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NOVEL DNA DIAGNOSTIC TECHNOLOGY IN PLANT DISEASE CONTROL USING *SEPTORIA TRITICI* AS A MODEL

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NOVEL DNA DIAGNOSTIC TECHNOLOGY IN PLANT DISEASE CONTROL USING *SEPTORIA TRITICI* AS A MODEL

by

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ABSTRACT

To detect and quantify *Septoria tritici* infection levels in wheat leaves, a polymerase chain reaction (PCR) assay was developed using the β-tubulin as target. Specific PCR primers were designed by aligning and comparing β-tubulin gene sequences from other fungi. PCR primer sets were selected after being tested against several fungi, and against *S. tritici*-infected and uninfected leaves of different cultivars, and from different localities. Single DNA fragments were amplified from *S. tritici*, whereas no products were generated from DNA of the host plant, or other micro-organisms associated with wheat leaves.

Using agarose gel analysis, *ca* 2 pg *S. tritici* DNA could be detected in each assay. This corresponds with approximately hundred *S. tritici* spores per leaf. For rapid quantification of PCR amplified products, a fluorometric microtitre plate formatted PicoGreen assay was developed; this could detect as little as 10 pg *S. tritici* DNA in the presence of 200 ng wheat leaf DNA.

The PCR/PicoGreen assay was applied successfully to study the colonisation, infection and subsequent disease development of *S. tritici* on wheat, both under controlled conditions in the glasshouse, as well as in the field. The effect of different fungicide treatments on the epidemics could be measured with the quantitative PCR/PicoGreen assay.

A multiplex PCR assay was developed to detect *S. tritici*, *S.nodorum*, *Puccinia striiformis* and *P. recondita* simultaneously in infected wheat leaves, ears and grain. The individual pathogens could be quantified in separate assays, allowing measurements of disease resistance for different wheat cultivars. Assays based in this work are now available commercially for pre-symptomatic detection of these four key foliar pathogens of wheat in the UK. This novel DNA diagnostic technology should enable improvements in the precision of disease control measures.

SUMMARY

Background

Foliar diseases of wheat are important, and largely controlled by fungicides, which account for about 30% of grower's variable costs. Up to one third of fungicide applications are badly timed, and managed disease control strategies are more cost effective than prophylactic spraying. Integration of the mainly preventative strobilurin fungicides into managed disease control requires a curative triazole mixture partner. Fungicide mixtures are also a cornerstone of strategies to combat resistance, which has already emerged to strobilurins and triazoles in some diseases, including cereal powdery mildews. Accurate information on pre-symptomatic disease levels is important, therefore, to ensure that timing and dose rates of these fungicide mixtures are optimal. This project sought to develop a robust, rapid and accurate DNA diagnostic to detect and quantify pre-symptomatically four key pathogens of wheat.

The **P**olymerase **C**hain **R**eaction (PCR) offers a simple way to specifically increase amounts of DNA to readily detectable levels. PCR has generated many novel opportunities to improve disease diagnosis, and although considerable advances have been made using PCR to detect human and other animal diseases, exploiting PCR to improve plant health has been much slower, especially for fungal diseases. Unlike immunology, the specificity of PCR diagnostics resides in unique DNA sequences, and not antibodies. Consequently, the initial step in developing any PCR diagnostic assay requires identification of some unique, but relevant, DNA sequences which can be used to prime PCR production of unlimited copies of the desired DNA sequence. In many cases detection of fungal species uses **I**nternally **T**ranscribed **S**pacer (ITS) primer sequences of ribosomal DNA, but in our experience these are not always specific, and can give DNA products in the absence of disease. Consequently, we have focussed on the β-tubulin gene, which occurs in every cell nucleus, as the diagnostic target.

Development of diagnostic assay for *Septoria tritici*

Although the β-tubulin gene is very similar throughout all higher organisms, sufficient diversity exists, especially in regions of the gene that do not code for the protein, between even very closely related species to generate the specificity needed for diagnostics. To detect and quantify *Septoria tritici* infection levels in wheat leaves, a PCR assay was developed using specific PCR primers designed by aligning and comparing the complete *S. tritici* β-tubulin gene sequence with β-tubulin gene sequences from other fungi. PCR primer sets were selected after being tested against several fungi, and against *S. tritici*-infected and uninfected leaves of different cultivars, and from different localities. Single DNA fragments were amplified from *S. tritici* , whereas no products were generated from DNA of the host plant, or other micro-organisms associated with wheat leaves.

Quantification

Because positive diagnosis of *S. tritici*- infected leaves only generated a single PCR product, a simple quantification protocol was devised using the fluorescent cyanine dye PicoGreen, which only fluoresces in the presence of double stranded DNA. PicoGreen was simply added when the PCR diagnostic step was completed, and fluorescence was then measured in a luminometer in a micro-titre plate format. Quantification required testing samples in duplicate, and generating a standard curve with each assay set. There was a good correlation between fluorescence and biomass in terms of DNA amounts. This approach to quantification avoided complicated procedures surrounding competitive PCR, which involve many pipeting steps and construction of a unique competitive internal standard sequence.

Optimisation of the PCR conditions included reducing primer levels. This increased sensitivity as primer-dimer formation was reduced, and consequently background fluorescence. PCR cycle number was also important, and up to 40 cycles were needed to quantify accurately small infection levels. The lower limit of detection using PicoGreen was about 1,000 *S. tritici* spores. Analysis of PCR products by agarose gel electrophoresis allowed detection of only 200 spores, but direct fluorescence measurement was quantitiative, robust, quick, and carried out in a micro-titre plate.

Compared with detection of *S. tritici* by immunoassay, PCR diagnosis was some 100-fold more sensitive. Pre-symptomatic detection was possible within three days after inoculation of wheat with *S. tritici*, whereas immunoassay only detected the beginnings of pycnidial formation some 10 to 14 days after infection. At this time fungicides have little or no eradicant activity against *S. tritici.*

Limitations to assay throughput

Extraction of DNA template from leaf samples remains a “bottleneck”, and restricts sample preparation to 10 per hour. Leaves are powdered in liquid nitrogen using a pestle and mortar, extracted with buffer, and DNA precipitated with isopropanol. Although time-consuming, the method is simple and avoids use of harmful solvents such as phenol.

Diagnostic for *Septoria nodorum*, brown and yellow rust

Because development of a quantitative diagnostic assay for *S. tritici* was successful, effort was made to add diagnostic assays for *S. nodorum*, brown and yellow rusts. Specific PCR primer sets for each disease were designed from either published sequence data (*S.nodorum),*or sequence analysis of PCR fragments produced using degenerate β-tubulin primers (rusts). Because specific primer pairs, including *S. tritici*, had the same annealing properties, but each pair generated a different size product, it was possible to multiplex detection of all four diseases in a single PCR reaction. Separation of the PCR products by agarose gel electrophoresis allowed detection, but not fluorescent quantification. To quantify each disease a separate PCR reaction was needed, so that only a single double-stranded DNA product was amplified.

Practical evaluation of diagnostic assays

Evaluation of these PCR diagnostic methods was achieved through analysis of experiments carried out either in a growth room, or under field conditions. In a controlled environment, and following inoculation at defined sites on leaves, the spread of *S. tritici* was followed until lesions appeared. The pathogen was detected inside the leaf within three days, but for the remainder of the biotrophic phase there was little further increase in pathogen biomass. Only when pycnidial initials begin to form did the amount of *S. tritici* increase. Colonisation of different leaf layers throughout the whole period of crop growth for the winter wheat cultivars Riband and Cadenza, was followed under field conditions. As early as November leaves were infected with *S. tritici*. Depending on rainfall, temperature and the emergence of new leaf layers, the *S. tritici* epidemic continued to spread with variable speed into new leaf layers. Depending on the temperature, the presence of *S. tritici* could be detected 2-5 weeks before symptoms were visible in the field.

The diagnostic was also used to assess fungicide efficacy against *S. tritici* in a crop of Cadenza treated with either epoxiconazole (Opus), azoxystrobin (Amistar) or a mixture of these two fungicides. Both fungicides controlled disease but epoxiconazole was more effective than azoxystrobin, especially in leaf layers infected at the time of spraying (GS 31). This no doubt reflected the better eradicant activity of epoxiconazole compared to largely preventative activity of azoxystrobin.

Quantification of the four wheat diseases in a series of wheat cultivars exposed to natural infection at just one site, generally produced a good correlation with NIAB recommended varietal disease ratings. Exceptions were confined to yellow rust. No rust was detected on some very susceptible cultivars, presumably because of the absence of the matching virulent rust race at that site.

Samples were not only collected for diagnosis from leaf layers, but in some cases from the ears and grain. As expected only the seed borne *S. nodorum* was detected in grain, even though it was not detected in leaves of the same cultivar. The other diseases were confined to the ears and foliage.

Practical implications

This research has generated rapid and robust DNA diagnostic methods, which accurately detect and quantify four important foliar diseases of wheat. Tests can be completed in one day, and can be used equally well for testing leaf samples collected from either field crops or greenhouse plants. False positive results are not a problem. The methods have already been shown to be useful in various research scenarios, including evaluating the effectiveness of fungicide treatments, and assessment of varietal resistance, and epidemiological studies. Various PCR primer sequences and assays are now available in kit form from Adgen limited (Nellies Gate, Auchincruive, Ayr KA6 5HW, Scotland). Although this represents a step towards commercializing this diagnostic technology for the benefit of cereal growers, translating diagnostic measurements into treatment thresholds to guide spray timing and fungicide dose rate remains a stumbling block and needs further research to translate DNA diagnostic technology into a useful decision support tool. Nevertheless, this work has successfully provided some novel technology, which should enable improvements in the precision of disease control measures.

