

Rapid detection and diagnosis of *Septoria tritici* epidemics in wheat using a polymerase chain reaction/PicoGreen assay

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B.A. FRAAIJE, D.J. LOVELL, E.A. ROHEL AND D.W. HOLLOWOMON. 1999. In order to detect and quantify *Septoria tritici* infection levels in wheat leaves, a polymerase chain reaction (PCR) assay was developed using the β -tubulin gene as target. Specific PCR primers were designed by aligning and comparing β -tubulin sequences from other fungi. The final primer set was selected after being tested against several fungi, and against *S. tritici*-infected and uninfected wheat leaves from different localities. A single DNA fragment (496 bp) was 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, approximately 2 pg *S. tritici* genomic DNA could be detected in each assay. However, for rapid quantification of PCR-amplified products, a fluorometric microtitre plate-formatted PicoGreen assay was used; 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 colonization, infection and subsequent disease development of *S. tritici* on wheat, both under controlled conditions in the glasshouse and in the field.

INTRODUCTION

Septoria tritici Roberge in Desmaz. (teleomorph *Mycosphaerella graminicola* (Fuckel) Schröter in Cohn), causal agent of leaf blotch, is a widespread and economically important pathogen of bread wheat (*Triticum aestivum* L.) and durum wheat (*T. turgidum* L.). The disease occurs particularly in countries with a humid, temperate-to-cool climate (Wiese 1987). In the UK, as a result of *S. tritici* infection, an average annual yield loss of £35 million has been reported for the period 1985–89 (Cook *et al.* 1991). The primary sources of inoculum of leaf blotch are stubbles that produce airborne sexual ascospores (Brown *et al.* 1978), while rainsplash-dispersed asexual pycnidiospores are most important for rapid disease development on upper leaves (Royle *et al.* 1986). Control measures are primarily focused on the application of foliar fungicide sprays and, recently, on the deployment of resistant germplasm. For optimum, cost-effective control,

fungicides are applied to protect the upper three leaves, which provide most of the grain-filling capacity. Fungicide choice (e.g. with curative and/or protective mode of action) and timing depend on the inoculum level and disease risk to 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, inhibiting ergosterol biosynthesis, are effective. Recently, fungicides with a novel mode of biochemical action, famoxadone (Joshi and Sternberg 1996) and strobilurins (Godwin *et al.* 1992), which inhibit mitochondrial respiration, have been developed. To ensure maximum effect of these new fungicides, which have mainly 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 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 test should detect *S. tritici* earlier in its latent phase, allowing

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well-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.

Polymerase chain reaction (PCR) assays are rapid techniques with high specificity and sensitivity, which have already 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), *Pseudocercospora herpotrichoides* (Nicholson *et al.* 1997), *Rhizoctonia solani* (Nicholson and Parry 1996), *Stagonospora nodorum* and *S. tritici* (Beck and Ligon 1995). Most of the primer sequences have been deduced from variable 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, were able to detect DNA of *S. tritici* and *S. nodorum* 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 Holmström *et al.* (1993) and Nikiforov *et al.* (1994), were too laborious and time-consuming.

The aim of the present research was to develop a robust and quantitative PCR-based diagnostic assay using the β -tubulin gene as target for the detection and quantification of *S. tritici* infection levels in wheat leaves. It is hoped that eventually, assays can be developed which improve fungicide efficacy, primarily through more accurate timing of fungicide application before leaf blotch is visible in the field. In this paper, the development is reported of a PCR assay using β -tubulin-derived primers, which can be used for specific detection of *S. tritici* in wheat leaves. The amount of PCR-amplified products can be quantified directly in microtitre plate wells with a fluorometer using the dye, PicoGreen, which fluoresces specifically upon binding with dsDNA (Singer *et al.* 1997). The potential application of this PCR/PicoGreen assay to monitor the infection process of *S. tritici* in a single leaf under controlled conditions, as well as epidemics in the field, is demonstrated and discussed.

MATERIALS AND METHODS

Fungal species and isolates

The fungal species and DNA used in this study are listed in Table 1. After 8 d of incubation at 18 °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. DNA from the remaining fungi were obtained directly from colleagues. To inoculate wheat leaves, dilutions of *S. tritici*

Table 1 Fungal species and isolates tested

Fungal strains and species	Source	Origin
<i>Gaeumannomyces graminis</i>	Wheat	UK
<i>Pseudocercospora herpotrichoides</i> (R-type)	Wheat	UK
<i>Puccinia striiformis</i>	Wheat	UK
<i>Rhynchosporium secalis</i>	Barley	UK
<i>Septoria tritici</i> ST16	Wheat	UK
<i>Septoria tritici</i> α 12-3B.8	Wheat	USA
<i>Stagonospora nodorum</i>	Wheat	UK

ST16 spore suspensions were made in distilled water supplemented with 0.05% (v/v) Tween-20.

