall, the optimal number of clusters for the isolates was confirmed to be six using a successive K-means approach (Figure S3), and these clusters are denoted by the branch colouring in Figure 3. Each of the six clusters contained isolates from more than one cropping region.

The cultivars were grouped into two major clusters based on the tan spot disease response (Figure 3). The cluster of the more susceptible cultivars consisted of all three SVS cultivars (Justica CL Plus, Correll, and Yitpi) and the two moderately susceptible to susceptible (MSS) cultivars (Trojan and GBA Sapphire). These five cultivars all harbour the wheat ToxA sensitivity gene *Tsn1* as they were the only cultivars of the 16 to exhibit necrosis following ToxA infiltration (Table S2). Conversely, the cluster of the more resistant cultivars that had lower disease scores comprised those cultivars rated as MS, MRMS, and MR. Again, a successive K-means approach identified six clusters to be the optimal number for the cultivars (Figure S3) as shown by the branch colours (Figure 3). The cultivar Hydra (MRMS) was the only cultivar in the clade with the lowest overall disease scores, while the second most resistant cluster consisted of five cultivars: Magenta and Wyalkatchem (MR), and Emu Rock, Mace, and Zen (MRMS), all of which are insensitive to ToxA (Table S2).

To examine the significance of the contribution of isolate and cultivar to the tan spot disease response, Wald tests of the fixed effects in the LMMs were used to test the significance. The analysis demonstrated that both cultivar and isolate have significant effects ($p < 0.0001$) on the tan spot disease severity response (Table S3). The variance explained by cultivar and isolate was 62.7% and 10.4%, respectively. A significant interaction between cultivar and isolate attributed to 9.8% phenotypic variance was also demonstrated in this study. PCA revealed that 56.0% of the variation could be explained by the principal component 1 (PC1), while only 8.6% of the variation could be explained by the principal component 2 (PC2; Figure S4).

3.2 | Validation of differences in isolate aggressiveness

To validate the differences in isolate aggressiveness as observed from the hierarchical cluster analysis, a subset of 10 isolates and three cultivars were selected for further evaluation of the tan spot disease response. Four isolates from the least aggressive cluster, and six isolates from the two most aggressive clusters were selected. The cultivars Mace, Westonia, and GBA Sapphire were selected as representative cultivars of MRMS, MS, and MSS disease response ratings.

The overall tan spot disease response obtained from the 10 isolates ranged from 2.17 to 3.33, with significant differences in aggressiveness observed. The most notable difference was between isolate S6561 (less aggressive) compared to isolates W0152U and

