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# PCR-based assays to assess wheat varietal resistance to blotch (*Septoria tritici* and *Stagonospora nodorum*) and rust (*Puccinia striiformis* and *Puccinia recondita*) diseases

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## Abstract

A multiplex Polymerase Chain Reaction (PCR) assay was developed to detect and quantify four fungal foliar pathogens in wheat. For *Septoria tritici* (leaf blotch) and *Stagonospora nodorum* (leaf and glume blotch), the $\beta$-tubulin gene was used as the target region. Diagnostic targets for *Puccinia striiformis* (stripe or yellow rust) and *P. recondita* (brown rust) were obtained from PCR products amplified with $\beta$-tubulin primer sequences. Final primer sets were designed and selected after being tested against several fungi, and against DNA of infected and healthy wheat leaves. For detection of the four pathogens, PCR products of different sizes were amplified simultaneously, whereas no products were generated from wheat DNA or other non-target fungi tested. The presence of each of the diseases was wheat tissue- and cultivar specific. Using real-time PCR measurements with the fluorescent dye SYBR Green I, PCR-amplified products could be quantified individually, by reference to a standard curve generated by adding known amounts of target DNA. Infection levels for each of the diseases were measured in the flag leaf of 19 cultivars at Growth Stage (GS) 60–64 in both 1998 and 1999. The infection levels for the cultivars were ranked, and showed, with a few exceptions, a good correlation with the NIAB Recommended List for winter wheat, which is based on visual assessment of symptoms. With PCR, the presence of the different pathogens was accurately diagnosed and quantification of pre-symptomatic infection levels was possible. Although sampling and DNA detection methods need further optimisation, the results show that multiplex PCR and quantitative real-time PCR assays can be used in resistance screening to measure the interaction between different pathogens and their hosts at different growth stages, and in specific tissues. This should enable an earlier identification of specific resistance mechanisms in both early-stage breeding material and field trials.

## Introduction

Blotch caused by *Septoria tritici* Roberge in Desmaz. (teleomorph *Mycosphaerella graminicola*) and *Stagonospora nodorum* (Berk.) E. Castellani and E.G. Germano (teleomorph *Phaeosphaeria nodorum*), and rusts caused by *Puccinia recondita* Roberge ex Desmaz. f. sp. *tritici* (brown rust) and *Puccinia striiformis* Westend. f. sp. *tritici* (yellow or stripe rust) are world-wide yield reducing diseases of wheat (Wiese, 1987). In the UK, epidemics of these diseases caused an average annual yield loss of £52.4 million during the period 1985–1989 (Cook et al., 1991). To prevent economically damaging epidemics, integrated disease management, combining good cultural practices, resistance breeding and the appropriate use of fungicide inputs is needed.

Because of the high levels of genetic variation of many pathogens and their ability to generate new pathotypes through mutation and DNA-recombination,

resistance based on single genes can be overcome rapidly (Bayles, 1997). Therefore, the main strategies of longer-term resistance breeding include pyramiding of non-durable race-specific resistance genes, and the use of durable quantitative resistance genes conferring partial resistance (Messmer et al., 2000). However, because of desirable additional traits, like high yield, short straw and resistance to multiple diseases, crossing programmes are not straightforward (Johnson, 1992). In the UK, wheat cultivars with moderate to good resistance to mildew (*Blumeria graminis* f. sp. *tritici*), yellow rust, brown rust, earblight (*Fusarium* spp.), eyespot (*Tapesia* spp.) and Septoria diseases are available (Anonymous, 1997–2000). However, none of these cultivars have multiple resistance to all diseases, and fungicides are still required to control *S. tritici*, the most important foliar pathogen of commercially grown winter wheat in the UK.

For optimum, cost-effective control, fungicides are applied at different growth stages to prevent and/or delay disease development on the upper three leaves, which provide most of the grain-filling capacity. Fungicide choice (e.g. with eradicant and/or protectant properties) and timing depend on the inoculum level and disease risk to a crop (Cook et al., 1999). Results obtained with ELISA showed that accurate presymptomatic detection and quantification of *S. tritici* can improve disease control through better timing, choice and dose-rate of fungicide sprays (Kendall et al., 1998). However, a more sensitive method using PCR, which detects *S. tritici* earlier in its latent phase (Fraaije et al., 1999), might allow better-timed fungicide sprays to eradicate the disease. This would also enhance current risk assessment models (Lovell et al., 1997) that might lead to more profitable disease control with less impact on the environment.

PCR-based assays are rapid techniques with high specificity and sensitivity, which have been used for detection and identification of several other wheat pathogenic fungi, including *S. nodorum* (Beck and Ligon, 1995) and *Tapesia* spp. (Nicholson et al., 1997). Furthermore, they have been applied to analyse genetic variation of pathogens (Park et al., 2000), mating types (Foster et al., 1999), fungicide efficacy (Doohan et al., 1999), fungicide resistance (Koenraadt and Jones, 1992) and host plant resistance (Willits and Sherwood, 1999).

Our objective was to develop quantitative PCR-based diagnostic assays using the $\beta$-tubulin gene as a target for detection of *S. tritici*, *S. nodorum*, *P. striiformis* and *P. recondita* infection levels in wheat leaves and ears. Because fungal $\beta$-tubulin gene sequences are well conserved, it is relatively easy to obtain DNA sequences and design specific primers (Fraaije et al., 1999). By comparing infection levels of different cultivars, we hope to establish the value of PCR assays in the assessment of wheat varietal resistance for each disease. In this paper, the potential application of PCR diagnostics to monitor wheat variety resistance to epidemics of different pathogens in the field is demonstrated and discussed.

## Materials and methods

### *Fungal species and isolates*

The fungal species and isolates used in this study are listed in Table 1. After eight days incubation at 18 °C, mycelia and spores from *S. nodorum* and *S. tritici* isolates were collected from Czapek Dox agar (modified) plates (Oxoid). Urediniospores from *P. striiformis* and *P. recondita* were collected from the field trials and material from other fungal pathogens were kindly provided by J. Butters, J. Babij, T. Hunter and T. Joseph-Horne (IACR-Long Ashton Research Station, Long Ashton, UK).

*Table 1.* Fungal species and isolates tested

| Fungal isolates | Origin | Source |
|---|---|---|
| *Septoria tritici* ST16 | UK | Wheat |
| *Septoria tritici* $\alpha$12-3B.8 | USA | Wheat |
| *Seotoria tritici* M72 | France | Wheat |
| *Stagonospora nodorum* SN1 | UK | Wheat, cv Longbow |
| *Stagonospora nodorum* SN2 | UK | Wheat, cv Norman |
| *Stagonospora nodorum* JH1 | UK | Wheat |
| *Puccinia striiformis* PS1 | UK | Wheat, cv Maverick |
| *Puccinia striiformis* PS2 | UK | Wheat, cv Brigadier |
| *Puccinia striiformis* PS3 | UK | Wheat |
| *Puccinia recondita* PR1 | UK | Wheat, cv Riband |
| *Puccinia recondita* PR2 | UK | Wheat, cv Buster |
| *Blumeria graminis* f.sp. *tritici* W26 | UK | Wheat |
| *Gaeumannomyces graminis* T7 | UK | Wheat |
| *Tapesia yallundae* 22-432-1 | UK | Wheat |
| *Tapesia acuformis* 202-3 | UK | Wheat |
| *Rhynchosporium secalis* 1130 | UK | Barley |

*Wheat leaf, ear and grain samples*

During the growing seasons of 1997–1998 and 1998–1999, 19 wheat cultivars were grown in small field plots (12 × 4 m) at IACR-Long Ashton Research Station, SW-England. Field samples consisting of five flag leaves or ears were taken at Zadoks growth stages (GS) 60–92 (Tottman, 1987) throughout the growing season in 1998 and 1999. To analyse grains at GS 92, husks were separated and grains washed with 70% (v/v) ethanol. After soaking in a 10% (w/v) hypochlorite solution for 2 min, grains were rinsed in sterile water and dried. For DNA extraction, ten grains per sample were processed.