Future research

Future developments in diagnostic technology involving ‘real-time’ PCR and fluorescent probes will improve the accuracy, speed, dynamic range and sample throughput of measuring pre-symptomatic infection levels. But a key requirement to translate these diagnostic methods into useful decision making tools is to determine the relationship between yield benefit, and diagnostic infection level at the time of treatment. Coupling this with the dose rate needed for effective disease control at different infection levels will allow more rational use of fungicides. Clear sampling protocols will need to be developed based on spatial and temporal distribution of each pathogen. Ideally, these protocols will differ between diseases, but different protocols will be impractical, and a common sampling protocol for foliar diseases will need to be defined, through analysis of the degree of heterogeneity within pathogen populations. Ultimately, results from commercially acceptable DNA diagnostics will need to be incorporated into advanced decision support systems, such as DESSAC, which can also advise farmers when to sample a crop at risk from infection. In this way the technology developed in this project will eventually benefit growers through more effective control of foliar diseases, and with less impact on the environment.

BACKGROUND OF RESEARCH INITIATIVE

Foliar diseases of wheat are important, and largely controlled by fungicides, which account for about 30% of grower's variable costs. Up to one third of fungicide applications are badly timed, and managed disease control strategies are more cost effective than prophylactic spraying. Integration of the mainly preventative strobilurin fungicides into managed disease control requires a curative triazole mixture partner. Fungicide mixtures are also a cornerstone of strategies to combat resistance, which has already emerged to strobilurins and triazoles in some diseases. Accurate information on pre-symptomatic disease levels is important, therefore, to ensure that timing and dose rates of these fungicide mixtures are optimal. This project sought to develop a robust, rapid and accurate DNA diagnostic to detect and quantify pre-symptomatically four key pathogens of wheat.

INTRODUCTION

Septoria tritici Roberge in Desmaz. (teleomorph *Mycosphaerella graminicola* (Fuckel) Schröt. in Cohn), the causal agent of Septoria tritici blotch, and *Stagonosporum* (*Septoria*) *nodorum* (Berk.) Castellani and Germano (teleomorph *Phaeosphaeria nodorum* (Muller) Hedjaroude), causing Stagonospora nodorum blotch, are widespread and economically important pathogens of bread wheat (*Triticum aestivum L.*) and durum wheat (*T. turgidum L.)*. In the UK, *S. tritici* infection caused an average annual yield loss of £35 million during the period 1985-1989 (Cook *et al.* 1991). And in 1999, economic losses from Septoria diseases totalled £24 million according to MAFF's survey on the economic impact of different diseases. Discrimination between S. tritici and S. nodorum blotch relies on differences in symptom morphology - lesion size, shape, the distribution of pycnidia on leaves and other plant organs, and pycnidial colour (Eyal, 1999). Despite differences in their physiology and development, *S. tritici* and *S. nodorum* have similar life cycles. The primary inoculum source of leaf blotch are airborne sexual ascospores released from stubble (Brown *et al.* 1978), while rain splash-dispersed asexual pycnidiospores are most important for rapid disease development on upper leaves (Royle *et al.* 1986). Immediately after infection and during early stages of hyphal colonization and initiation of pycnidia within leaf tissue, there are no visible signs that infection has occurred. These latent periods differ; under optimum conditions for *S. nodorum* it can be 7-10 days, while for *S. tritici* it can be 14-21 days.

Measures to control Septoria diseases are primarily focussed on the application of foliar fungicide sprays and, recently, on the deployment of partially resistant germplasm. For optimum, cost-effective control, fungicides must protect the upper three leaves, which provide most of the grain-filling capacity, although applications as early as GS31, reducing initial infection levels in the crop, are often beneficial. Timing and fungicide choice (e.g. with curative and/or protective mode of action) depend on the inoculum level and disease risk of a crop (Royle *et al.* 1995). However, as soon as pycnidia are initiated in the upper three leaves, none of the currently available fungicides with eradicant properties, even the most active triazoles (ergosterol biosynthesis inhibitors) are effective. Recently, fungicides with a novel biochemical action, famoxadone (Joshi and Sternberg 1996) and strobilurins (Godwin *et al.* 1992), which both inhibit

mitochondrial respiration, have been developed. To ensure maximum effect of these new fungicides, which mainly have systemic protective properties and some curative activity, new decision-making strategies about spray timing and resistance management are needed (Knight *et al.* 1997). Results obtained with ELISA showed that an accurate pre-symptomatic 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 test based on DNA diagnostics should detect *S. tritici* earlier in its latent phase, allowing well-timed fungicide sprays to eradicate the disease. This would also enhance current risk assessment models (Lovell *et al.* 1997) and lead to more profitable disease control with less impact on the environment by reducing the fungicide inputs.

Polymerase chain reaction (PCR) assays are rapid techniques with high specificity and sensitivity. PCR has been used for identification and detection of several wheat pathogenic fungi, including *Fusarium* species (Doohan *et al.* 1998), *Gaeumannomyces graminis* (Schesser *et al.* 1991), *Microdochium nivale* (Nicholson *et al.* 1996), *Pseudocercosporella herpotrichoides* (Nicholson *et al*. 1997), *Rhizoctonia solani* (Nicholson and Parry 1996), *Stagonospora nodorum* and *S. tritici* (Beck and Ligon 1995). Most of the PCR primer sequences were deduced from variable ribosomal DNA (rDNA) internal transcribed spacer regions (ITS) or from products amplified by random amplified polymorphic DNA (RAPD) assays. Beck *et al.* (1996), using ITS-derived primers, detected DNA of *S. tritici* and *S. stagonospora* in extracts of wheat leaves, which did not show any symptoms. However, microtitre plate formatted ELISA-type methods to quantify the PCR products, involving DNA capture steps and labelling reactions, as described by Holmstrøm *et al.* (1993) and Nikiforov *et al.* (1994), were too laborious and time-consuming. New advances in DNA diagnostics in which single tube PCR assays detect more than one disease, are emerging as the main DNA diagnostic format. The PCR-amplified products can be measured directly with real-time PCR technology using DNA intercalator or probe based fluorimetric assays. This offers real benefits in diagnostics through simple, rapid and specific high-throughput quantification.

OBJECTIVE

The project aimed to develop generic, quantitative microtitre plate-formatted DNA diagnostic for the detection of plant diseases, using β-tubulin as the target. Although the β-tubulin gene is well conserved amongst different fungal species, the presence of less conserved regions within the gene, e.g. introns, allows the design of species-specific primers for the polymerase chain reaction (PCR). Because of the economic impact, *S. tritici* was chosen as model pathogen for this project. A DNA diagnostic will allow studying the colonization, infection and subsequent disease development of *S. tritici* on different wheat cultivars, both under controlled conditions in the glasshouse, as well as in the field. The research will provide a tool to improve fungicide efficacy through more rational precise timing of applications before leaf blotch is visible in the field. Depending on the progress of the project, PCR assays for the detection of *S. nodorum*, *Puccinia striiformis* (yellow rust) and *Puccinia recondita* (brown rust) will also be developed. With these assays wheat varietal resistance levels for all the important foliar diseases can be studied.

MATERIALS AND METHODS

Fungal strains

The fungal isolates and DNA used in this study and their sources are listed in Table 1. After 8 days incubation at 18^{o}C, mycelia and spores from *Rhynchosporium secalis*, *S. nodorum* and *S. tritici* isolates were collected from Czapek Dox Agar (modified) plates (Oxoid), directly by scraping or after adding distilled water. Fungal biomass and/or DNA from the remaining fungi, e.g. *P. striiformis* and *P. recondita*, were obtained directly from colleagues. To inoculate wheat leaves, dilutions of *S. tritici* ST16 spore suspensions were made in distilled water supplemented with 0.05 % (v/v) Tween-20.

Table 1 Fungal isolates tested.

Fungal isolates	Origin	Source
Septoria tritici ST16	UK	Wheat
Septoria tritici α12-3B.8	USA	Wheat
Stagonospora nodorum ***SN1***	UK	Wheat, cv Longbow
Puccinia striiformis PS1	UK	Wheat, cv Maverick
Puccinia striiformis PS2	UK	Wheat, cv Brigadier
Puccinia recondita PR1	UK	Wheat, cv Riband
Puccinia recondita PR2	UK	Wheat, cv Buster
Gaeumannomyces graminis T7	UK	Wheat
Rhynchosporium secalis 1130	UK	Barley
Rhynchosporium secalis 812	UK	Barley
Tapesia acuformis 202-3	UK	Wheat
Tapesia yallundae 22-432-1	UK	Wheat

Wheat leaf, ear and grain samples from the field

During the growing seasons of 1997-1999 wheat leaves with and without visual symptoms, showing lesions with pycnidia, and treated or not with fungicides, were collected from different leaf layers and fields throughout England (Table 2). During 1997 and 1998 also, leaf samples from at least 20 different wheat cultivars were tested to study varietal resistance for *S. tritici*, *S. nodorum*, *P. striiformis* and *P. recondita*. To analyse grains, husks were separated and grains were washed with 70% (v/v) ethanol. After soaking the grains in a 10% hypochlorite solution for 2 min, grains were rinsed in sterile water and dried. For DNA extraction, ten grains per sample were processed. Additionally, for two field-sown crops, cv Cadenza and Riband, the fungal colonization of successive leaf layers by *S. tritici* was studied during the entire growing season. The effect of different fungicide applications on the epidemics of *S. tritici* on the top three leaves of these plots was also followed in time by PCR testing. Samples consisting of 10 leaves of each leaf layer were taken at weekly intervals from these plots.

