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Quantitative disease resistance assessment by real-time PCR using the *Stagonospora nodorum*-wheat pathosystem as a model

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Measuring disease resistance accurately and reproducibly is a key requirement for the introgression of partial resistance genes into breeding lines. Here, a qPCR protocol is used to measure fungal biomass, using the wheat-*Stagonospora nodorum* pathosystem as a model. Seven cultivars of differing reported resistance levels were used. Fungal biomass taken at 220°C thermal days after inoculation accurately predicts the final grain weight loss. It is concluded that a test based on qPCR methods is specific, quantitative, rapid and objective. Such tests could provide useful and economic tools in the development of robustly resistant crop cultivars.

Keywords: breeding for disease resistance, glume blotch of wheat, stagonospora leaf blotch, *Triticum aestivum*

Introduction

The detection and identification of pathogens and the quantification of disease is a central preoccupation of plant pathology. Traditional methods, based on visual and microscopic assessment, are well accepted but require high levels of training and have a significant degree of subjectivity. The promise of molecular methodologies – particularly monoclonal antibodies and polymerase chain reaction – has long been touted (Schots *et al*., 1994) but, with few exceptions, has hitherto failed to translate into routine use. Methods based on the polymerase chain reaction would appear to fulfil all the required criteria, in that applications can be designed that are highly sensitive and can have any level of discrimination from race to genus (Martin *et al*., 2000). Thus, although there are numerous examples of PCR being used to identify pathogens, such studies generally only offered qualitative diagnoses. The development of reliable methods of quantitative PCR has opened up many further possibilities on both foliar and root pathogens (Doohan *et al*., 1999; Nicholson *et al*., 2002, 2003; Mumford *et al*., 2006). Quantification of pathogens, allied to the inherent capability to detect and identify a given taxon range, brings many old and recalcitrant problems within the purview of the technology. The challenge now is to design procedures that can develop new capabilities for use in the plant breeding and crop protection arenas.

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Breeding for disease resistance is the most environmentally favourable method of crop protection. Most sources of resistance to most pathogens are partial. Hence, both the selection of donor material and the introgression of resistance alleles into breeding lines require a quantitative assessment of disease resistance. The primary concern is that the pathogen reduces either or both the yield and quality of the crop. Direct and accurate assessment of the yield penalty produced by the pathogen requires large plot sizes and multiple replications at different locations and different years. This is a space- and time-consuming procedure that is not practical for use on the very large scale used in current plant breeding methodologies. Hence traditional methodologies have substituted visual assessment of symptoms as a proxy for yield measurements, which inevitably introduces an element of subjectivity. The correlation between symptom expression and yield penalty has rarely been rigorously established.

The goal of this study was to compare four methods of disease assessment and to determine the levels of correlation between them. The baseline comparator was the reduction in the weight of 100 grains resulting from pathogen inoculation of a range of wheat cultivars. The hundred grain weight is a very well established combined assessment of yield and basal quality in a variety of cereal pathosystems (Knott & Talukdar, 1971; Nass & Johnston, 1985; Jiang *et al*., 2006). Two traditional proxy methods were used, the visual assessment of necrotrophic leaf blotch symptoms and of the glume blotch symptom on the head. In addition, a quantitative PCR was used to measure pathogen biomass at both stages of the flag leaf development.