*Visual assessment of* S. tritici *in leaf layers of different cultivars*

In 1999 at GS 62, ten whole plants per cultivar were sampled at random and the top three leaves visually assessed for symptoms of *S. tritici* using a leaf grid aid as described by Parker et al. (1995a).

*DNA extraction*

DNA was extracted directly from mycelium and wheat leaf, ear and grains by crushing samples to powder in liquid nitrogen using a pestle and a mortar. DNA extractions, with adjustment of the volumes of the different components of the extraction buffer so that DNA sample mixtures could be poured, and DNA concentration measurements with the dye PicoGreen (Molecular Probes, Leiden, The Netherlands) were done as described previously (Fraaije et al., 1999).

*Standard PCR protocol*

Standard PCR was carried out in a Biometra T3 thermocycler (Anachem, UK) with 0.5 units of Red Hot DNA polymerase (ABgene, Epsom, UK) using 20 mM $(NH_4)_2SO_4$, 75 mM Tris–HCl, pH 9.0, 0.01% (w/v) Tween 20, 1.5 mM $MgCl_2$, containing 125 μM of each dNTP, 0.5 μM primers and 1 ng template DNA in a final volume of 100 μl. For testing wheat samples, 0.2 μM primers and 200 ng of template DNA per PCR reaction of 40 μl were used. The PCR conditions were 94 °C for 3.5 min, followed by 35 cycles at 94 °C for 30 s, 65 °C for 1 min and 72 °C for 1.5 min, with a final DNA extension at 72 °C for 8.5 min. PCR products were separated in ethidium bromide-stained 1.3% (w/v) agarose gels run in 1X Tris–Borate–EDTA buffer and exposed to UV light to visualise DNA fragments (Sambrook et al., 1989).

*Cloning of fungal β-tubulin gene sequences*

In order to amplify and clone part of the β-tubulin gene from of *S. tritici*, *P. striiformis* and *P. recondita*, primers BAF6 and BAF2 (Table 2) derived from conserved regions of the β-tubulin gene of *S. nodorum* (EMBL S56922) were used. After PCR, excess primers were removed with the High Pure PCR Product Purification Kit (Boehringer Mannheim, Germany) and products directly ligated and cloned into the pGEM-T easy vector (Promega Corperation, Madison, WI). Plasmids were transformed into *Escherichia coli* JM109 cells (Stratagene, La Jolla, CA) according to a standard protocol (Sambrook et al., 1989), and plasmid DNA was extracted using the RPM kit (Bio101 Inc, Carlsbad, CA). Finally, the nucleotide sequence was determined using the dideoxy chain termination method reaction (Sanger et al., 1977).

*Primer design*

Specific primers were designed from the β-tubulin gene sequence of *S. nodorum* and from sequences of PCR-amplified fragments of *S. tritici*, *P. striiformis* and *P. recondita*. Reverse and forward 24-mer primers with unique 3′-ends, high GC content and melting temperatures of 65–70 °C were designed. This was done by aligning and comparing corresponding β-tubulin

*Table 2*. Primer sequences

| Primer designation[1] | Sequence (5′–3′) |
|---|---|
| BAF6 (f) | ACCCACAACCGCCAACATGCGTGA |
| BAF2 (r) | CGTACCGGGCTCGAGATCGACGAG |
| BT3 (r) | GCTGATGAGAAGTGTTCCC |
| STIF2 (f) | ACTCACAATCCTCATTCGACGCGA |
| BAF4ST (r) | GACCAATTCGGCACCCTCAGTGTA |
| SNSP7 (f) | CCGGTCAGCTCAACTCTGACCTGA |
| CONS1 (r) | CCAATGCAAGAAAGCCTTGCGCCT |
| YRNT1 (f) | CTTCAAGATCGGTGGCCTGACCGA |
| YRNT2 (r) | GTGAGCTGTGAAGGGATCGCGGGA |
| YRNT4 (r) | CAAGTCAAACGCATCCTCGGCATG |
| BR3 (f) | TCCCAAAGCAAGCCCAAATACACG |
| BR2 (r) | GAATGTTTCACAGCAGCTGCTGGT |

[1]Primer designation; primer orientation forward (f) or reverse (r).

sequences of other fungi, including *Aspergillus nidulans* (M17519, 17520), *Colletotrichum graminicola* (M34491, 34492), *Epichloe typhina* (X52616), *Erysiphe graminis* f. sp *hordei* (M30927), *Fusarium poae* (AF006365), *Mycosphaerella pini* (AF044975), *Neurospora crassa* (M13630), *Rhynchosporium secalis* (X81046) *Trichoderma viride* (Z15055) and *Venturia inaequalis* (M97951). Final primer sets for detection of *S. tritici, S. nodorum, P. striiformis* and *P. recondita* were selected for specificity, sensitivity and product size after being tested against DNA of several other fungi (Table 1), and against DNA of infected and healthy wheat leaves from different localities.

*Quantitative fluorimetric real-time PCR measurements using SYBR Green I*

SYBR Green I (Molecular Probes, Leiden, The Netherlands) is a dye which fluoresces when bound with double stranded (ds) DNA and can be used to measure the accumulation of DNA products during PCR (Schneeberger et al., 1995). The amount of PCR amplified products from each pathogen in the leaf samples tested was quantified using calibration curves. These were generated by spiking 100 ng DNA samples from healthy wheat leaves (control) with different amounts of genomic DNA of *S. tritici*, *S. nodorum*, *P. striiformis* and *P. recondita*, ranging from 0.5 pg to 33.5 ng, which were run simultaneously in each real-time PCR assay. For each reaction, 150 μM of each dNTP, 0.3 μM of each primer, 0.35 unit Red Hot DNA polymerase, and 1X SYBR Green I (Molecular Probes, Leiden, The Netherlands), 1 : 12.5 diluted, were used. For each reaction, 100 ng of template DNA per sample was tested. Assays (25 μl) were performed in capped MicroAmp Optical 96-well reaction plates (PE Applied Biosystems, Fosters City, CA). Amplification and detection were performed in a PRISM 7700 Sequence Detection System (PE Applied Biosystems) under the following conditions: 1 cycle at 50 °C for 5 s, 1 cycle at 94 °C for 2 min, and followed by 40 cycles at 94 °C for 30 s, 65 °C for 45 s and 72 °C for 1 min. The increase of fluorescent emission signal from SYBR Green I ($\Delta Rn$) was registered at 72 °C. The Sequence Detector Software version 1.6.3 (PE Applied Biosystems) was used to analyse the data. Samples were regarded as positive at any given cycle when $\Delta Rn$ of the sample exceeded at least ten times the standard deviation of the fluorescent emission of the no-template control reaction. This threshold was set manually and the cycle reaching this point is called the cycle threshold (Ct). All samples were run in duplicate and samples detected earlier than control samples (DNA from uninfected wheat leaves) were regarded as positive.

*Analysing wheat variety resistance*

To validate the quantitative results of the real-time PCR measurements, the presence of the four foliar diseases on five flag leaves of 19 cultivars was also visually assessed at GS 60–64. Infection levels of *S. tritici* on the flag leaves were measured by estimating the average number of pycnidia-bearing lesions per leaf. Results of the real-time PCR measurements were compared with visual assessment and with information from the NIAB Recommended List of winter wheat (Anonymous, 1997–2000).