Wheat leaf field samples

During the growing season of 1997/1998, wheat leaves with and without visual symptoms, showing lesions with pycnidia, were collected from different fields throughout England (Table 2). For one field-sown crop, cv. Riband, the fungal colonization of successive leaf layers by *S. tritici* was studied early in the growing season (Zadoks growth stage (GS) 12–15 (Tottman 1987)). Samples consisting of 10 leaves of each leaf layer were taken at weekly intervals from this plot. To

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 *Septoria tritici* ST16.

obtain healthy leaves, seedlings of different cultivars were grown in a growth room at 18 °C with a 12 h/12 h light/dark alternation.

Infection of wheat leaves by *S. tritici*

Seedlings of different cultivars at GS 11, eight per pot, were inoculated by placing two droplets of 6 µl spore suspension (about 10⁵ spores ml⁻¹) of *S. tritici* on each second leaf emerged (leaf 2). Immediately after inoculation, pots were placed in water-saturated trays and covered with polyethylene bags. The seedlings were grown in two controlled-environment cabinets, set at 12 and 18 °C with a 12 h/12 h light/dark alternation. Leaf samples, consisting of equally sized parts of four different leaves containing the inoculation spots, were taken daily while keeping the environment water-saturated. After extracting DNA from the leaves, approximately 50 ng DNA was tested in the PCR/PicoGreen assay to quantify *S. tritici* biomass.

DNA extraction

DNA was extracted directly from mycelium or single leaves by powdering the samples in liquid nitrogen using a pestle and a mortar; 40 µl 1% (v/v) β-mercapthoethanol, 400 µl TEN buffer (500 mmol l⁻¹ NaCl, 400 mmol l⁻¹ Tris-HCl, 50 mmol l⁻¹ EDTA, pH 8.0) and 400 µl 2% (w/v) SDS were added to each sample while mixing. For larger wheat leaf samples, the volumes of the different components were adjusted until the mixture could be poured. After incubating the mixture for 30 min at 70 °C, 400 µl ice-cold ammonium acetate (7.5 mol l⁻¹) was mixed with the heat-treated sample and the total suspension kept on ice for 30 min. After centrifugation at 10 000 rev min⁻¹ (9400 g) for 10 min, an equal volume of cold (-20 °C) isopropanol was added to the supernatant fluid and the extract shaken at room temperature for 15 min. After centrifugation at 6000 rev min⁻¹ (3400 g) for 5 min, DNA pellets were washed with ice-cold 70% (v/v) ethanol 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 about 25 µg.

Standard PCR protocol

A standard PCR was carried out on a Biometra T3 thermocycler (Biotron GmbH, Göttingen, Germany) with 0.5 units of Red Hot DNA polymerase (Advanced Bioenzymes Ltd) using 20 mmol l⁻¹ (NH₄)₂SO₄, 75 mmol l⁻¹ Tris-HCl, pH 9.0, 0.01% (w/v) Tween-20, 1.5 mmol l⁻¹ MgCl₂, containing 125 µmol l⁻¹ of each dTTP, dATP, dCTP and dGTP, 0.5 µmol l⁻¹ 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 °C for 3.5 min, followed by 40 cycles at 94 °C for 30 s, 65 °C for 1 min and 72 °C for 1.5 min. The PCR was terminated with a DNA extension at 72 °C for 8.5 min. Gel electrophoresis was used to analyse PCR amplified products; PCR products in a 10 µl sample were separated on a 1.3% (w/v) agarose gel containing ethidium bromide and exposed to u.v. light to visualize DNA fragments.

Primer design

In order to design specific primers, different parts of the coding sequence of the β-tubulin gene of *S. tritici* (Payne *et al.* unpublished) were used. Reverse and forward 24-mer primers with unique 3'-ends 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*. 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, results of the 'β-tubulin' primers were compared with the *S. tritici*-specific 'ITS' primers described by Beck and Ligon (1995).