An early qPCR study used *Mycosphaerella graminicola* infection of wheat and compared fungal biomass with the cultivar rating for septoria (tritici) leaf blotch (Fraaije *et al*., 2001). A more recent study used qPCR to detect presymptomatic fungal biomass in order to best direct fungicide applications (Guo *et al*., 2006). The current study compares fungal biomass not only against the historical performance of the cultivar but also against the actual performance in these infections. The pathogen chosen was *Stagonospora* (syn. *Septoria*) *nodorum* [teleomorph: *Phaeosphaeria* (syn. *Leptosphaeria*) *nodorum*], cause of stagonospora nodorum blotch and glume blotch of wheat and related cereals (Solomon *et al*., 2006a). *Septoria nodorum* is a model necrotrophic pathogen with a simple life-style involving rapid epidermal penetration, proliferation in the mesophyll and the development of pycnidia. The asexual lifecycle typically takes a week to 10 days (Solomon *et al*., 2006b). In addition to the elaboration of black pycnidia, symptom expression includes necrotic and chlorotic lesions around the site of infection, contributed to by the secretion of various host-specific toxins (Liu *et al*., 2004a,b, 2006; Friesen *et al*., 2006). There is no evidence in stagonospora nodorum blotch of a transient biotrophic or quiescent phase such as described for *Leptosphaeria maculans* and *Mycosphaerella graminicola* (Howlett *et al*., 2001; Palmer & Skinner, 2002). The rationale was to choose a pathogen with the most direct apparent connections between biomass, symptoms and yield penalty as a first test case. If good correlations between biomass and yield penalty can be established for this disease, the more problematic pathogens that are slow and/or include symptomless phases would become candidates for a similar study.

Materials and methods

Seven wheat lines, including three current cultivars and four advanced breeding lines with different levels of resistance to stagonospora nodorum blotch were used in this study. These lines had previously been assessed on a 9 point resistance score; the most susceptible line was Millewa, scored as 2, and the most resistant was 6HRWSN125, scored as 7. Other lines included EGA Blanco (score 6), Carnamah (score 5), 7HRWSN108 (score 5), WAWHT2074 (score 3) and WAWHT2234 (score 3). The experiment was sown in an irrigated field nursery in a strip-plot design of infected and uninfected main plots and four replications in 2006 in South Perth, Western Australia. Lines within main plots were sown in the second week of May as paired 10 cm rows of up to 10 seeds per row sown 10 cm apart, separated by 30 cm centres from adjacent rows. Infected and uninfected main plots were separated by 0·5 m wide rows of barley. The entire experimental design was further replicated, with one set used for conventional disease assessments and the other for qPCR. In this way, the removal of leaf material for DNA extraction did not affect the progress of disease or the accrued yields. Infection was established at full spike emergence (Feekes stage 10·3) by spraying spikes to run-off with a mixed conidial suspension (10^6 conidia mL^{-1} with 0·5% gelatine) of *S. nodorum* produced from grain cultures (Fried, 1989) of ten isolates obtained from the culture collection maintained by the Department of Agriculture and Food, Western Australia. High humidity was created by watering the site just before inoculation and covering individual sub-plots with plastic bags secured over PVC rings (15 cm high, 30 cm diameter) for 48 h after inoculation. Before covering the plots, plastic bags were misted internally with water. Inoculated plants were shaded from direct sunlight during moist incubation by further covering the plastic bags with shade cloth bags (84–90% cover factor).

Percentage leaf area diseased was assessed on flag leaves at 150°C thermal days (sum of mean 24 h daily temperatures) after inoculation and flag leaf samples were taken for later qPCR analysis. A second visual assessment was made at 220°C thermal days together with a second set of leaf samples. Percentage glume infection was measured at 370°C thermal days after inoculation. The rating scales for glume and leaf infection to SNB was based on a percentage scale from 0% (highly resistant) to 100% (highly susceptible) (James, 1971). Control plots which did not receive inoculations were assessed similarly. No disease was observed on these plants. Glumes were hand harvested at maturity and hundred grain weights were measured in both infected and uninfected plots. Relative grain weight was calculated as the percentage of grain weight from infected plots to that of uninfected plots.

DNA was extracted from leaf tissue as follows. Infected leaves (typically six), were collected at the appropriate time and frozen immediately in liquid nitrogen prior to storage at –80°C. The leaves were pulverized using a mortar and pestle with a small amount of material then placed in a pre-weighed 2 mL tube. The tube containing the sample was then re-weighed and the sample weight collected. The DNA was extracted from the material using a BioSprint 15 (Qiagen) and quantified on a Nanodrop ND-1000 spectrophotometer. The integrity of the DNA was confirmed by gel electrophoresis.