## Results

*Diagnostic DNA sequences of* S. tritici*,* S. nodorum*,* P. striiformis *and* P. recondita

Primer set BAF6/BAF2 produced, as expected, a single fragment of 444 bp of the $\beta$-tubulin gene for *S. nodorum*. For *S. tritici* ST16, a fragment of 612 bp encoding for the first 596 bp of the $\beta$-tubulin gene was generated using BAF6/BAF2. With primer set BAF6/BT3 (Table 2) an additional 243 bp was amplified and sequenced. Together with other sequences (A. Payne, unpublished results), the complete $\beta$-tubulin gene of *S. tritici* consisting of 1724 bp was determined (EMBL AJ310917). The $\beta$-tubulin gene contained six introns and encodes for 447 amino acids. When the BAF6/BAF2 $\beta$-tubulin gene fragment of *S. tritici* isolate $\alpha$12–3B.8 was sequenced, an additional 6 bp (CACGTG) between nucleotide positions 348 and 349 were identified in the fifth intron. All *P. striiformis* and *P. recondita* isolates produced two to four PCR products with primer set BAF6/BAF2. For both species, fragments of two different $\beta$-tubulin genes could be identified after sequencing. However, highly polymorphic sequences in both the coding sequences and intron regions of the $\beta$-tubulin genes among the isolates prevented design of specific primers for the two *Puccinia* species. Fortunately, for *P. striiformis* and *P. recondita*, an additional fragment of 548 bp (AJ310915) and 486 bp (AJ310916), respectively, was amplified

with BAF6/BAF2. These fragments shared no homology with any other fungal genes in DNA sequence databases, but were specific and conserved, and could be used to design specific primers.

*Development of PCR assays*

Several primer sets directed against different regions of the $\beta$-tubulin gene of *S. tritici* and *S. nodorum*, and against the diagnostic PCR fragments of *P. striiformis* and *P. recondita* were tested for specificity at different $MgCl_2$ concentrations (0.5–5.0 mM) and annealing temperatures (55–68 °C). Primers that produced single products from target DNA and no products from healthy wheat leaves, uninfected leaves originating from different localities and the other fungi tested were selected. Primer sets SNSP7F/CONS1R, YRNT1/YRNT2 and BR3/BR2 (Table 2) amplified single products from *S. nodorum* (464 bp), *P. striiformis* (351 bp) and *P. recondita* (300 bp) (Figure 1). For detection of *S. tritici*, primer set STIF2/BAF4ST was used (Table 2). This primer set amplifies the variable fifth intron of the $\beta$-tubulin gene of *S. tritici* and, depending on sequences of the fifth intron present in field strains, products can be either 555 and/or 561 bp long (Figure 1). Having similar annealing temperatures of 65–70 °C, but amplifying products of different sizes, all primers could be used together to detect four pathogens simultaneously in one PCR/gel electrophoresis assay (see Figure 2). Based on intensities of the stained PCR products in agarose gel electrophoresis, the sensitivity of the PCR was not affected when a single or two, three or four different DNA targets were simultaneously amplified (data not shown).

*Multiplex detection of four diseases in different leaf layers and plant tissues*

The presence of the four pathogens in different leaf layers of six cultivars at GS 60–61 was studied in 1999 (Figure 2a). Based on differences in the intensity of the ethidium-bromide stained diagnostic PCR products, different amounts of pathogen DNA were detected in the samples. Infection of *S. nodorum* and *S. tritici* was detected in all cultivars, whereas the presence of *P. striiformis* and *P. recondita* was more cultivar-specific. Buster with all leaf layers infected was most susceptible to *P. recondita,* while both *P. striiformis* and *P. recondita* were detected in Soissons and Riband. For most pathogens, highest infection levels were detected in leaf 3, with decreasing amounts in leaf 2 and flag leaf, indicating a vertical transmission. Results of visual assessment of *S. tritici* in the top three leaf layers of different cultivars at GS 62, showing increasing infection levels in subsequent leaf layers (Table 3), also indicated vertical transmission and confirmed the PCR results.

To follow the epidemiology of all four pathogens in late season during grain filling, we also checked if they could be detected in the flag leaf, ear and grain of six cultivars (Figure 2b). In all cultivars, based on the intensity of the stained PCR products, high levels of *S. nodorum* were detected in the ear and grain. No other pathogens, including *S. tritici*, could be detected in the grain. High infection levels of *S. tritici* and *P. recondita*

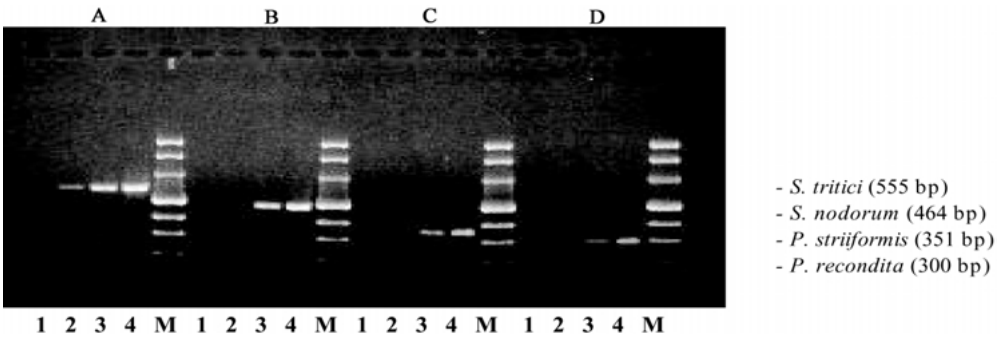


*Figure 1*. Ethidium bromide-stained agarose gel of PCR amplified products. Primer sets STIF2/BAF4ST, SNSP7F/CONS1R, YRNT1/YRNT2 and BR3F/BR2R were used in a single reaction to detect different amounts of DNA of *S. tritici* (A), *S. nodorum* (B), *P. striiformis* (C) and *P. recondita* (D), respectively, in 200 ng DNA samples of uninfected leaves. A, Lanes: 1, control; 2, 23 pg; 3, 230 pg; 4, 2, 3 ng DNA of *S. tritici* added; B, Lanes: 1, control; 2, 3.6 pg; 3, 36 pg; 4, 360 pg DNA of *S. nodorum* added; C, Lanes: 1, control; 2, 13 pg, 3, 130 pg; 4, 1.3 ng DNA of *P. striiformis* added; D, Lanes: 1, control; 2, 6.8 pg; 3, 68 pg; 4, 680 pg DNA of *P. recondita* added; M, DNA ladder VIII (Boehringer, Mannheim, Germany).

