Leaf yellowing of the wheat cultivar Mace in the absence of yellow spot disease

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ABSTRACT

The wheat variety Mace is currently dominating the southern wheat growing regions of Australia. It is high yielding in most environments and resistant to many diseases including yellow spot (also known as tan spot). However, observations of foliar yellowing of Mace have recently been reported in the field. This has raised concerns over a possible breakdown of resistance to yellow spot, which is caused by the necrotrophic fungal pathogen *Pyrenophora tritici-repentis*. West Australian field samples of yellowing Mace leaves were evaluated for *P. tritici-repentis* infection, and this pathogen was determined to be absent. Instead, *Alternaria* spp. were isolated from the wheat leaves. Pathogenicity assays showed that the recovered *Alternaria* spp. were unable to cause disease symptoms on Mace. Furthermore, spontaneous foliar lesions were observed in Mace grown in the absence of pathogens. It is therefore likely that such yellowing is a physiological trait, which will not respond to fungicide application. A marginal impact on yield cannot be excluded.

Additional key words:

Tan spot

Yellow leaf spot

Pyrenophora tritici-repentis

Alternaria

Triticum aestivum

Leaf blight

INTRODUCTION

The wheat variety Mace (*Triticum aestivum* L.), derived from a cross between Wyalkatchem and Stylet (AGT 2013a, b) has been rapidly adopted by Australian growers since its release in 2008. Mace has been widely tested in National Variety Trials and leads the commercial varieties for yield (Young 2013; Wheeler 2012). Prior to the release of Mace, its major parent Wyalkatchem was the dominant variety sown across southern Australian regions. However, compared to Wyalkatchem, Mace shows enhanced sprouting tolerance, improved grade quality (from Australian Premium White (APW) to Australian Hard (AH)), higher and more stable grain yields, a reasonably robust disease resistance profile, and expanded zonal adaptation (AGT 2013a, b; DAFWA 2013d). The reaction of growers has been swift, and Mace has rapidly become the leading wheat variety in Southern and Western Australian regions. In the 2012/2013 season, Mace comprised 41.4% of the area sown to wheat in the 2012/2013 season, followed by Wyalkatchem at 14.5% in Western Australia (WA) (DAFWA 2013d). This increased to 53.4% in the 2013/2014 season, with Calingiri second placed at 9.5% (DAFWA 2014). The current area sown to Mace in the Esperance port zone is estimated at 75% (South East Premium Wheat Growers Association, personal communication, 04 April 2014).

The foliar disease yellow spot (known as tan spot outside Australia) is the most economically damaging wheat disease in Australia (Murray and Brennan 2009), and is caused by the

necrotrophic fungus *Pyrenophora tritici-repentis* (Died.) Drechs. [anamorph: *Drechslera tritici-repentis* (Died.) Shoem.]. Mace is rated as moderately resistant to moderately susceptible (MR-MS) against yellow spot disease (DAFWA 2014; SARDI 2014). Only a handful of varieties, including Wyalkatchem, have the next highest disease rating of moderately resistant (MR), and none are rated as more resistant.

There has been a recent spate of reports of yellow blotching on Mace leaves in Western Australia (WA) and this has led to concern over a possible breakdown of resistance against yellow spot (DAFWA 2013a, b; AGT 2013c). Reports from the field have described chlorotic blotches scattered throughout the leaf canopy, although particularly extensive on the lower leaves, and this is often followed by the development of mild necrotic areas.

Here, we examine Western Australian field samples of Mace leaves exhibiting such yellowing, for the presence of *P. tritici-repentis*, in order to determine whether there has been a breakdown of yellow spot resistance and if fungicide application is appropriate.