Table 2 Wheat leaf samples tested

Samples	Wheat cultivar	Symptoms	Locality
1	Unknown	+	Gloucestershire
2	unknown	+	Isle of Wight
3	unknown	+	Somerset
4	unknown	-	Somerset
5	unknown	+	Somerset
6	unknown	-	Somerset
7	unknown	+	Gloucestershire
8	unknown	-	Gloucestershire
9	unknown	-	Devon
10	unknown	+	Devon
11	Riband*	-	Lincolnshire
12	Spark†	-	Lincolnshire
13	Hereward*	-	Lincolnshire
14	Riband†	-	North Somerset
15	Cadenza†	-	North Somerset
16	Cadenza*	-	North Somerset
17	Cadenza	-	glasshouse
18	Cadenza‡	+	glasshouse

***** Wheat cultivar treated with fungicides.

† Wheat cultivar not treated with fungicides.

‡ Wheat cultivar inoculated with *S. tritici* ST16

Infection of wheat leaves by *S. tritici* under controlled conditions

To obtain healthy leaves, seedlings of different cultivars were grown in a growth room at 18^{o}C with a 12 h/12 h light/dark alternation. Seedlings of different cultivars at GS11 (Zadoks growth stage (Tottman 1987)), 8 per pot, were inoculated by placing two 6 µl droplets of spore suspension (*ca* 10^5 spores ml^{-1}) of *S. tritici* on each emerged second leaf (leaf 2). Immediately after inoculation, pots were placed in water-saturated trays and covered with polyethylene bags. The seedlings were grown in controlled-environment cabinets, at either 12 or 18^{o}C, and with a 12h/12h light/dark alternation. Leaf samples, consisting of equally sized parts of four different leaves containing the inoculation spots, were taken daily, whilst keeping the environment water-saturated. After extracting DNA from the leaves, a fixed amount of template DNA was tested in the PCR/PicoGreen assay to quantify *S. tritici* DNA.

DNA extraction

DNA was extracted directly from mycelium or single leaves by powdering the samples in liquid nitrogen using a pestle and a mortar. Subsequently, 840 µl DNA extraction buffer consisting of 40 µl 1% (v/v) β-mercapthoethanol, 400 µl TEN buffer (500 mM NaCl, 400 mM Tris-HCl, 50 mM EDTA; pH 8.0) and 400 µl 2% (w/v) SDS was added to each sample while mixing. For larger wheat leaf samples, the amount of extraction buffer to be added was increased until the mixture could be poured. After incubating the mixture for 30 min at 70^{o}C, 400 µl ice-cold ammonium acetate (7.5 M) was mixed with the heat-treated sample and the total suspension kept on ice for 30 min. After centrifugation at 10,000 rpm for 10 min, an equal volume of cold (-20^{o}C) isopropanol was added to the supernatant and the extract shaken at room temperature for 15 min. After centrifugation at 6,000 rpm for 5 min, DNA pellets were washed with ice-cold 70% (v/v) ethanol, centrifuged again and dissolved in 500 µl sterile distilled water. For every sample, the DNA concentration was measured with the PicoGreen assay. The DNA yield of a moderate-sized leaf grown in the glasshouse was *ca* 25 µg.

Cloning of fungal β-tubulin gene sequences

In order to amplify and clone part of the β-tubulin gene, primers BAF6 and BAF2 (Table 3) derived from the DNA sequence of the β-tubulin gene from *S. nodorum* were used using low annealing temperatures.
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 Corporation, Madison, Wisconsin). Plasmids were transformed into *Escherichia coli* JM109 cells (Promega Corporation, Madison, Wisconsin) according a standard protocol (Sambrook *et al.* 1989) and plasmid DNA extracted using the RPM kit (Bio101 Inc., Carlsbad, CA). Finally, sequences were analysed in a dideoxy chain termination method reaction (Sanger *et al.* 1977).

Primer design

To design specific primers different parts of the coding sequence of the β-tubulin gene of *S. tritici* (Payne *et al.* unpublished), *S. nodorum* (Cooley *et al.* 1991), *Puccinia striiformis* (Fraaije unpublished) and *Puccinia recondita* (Fraaije unpublished) were used. Reverse and forward 24-mer primers with unique 3'-ends and melting temperatures of around 65^{o}C were designed by aligning and comparing corresponding β-tubulin sequences of other fungi, such as *Aspergillus nidulans*, *Erysiphe graminis*, *Neurospora crassa*, *R. secalis* and *S. nodorum* (EMBL database). To determine the sensitivity and specificity of the 'β-tubulin' primers and the PCR/PicoGreen assay, DNA from several fungi, inoculated wheat leaves and samples from different geographical locations, with and without symptoms, were tested. Subsequently, for *S. tritici*, results of the 'β-tubulin' primers were compared with the 'ITS' primers described by Beck and Ligon (1995).

Table 3 Primer sequences used in this study

Primer designation[a]	Sequence (5'-3')	Target organism
BAF6 (f)	ACCCACAACCGCCAACATGCGTGA	*S. nodorum*
BAF2 (r)	CGTACCGGGCTCGAGATCGACGAG	*S. nodorum*
E1 (f)	CGGTATGGGAACACTTCTCATCAG	*S. tritici*
STSP2 (r)	GTAACGACCGTTGCGGAAATCGCT	*S. tritici*
STIF2 (f)	ACTCACAATCCTCATTCGACGCGA	*S. tritici*
BAF4ST (r)	GACCAATTCGGCACCCTCAGTGTA	*S. tritici*
SNSP7 (f)	CCGGTCAGCTCAACTCTGACCTGA	*S. nodorum*
CONS1 (r)	CCAATGCAAGAAAGCCTTGCGCCT	*S. nodorum*
YRNT1 (f)	CTTCAAGATCGGTGGCCTGACCGA	*P. striiformis*
YRNT2 (r)	GTGAGCTGTGAAGGGATCGCGGGA	*P. striiformis*
BR3 (f)	TCCCAAAGCAAGCCCAAATACACG	*P. recondita*
BR2 (r)	GAATGTTTCACAGCAGCTGCTGGT	*P. recondita*

[a] Primer designation; primer orientation forward (f) or reverse (r) between brackets

Standard PCR protocol

A standard PCR was carried out on a Biometra T3 thermocycler (Anachem) with 0.5 units of red hot DNA polymerase (ABgene, 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 dTTP, dATP, dCTP and dGTP, 0.5 µM primers and template DNA in a final volume of 40 µl. For testing wheat leaf samples, 200 ng of template DNA per reaction was always used. The PCR conditions were 94^{o}C for 3.5 min, followed by 40 cycles at 94^{o}C for 30s, 65^{o}C for 1 min and 72^{o}C for 1.5 min. The PCR was terminated with a final DNA extension at 72^{o}C for 8.5 min. Gel electrophoresis was used to analyse PCR amplified products. PCR products in a 10 µl sample were separated on an 1.3 % (w/v) agarose gel containing ethidium bromide and exposed to u.v. light to visualise DNA fragments.

Fluorometric pre- and post-PCR DNA quantification by PicoGreen

PicoGreen (Molecular Probes, Leiden, The Netherlands) is a cyanine dye which specifically binds with dsDNA (Singer *et al.* 1997) and can be used for quantitative pre- and post-PCR evaluation of DNA products (Ahn *et al.* 1996). The PCR amplified DNA of *S. tritici* of wheat leaf samples was quantified using appropriate calibration curves. These were generated using wheat leaf DNA samples spiked with different amounts of genomic DNA of *S. tritici* which were run simultaneously in each experiment using the PCR/PicoGreen assay. From each crude DNA extract or PCR assay, 3 µl of sample was incubated at room temperature in a well of microtitre plate with 150 µl PicoGreen solution (1:400 dilution in 1 mM EDTA, 10 mM Tris-HCl, pH 8.0). Fifteen min after excitation at 480 ± 2.5 nm, fluorescence was measured at 523 ± 2.5 nm using a Perkin Elmer LS50B luminescence spectrometer with microtitre plate attachment (Perkin Elmer, Seer Green, UK).

RESULTS

Extraction of template DNA

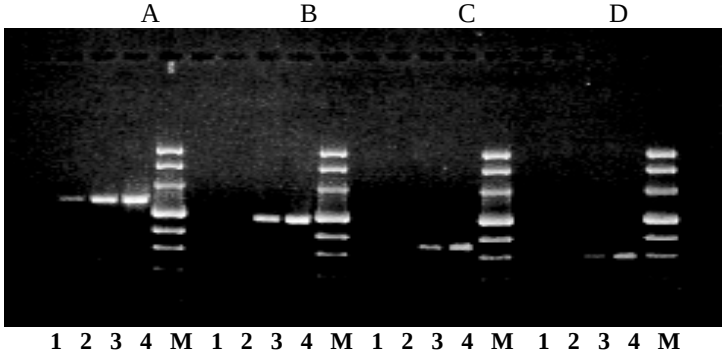
The first step in developing a PCR assay to detect *S. tritici* in wheat leaves was to find a simple method to extract template DNA from wheat leaf samples. A method was finally developed based on grinding the leaves with liquid nitrogen using a pestle and mortar, and subsequent extraction of DNA using an extraction buffer, ammonium acetate, isopropanol and ethanol (see material and methods). Although extraction is laborious, it is simple to perform without using the hazardous chemicals phenol and chloroform.