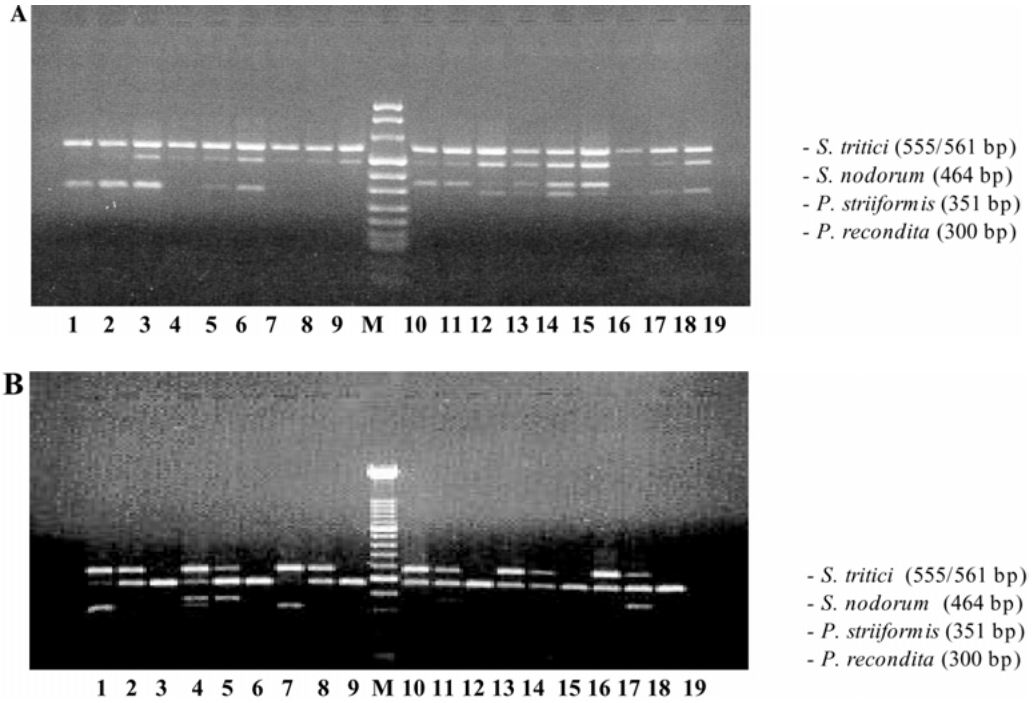


*Figure 2*. Multiplex PCR detection of *S. tritici*, *S. nodorum*, *P. striiformis* and *P. recondita*. Ethidium bromide-stained agarose gel of multiplex PCR amplified products. A, Leaf 3, leaf 2 and flag leaf samples of different cultivars at GS 60–61 in 1999. Ethidium bromide-stained agarose gel of multiplex PCR amplified products. Lanes: 1, cv Buster flag; 2, Buster leaf 2 (L2); 3, Buster leaf 3 (L3); 4, Consort flag; 5, Consort L2; 6, Consort L3; 7, Rialto flag; 8, Rialto L2; 9; Rialto L3; 10, Riband flag; 11, Riband L2; 12, Riband L3; 13, Soissons flag; 14, Soissons L2; 15, Soissons L3; 16, Spark flag; 17, Spark L2; 18, Spark L3; 19, Control, water; M, DNA ladder VIII (Boehringer, Mannheim, Germany). B, Samples of flag leaf (GS 77–83), ear (GS 85–91) and grain (GS 92) in 1999. Lanes: 1, cv Buster flag; 2, Buster ear; 3, Buster grain; 4, Soissons flag; 5, Soissons ear; 6, Soissons grain; 7, Consort flag; 8, Consort ear; 9, Consort grain; 10, Riband flag; 11, Riband ear; 12, Riband grain; 13, Claire flag; 14, Clair ear; 15, Claire grain; 16, Savannah flag; 17, Savannah ear; 18, Savannah grain L3; 19, Control, Spark uninfected leaf from glasshouse; M, 100 bp DNA ladder XIV (Boehringer, Mannheim, Germany).

*Table 3*. Visual assessment of *S. tritici* infection levels in the top three leaf layers at GS62 in 1999

| Cultivar | Leaf 3 | Leaf 2 | Flag leaf |
|---|---|---|---|
| Abbot | 13.5 (0.0–100) | 1.2 (0.0–4.0) | 0.2 (0.0–1.0) |
| Buster | 10.4 (0.5–60) | 0.8 (0.0–3.0) | 0.2 (0.0–1.0) |
| Cantata | 50.7 (18–100) | 6.5 (0.0–25) | 1.1 (0.0–3.0) |
| Charger | 4.6 (1.0–17) | 0.4 (0.0–3.0) | 0.4 (0.0–2.0) |
| Consort | 20.9 (1.0–50) | 6.2 (1.0–15) | 1.5 (0.1–7.0) |
| Equinox | 26.6 (5.0–100) | 3.3 (1.0–6.0) | 1.4 (0.1–4.0) |
| Harrier | 10.9 (0.2–30) | 2.5 (0.0–6.0) | 0.4 (0.0–1.0) |
| Madrigal | 21.4 (4.0–70) | 4.0 (1.0–8.0) | 0.9 (0.0–3.0) |
| Reaper | 17.3 (1.0–50) | 3.6 (0.5–7.0) | 2.4 (0.0–5.0) |
| Rialto | 4.3 (1.0–20) | 2.2 (0.0–4.0) | 0.5 (0.0–1.0) |
| Riband | 28.0 (2.0–75) | 8.7 (0.5–22) | 1.6 (0.0–3.0) |
| Savannah | 13.6 (2.0–50) | 3.2 (1.0–6.0) | 0.7 (0.0–2.0) |
| Soissons | 60.5 (15–100) | 6.9 (0.2–25) | 0.4 (0.0–1.0) |
| Spark | 2.1 (0.2–5.0) | 1.2 (0.5–3.0) | 0.5 (0.0–2.0) |

For each leaf layer, ten plants per cultivar were analysed, the average percentage of infected leaf area is shown. The range of percentages of infected leaf area for individual leaves in parentheses.

were detected in the flag leaves, while *P. striiformis* was mainly detected in the ears of Soissons and Savannah.

### *Quantification of PCR products with fluorometric real-time PCR measurements*

To quantify PCR products with non-sequence specific DNA-intercalating dyes, like SYBR Green I, only single fragments should be amplified (Witwer et al., 1997). For each pathogen, therefore, with the exception of *P. striiformis*, individual real-time PCR assays were performed using the same primer sets as used for the multiplex PCR. Primers YRNT1 and YRNT4 (Table 2), amplifying a 417 bp product, were used to detect *P. striiformis*, while, for detection of *P. recondita*, the annealing and extension steps were decreased to 40 s to reduce background amplification of non-target DNA and primer dimer formation (data not shown). For

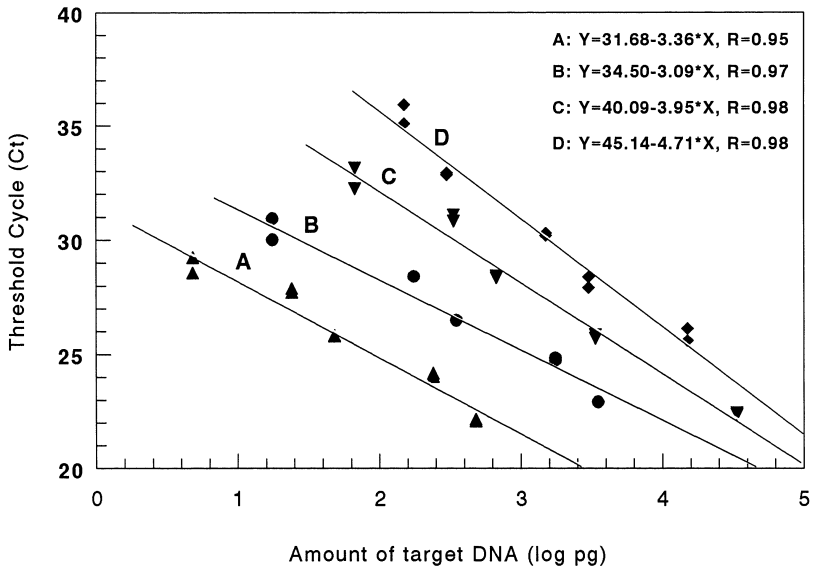


*Figure 3*. Real-time PCR calibration curves for quantification of DNA infection levels of different pathogens in wheat leaves. Calibration samples were obtained by spiking wheat leaf DNA samples (100 ng) with different amounts of genomic target DNA of *S. tritici* (A), *S. nodorum* (B), *P. striiformis* (C) and *P. recondita* (D).

detection of all pathogens, amount of input target DNA added to 100 ng of wheat leaf DNA was linearly correlated with the Ct (Figure 3). The sensitivity was approximately 2, 6, 40 and 60 pg of genomic DNA per sample for detection of *S. tritici*, *S. nodorum*, *P. striiformis* and *P. recondita*, respectively (Figure 3), because wheat DNA, alone, or spiked with less target DNA, could produce signals with identical Ct and shape. The variation in sensitivity can be explained by the size of the PCR product, larger dsDNA fragments bind more SYBR Green I dye, and differences in background fluorescence caused by weak non-specific amplification and primer dimer formation during PCR. Using the *P. recondita* PCR assay, weak amplification of non-target DNA, was sometimes visualised in agarose gel electrophoresis when uninfected wheat leaf field samples were tested (data not shown). In the *P. striiformis* PCR assay more primer dimers were generated without template DNA than in the presence of 100 ng of healthy wheat leaf DNA (data not shown). This is probably due to competition between primers, non-target DNA and target-DNA in primer binding during the PCR annealing step.