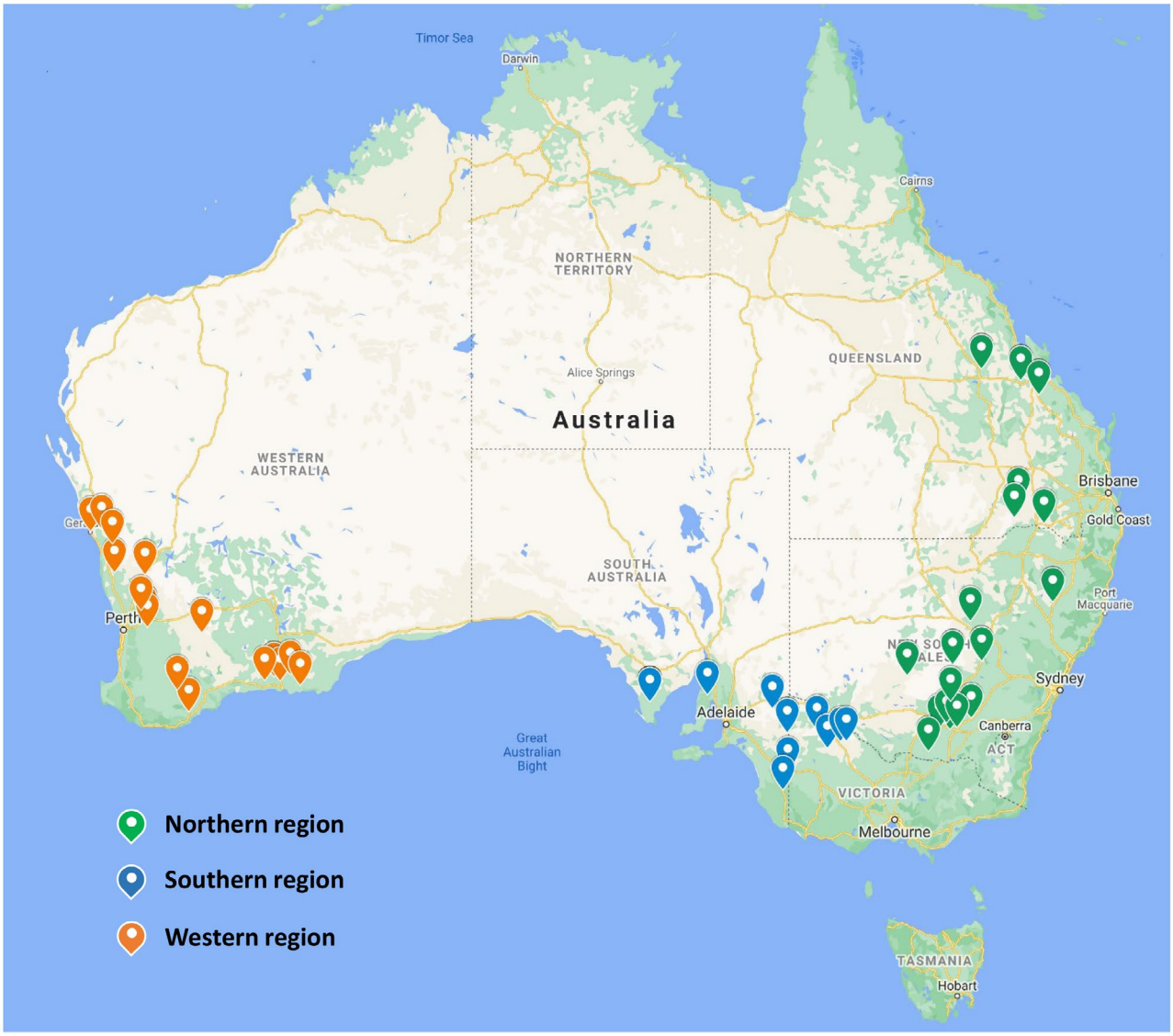

FIGURE 1 Distribution of the 57 *Pyrenophora tritici-repentis* isolates across the three major growing regions in Australia. Icon colour denotes the different cropping regions. Image generated using Google Maps [Colour figure can be viewed at wileyonlinelibrary.com]

Q4034 (more aggressive; Figure 4a). For the cultivars, a significant difference in the disease responses of Mace and Westonia compared to cultivar GBA Sapphire was observed, with the largest difference between Mace (2.00) and GBA Sapphire (3.85; Figure 4b). When the individual disease response of each isolate was examined on the three cultivars, the responses were most variable on GBA Sapphire (ranging from 2.83 to 4.67) and Westonia (1.50–3.50) compared to Mace (1.67–2.67; Figure 4c). For example, isolate WAI2300 was the most aggressive on GBA Sapphire while on Westonia, the most aggressive isolate was isolate Q4034 (Figure 4c). Even though the difference between the highest and lowest disease scores were comparable between GBA Sapphire and Westonia (1.84 and 2.00, respectively), GBA Sapphire displayed a more susceptible response with nine of the isolates inducing disease scores >3.0, while only isolates W1052U and Q4034 produced disease scores of more than 3.0 on cultivar Westonia (Figure 4c).

The Wald test of the LMM using the subset of 10 Ptr isolates against three wheat cultivars also demonstrated that cultivar and isolate, as well as the interaction between cultivar and isolate, have significant effects on tan spot disease severity ($p < 0.0001$; Table S4). The variations attributed to cultivar, isolate, and their interaction were 61.2%, 12.5%, and 11.1%, respectively. This result from the subset is in good agreement with that from the initial larger data set of 57 isolates and 16 cultivars.