Specificity of β-tubulin primers for *S. tritici*

Several sets of primers directed against different regions of the β-tubulin gene of *S. tritici* were tested for specificity at different $MgCl_2$ concentrations and annealing temperatures. Many primer combinations generated multiple products, often caused by amplification of wheat DNA (results not shown).

However, three combinations of primers, E1/STSP2R, BAF4ST/STIF2 and CP2/STIF2 (Table 3), produced a single fragment from genomic DNA of *S. tritici* of 496, 555 and 637 bp, respectively, whereas that from wheat and the other fungi tested was not amplified. The PCR product amplified with primer set BAF4ST/STIF2 is shown in Figure 1.

Figure 1 Ethidium bromide-stained agarose gel of PCR amplified products. Primer sets STIF2/BAF4ST, SNSP7F/CONS1R, YRNT1/YRNT2 and BR3F/BR2R were used 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.



S. tritici (555 bp); S. nodorum (464 bp); P. striiformis *(351 bp);* P. recondita *(300 bp)*

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;

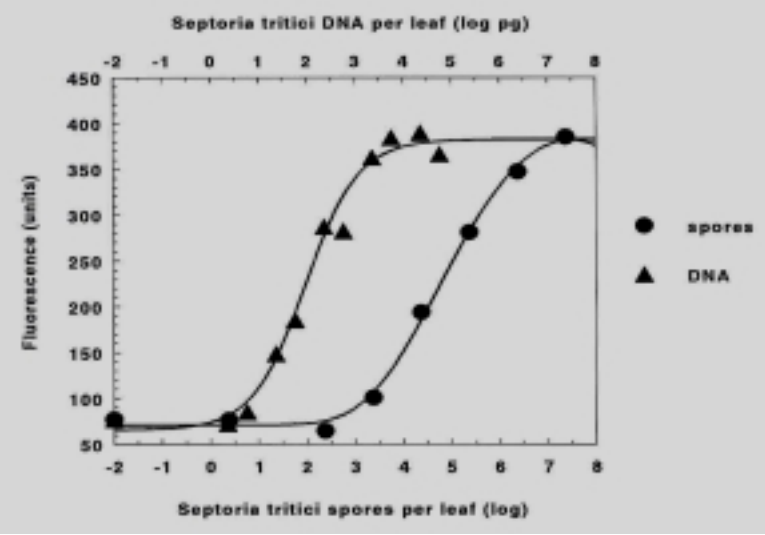
D, Lanes: 1, control; 2, 6.8 pg; 3, 68 pg; 4, 680 pg DNA of *P. recondita* added;

M, DNA ladder VIII (Boehringer, Mannheim, Germany)

Sensitivity and specificity of the PCR/PicoGreen assay

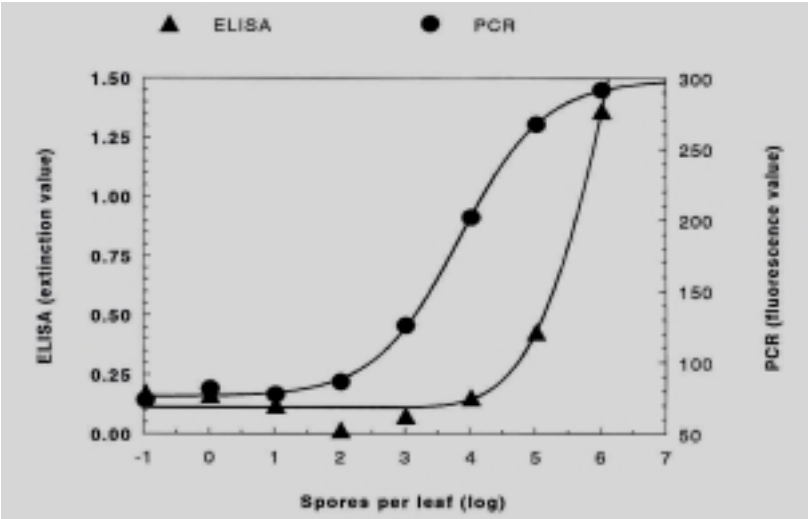
Because of the amplification of a single DNA fragment, PCR products could be quantified directly with the PicoGreen assay. With this assay, as few as 1000 spores per leaf, or *ca* 10 pg of *S. tritici* DNA per 200 ng wheat leaf DNA, were detected (Fig. 2).

Figure 2 Detection and quantification of *S. tritici* in DNA samples of inoculated wheat leaves and in spiked DNA samples of wheat leaves by the PCR/PicoGreen assay using primers E1 and STSP2.



ELISA detection (kit kindly supplied by DuPont, UK) of *S. tritici* was 10-100 fold less sensitive than PCR (Fig. 3). The background fluorescence, primarily caused by the amount of wheat leaf DNA and primer-dimer formation during the PCR, was always below 50 and 90 units, for samples without target DNA and DNA obtained from uninfected wheat leaves, respectively.

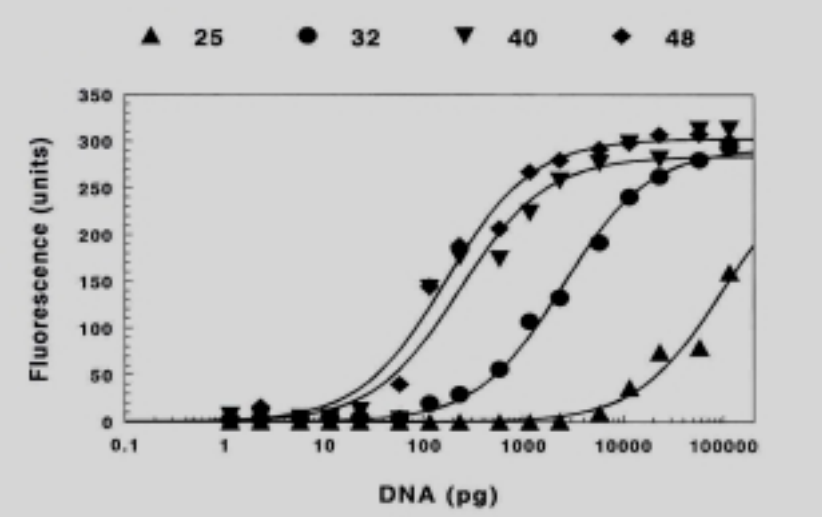
Figure 3 Detection and determination of detection thesholds of ELISA and PCR in *S. tritici* inoculated wheat leaves.



Further optimization of PCR/PicoGreen assay

At 200 ng per reaction, the amount of template DNA was already optimal with regard to the signal/dsDNA background ratio in the PCR/PicoGreen assay. However, the background fluorescence signal of the PCR/PicoGreen assay could be reduced by decreasing the primer concentration from 0.5 to 0.2 µM (results not shown). This allowed 5 µl of the PCR sample, instead of 3 µl, to be tested in the PicoGreen assay without exceeding the upper detection limit of *ca* 200 ng DNA per well. The sensitivity of the PCR/PicoGreen assay was further influenced by the number of PCR cycles conducted. For different cycle numbers, the calibration curves generated by the PCR/PicoGreen assay are shown in Fig. 4.

Figure 4 Calibration curves for quantification of *S. tritici* in wheat leaves generated by the PCR /PicoGreen assay using different cycle numbers for PCR. The different cycle numbers are shown for each curve.

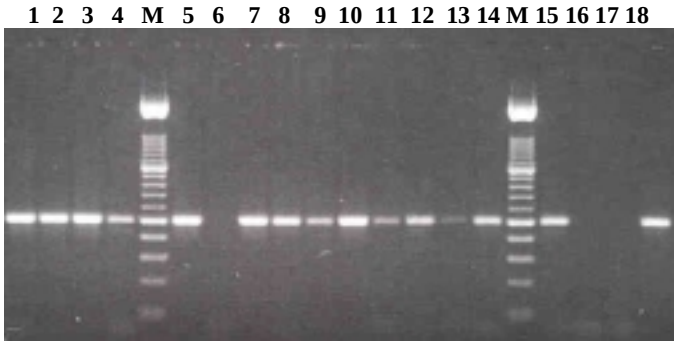


For each cycle number, the fluorescence value was corrected for background fluorescence, because after many cycles (> 30) the background increased significantly due to primer self-annealing and non-specific amplification. For an accurate and reliable quantification of *S. tritici* in wheat leaf tissue, only the linear part of a calibration curve can be used. To quantify small amounts of *S. tritici* DNA (< 1 ng) in wheat leaves, 40 cycles are required, whereas fewer cycles are needed for quantification of larger amounts of *S. tritici* DNA (>1 ng) present in leaves with symptoms.