*Wheat variety resistance testing*

Flag leaf samples from all 19 cultivars sampled in 1998 (GS 64) and 1999 (GS 60–61) were analysed with visual assessment (Table 4), multiplex PCR/gel electrophoresis (Figure 4) and real-time PCR measurements (Figure 5). *Septoria tritici* was detected in all cultivars with visual assessment and PCR. Results obtained with real-time PCR measurements showed a good correlation between *S. tritici* infection level and amount of PCR-amplified products (Figure 6). Less than 2 pg of *S. tritici* DNA was present in leaves without symptoms, e.g. Abbot, Claire and Savannah in 1999 (Figures 4 and 5), while leaves with symptoms, depending on the number of lesions (Table 4), contained 3–68 pg of *S. tritici* DNA per sample (Figure 6). For 14 out of the 19 cultivars tested, *S. tritici* DNA infection levels in the flag leaves determined by real-time PCR were higher in 1998 than in 1999 (Figure 5). Higher incidence of *S. tritici* infection in 1998 was also confirmed with visual assessment, where, in comparison with 1999, 13 out of the 17 samples showed higher infection levels (Table 4). Only Soissons in 1999, having a DNA infection level $>20$ pg, showed *S. nodorum* symptoms, while *S. nodorum* DNA levels $<20$ pg were detected in flag leaves of cultivars without symptoms. For *P. striiformis*, infection levels $>100$ pg DNA were detected in Abbot, Harrier, Reaper, Riband, Savannah and Soissons, whereas infection levels $>100$ pg of *P. recondita* DNA were only detected in Buster, Soissons, Consort and Charger (Figure 5). Brown rust was difficult to diagnose, but the presence

*Table 4*. Visual assessment of flag leaf samples tested in PCR

| Wheat cultivar | Diseases in 1998[1] at GS 64 | Number of lesions in 1998[2] | Diseases in 1999[1] at GS 60–61 | Number of lesions in 1999[2] |
|---|---|---|---|---|
| Aardvark | STB | 11.2 (0–50) | STB | 1.4 (0–3) |
| Abbot | STB | 1.8 (0–6) | YR, BR | 0 |
| Buster | STB, BR | ND | STB, BR | 4.2 (0–15) |
| Cantata | STB | ND | STB | 13.6 (0–60) |
| Charger | STB | 6.0 (3–10) | STB | 0.6 (0–2) |
| Claire | STB | 0.2 (0–1) | | 0 |
| Consort | STB | 46.0 (10–150) | STB | 15.2 (2–28) |
| Equinox | STB | 56.8 (2–150) | STB | 13.4 (4–33) |
| Harrier | STB | 7.2 (2–13) | STB, YR | 1.4 (0–3) |
| Madrigal | STB | 37 (0–80) | STB | 18.0 (5–30) |
| Malacca | STB | 4.6 (1–11) | STB | 7.2 (1–30) |
| Reaper | STB | 12.4 (7–30) | STB, YR | 13.4 (0–24) |
| Rialto | STB | 4.2 (1–11) | STB | 0.4 (0–1) |
| Riband | STB | 63.6 (15–150) | STB | 2.0 (0–6) |
| Savannah | STB, YR | 6.0 (1–20) | YR | 0 |
| Shamrock | STB | 41.4 (10–90) | STB | 3.2 (2–28) |
| Shango | STB | 2.2 (0–5) | STB | 3.0 (0–6) |
| Soissons | STB | 38 (2–60) | YR, SNB, BR | 0.6 (0–2) |
| Spark | STB | 0.8 (0–4) | STB | 2.0 (0–6) |

[1]Diseases; STB, SNB, YR, BR symptoms caused by *S. tritici*, *S. nodorum*, *P. striiformis* and *P. recondita*, respectively. Five flag leaves per cultivar were visually assessed.
[2]Average number of *Septoria tritici* pycnidia-bearing lesions per leaf; ND, not detected because of senescence; range of lesion numbers found in individual leaves among the five leaves tested are in parentheses.

of yellow rust, particularly in 1999, was confirmed with visual assessment in Abbot, Harrier, Reaper, Savannah and Soissons (Table 4). For each disease, most cultivars tested showed similar susceptibilities in 1998 and 1999. For most cultivars, the resistance ratings from the NIAB recommended list for winter wheat (Anonymous, 1997–2000) corresponded well with quantitative disease data obtained with visual assessment (Tables 3 and 4) and real-time PCR (Figure 5). Exceptions were low *S. tritici* resistance ratings for Charger and Rialto, low yellow rust resistance ratings for Madrigal, Equinox and Rialto, and the high brown rust resistance rating for Charger. Claire, with resistance ratings of 7, 7, 9 and 9 for *S. tritici*, *S. nodorum*, *P. striiformis* and *P. recondita*, respectively, showed the lowest infection levels for all four diseases, and only small amounts of *S. tritici* and *S. nodorum* were detected in flag leaves (Figure 5). In comparison with Claire, Soissons with lower resistance ratings for all diseases showed higher flag leaf infection levels for each pathogen.

## Discussion

Multiplex PCR assays have been used for genotyping plant genetic resources (Mitchell et al., 1997) and for detection of microbes (see Bubert et al., 1999). However, for quantitative detection using end-point measurements, accuracy can be low due to template-specific over-amplification (Polz and Cavanaugh, 1998). Furthermore, unlike real-time PCR, products have to be quantified in the linear part of the PCR calibration curve, before PCR components are running out, limiting its dynamic range (Higuchi et al., 1993).

Because of end-point measurements, infection levels in some samples might be under-estimated with PCR/gel electrophoresis. However, the quantitative data obtained with real-time PCR correlated generally well with the results of PCR/gel electrophoresis and visual assessment when leaves were tested for the presence of the four diseases (Table 4, Figures 4 and 5). To check for inhibition in real-time PCR, we spiked each sample with 1.75 ng of genomic DNA of *S. nodorum*. The Ct of the spiked samples ranged from 22.96 to 24.75, with an average Ct of 23.99, indicating that no PCR inhibition occurred (see Figure 3).

When comparing infection levels of the four diseases, higher levels of *P. striiformis* were observed in most samples in 1999, while 1998 was more favourable for *S. tritici* epidemics (Figure 5). In both years, with the exception of Soissons in 1999, only pre-symptomatic infection levels of *S. nodorum* were detected in flag leaves (Table 4). Higher infection levels of *S. nodorum* were detected later in the season in flag leaves, ears and grains (Figure 2b), confirming the seedborne nature of *S. nodorum*, and its greater ability to colonise more matured and senescent leaf tissue (Wainshilbaum and Lipps, 1991).