3.3 | *ToxA* expression during infection

The expression of the Ptr *ToxA* gene was examined during infection of cultivars Mace, Westonia (both ToxA-insensitive), and GBA Sapphire (ToxA-sensitive) with isolate S6561 (less aggressive) and isolate Q4034 (more aggressive). *ToxA* expression was in general

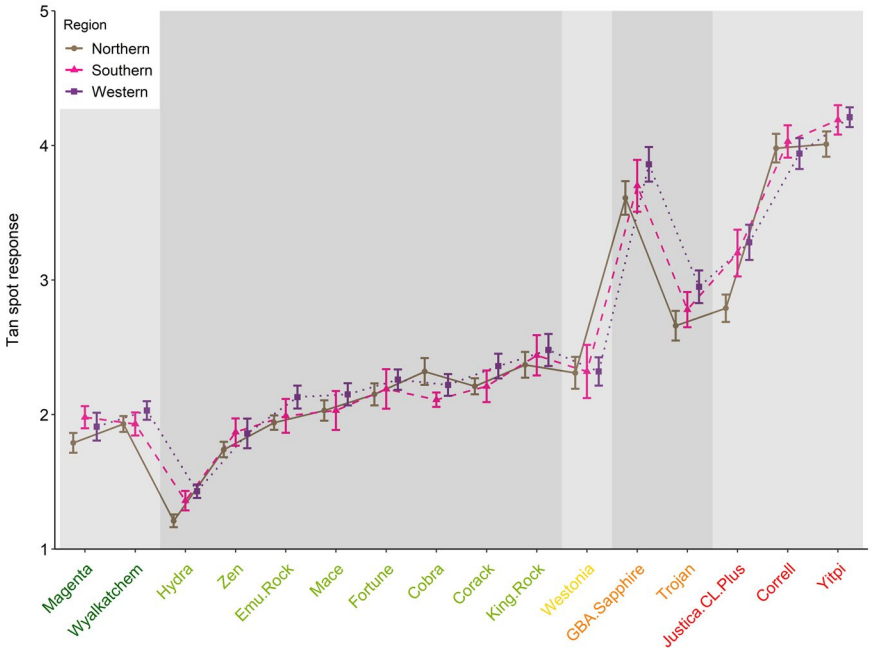

FIGURE 2 Tan spot disease response of 16 Australian bread wheat cultivars to 57 *Pyrenophora tritici-repentis* isolates from the northern, southern, and western Australian cropping regions. Font colours of cultivar names from left (green) to right (red) denote the different tan spot disease ratings of the wheat panel: MR, MRMS, MS, MSS, and SVS. Error bars represent 1 *SEM*. MR, moderately resistant; MRMS, moderately resistant to moderately susceptible; MS, moderately susceptible; MSS, moderately susceptible to susceptible; *SEM*, standard error of mean; SVS, susceptible to very susceptible [Colour figure can be viewed at wileyonlinelibrary.com]

most strongly up-regulated at 3 dpi and down-regulated after 5 dpi (Figure 5). A comparable expression profile across the 7 day period was observed for both isolates on cultivars GBA Sapphire and Westonia. In cultivar Mace, a higher gene expression level of *ToxA* was detected at 3 dpi for the more aggressive isolate Q4034 compared to isolate S6561. Overall, there was no clear pattern in the expression profile of the *ToxA* gene between ToxA-sensitive and -insensitive cultivars, but intriguingly, differences were observed between the ToxA-insensitive cultivars (Mace and Westonia).