Wheat leaf sample testing

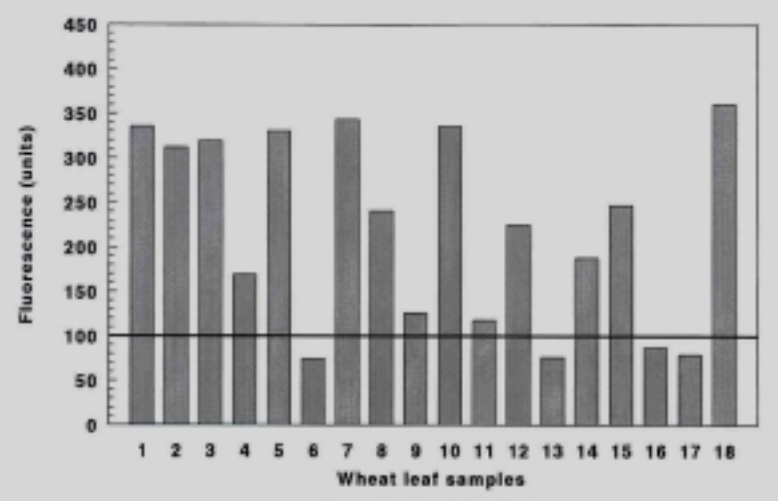
The specificity of the primers and sensitivity of the PCR/PicoGreen assay was further demonstrated for field samples collected from different localities (Table 2). Using an annealing temperature of 60^{o}C, the 'ITS' primers produced two products, a 345 bp fragment specific for *S. tritici* and a second product of *ca* 280 bp, which was also amplified from 'healthy' wheat leaf samples (data not shown). This demonstrated that where the same samples were also tested using the 'β-tubulin' primers, there was only one product specific for *S. tritici* (Fig. 5).

Figure 5 Detection of *Septoria tritici* in wheat leaves by PCR using primers E1 and STSP2. Size of the amplified product is 496 bp. Lane numbers represent the sample number of the samples shown in Table 2. M, DNA ladder XIV (Boehringer, Mannheim, Germany).



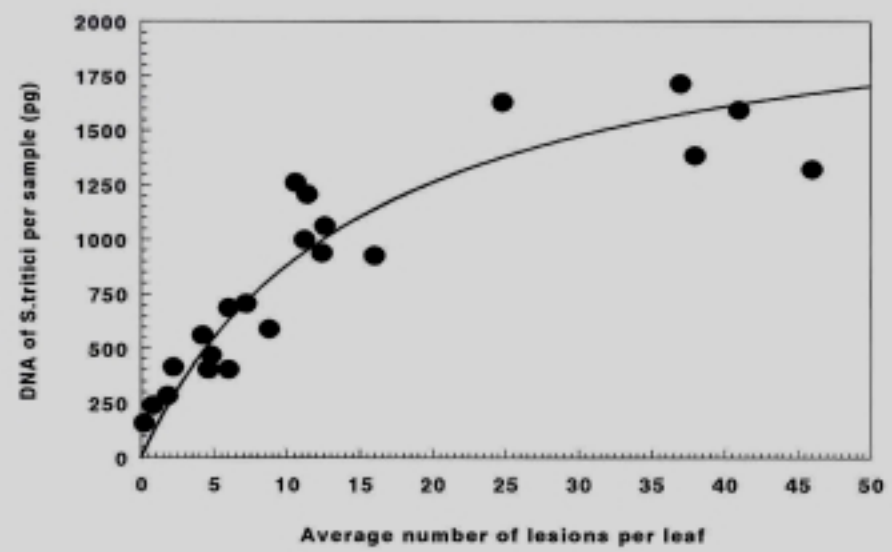
Results obtained with the PicoGreen assay showed that the PCR products could be quantified. Leaves with lesions containing pycnidia produced high fluorescence values (≥ 300 units) in the PCR/PicoGreen assay (Fig. 6).

Figure 6 PicoGreen dsDNA quantification of PCR products using primers E1 and STSP2. Lane numbers represent the sample number of the samples shown in Table 2.



After testing more samples, a clear relation between the number of pycnidia-bearing lesions and *S. tritici* biomass expressed in pg of DNA was obtained (Fig. 7). Infected leaves, which had not developed symptoms, generated low fluorescence values (100-300 units) in the linear part of the calibration curve (Fig. 2). For some samples, which were either negative or weakly positive, spiking these samples with ca 25 pg *S. tritici* DNA checked the possibility of inhibition of the PCR. After spiking, all samples were found to be positive, indicating that no inhibition occurred. Additionally, the amplification of the 280 bp wheat DNA fragment from the *S. tritici*-negative samples with the 'ITS' primers also showed that the PCR was not inhibited (data not shown).

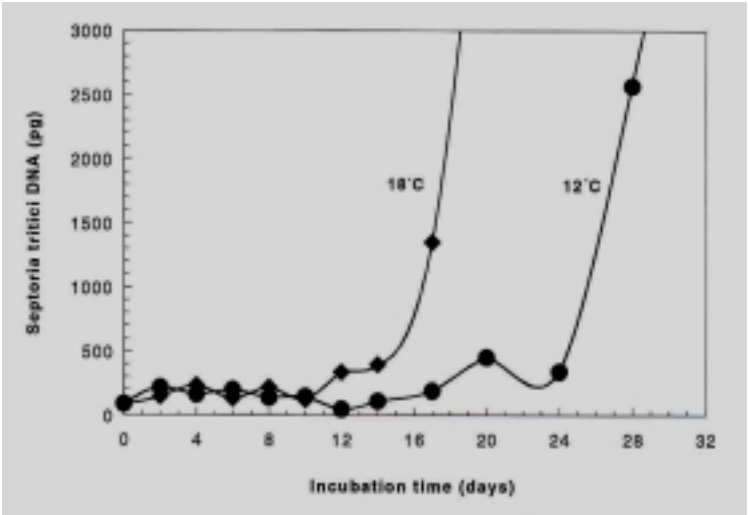
Figure 7 Relation between the amount of visual symptoms and infection levels of *S. tritici* measured with the PCR/PicoGreen assay.



Quantitative detection of *S. tritici* in inoculated wheat leaves during disease development under controlled conditions

The amount *S. tritici* biomass in leaf 2 of cv Riband and Axona was measured during infection using the PCR/PicoGreen assay (Fig. 8). At 18°C, the first symptom was discoloration, which emerged 6 days after inoculation. After 14 days, necrotic lesions were visible and pycnidia subsequently developed 17 days after inoculation. At 12°C, pycnidia emerged only 24 days after inoculation, when significant necrosis and discoloration were not visible. A slight increase of fungal biomass was measured immediately after inoculation. This increase in biomass was higher for Axona, possibly leading to an earlier and more intense discoloration. Then biomass remained steady, or even slightly decreased, but following necrosis and pycnidia production, the amount of biomass increased exponentially.

Figure 8 Measurement of biomass of *S. tritici* during infection of wheat leaves at 12 and 18°C

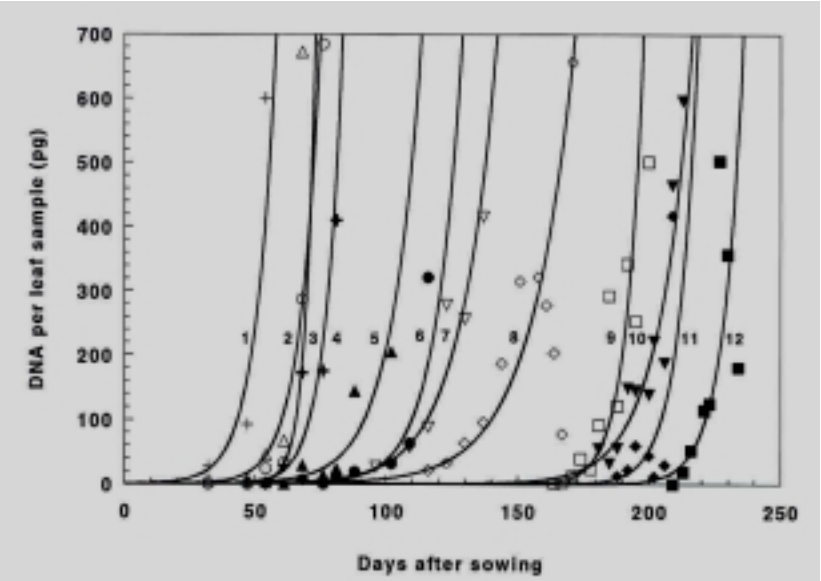


Quantification of *S. tritici* colonization and infection of field sown crops

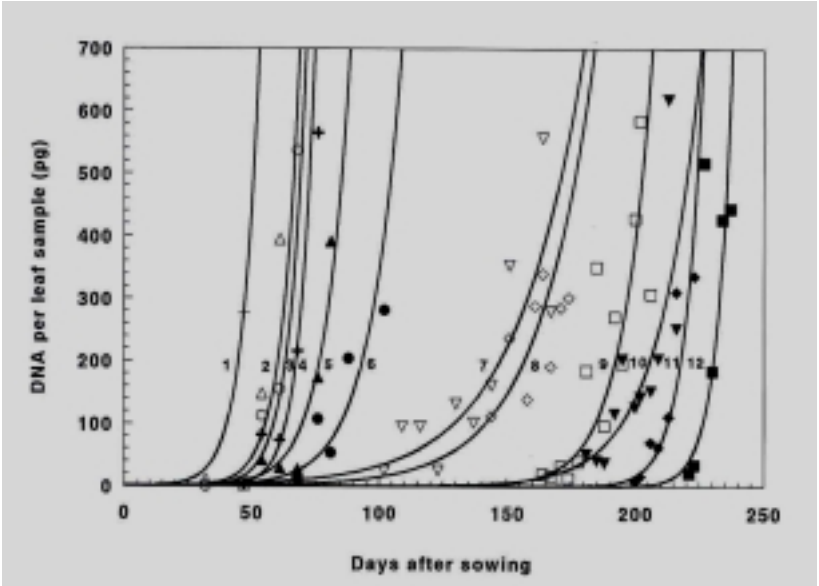
For cv. Cadenza and Riband, the colonization of *S. tritici* in all leaf layers was studied in the field with the PCR/PicoGreen assay during the entire growing season (Fig. 9). Already, as early as November leaves were infected with *S. tritici*. Depending on rainfall, temperature and the emergence of new leaf layers, the epidemics of *S. tritici* continued to spread with variable speed into new leaf layers. Depending on the temperature, the presence of *S. tritici* could be detected 2-5 weeks before symptoms were visible in the field.