Wet springs and early summers of 1998 and 1999 were responsible for the high infection levels of *S. tritici* detected in this study. For Abbot, Claire, and Savannah, detection of *S. tritici* was pre-symptomatic in 1999 (Table 4). The relatively large difference in infection levels for Soissons between 1998 and 1999 is probably due to its early maturing, and, therefore, greater potential for disease escapes (Peters et al., 1996). For some cultivars, particularly, Aardvark in 1998, infection levels might be overestimated because of uneven distribution of lesions in the five flag leaves tested (Table 4). In 1998 and 1999, only low *S. tritici* infection levels were found in Rialto (Tables 3 and 4), which has a low

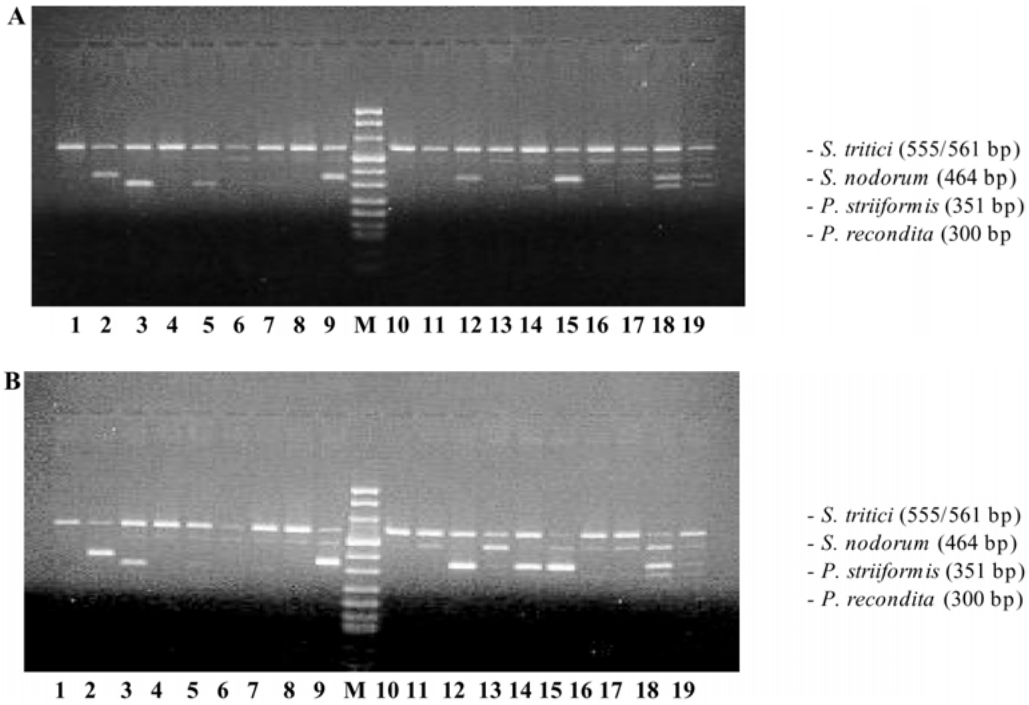


*Figure 4.* Wheat varietal resistance testing. Ethidium bromide-stained agarose gel of products amplified with multiplex PCR. Five flag leaves of different cultivars from field trials in Long Ashton at GS 64 in 1998 (A) and at GS 60–61 in 1999 (B) were tested. Lanes: 1, cv Aardvark; 2, Abbot; 3, Buster; 4, Cantata; 5, Charger; 6, Claire; 7, Consort; 8, Equinox; 9, Harrier; 10, Madrigal; 11, Malacca; 12, Reaper; 13, Rialto; 14, Riband; 15, Savannah; 16, Shamrock; 17, Shango; 18, Soissons; 19, Spark; M, DNA ladder VIII (Boehringer, Mannheim, Germany).

*S. tritici* resistance rating of 5. Because relatively high levels of *S. nodorum* were found in Rialto (Figure 5), the difficult discrimination between visual symptoms of *S. tritici* and *S. nodorum* may contribute to the low resistance rating for *S. tritici*. Although Claire was found to be most resistant in this study, in comparison with other cultivars, relatively high infection levels were found in the early season (unpublished results), indicating adult plant-based resistance (Kema and Silfhout, 1997).

The presence of high levels of brown and yellow rust in certain varieties (Figure 5) can be explained by a cultivar-specific resistance response to different rust pathotypes (McIntosh et al., 1995). The discrepancy between the lack of yellow rust infections and low resistance ratings for Madrigal (3), Equinox (5) and Rialto (5), all carrying the *Yr6* resistance gene, might be due to the absence in SW-England of specific rust pathotypes with the *Yr6*-matching virulence gene (Bayles and Stigwood, 1999).

Improved understanding about host pathogen interactions, crop architecture and more knowledge of genetics of resistance should enable plant breeders to make crosses with more durable resistance sources (Eyal, 1999). PCR can be used to study competition between pathogens and to measure host pathogen interactions at different growth stages and in different plant tissues, so that specific resistance mechanisms can be identified earlier and more accurately in field trials. With multiplex PCR, more than one organism can be detected at the same time without relying on less accurate visual disease assessment (Parker et al., 1995b). Real-time PCR assays can be further used to quantify infection levels of different diseases in order to allow well-targeted fungicide applications to eradicate, or to prevent, in case of pre-symptomatic detection, epidemics in crops at risk.

To improve assays described in this study, we plan to establish sampling protocols for each disease reflecting in-field infection development (Parker et al., 1997). We

are also developing assays based on real-time PCR and fluorescent DNA probes, for example, TaqMan probes (Livak et al., 1995) Molecular Beacons (Tyagi and Kramer, 1996) and Scorpions (Thelwell et al., 2000), to detect and quantify different pathogens simultaneously (Shin et al., 1999). By using sequence-specific probes and internal controls, the sensitivity and accuracy of the assays can be further improved, as recently shown for detection of the plant pathogenic fungi *Diaporthe phaseolorum* and *Phomopsis longicolla* (Zhang et al., 1999), and the bacterium *Ralstonia solanacearum* (Weller et al., 2000).

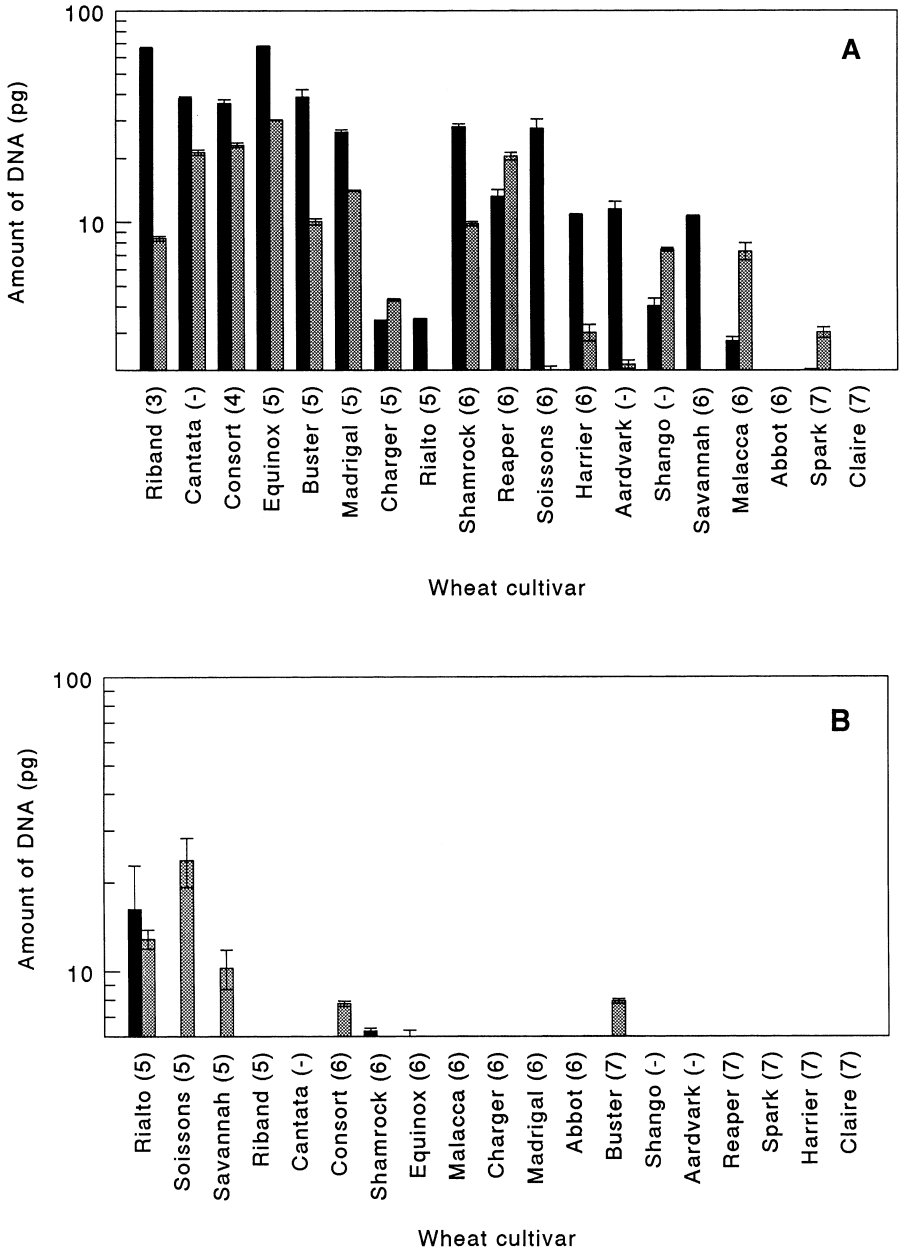


*Figure 5.* (*Continued*)