MATERIALS AND METHODS

Plant and fungal material

Samples of cv. Mace leaves (*Triticum aestivum* L.) exhibiting yellow blotching were collected from five West Australian field sites at Perth, Toodyay (approx. 90 km NE of Perth), Cunderdin (approx. 150 km NE of Perth), Gibson (approx. 700 km SE of Perth) and Esperance (approx. 730 km SE of Perth) during the 2012/13 growing season. Three separate fields at Cunderdin were sampled (designated as i, ii and iii). Leaves were cut into 0.5 cm^2 sections, placed on to water agar plates (agar 15 g l^{-1}) supplemented with antibiotics (to a final concentration of ampicillin 100 mg l^{-1}, neomycin 50 mg l^{-1} and streptomycin 30 mg l^{-1}) and incubated at 22 ^{o}C under 12 h cycles of light until fungal hyphae emerged. Hyphae were excised as agar plugs and transferred on to V8PDA plates (Campbell's V8 juice 150 ml l^{-1},

potato dextrose agar 10 g l^{-1}, $CaCO_3$ 3 g l^{-1}, agar 15 g l^{-1}). After 5 days, sporulation was induced as previously described (Moffat et al. 2014). Single spore re-isolation was performed to ensure isolate purity.

Seeds of cv. Mace were obtained from the Australian Winter Cereals Collection (AWCC), and were sown in pots (10 cm in diameter) containing P500 perlite and Grade 2 vermiculite (The Perlite and Vermiculite Factory, Australia). Plants were grown at 21 ^{o}C under a 12 h day/night cycle in a controlled growth chamber, and were supplemented with Thrive all-purpose soluble fertiliser as per the manufacturer's recommendations (Yates, Australia).

Molecular techniques

Genomic DNA was extracted from fungi and wheat leaves using the Biosprint 15 DNA kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol.

Pathogen specific primers were used for PCR detection of *Pyrenophora tritici-repentis* (primers PtrUnique_F2 and PtrUnique_R2 (product size of 490 bp) (Antoni et al. 2010)) from wheat leaf tissue. Thermal cycling conditions were 94 ^{o}C/2 min; (94 ^{o}C/5 s, 57 ^{o}C/30 s, 72 ^{o}C/1 min) x 35; 72 ^{o}C/5 min. For further confirmation, quantitative PCR (qPCR) was performed to detect relative amounts of *P. tritici-repentis* DNA within starting leaf material, using pathogen specific primers (PtrMulti_F and PtrMulti_R) to amplify 150 bp of a multicopy region. Each 20 µl qPCR reaction consisted of 50 ng DNA, 10 µl QuantiTect SYBR Green PCR mix (Qiagen) and 300 nM of primers. Thermal cycling conditions were 95°C/15 min; (94°C/15 s, 55°C/30 s, 72°C/30 s) x 35 and were performed using a CFX96 Real-Time PCR Detection System (Bio-Rad). Samples were analysed in triplicate with two technical replicates.

In order to identify fungal species, primers ITS1 and ITS4 (White 1990) were used to amplify the highly variable internal transcribed spacer (ITS) regions ITS1 and ITS2 surrounding the 5.8S rRNA gene in a 20 µl PCR reaction. Amplicons were visualised on a 1.5% agarose gel,

extracted using a QIAquick Gel Extraction Kit (Qiagen) and eluted in 50 µl elution buffer. Sequencing reactions were performed using the BigDye Terminator v3.1 Cycle Sequencing Kit (Life Technologies) as per manufacturer's protocol and chromatograms were determined using a 3730 DNA Analyser (Life Technologies). The resulting sequences were searched against nucleotide BLAST databases (NCBI) to determine fungal species and are detailed in Supporting Information.

All primer sequences are shown in Table 1.

Pathogenicity assays

For detached leaf assays, leaves of two-week old Mace, grown in a controlled growth chamber, were cut into 7 cm sections (with the tips removed) and both ends were embedded adaxial side up in benzimidazole agar (benzimidazole 70 mg l^{-1}, agar 15 g l^{-1}). Mycelial plugs were cut from fungal colonies growing on V8PDA plates, and placed mycelial side down on to the embedded leaves. To aid attachment, a 2 µl drop of 0.02% Tween-20 was pipetted onto each leaf prior to placement of the mycelial plug. Leaves were incubated under 12 h light cycles at 22 ^{o}C, to enable disease development. Symptom severity was assessed after 5, 7 and 10 days.