Fluorometric preand 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 by spiking wheat leaf DNA samples (200 ng of DNA) with different amounts of genomic DNA of *S. tritici*, ranging from 2 pg to 23 ng, which were run simultaneously in each experiment using the PCR/PicoGreen assay. From each crude DNA extract or PCR assay, 3 µl of sample were incubated at room temperature in a well of a microtitre plate (Perkin Elmer) with 150 µl PicoGreen solution (1:400 dilution in 1 mmol l⁻¹ EDTA, 10 mmol l⁻¹ Tris-HCl, pH 8.0). Fifteen minutes 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

Specificity of β-tubulin primers

Several sets of primers directed against different regions of the β-tubulin gene of *S. tritici* were tested for specificity at

Table 3 ' β -tubulin' primer sequences E1 (forward primer) and STSP2R (reverse primer) of *Septoria tritici* aligned with corresponding partial β -tubulin sequences of other fungi (data obtained from EMBL database)

Forward primer (5'-3')	Reverse primer (5'-3')
1. CGGTATGGGAACACTTCTCATCAG	1. GTAACGACCGTTGCGGAAAATCGCT
2. CGGTATGGGTACTCTTTTGATCTC	2. GTAGCGGCCGTTGCGGAAGTCAGA
3. CGGTATGGGTACGTTATTAATTTTC	3. GTAACGACCATTTTCGGAAGTCGGA
4. CGGTATGGGTACCCTCCTTATCTC	4. GTAACGACCGTTGCGGAAGTCAGA
5. TGGTATGGGTACGCTTTTGATCTC	5. GTAGCGACCGTTGCGGAAGTCAGA
6. CGGTATGGGTACGCTTTTGATCTC	6. GTAGCGACCGTTACGGAAAATCGGA

1, *Septoria tritici*; 2, *Aspergillus nidulans*; 3, *Erysiphe graminis*; 4, *Neurospora crassa*; 5, *Stagonospora nodorum*; 6, *Rhynchosporium secalis*.

different $MgCl_2$ concentrations (0.5 – 5.0 mmol l^{-1}) and annealing temperatures (55 – 68 °C). Many primer combinations generated multiple products, often caused by amplification of wheat DNA (results not shown). However, primers E1 and STSP2R (sequences in Table 3) produced a single fragment of 496 bp from genomic DNA of *S. tritici*, whereas that from wheat and the other fungi tested was not amplified (Fig. 1a). The quantitiveness of the PCR assay is shown in Fig. 1(b), where approximately 2 pg genomic DNA of *S. tritici* 200 ng $^{-1}$ DNA of uninfected wheat leaves could be visualized with ethidium bromide after gel electrophoresis.

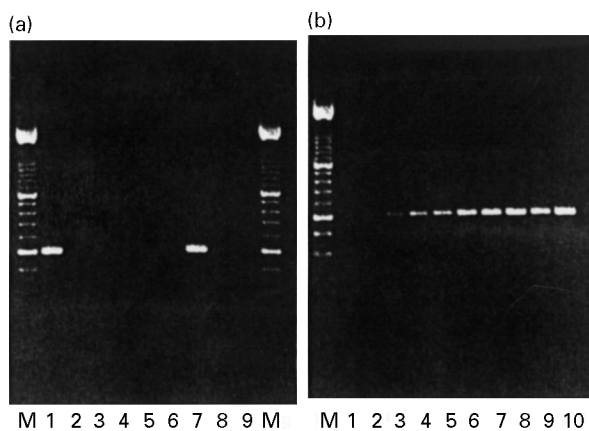


Fig. 1 Ethidium bromide-stained agarose gel of PCR-amplified products using the ' β -tubulin' primers E1 and STSP2R. (a) Different fungal isolates and wheat. Lanes: 1, *Septoria tritici* ST16; 2, *Stagonospora nodorum*; 3, *Rhynchosporium secalis*; 4, *Gaeumannomyces graminis*; 5, *Pseudocercospora herpotrichoides*; 6, *Puccinia striiformis*; 7, *S. tritici* α 12-3B.8; 8, wheat cv. Spark (glasshouse, uninoculated); 9, water control; M, 100 bp DNA ladder. (b) Detection of different amounts of *S. tritici* DNA in 200 ng DNA samples of uninfected leaves. Lanes: 1, cv. Spark (glasshouse); 2, 2.3 pg; 3, 5.8 pg; 4, 23 pg; 5, 58 pg; 6, 0.23 ng; 7, 0.58 ng; 8, 2.3 ng; 9, 5.8 ng; 10, 23 ng DNA of *S. tritici* ST16 added; M, 100 bp DNA ladder

Sensitivity and specificity of the PCR/PicoGreen assay

As a single DNA fragment could be amplified, PCR products could be quantified directly with the PicoGreen assay. With this assay, as few as 1000 spores per leaf, or about 10 pg *S. tritici* DNA 200 ng $^{-1}$ wheat leaf DNA, were detected (Fig. 2). 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.