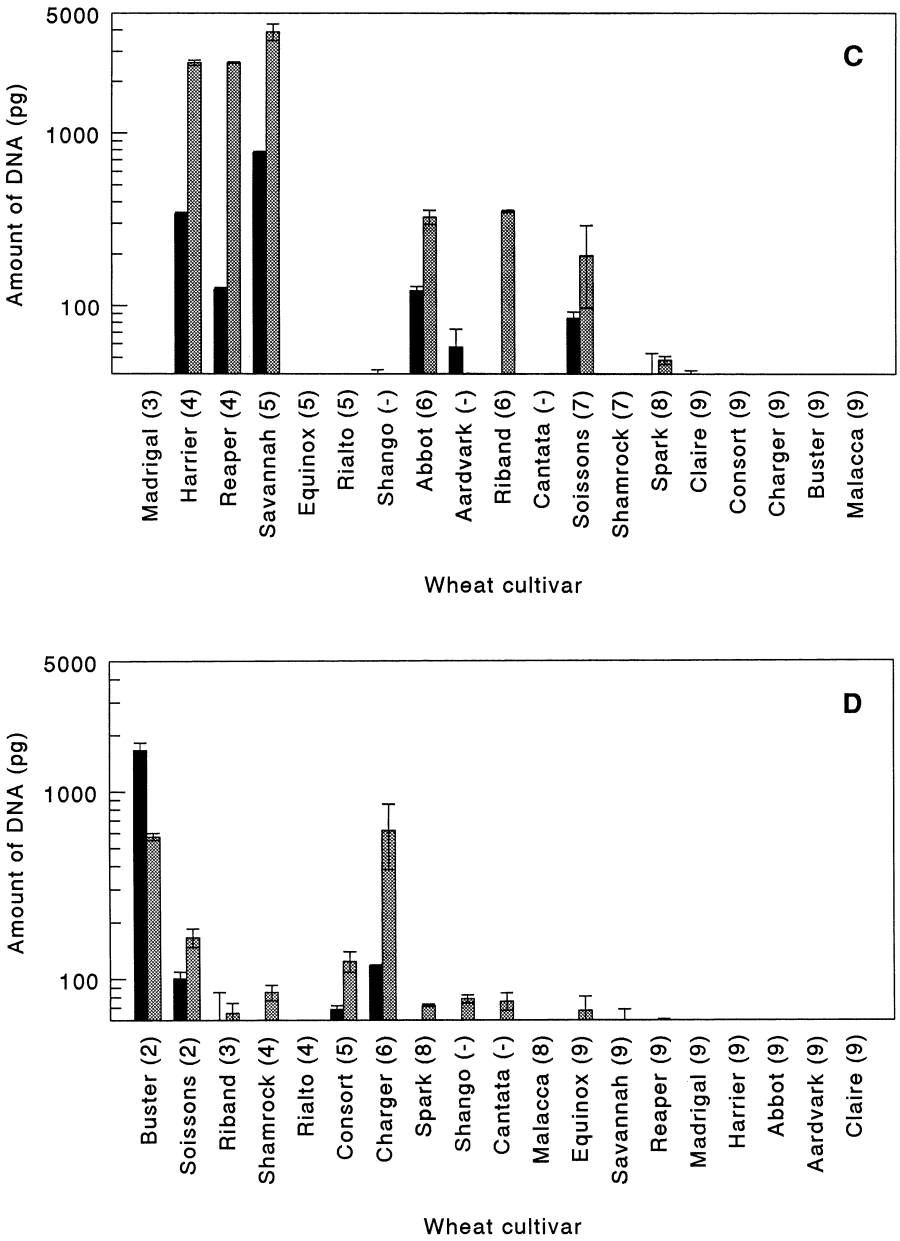


*Figure 5*. Wheat varietal resistance testing. DNA infection levels of each pathogen in flag leaves of different cultivars at GS 64 (1998) and GS 60–61 (1999) were measured with real-time PCR. Infection levels are expressed in pg of genomic pathogen DNA present in 100 ng of DNA extracted from five flag leaves. For each cultivar, the NIAB resistance ratings are shown between brackets; -, NIAB resistance rating unknown. Detection of *S. tritici* (A), *S. nodorum* (B), *P. striiformis* (C) and *P. recondita* (D). Black bar, 1998; hatched bar, 1999. Vertical bars represent standard error of means.

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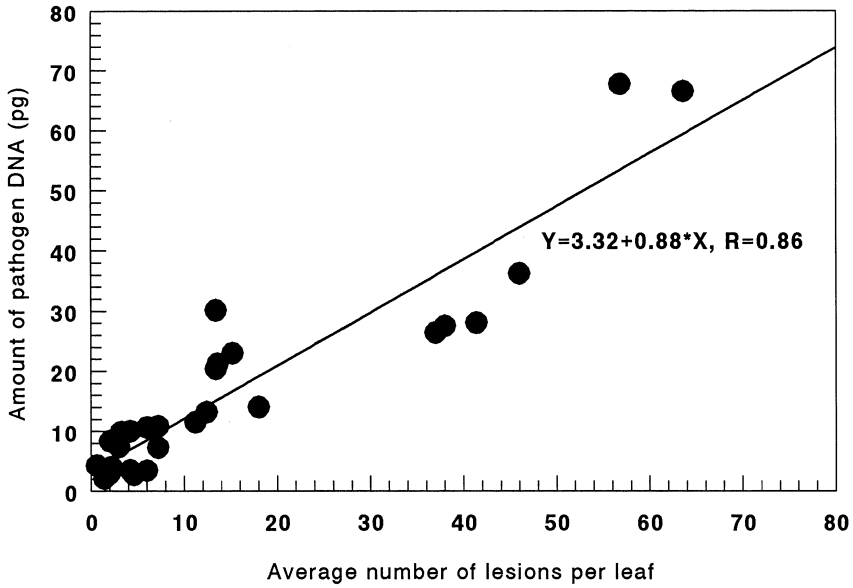


*Figure 6.* Relation between DNA infection levels and the average number of *S. tritici* pycnidia-bearing lesions per leaf. All flag leaf samples presented in Table 4 were tested, with the exception of Buster and Cantata in 1998.

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## References

Ahn SJ, Costa J and Emanuel JR (1996) PicoGreen quantification of DNA: effective evaluation of pre- or post-PCR. Nucleic Acids Research 24: 2623–2625

Anonymous (1997–2000) Cereal variety handbooks 1997–2000. NIAB recommended lists for winter wheat. National Institute of Agricultural Botany, Cambridge, UK.

Bayles RA (1997) Disease development – the interaction of variety resistance and pathogen. Aspects of Applied Biology 50: 249–254

Bayles RA and Stigwood PL (1999) Yellow rust of wheat. In: Clarkson JDS (ed.) UK cereal pathogen virulence survey 1999 Annual Report (pp 29–35) The United Kingdom Cereal Pathogen Virulence Survey Committee, Cambridge, UK.