For whole plant spray infection assays, inoculum was prepared consisting of 8 x10^5 conidia ml^{-1} (for *Alternaria* spp.) or 3000 conidia ml^{-1} (for *P. tritici-repentis*) in 0.25 % gelatin. Two-week old plants were sprayed evenly using a spray bottle until run-off, and were incubated in a misting chamber for 24 h with continuous moisture supplied by a humidifier. Plants were visually assessed for disease severity 7 days post-inoculation.

RESULTS

Field samples of Mace leaves exhibiting yellow blotching were tested via PCR for the presence of *Pyrenophora tritici-repentis*, the dominant wheat fungal pathogen in Australia. A faint band was detected from the chlorotic Mace leaves obtained from one site only (Cunderdin, site i) (Figure 1a). ITS primers were able to amplify products from genomic DNA from all leaf DNA samples. In order to confirm the absence of the yellow spot pathogen at the Gibson and Toodyay sites, a highly sensitive quantitative PCR (qPCR) method was utilised to detect relative amounts of *P. tritici-repentis* DNA within the starting leaf material (Figure 1b). *P. tritici-repentis* was not detected within these yellowing Mace leaves.

In order to determine the presence of other fungal pathogens, without placing limits on the detection of a particular species, yellowing Mace leaves were sectioned and placed on water agar supplemented with antibiotics. Fungi emerging from the leaves were isolated via a single spore and subjected to diagnostic sequencing of the ITS fungal barcode region (Table 2). Nucleotide sequences identified ITS1, 5.8S ribosomal RNA gene and ITS2 only of *Alternaria* spp. (and its teleomorph *Lewia* spp.), although the ITS sequence was unable to distinguish one particular species (Supporting Information).

To determine whether these *Alternaria* spp. were the causative agents of the foliar lesions, two types of pathogenicity assays were employed. Firstly, detached leaf assays were performed, whereby V8PDA agar plugs of recovered *Alternaria* spp. from Gibson and Toodyay were placed on Mace leaves and monitored for symptom development. Leaves remained green and were still symptom-free at 10 days post-inoculation (Figure 2a). *P. tritici-repentis* was included as a control and as expected induced necrosis.

Secondly, *Alternaria* isolates (G1 and T1) were induced to sporulate and five-week old Mace plants were inoculated with the resulting conidia. No significant differences in symptoms were observed between inoculated and control plants (Figure 2b). The isolates were unable to infect and sporulate on the plants, indicating they are not pathogenic on Mace. However, spontaneous leaf blotching and yellowing was observed even on the uninfected Mace plants

(Figure 2b). Therefore, to determine if this was due to a reaction to the 0.25% gelatin used in the spore suspensions. Mace plants were grown in a controlled growth chamber in the absence of any pathogens. Yellowing of leaves was observed from 2.5 weeks (Figure 2c).

DISCUSSION

Here, we determined that foliar yellowing of Mace was not the result of a major fungal wheat pathogen, and thus is unlikely to respond to fungicide. Although we observed prevalence of *Alternaria* spp. from field sampled Mace leaves, we were unable to establish a causal relationship between *Alternaria* spp. and leaf yellowing, and thus could not fulfil Koch's postulates.

Previous reports have described *Alternaria* spp. within Australian wheat. Alternaria spores have been detected in air sampled above wheat crops and grain sheds in rural towns of NSW and Alternaria has been isolated from grain and wheat leaves (Mitakakis et al. 2001; Shipton and Chambers 1966). *A. alternata* was identified as the predominant *Alternaria* species in QLD and NSW, and *A. infectoria* the most prevalent in WA and SA (Webley et al. 1997; Webley et al. 1995).

Most species of *Alternaria* are considered to be saprophytic fungi which reside in the soil or on decaying plant matter (Thomma 2003), and are found as surface contaminants on grains and on dead or dying plant tissue. This is likely to be the case described herein. However, other *Alternaria* species are plant pathogens. An increase in the incidence levels of *A. infectoria* on wheat in Argentina has been associated with black point (a black discolouration of wheat grains) (Perello et al. 2008), and the disease termed Alternaria leaf blight has been described on wheat, predominantly in India and South East Asia (Singh et al. 2004; Singh et al. 2008). Various *Alternaria* spp. have been isolated from wheat leaves displaying such leaf blight symptoms, including *A. alternata, A. arborescens, A. tenuissima* and *A. triticina*

(Vergnes et al. 2006). However, only *A. triticina* was shown to induce foliar lesions on the wheat varieties tested. The authors suggest that the high recovery of *Alternaria* spp. from wheat may be explained by the ability of *Alternaria* species to grow saprotrophically. Additionally, *A. triticina* has a limited host range among bread wheat varieties which further restricts those cultivars it can infect (Vergnes et al. 2006).