The quantification of *S. nodorum* within the leaf tissue was undertaken using a SYBR Green-based qPCR assay. For the quantification of *S. nodorum*, 3-fold, 10-fold and 30-fold dilutions were taken from each extraction and analysed in technical duplicates using a Rotogene 3000 (Corbett Research). The primers, StagoUniqueF (5′-GTCACCGCATTACCAAAGTT-3′) and StagoUniqueR (5′-GGAAACTGGAACTGGAACAA-3′) were designed within the first intron of the SNOG_01116·1 open reading frame using VectorNTI software (Invitrogen). SNOG_01116·1 is an anonymous single-copy gene without any significant similarity to any other sequences in the GenBank databases (GenBank EAT92611·1). Extensive studies using uninfected wheat and wheat infected with a range of other pathogens conclude that these primers are specific to *S. nodorum*. The primers were designed to bind to intron-exon boundaries so as to reduce even further the chances of amplifying DNA from an organism carrying a

Table 1 Hundred grain weight (HGW) of *Stagnospora nodorum*-infected and uninfected wheat lines, relative grain weight (RGW) and reported cultivar score

Wheat lines	HGW (uninfected)	HGW (infected)	%RGW[a]	Cultivar score[b]
6HRWSN125	3·6	2·3	63·6	7
7HRWSN108	3·4	2·0	58·4	5
EGA Blanco	3·7	2·0	54·5	6
Carnamah	4·1	1·8	44·5	5
WAWHT2074	4·2	1·5	34·6	3
WAWHT2234	3·8	1·4	36·8	3
Millewa	2·9	1·2	42·8	2
Mean	3·7	1·7	45·9	

[a]Percentage ratio of HGW uninfected to HGW infected; $P < 0{\cdot}001$; LSD $P < 0{\cdot}05 = 0{\cdot}4$.
[b]Score 1–9 scale where 9 is most resistant.

Table 2 Coefficients of determination (R^2) between stagnospora nodorum disease scores and relative wheat grain weight. All R^2 values shown were determined to be significant ($P < 0{\cdot}05$)

	FLN1[a]	qPCR1[b]	FLN2[a]	qPCR2[b]	Glume blotch
qPCR2	0·7	0·7	0·9		0·8
Glume blotch	0·6	0·6	0·8	0·8	
RGW[c]	−0·5	−0·6	−0·8	−0·9	−0·9

[a]FLN1/2 = flag leaf necrosis measurement at 150°C and 220°C thermal days, respectively.
[b]qPCR1/2 = quantitative PCR measurement at 150°C and 220°C thermal days, respectively.
[c]RGW = relative grain weight.

related gene. The reaction mixture contained 5 μL diluted primers (0·3 μM final concentration), 10 μL iQ SYBR Green Supermix (BioRad) and 5 μL of the diluted DNA. The conditions for amplification were 95°C 3 min, (95°C 10 s, 57°C 10 s, 72°C 20 s) × 40. The amount of *S. nodorum* DNA within the infected leaves was determined by comparing the data to a standard curve generated using the same primers on varying concentrations of purified *S. nodorum* genomic DNA. The calculated concentration was normalized using the fresh weight of the starting material and this number was then used for the subsequent correlation analyses. Subsequent statistical and correlation analyses were undertaken using JMP IN 5·1 (SAS) and Microsoft Excel.

Results

The methods for wheat cultivation, inoculation with *S. nodorum* and disease assessments were as close as practicable to the standard procedures used to make stagonospora nodorum leaf and glume blotch resistance evaluations, during the identification of elite sources of resistance for breeding of novel cultivars by the Department of Agriculture and Food, WA (Loughman *et al.*, 1996). The ratio of hundred grain weight for matched inoculated and uninoculated samples (RGW; relative grain weight) shows that the pathogen caused significant yield losses in all lines (Table 1). The average grain weight after infection ranged from 64 to 35% of the uninoculated controls, indicating the high losses that can be experienced with even the most resistant current cultivars (Bhathal *et al.*, 2003). The rank order of grain weight with and without infection showed no significant correlation but the highest grain weight cultivars in the absence of disease (2074, Carnamah and 2234) were three of the four lowest grain weight cultivars after infection. Millewa grain weight was lowest both with and without infection.