Beck JJ and Ligon JM (1995) Polymerase chain reaction assays for the detection of *Stagonospora nodorum* and *Septoria tritici* in wheat. Phytopathology 85: 319–324

Bubert A, Hein I, Rauch M, Lehner A, Yoon B, Goebel W and Wagner M (1999) Detection and differentiation of *Listeria* spp. by a single reaction based on multiplex PCR. Applied and Environmental Microbiology 65: 4688–4692

Cook RJ, Hims MJ and Vaughan TB (1999) Effects of fungicide spray timing on winter wheat disease control. Plant Pathology 48: 33–50

Cook RJ, Polley RW and Thomas MR (1991) Disease-induced losses in winter wheat in England and Wales 1985–1989. Crop Protection 10: 504–508

Doohan FM, Parry DW and Nicholson P (1999) *Fusarium* ear blight of wheat: the use of quantitative PCR and visual disease assessment in studies of disease control. Plant Pathology 48: 209–217

Eyal Z (1999) The septoria tritici and stagonospora nodorum blotch diseases of wheat. European Journal of Plant Pathology 105: 629–641

Foster SJ, Singh G, Fitt BDL and Ashby AM (1999) Development of PCR based diagnostic techniques for the two mating types of *Pyrenopezia brassicae* (light leaf spot) on winter oilseed rape (*Brassica napus* spp. *oleifera*) Physiological and Molecular Plant Pathology 55: 111–119

Fraaije BA, Lovell DJ, Rohel EA and Hollomon DW (1999) Rapid detection and diagnosis of *Septoria tritici* epidemics in wheat using a polymerase chain reaction/PicoGreen assay. Journal of Applied Microbiology 86: 701–708

Higuchi R, Fockler C, Dollinger G and Watson R (1993) Kinetic PCR analysis: real-time monitoring of DNA amplification reactions. Biotechnology 11: 1026–1030

Johnson R (1992) Reflections of a plant pathologist on breeding for disease resistance, with emphasis on yellow rust and eyespot of wheat. Plant Pathology 41: 239–254

Kema GHJ and Silfhout CH (1997) Genetic variation for virulence and resistance in the wheat-*Mycosphaerella graminicola* pathosystem III. Comparative seedling and adult plant experiment. Phytopathology 87: 266–272

Kendall SJ, Hollomon DW and Selley A (1998) Immunodiagnosis as an aid to the timing of fungicide sprays for the control of *Mycosphaerella graminicola* on winter wheat in the UK. In: Proceedings Brighton Crop Protection Conference – Pests and Diseases 1998, Vol 2. (pp 701–706) British Crop Protection Council, Farnham, UK

Koenraadt H and Jones AL (1992) The use of allele-specific oligonucleotide probes to characterize resistance to benomyl in field strains of *Venturia inaequalis*. Phytopathology 82: 1354–1358

Livak KJ, Flood SJA, Marmero J, Giusti W and Deetz K (1995) Oligonucleotides with fluorescent dyes at opposite ends provide a quenched probe system useful for detecting PCR products and nucleic acid hybridization. PCR Methods and Applications 4: 357–362

Lovell DJ, Parker SR, Hunter T, Royle DJ and Coker RR (1997) Influence of crop growth and structure on the risk of epidemics by *Mycosphaerella graminicola* (*Septoria tritici*) in winter wheat. Plant Pathology 46: 126–138

McIntosh RA, Wellings CR and Park RF (1995) Wheat rusts: an atlas of resistance genes. CSIRO, Australia, and Kluwer Academic Publishers, Dordrecht, The Netherlands

Messmer MM, Seyfarth R, Keller M, Schachermayr G, Winzeler M, Zanetti S, Feuillet C and Keller B (2000) Genetic analysis of durable leaf rust resistance in winter wheat. Theoretical and Applied Genetics 100: 419–431

Mitchell SE, Kresovich SK, Jester CA, Hernandez CJ and Szewc-McFadden AK (1997) Application of multiplex PCR and fluorescence-based, semi-automated allele sizing technology for genotyping plant genetic resources. Crop Science 37: 617–624

Nicholson P, Rezanoor HN, Simpson DR and Joyce D (1997) Differentiation and quantification of the cereal eyespot fungi *Tapesia yallundae* and *Tapesia acuformis* using a PCR assay. Plant Pathology 46: 842–856

Park RF, Jahoor A and Felsenstein FG (2000) Population structure of *Puccinia recondita* in Western Europe during 1995, as assessed by variability in pathogenicity and molecular markers. Journal of Phytopathology 148: 169–179

Parker SR, Perry J and Royle DJ (1995a) Reliable measurement of disease severity. Aspects of Applied Biology 43: 205–214

Parker SR, Shaw MW and Royle DJ (1995b) The reliability of visual estimates of disease Severity on cereal leaves. Plant Pathology 44: 856–864

Parker SR, Shaw MW and Royle DJ (1997) Measurements of spatial patterns of disease in winter wheat crops and the implications for sampling. Plant Pathology 46: 470–480

Peters BA, Loughman R and Di Prinzio P (1996) Leaf development in relation to infection by *Stagonosporum nodorum* and *Septoria tritici* in wheat. Australian Journal of Agricultural Research 47: 1169–1179

Polz MF and Cavanaugh CM (1998) Bias in template-to-product ratios in multitemplate PCR. Applied and Environmental Microbiology 64: 3724–3730

Sambrook J, Fritsch EF and Maniatis T (1989) Molecular cloning – a laboratory manual, 2nd edn. Cold Spring Harbor Laboratory Press, Plainview, NY, USA

Sanger F, Nicklen S and Coulson AR (1977) DNA sequencing with chain termination inhibitors. Proceedings National Academy of Sciences USA 74: 5463–5467

Schneeberger C, Speiser P, Kury F and Zeillinger R (1995) Quantitative detection of reverse transcriptase-PCR products by means of a novel and sensitive DNA stain. PCR Methods and Applications 4: 234–238

Shin JH, Nolte FS, Holloway BP and Morrison CJ (1999) Rapid identification of up to three *Candida* species in a single reaction tube by 5′exonuclease assay using fluorescent DNA probes. Journal of Clinical Microbiology 37: 165–170

Thelwell N, Millington S, Solinas A, Booth J and Brown T (2000). Mode of action and application of Scorpion primers to mutation detection. Nucleic Acids Research 28: 3752–3761

Tottman DR (1987) The decimal code for the growth stages of cereals, with illustrations. Annals of Applied Biology 110: 441–454

Tyagi S and Kramer FR (1996) Molecular Beacons: probes that fluoresce upon hybridization. Nature Biotechnology 14: 303–308

Wainshilbaum SJ and Lipps PE (1991) Effect of temperature and growth stage of wheat on development of leaf and glume blotch caused by *Septoria tritici* and *S. nodorum*. Plant Disease 75: 993–998

Weller SA, Elphinstone JG, Smith NC, Boonham N and Stead DE (2000) Detection of *Ralstonia solanacearum* strains with a quantitative, multiplex, real-time, fluorogenic PCR (TaqMan) assay. Applied and Environmental Microbiology 66: 2853–2858

Wiese MV (1987) *Compendium of wheat diseases*, 2nd edn. APS press, St. Paul, USA

Willits DA and Sherwood JE (1999) Polymerase Chain Reaction detection of *Ustilago hordei* in leaves of susceptible and resistant barley varieties. Phytopathology 89: 212–217

Wittwer CT, Herrmann MG, Moss AA and Rasmussen RP (1997) Continuous fluorescence monitoring of rapid cycle DNA amplification. Biotechniques 22: 130–138

Zhangh AW, Hartman GL, Curio-Penny B, Pedersen WL and Becker KB (1999) Molecular detection of *Diaporthe phaseolorum* and *Phomopsis longicolla* from soybean seeds Phytopathology 89: 796–804.