Figure 9 Monitoring of *S. tritici* infection levels on different leaf layers in the field. A, Cultivar Cadenza; B, Cultivar Riband

A



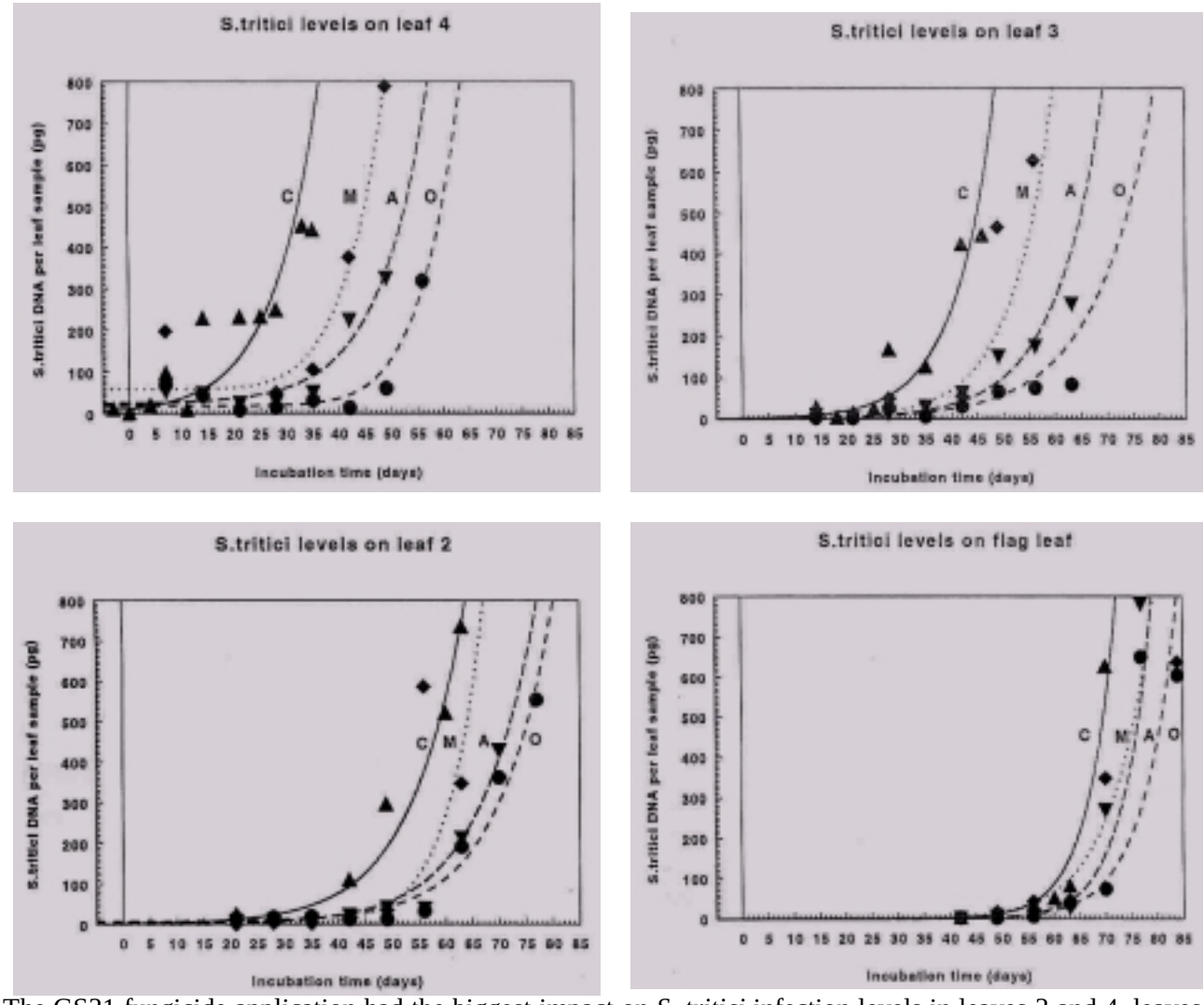
B



Fungicide efficacy testing

During the 1997/1998 growing season the effects of different fungicide applications on *S. tritici* epidemics in the field was measured with DNA diagnostics. The previous mentioned crop of cv Cadenza, grown nearby at Long Ashton Research Station, was treated at GS31 with Opus (epoxiconazole; 1L/ha), Amistar (azoxystrobin; 1L/ha) or with the mixture of Opus (0.25 L/ha) and Amistar (0.5L/ha). The last treatment was repeated at the same plot around GS 37. Samples were also collected from untreated control plots. The infection levels of *S. tritici* on leaf 4, 3 and 2 and the flag leaf were measured in time with the PCR/PicoGreen assay (Fig. 10).

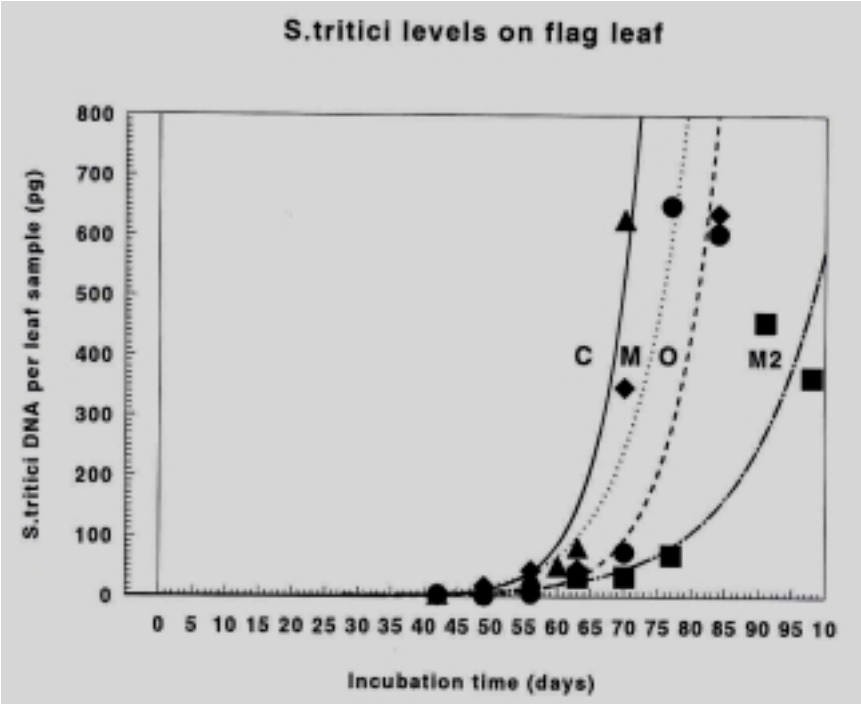
Figure 10 The effect of different fungicide treatments on *S. tritici* infection levels on different leaf layers of cv Cadenza in the field. C, Control, no fungide applied; M, Mixture of Opus (0.25 L/ha) and Amistar (0.5 L/ha); A, Amistar (1 L/ha); O, Opus (1 L/ha). Fungicides applied on 12/3/98 (GS31) (day 0).



The GS31 fungicide application had the biggest impact on *S. tritici* infection levels in leaves 3 and 4, leaves that either had emerged (leaf 4), or were just beginning to emerge (leaf 3). However, in comparison with the untreated plot, the epidemic of *S. tritici* on leaf 2 and the flag leaf was delayed long after this fungicide

application. At full dose rate Opus controlled *S. tritici* better than Amistar, probably due to its better eradicative property as low infection levels of *S. tritici* on the target leaves were already present at the time of application. Although the first application of the Opus (1/4 dose)/Amistar (1/2 dose) mixture was less effective than full dose rate applications of either fungicide alone, the second treatment gave the flag leaf extra protection (Fig. 11).