Alternaria species are well known for the production of toxic secondary metabolites, some with mammalian toxicity, although this is generally associated with *A. alternata* (Brugger et al. 2006; Fehr et al. 2009; Lehmann et al. 2006; Pfeiffer et al. 2007; Schreck et al. 2012). However, *A. infectoria* strains isolated from wheat kernels in Argentina have recently been reported to produce the mycotoxins alternariol (AOH) and alternariol monomethyl ether (AME) on semi-synthetic media (Oviedo et al. 2013). Therefore, although not determined to be the causal agent of the observed leaf yellowing of Mace, *A. infectoria* should be considered as a pre-harvest contaminant with a potential risk of mycotoxin contamination. The ITS sequences presented herein did include matches to *A. infectoria* (and *Lewia infectoria*) from all sites examined.

Recent attention has been focused around Mace as it currently dominates the Southern and Western Australian wheat growing regions. However, there have been reports of yellowing in other wheat varieties that also appear not to be the result of disease. For example, Kord CL Plus typically exhibits yellowing, as do other varieties with a similar pedigree (such as Axe, Corell, Gladius, Grenade CL Plus and Justica CL Pus, which all have RAC875 in their pedigrees) (Birchip Cropping Group 2013; Wallwork 2011; AGT 2013c). Foliar yellowing, described as “frame yellows”, has also been observed in crops of Yitpi (Wallwork 2011).

The impact of such yellowing wheat leaves on yield has been examined. Trial sites of breeding and established lines at 8 locations across southern Australia (including WA, Vic and SA) were assessed during the 2008/2009 season (AGT 2013c). At four of the eight trial sites there was no significant effect of yellowing on yield. However, at three high yielding

(and relatively stress-free sites), varieties with yellowing yielded less than those without. At the eighth site (Kumarl, WA), which suffered terminal heat and water stress, yellowing varieties actually yielded more than non-yellowing. The authors speculate that at the Kumarl site where water was limited, a loss of green leaf area may have been advantageous, whilst at the four high yielding sites the reduction in green leaf area led to a reduction in yield.

Foliar yellowing appears to be due to a spontaneous physiological phenomenon, which is unlikely due to micronutrient deficiencies of iron or zinc, since no content differences were observed between affected and unaffected leaves (AGT 2013c). Yellowing crops were also not determined to be nitrogen deficient and soils had adequate nitrogen (AGT 2013c; Birchip Cropping Group 2013). Field observations demonstrate that yellowing is more prevalent in wet winters and under extended periods of cool wet conditions (Birchip Cropping Group 2013; Wallwork 2011). Although the cause remains elusive, it seems reasonable that leaf yellowing is a genetically inherited physiological trait since it is observed in varieties of similar pedigrees. However, the environmental conditions which trigger this yellowing have yet to be determined. With such vast areas being sown to Mace, it is not good risk management strategy to depend so much on one variety. Indeed, a major weakness of Mace is the potential threat of stem rust infection, if the two major genes that it possesses (*Sr15* and *Sr38*) were to both break down (DAFWA 2013c). Furthermore, the possibility of secondary infection resulting from these spontaneous lesions cannot be excluded, so growers need to remain vigilant.

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334 **Table 1.** Primers used throughout this study.

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Primer name	Sequence
PtrUniqueF2	GGACTTTGGCTTTCTATTGTGC
PtrUniqueR2	CTTGGTGAATGGTGAAGATGG
PtrMulti_F	GTAAGCCCGAGCAGAAGGAC
PtrMulti_R	CCATAGGCGACCGAGTAGAG
ITS1	TCCGTAGGTGAACCTGCGG
ITS4	TCCTCCGCTTATTGATATGC

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338 **Table 2.** Isolates recovered in this study.