4 | DISCUSSION

Evaluation of 57 Ptr isolates from across the three Australian cropping regions demonstrated that all the Ptr isolates collected were virulent and able to cause disease. However, there was no obvious distinction in virulence levels among the isolates, instead subtle differences in aggressiveness were observed. The majority of the phenotypic variation in the disease response (62.7%) could be explained by the cultivar, while only 10.4% was attributed to the isolate. This agrees with a previous study in the wheat–*Parastagonospora nodorum* pathosystem, in which only a small difference in disease scores was reported between different pathogen genotypes compared to larger differences recorded between cultivars (Phan et al., 2020). The absence of distinct virulence patterns is very likely associated with a single or predominant race of the Ptr population in Australia. However, this observation would need to be validated with a race classification study of the Australian Ptr population. Furthermore, evaluating the virulence of Ptr isolates collected over a wide temporal range would help answer if a variation in virulence has occurred over time, as has been reported in *P. nodorum*, whereby an association of pathogen population diversity to the most widely grown cultivars was shown (Phan et al., 2020). The interaction between the cultivar and the Ptr isolate was significant, accounting for 9.8% of the total variance of the disease response, which suggests that the cultivar effect on the disease response differed markedly among the isolates. A recent study examining the virulence and cultivar response of 12 race 1 Ptr isolates from Oklahoma, USA against 11 wheat cultivars also found a significant interaction between cultivar and isolate (Kader et al., 2021). The PCA of the 57 isolates against 16 cultivars indicated that in general the isolate groups are in good agreement with those from the hierarchical cluster analysis. A biplot of the first two principal components from the PCA analysis revealed that each cultivar contributed similarly to the PC1 with 56% of variation explained. In contrast, the cultivars Hydra, Magenta, Zen, Westonia, Cobra, Mace, Fortune, Yitpi, GBA Sapphire, and Correll showed much stronger contribution to the PC2, with 8.6% of explained variation, than the other cultivars. Additionally, there appeared to be no obvious pattern between isolate aggressiveness and cropping region. Therefore, in the context of wheat breeding programmes, the selection of representative isolates for testing germplasm should be based on aggressiveness, rather than purely by collection location, in order to optimize screening for resistance.

Further work to determine the level of genetic diversity of Ptr within Australia is necessary. Population studies of Ptr using molecular markers have generally observed a moderate degree of genetic variability. For example, a previous study which assessed 67 Australian Ptr isolates from the three cropping regions found an average gene diversity (H_S) of 0.36 based on 12 microsatellites, which was similar to the H_S levels determined for Ptr isolates from North America (0.43), South America (0.38), and Europe (0.44) (Gurung et al., 2013). This is comparable to an earlier assessment of 80 Ptr isolates from Canada, Azerbaijan, Algeria, and Syria using 31 microsatellite markers that estimated the genetic diversity of the collection to be 0.38 (H_T) (Aboukhaddour et al., 2011). Thus, given the