Figure 11 The effect of an additional fungicide spray on *S. tritici* infection levels on the flag leaf of cv Cadenza. C, Control, no fungide applied; M, Mixture of Opus (0.25 L/ha) and Amistar (0.5 L/ha); O, Opus (1 L/ha); all applied on 12/3/98 (GS31); M2, extra application of the mixture of Opus (0.25 L/ha) and Amistar (0.5 L/ha) on 8/5/98 (GS37) (day 57)



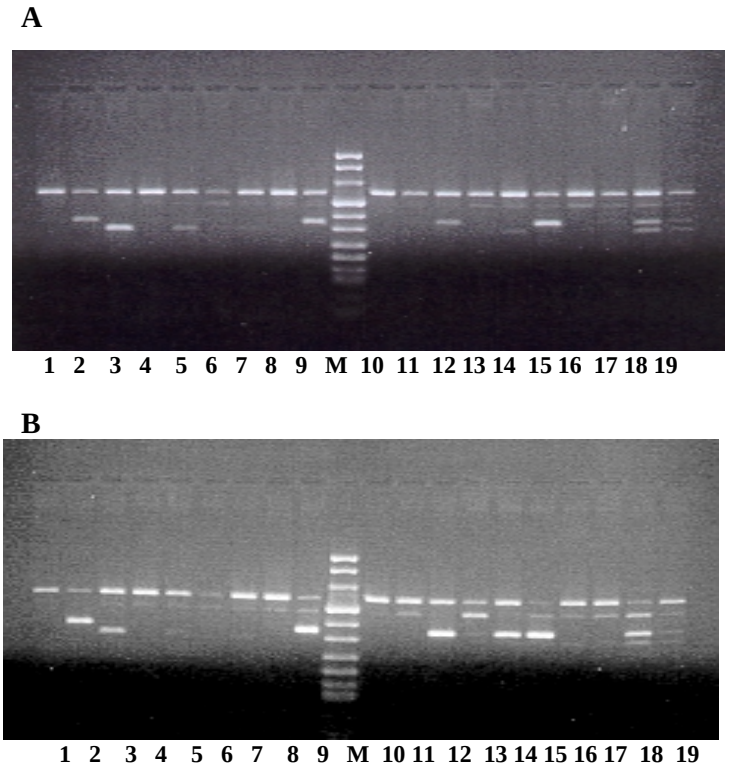
Simultaneous detection of *S. tritici, S. nodorum, P. striiformis* and *P. recondita*.

For *S. nodorum*, like *S. tritici*, several sets of primers directed against different regions of the β-tubulin gene were tested for specificity, at different $MgCl_2$ concentrations and annealing temperatures. Only primer set SNSP7F/CONS1R (Table 3), amplifying a fragment of 464 bp (Fig. 1), was specific enough to allow a reliable detection and quantification of *S. nodorum* in infected wheat leaves. Based on fragments amplified using primer set BAF6 and BAF2, *P. striiformis* and *P. recondita* have at least two different β-tubulin genes. The presence of more than one β-tubulin gene, and the variability observed in the DNA sequence of these genes in different isolates, prevented development of a specific diagnostic for these two rust pathogens. Fortunately, for both *P. striiformis* and *P. recondita* an additional, and specific, DNA fragment was amplified with primer set BAF6 and BAF2, and the sequence of these fragments was used to design specific primers (Fraaije *et al.*, unpublished). For *P. striiformis*, a single product of 351 bp was amplified with primer set YRNT1/YRNT2, while a 300 bp product was generated from *P. recondita* with primers BR3F and BR2R (Fig. 1). Because primers BAF4ST, STIF2, SNSP7F, CONS1R, YRNT1, YRNT2, BR3F and BR2R all have

identical optimum annealing temperatures, but the sizes of amplified products are different, *S. tritici*, *S. nodorum*, *P. striiformis* and *P. recondita* could be detected simultaneously in one multiplex PCR assay combined with gel electrophoresis. Results of wheat varietal resistance testing of the flag leaf at GS42 during 1998 and 1999 are shown in Figure 12.

Figure 12 Wheat varietal resistance testing. Ethidium bromide-stained agarose gel of products amplified with multiplex PCR. Flag leaves of different cultivars from field trials in Long Ashton in 1998 (GS 64) (A) and 1999 (GS 60-61) (B) were tested.

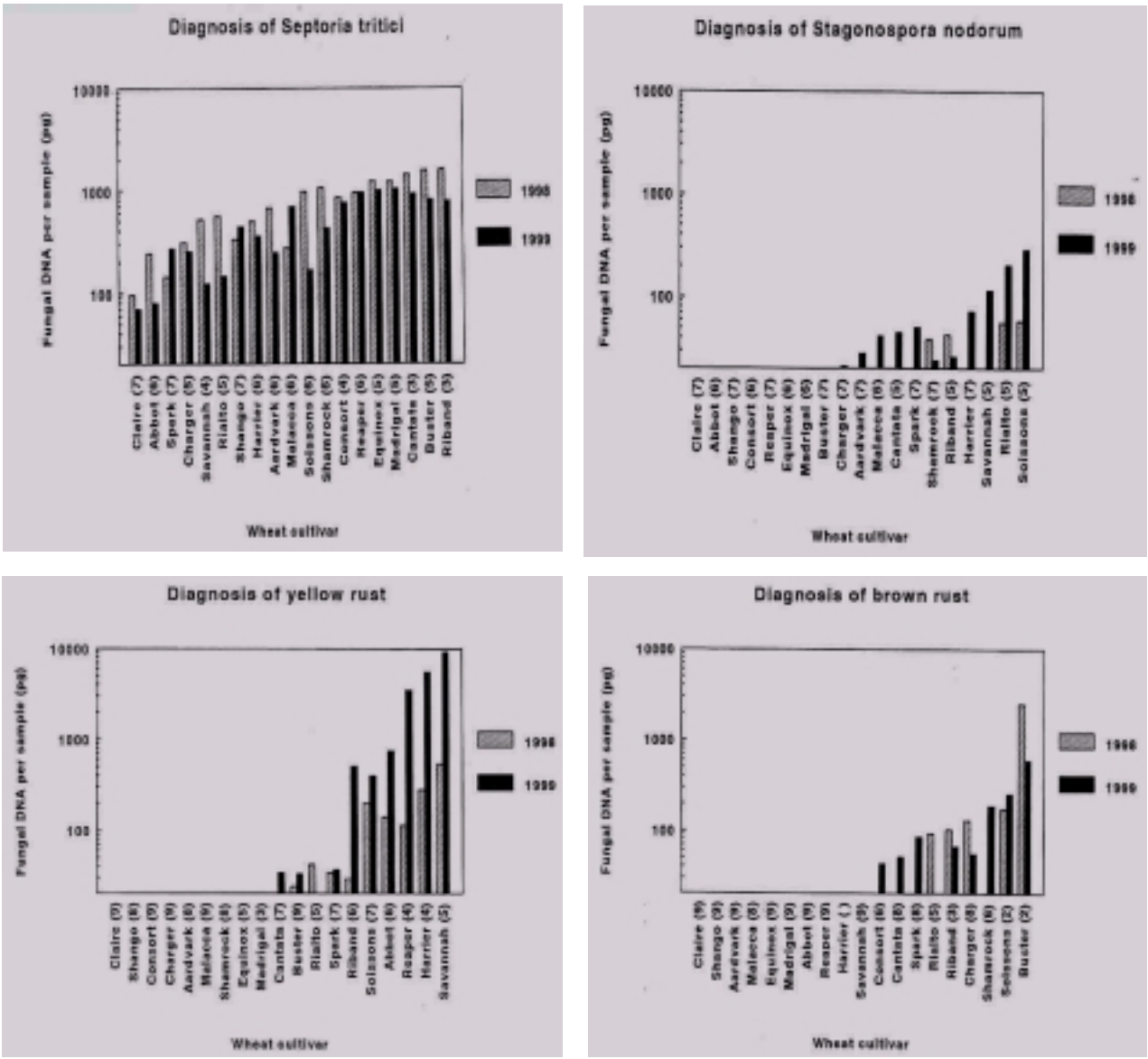
Lanes: 1, cv Aardvark; 2, Abbot; 3, Buster; 4, Cantata; 5, Charger; 6, Claire; 7, Consort; 8, Equinox; 9; Harrier; 10, Madrigal; 11, Malacca; 12, Reaper; 13, Rialto; 14, Riband; 15, Savannah; 16, Shamrock; 17, Shango; 18, Soissons; 19, Spark; M, DNA ladder VIII (Boehringer, Mannheim, Germany)



S. tritici (555/561 bp); *S. nodorum* (464 bp); *P. striiformis* (351 bp); - *P. recondita* (300 bp)

For quantification of the infection levels of each disease, PCR/PicoGreen assays have to be performed separately with each specific primer set for each pathogen. In general, there was a good correlation with the NIAB Recommended List of varietal disease resistance ratings (Fig. 13).

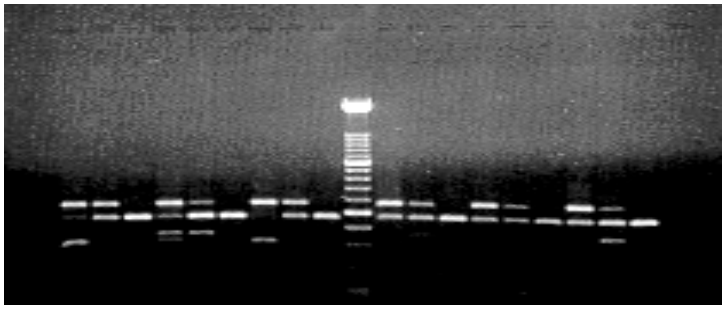
Figure 13 Wheat varietal resistance testing. Infection levels of foliar pathogens on flag leaves of different cultivars at GS 64 (1998) and GS 60-61 (1999) measured with the PCR/PicoGreen assay. Results expressed as the amount of genomic DNA in pg of each pathogen. The NIAB resistance rating of each cultivar is given between brackets. A, *S. tritici*; B, *S. nodorum*; C, *P. striiformis*; D, *P. recondita*. Striped bar, 1998; black bar, 1999.



No yellow rust was detected in the susceptible cultivars Madrigal and Equinox, however, this can be explained by the absence of corresponding specific pathogenic races in SW England. Infection by each foliar pathogen of various leaf layers, ear and grain could be studied and followed in time (Figs 14,15). As expected for a seed-borne fungal pathogen, high infection levels for *S. nodorum* were measured on the ear and in the grain.