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Isolate	Collection Site	Year	ITS
G1, G2, G3	Gibson, WA	2012	*Alternaria spp.*
T1	Toodyay, WA	2012	*Alternaria spp.*
C1, C2, C3	Cunderdin, WA	2013	*Alternaria spp.*

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Figure 1. Detection of the yellow spot pathogen from yellowing Mace leaves.

(a) Mace field leaf samples collected from Gibson (G), Toodyay (T), Esperance (E), Perth (P) and Cunderdin (C) were tested for pathogen presence via PCR, using *P. tritici-repentis* specific primers (PtrUniqueF2/R2, which amplifies a product of 490 bp). Three separate field sites at Cunderdin were samples (i, ii and iii). Two individual leave per site were examined (a and b). The ITS region was amplified as a control using ITS1/4 primers. Fungal DNA of *P. tritici-repentis* (Ptr) and the septoria nodorum blotch pathogen *Parastagonospora nodorum* (SNB) were included as controls, as well as DNA from uninfected wheat (W) and a no template control (NTC). PCR products were visualised by agarose gel electrophoresis.

(b) Quantitative PCR detection of *P. tritici-repentis*. Mace field leaf samples from Gibson (G) and Toodyay (T) were tested for *P. tritici-repentis* presence using a highly sensitive and specific multicopy probe (primers PtrMulti_F/R). Ptr-infected, SNB-infected and uninfected Mace leaves (Ctrl) were included as controls. The relative quantity of DNA was obtained from the mean of three replicates is shown. Error bars depict standard deviation.

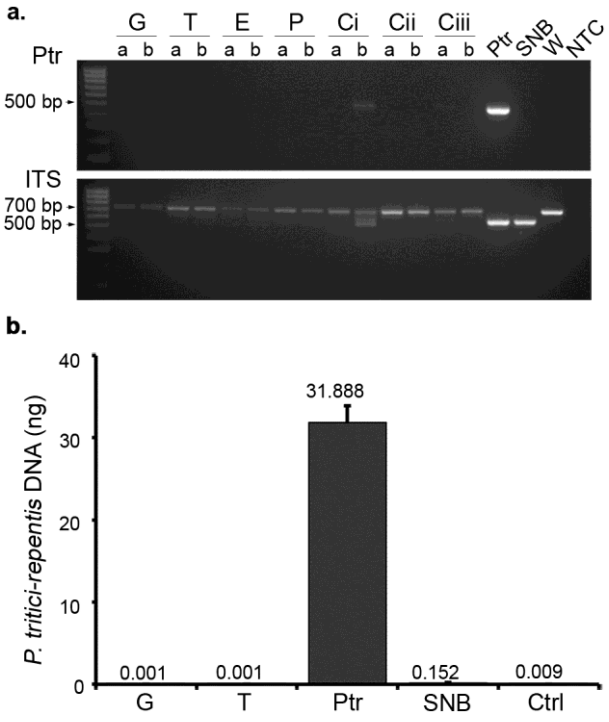

Figure 2. Pathogenicity assays of recovered *Alternaria* spp. inoculated on to Mace.

(a) Detached leaf assays of re-isolated *Alternaria* spp. collected from Gibson (G1) and Toodyay (T1) sites were unable to cause symptoms on Mace after 10 days. *P. tritici-repentis* (Ptr) was included as a positive control, and uninoculated leaves (Ctrl) as a negative control. Photographs were taken at 5, 7 and 10 days post-inoculation.

(b) Mace leaves inoculated with *Alternaria* spp (G1 and T1) conidia. *P. tritici-repentis* (Ptr) infection and inoculation with 0.25% gelatin (Ctrl) were included as controls. Photographs were taken 7 days post-inoculation and show representative leaf symptoms.

(c) Spontaneous lesions on Mace. Yellow blotching was observed on uninfected plants grown within a controlled growth chamber. Images were taken of 2.5-, 3-, 4- and 5-week old plants and show representative leaf symptoms.