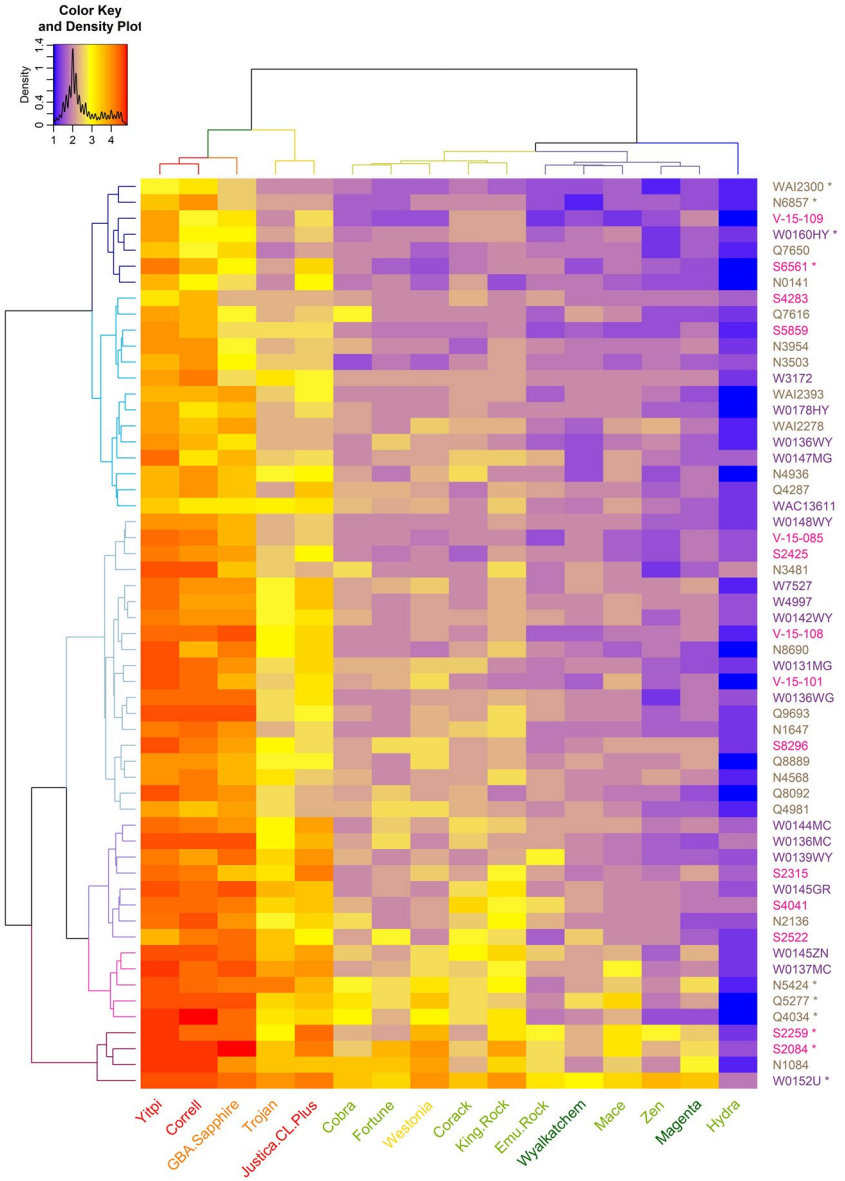

FIGURE 3 Heat map of the tan spot disease responses with hierarchical clustering of 16 wheat cultivars based on seedling assays using 57 *Pyrenophora tritici-repentis* isolates. Mean disease response is represented by the heat map colour key from blue (low disease score) to red (high disease score). The dendrograms depict the relationship among the groups of isolates (left) and cultivars (top). The isolate names are indicated on the right of the heat map and are coloured by cropping region: northern (brown), southern (pink), and western (purple). Asterisks indicate the subset of 10 isolates that were selected for further analysis [Colour figure can be viewed at wileyonlin elibrary.com]

ability of the pathogen to differentiate genetically, as well as the vast hectares now sown to MRMS wheat cultivars in Australia, a high selection pressure presumably exists for an increase in pathogenicity to occur. Therefore, monitoring virulence and assessing the current genetic diversity of the Ptr population within Australia is advisable.

The evaluation of the 16 wheat cultivars revealed the ToxA-insensitive cultivar Hydra as the most resistant cultivar to all isolates tested, while the most susceptible cultivars (GBA Sapphire, Trojan, Correll, Justica CL Plus, and Yitpi) all harboured the ToxA sensitivity gene *Tsn1* (Table S2). All 57 Ptr isolates were found to possess the *ToxA* gene, thus the removal of *Tsn1* from wheat breeding programmes is reasonable to avoid severe ToxA-induced necrosis (See et al., 2018). Indeed, a field trial over 2 years and four locations in the Western Australian wheatbelt found no yield penalties associated with ToxA-insensitive cultivars (Oliver et al., 2014). However, it has been suggested that plant effector sensitivity genes that confer susceptibility to a necrotrophic pathogen, such as *Tsn1*, may potentially confer resistance to a biotrophic pathogen, thus elimination of effector sensitivity genes may not be without risk (Faris & Friesen, 2020). Indeed, it is conceivable that removal of host effector sensitivity is likely to increase the selection pressure on the pathogen to overcome alternative host targets and thus may not provide durable resistance. The expression of the Ptr *ToxA* gene was demonstrated to be induced during infection on both ToxA-sensitive and -insensitive cultivars independent of the recognition of ToxA–*Tsn1*. A higher expression level of *ToxA* was detected on the ToxA-insensitive cultivar Mace in comparison to the ToxA-sensitive cultivar GBA Sapphire. Elevated *ToxA* expression in planta at 3 dpi on the ToxA-insensitive cultivars is in agreement with a previous study that showed that *ToxA* was highly up-regulated at 3 dpi on a ToxA-sensitive cultivar following Ptr infection (Rybak et al., 2017).