Visual disease assessments of leaf symptoms (measured as % flag leaf necrosis (FLN)) and samples for qPCR were taken at 150°C and at 220°C thermal days. Glume blotch measurements were made at 370°C thermal days and grain weight measurements were made after the plants had matured. Coefficients of determination (R^2) comparing each of these measurements are shown in Table 2. Correlation between the visual disease assessments and RGW improved as the season progressed. A significant correlation of –0·5 was observed between the first flag leaf measurement at 150°C thermal days and RGW. The R^2 between the second flag leaf necrosis measurement, taken at 220°C thermal days and RGW was –0·8. Correlations between the qPCR measurements taken at 150°C and 220°C and the glume blotch measurement taken at 370°C thermal days increased from –0·6 to –0·8.

A significant correlation of –0·7 was observed between the qPCR sample taken at 150°C thermal days and RGW. This indicates that qPCR has the potential to detect and quantify the pathogen before symptoms are fully developed. The correlation between the qPCR measurements taken at 220°C thermal days and RGW was –0·9.

Discussion

Stagonospora nodorum leaf and glume blotch is a major disease of wheat in many (Solomon *et al.*, 2006a), but not all (Bearchell *et al.*, 2005) wheat growing areas of the world. It remains a severe problem in Western Australia (Bhathal *et al.*, 2003). The pathogen caused large losses in this study, ranging from 33 to 66% as assessed by reduced average grain weight. The lines used represent some of the best sources of resistance currently available for introgression into new cultivars. The most resistant lines were still subject to major losses. Also, in the absence of disease, the more resistant cultivars tended to have lower grain weight than the more susceptible varieties. These observations highlight the need to maximize the efficiency of disease resistance measurement so that minor sources of resistance can be incorporated into breeding lines.

The breeding of crops with partial resistance to fungal diseases is a complex procedure involving the integration of diverse sets of information. For many of the major crops, the importance of a disease is entirely related to its potential to cause yield loss. However, the breeding of

crops involves the screening of hundreds or thousands of candidate lines. It is clearly impractical to obtain meaningful yield penalty estimates for large numbers of lines as the space and time requirements would render such a process uneconomic. As a result, plant breeders and pathologists have used a range of proxy measurements ranging from *in vitro* tests on detached leaves, through inoculated seedlings grown in glasshouses, to field grown plants. The relative cost of each of these methods, both in terms of equipment and time, must be weighed against the correlation with yield penalty.

Numerous studies have shown that detached leaf and seedling assays have only poor correlation with adult disease resistance to *S. nodorum* (Hewett, 1975; Scott & Benedikz, 1977; Rufty *et al*., 1981; Cooke & Fitzgerald, 1982; Karjalainen & Laitinen, 1982; Griffiths *et al*., 1985; Nelson & Marshall, 1990; Walther, 1990; Wicki *et al*., 1999a,b; Shah *et al*., 2000; Mebrate & Cooke, 2001; Czembor *et al*., 2003). Similar disappointing results have been obtained for other cereal pathogens and for other crops so that seedling assays for quantitative resistance have been largely abandoned in favour of assays of field grown adult crops. Such results indicate that different resistance genes can operate at the seedling and adult phase, but that it is adult-operating genes that have the most direct bearing on yield (Milus & Line, 1986; Badawy *et al*., 1991; McIntosh *et al*., 1995; Ballinger & Salisbury, 1996).