Figure 14 Detection of *S. tritici*, *S. nodorum*, *P. striiformis* and *P. recondita* on flag leaf (GS 77-83), ear (GS 85-91) and grain (GS 92) in 1999. Ethidium bromide-stained agarose gel of multiplex PCR amplified products.

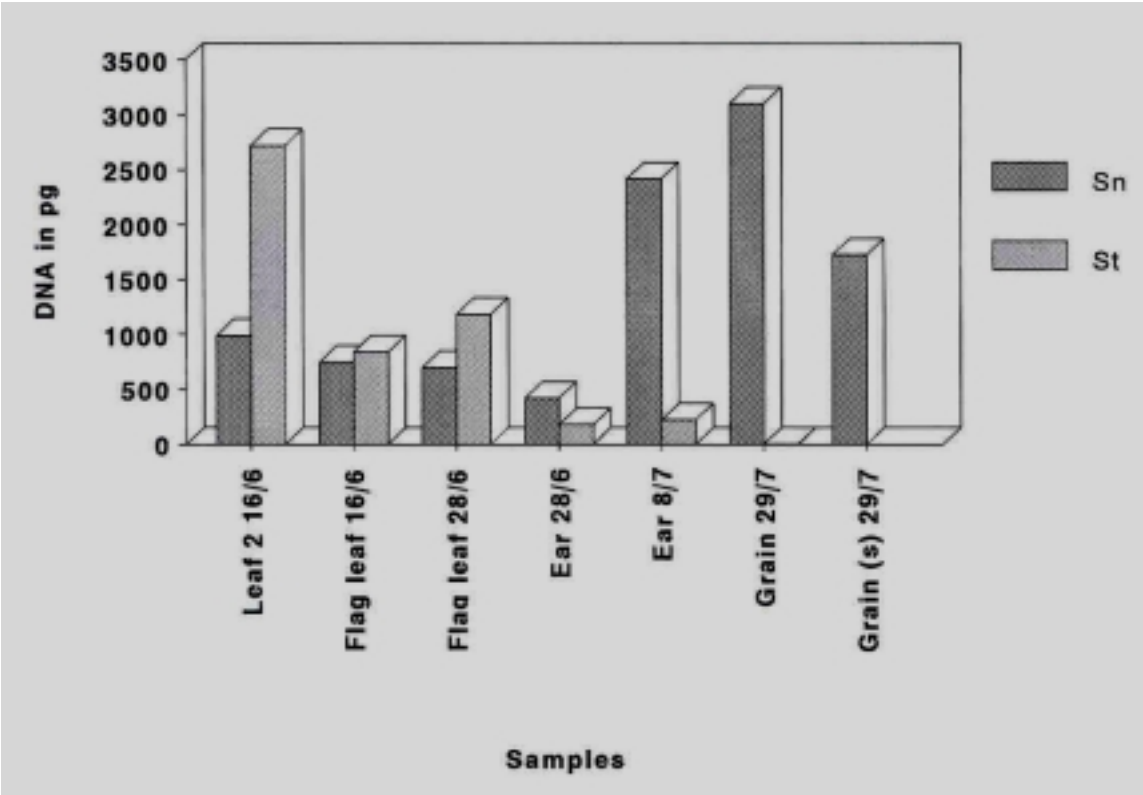
Lanes: 1, cv Buster flag; 2, Buster ear; 3, Buster grain; 4, Soissons flag; 5, Soissons ear; 6, Soissons grain; 7, Consort flag; 8, Consort ear; 9; Consort grain; 10, Riband flag; 11, Riband ear; 12, Riband grain; 13, Claire flag; 14, Clair ear; 15, Claire grain; 16, Savannah flag; 17, Savannah ear; 18, Savannah grain L3; 19, Control, Spark uninfected leaf from glasshouse; M, DNA 100 bp ladder XIV (Boehringer, Mannheim, Germany)



1 2 3 4 5 6 7 8 9 M 10 11 12 13 14 15 16 17 18 19

S. tritici (555/561 bp); *S. nodorum* (464 bp); *P. striiformis* (351 bp); - *P. recondita* (300 bp)

Figure 15 Detection and quantification of Septoria diseases on cultivar Soissons. Infection levels of *S. tritici* (St) and *S. nodorum* (Sn) on leaf 2, flag leaf, ear and grain at different GS in 1999. Infection levels measured with the PCR/PicoGreen assay. Grain (s), washed and sterilized



DISCUSSION

After performing PCR, the sensitivity of gel electrophoresis for detecting *S. tritici* was slightly higher than that for the PicoGreen assay. The PicoGreen assay detected *ca* 10 pg of *S. tritici* DNA per reaction, whereas the sensitivity of gel electrophoresis of *ca* 2 pg of DNA was equivalent to two spores or copies of the β-tubulin gene. However, unlike gel electrophoresis, the PicoGreen assay is quantitative, quick and easy to perform in a microtitre plate format, allowing high-throughput screening. To detect and quantify the infection levels of *S. tritici* in wheat leaf samples, a new calibration curve must be made for each experiment, because amplification efficacy can differ between PCR mixes. Furthermore, every sample should be tested in duplicate, adding a small amount of *S. tritici* DNA at an appropriate detection threshold level to check the sensitivity and reliability of the PCR/PicoGreen assay.

With regard to optimization, the dynamic range of the PCR/PicoGreen assay can be improved using 'real-time' PCR. Fluorescence is measured directly during 'real-time' PCR in a closed tube format, using thermostable dsDNA-binding fluorescent dyes, like SYBR Green I, which is similar to PicoGreen, or sequence-specific double dye-labelled fluorogenic oligonucleotide primers, probes or molecular beacons (Wittwer *et al*. 1997). Fluorescence with the double-labelled fluorophores can be measured when primers are incorporated into the PCR product (Nazarenko *et al*. 1997), when molecular beacons hybridise during annealing (Tyagi and Kramer 1996), or when probes after hybridization are cleaved by 5'-exonuclease activity of DNA polymerase (Livak *et al*. 1995).

For detecting and quantifying *S. tritici* during infection of wheat leaves under controlled conditions in the glasshouse, both ELISA (Kema *et al.* 1996) and the PCR/PicoGreen assay, as shown in this study, can be used. Both studies revealed the same pattern of development after inoculation. Until formation of necrotic lesions, biomass increased only slightly or even decreased, but then increased rapidly during necrosis and pycnidia formation. The use of fungal strains transformed with specific reporter genes, like β-glucuronidase (Oliver *et al.* 1993) or the green-fluorescent protein (Chalfie *et al*. 1994), are also good techniques for studying the *S.* tritici-wheat interaction (Rohel *et al.* in press) but cannot be used in field studies.

Our data show that the PCR/PicoGreen assay can be used for this purpose. For field samples, aspecific amplification products were not detected with the 'β-tubulin' primers, indicating that other micro-organisms on the leaf surface will not interfere with the test. This assay was also useful for studying colonization and subsequent further disease development of *S. tritici* on successive leaf layers caused by vertical movement of rainsplash-dispersed spores (Royle *et al*. 1986). As demonstrated in this study, the effect of fungicide treatments on epidemics of *S. tritici* in the field (Figs 10,11) and varietal resistance to leaf and glume blotch, yellow rust and brown rust (Fig. 13) can be measured with the PCR/PicoGreen assay.

This work has led to the development of a rapid and robust diagnostic method for the pre-symptomatic detection and quantification of four key foliar pathogens of wheat. Various PCR primer sequences and assays are now available in kit form from Adgen limited (Nellies Gate, Auchincruive, Ayr KA6 5HW, Scotland). Although this represents a step towards commercialising this diagnostic technology for the benefit

of cereal growers, translating diagnostic measurements into treatment thresholds to guide spray timing and fungicide dose rate remains a stumbling block and needs further research.

PRACTICAL IMPLICATIONS

This research has generated rapid and robust DNA diagnostic methods, which accurately detect and quantify four important foliar diseases of wheat. Tests can be completed in one day, and can be used equally well for testing leaf samples collected from either field crops or greenhouse plants. False positive results are not a problem. The methods have already been shown to be useful in various research scenarios, including evaluating the effectiveness of fungicide treatments, and assessment of varietal resistance. Improvement of disease control through a better definition of the relationship between pathogen levels, spray timing and fungicide choices requires further research to translate DNA diagnostic technology into a useful decision support tool. Nevertheless, this work has successfully provided some novel technology, which should enable improvements in the precision of disease control measures.

FUTURE RESEARCH NEEDS

Future developments in diagnostic technology involving 'real-time' PCR and fluorescent probes (Wittwer *et al.* 1997) will improve the accuracy, speed, dynamic range and sample throughput of measuring pre-symptomatic infection levels. But a key requirement to translate these diagnostic methods into useful decision making tools is to determine the relationship between yield benefit, and diagnostic infection level at the time of treatment. Coupling this with the dose rate needed for effective disease control at different infection levels will allow more rational use of fungicides. Clear sampling protocols will need to be developed based on spatial and temporal distribution of each pathogen. Ideally, these protocols will differ between diseases, but different protocols will be impractical, and a common sampling protocol for foliar diseases will need to be defined, through analysis of the degree of heterogeneity within pathogen populations. Ultimately, results from commercially acceptable DNA diagnostics will need to be incorporated into advanced decision support systems, such as DESSAC, which can also advice farmers when to sample a crop at risk from infection. In this way the technology developed in this project will eventually benefit growers through more effective control of foliar diseases, and with less impact on the environment.

ACKNOWLEDGEMENTS

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