ToxA has been shown to induce necrosis in a light-dependent manner through the alteration of photosystem I and photosystem II, leading to the accumulation of reactive oxygen species and cell

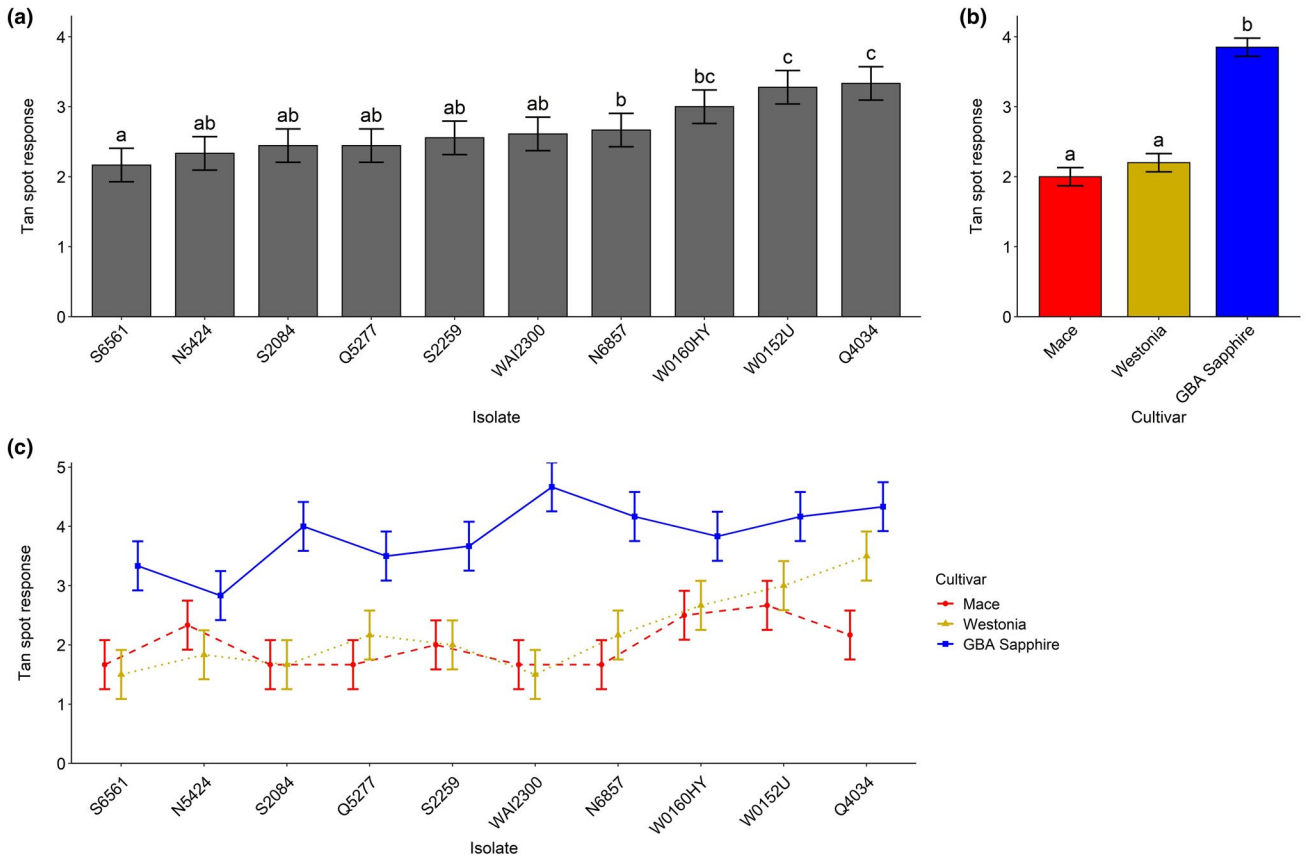

FIGURE 4 Tan spot disease infection by a subset of 10 *Pyrenophora tritici-repentis* isolates on three wheat cultivars (Mace, Westonia, and GBA Sapphire). (a) Mean disease response for each isolate, (b) mean disease response for each cultivar, and (c) mean disease response for each isolate by cultivar. Bar graphs and error bars represent the mean disease response and 0.5 × LSD with $\alpha = 0.05$. Different letters above bars represent significant differences between treatments at a significance level of 0.05. LSD, least significance difference [Colour figure can be viewed at wileyonlinelibrary.com]