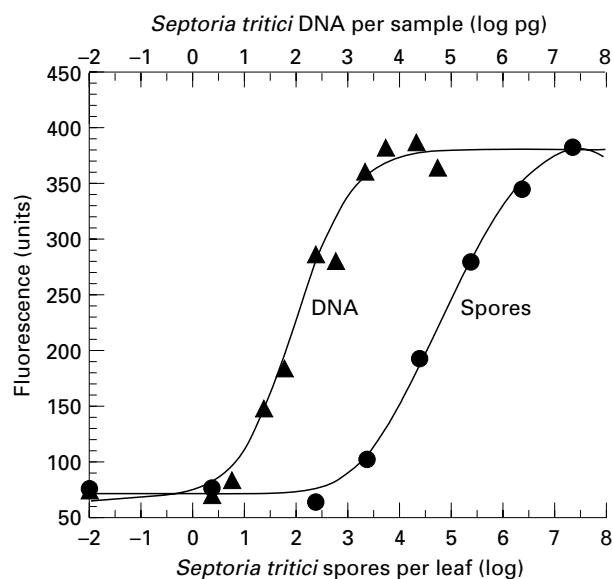


Fig. 2 Detection and quantification of *Septoria tritici* in DNA samples of inoculated wheat leaves and in spiked DNA samples of wheat leaves by the PCR/PicoGreen assay using ' β -tubulin' primers

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 °C, the 'ITS' primers produced two products, a 345 bp fragment specific for *S. tritici* and a second product of about 280 bp, which was also amplified from 'healthy' wheat leaf samples (Fig. 3a). This demonstrated that where the same samples were tested using the ' β -tubulin' primers, there was only one product specific for *S. tritici* (Fig. 3b).

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. 4). 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, the possibility of inhibition of the PCR was checked by spiking these samples with about 25 pg *S. tritici* DNA. After spiking, all samples were found to be positive, indicating that no inhibition had occurred. Additionally, the amplification of the 280 bp wheat DNA fragment from the *S. tritici*-negative samples (Fig. 3a) showed that the PCR was not inhibited.

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

The amount of *S. tritici* biomass in leaf 2 of cv. Riband was estimated during infection by the PCR/PicoGreen assay (Fig. 5). At 18 °C, the first symptom was discoloration, which emerged 6 d after inoculation. After 14 d, necrotic lesions were visible and pycnidia subsequently developed 17 d after

Fig. 3 Detection of *Septoria tritici* in wheat leaves by PCR. Ethidium bromide-stained agarose gels of PCR-amplified products. 'ITS' primers (a) compared with ' β -tubulin' primers (b). Lane numbers represent the sample number of the samples shown in Table 2. M, 100 bp DNA ladder

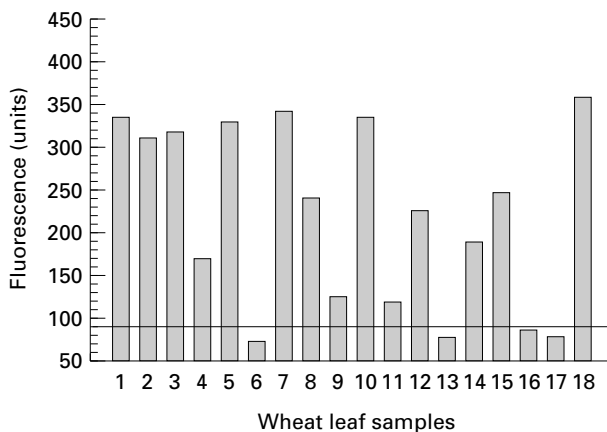
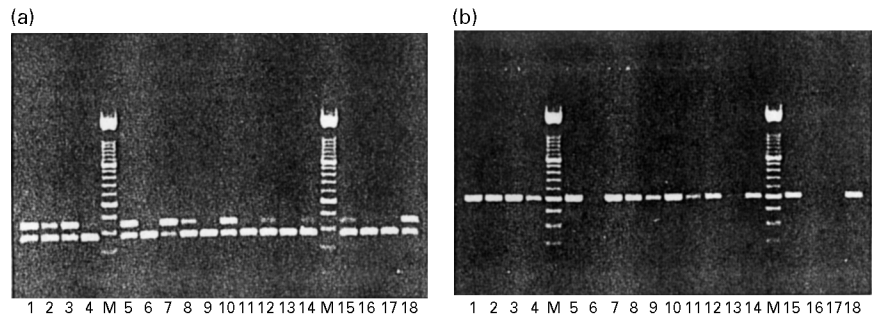