Visual assessments of disease resistance, such as the flag leaf necrosis and glume blotch coverage methods, require skill and experience and consume significant amounts of time and space. In this study, good correlations were observed between the second FLN (0·8) and the glume blotch score (0·9) and final yield penalty. Thus, both methods of disease assessment have been validated in this study. The degree of correlation indicates that *S. nodorum* causes disease by a combination of disruption of the photosynthetic capacity of the flag leaf and by adsorption of assimilates intended for the grain filling process.

The qPCR score taken at 220°C thermal days had a correlation with yield penalty of 0·9. Although this assay can be improved, and confounding effects may occur under some circumstances, this already indicates that qPCR can be used as an objective pre-harvest method of disease resistance measurement. The relative expense of the procedure compared to FLN, glume blotch and RGW was not evaluated in this experiment, but with suitable levels of automation it is estimated that the cost could be very competitive. The test can be carried out in a few hours if needed, but can also be performed on stored material. In addition to timing, cost and objectivity, a qPCR test platform could have other advantages and applications. The specificity of PCR means that multiple diseases could be simultaneously assayed. A crop is rarely assaulted by a single pathogen. Wheat infected with mixtures of *Stagonospora nodorum*, *Septoria tritici* and *Pyrenophora tritici-repentis* have been observed to confound visual assessments of disease resistance to each disease (Loughman *et al*., 1993). qPCR probes for each disease should be trivial to design as each pathogen has recently been sequenced. It should then be possible to use a single mixed infection to assess disease resistance to each pathogen and to model the effect of each disease on the overall yield penalty.

The correlation between the qPCR-measure biomass taken at 150°C thermal days and final yield penalty was lower (–0·6) but still significant. At this stage, visual disease symptoms had not fully developed. This observation suggests that a qPCR platform could be used to optimize the application of fungicides. Most fungicides work best when applied early in the infection cycle, prior to visual symptom expression (O'Reilly *et al*., 1988). In extensive agricultural systems, diseases are more sporadic and fungicides are not needed every year. Thus a qPCR monitoring process could help evaluate when fungicide applications would be needed and determine when spraying would be most economic (Guo *et al*., 2006). Furthermore, the genetic basis of fungicide resistance is increasingly being attributed to known DNA sequence changes (McCartney *et al*., 2003; Morzfeld *et al*., 2004; Kianianmomeni *et al*., 2007; Sierotzki *et al*., 2007). qPCR would allow the simultaneous detection and quantification of fungicide resistance-associated alleles.

Fungal diseases are known to interact with the nutritional status of the plant (Solomon *et al*., 2003). A dogma has arisen in which biotrophic pathogens are thought to be promoted by over-feeding whilst necrotrophs are supposedly associated with nutrient limited plants (Jensen & Munk, 1997; Hoffland *et al*., 1999, 2000). These studies are compromised because nutrient has a major impact on yield and the use of visual disease assessments blurs the ability to quantify pathogen loads. Biomass measurements may help distinguish the opposing effects.

Stagonospora nodorum was used in this study because of its short infection cycle and absence of even a transient biotrophic phase. This enabled the establishment of what the maximum level of correlation between biomass and yield penalty would be under the most favourable conditions. Most other pathogens have latent periods ranging from weeks to months. In these cases, the connection between visual disease and yield penalty is more tenuous and so qPCR could have a more critical role. An extreme example would be black leg of canola, caused by *Leptosphaeria maculans*, in which the damaging infection occurs at the cotyledon leaves stage (Hammond *et al*., 1985; Fitt *et al*., 2006). The pathogen may or may not cause significant symptoms at this point, and resistance can appear similar to susceptibility. Serious losses can occur if the pathogen penetrates the main stem. It then remains largely quiescent until the plant reaches maturity, whereupon it can form the stem-girdling cankers. If these eponymous black leg cankers cause lodging, the yield is severely reduced. Thus disease resistance measurements at the seedling stage could predict the potential for yield loss months later. A β-glucuronidase expressing strain was developed for this purpose (Oliver *et al*., 1993) but regulations regarding the use of genetically modified

strains have limited its use. qPCR might offer a general solution to this problem.

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