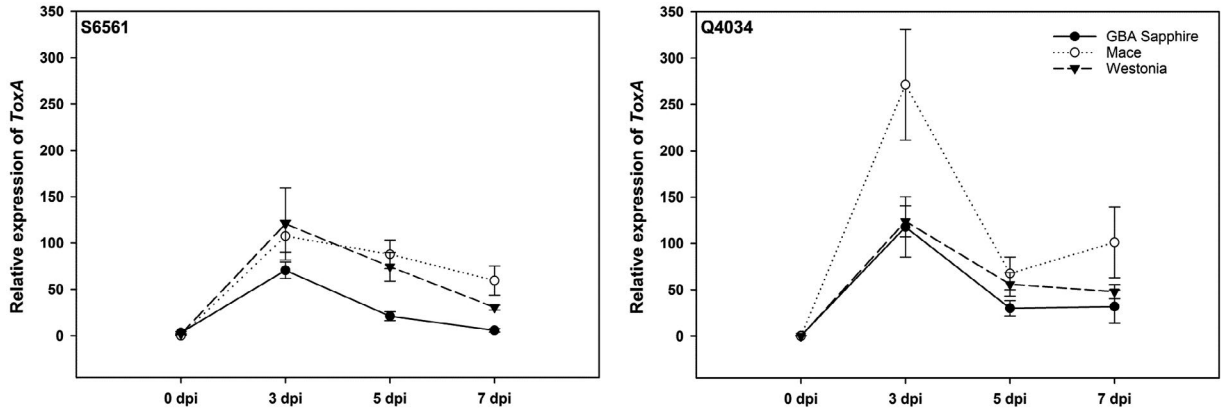

FIGURE 5 Relative expression of the *Pyrenophora tritici-repentis ToxA* gene throughout a leaf infection time-course. The expression of *ToxA* was examined in two isolates with low and high aggressiveness, represented by isolates S6561 and Q4034, respectively. The wheat cultivar GBA Sapphire is sensitive to ToxA while Mace and Westonia are both insensitive. Error bars are shown as the standard error of the mean

death (Manning et al., 2009). However, ToxA also acts as an elicitor, inducing a broad range of genes involved in plant defence early (≤24 h), in addition to genes involved in photosynthesis (i.e., receptors, pathogenesis-related, jasmonic acid and phenylpropanoid pathways, and oxidative stress; Pandelova et al., 2009). Variation in the expression of *ToxA* between different isolates on different wheat genotypes under the same photoperiod, as observed in this study, suggests that other factors may contribute to *ToxA* expression, and it would be of interest to investigate the biochemical and molecular host responses at later time points

(>24 h) given that the *ToxA* expression levels were greatest at 3 dpi.

Some cultivars exhibited a broad range of differences in their disease reactions following infection with the various Ptr isolates, such as GBA Sapphire, Trojan, and Justica CL Plus with disease scores ranging from 2.33 to 4.83, 1.99 to 4.33, and 1.83 to 4.16, respectively. Thus, for the evaluation of the host–pathogen interaction, it would be prudent to select cultivars such as these that displayed the most diversity in disease phenotypes. Fungal necrotrophic pathogens are known to produce an array of diverse host-selective toxins (Friesen et al., 2008; Rawlinson et al., 2019), many of which have yet to be characterized. The low scores may suggest the presence of certain resistance factors in these cultivars against corresponding virulence factors present in particular isolates. Careful selection of suitable wheat genotypes with a diverse qualitative nature of disease response, rather than those with resistance responses that have little potential in differentiating the host–pathogen interaction, may help dissect the more subtle interactions within the tan spot pathosystem.

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DATA AVAILABILITY STATEMENT

All data that support the findings of this study are presented herein.

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