Fig. 4 PicoGreen DNA quantification of PCR amplified products using ' β -tubulin' primers. DNA samples were obtained from uninfected leaves grown in the glasshouse and from naturally infected wheat leaves. For origin of samples see Table 2

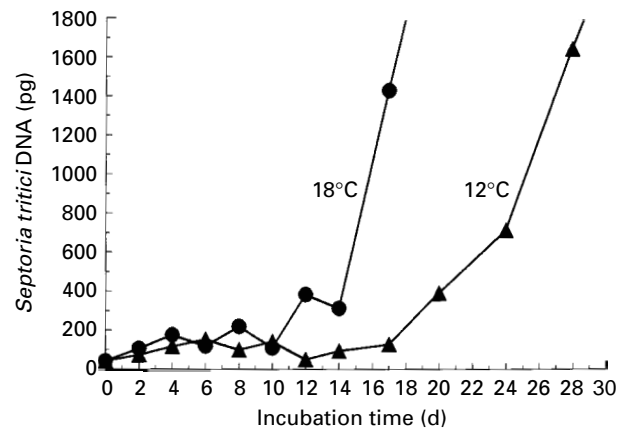


Fig. 5 Measurement of biomass of *Septoria tritici* during infection of wheat leaves at 12 and 18 °C

inoculation. At 12 °C, pycnidia emerged only 24 d after inoculation, when significant necrosis and discoloration were not visible. A slight increase in fungal biomass was measured immediately after inoculation. Biomass was steady for a while, or even slightly decreased, but following necrosis and pycnidial production, the amount of biomass increased exponentially.

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

The early colonization of *S. tritici* in different leaf layers, 2 to 5, was studied in the field with the PCR/PicoGreen assay during GS 12–15 (Fig. 6). Leaves 2 and 3 were infected first, followed by the successive layers of leaves 4 and 5. During the period studied, the presence of *S. tritici* could be detected more than 3 weeks before symptoms were visible in the field (data not shown).

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 $\mu\text{mol l}^{-1}$ (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 approximately 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

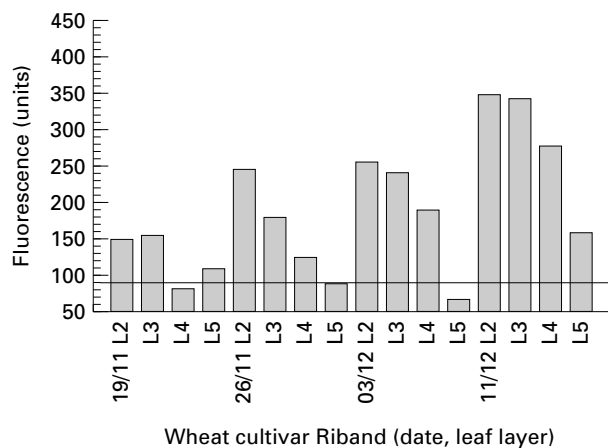


Fig. 6 Monitoring colonization of *Septoria tritici* on different leaf layers in the field during GS 12–15

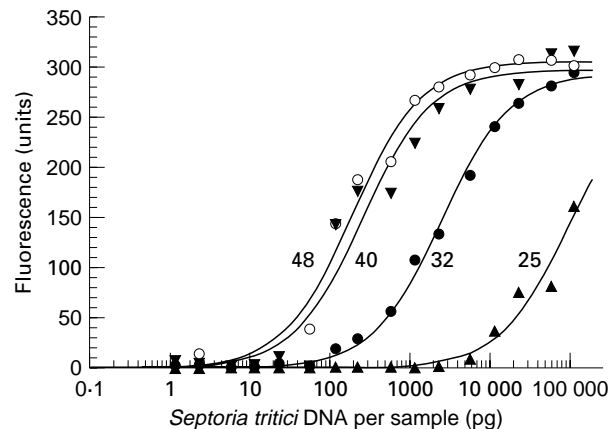


Fig. 7 Calibration curves for quantification of *Septoria tritici* in wheat leaves generated by the PCR/PicoGreen assay using different cycle numbers for PCR. The different cycle numbers are shown for each curve

are shown in Fig. 7. For each cycle number, the fluorescence value was corrected for background fluorescence because after many cycles (> 30), the background increased significantly because of 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.

DISCUSSION

After performing PCR, the sensitivity of gel electrophoresis for detecting *S. tritici* was slightly greater than that for the PicoGreen assay. The PicoGreen assay detected approximately 10 pg *S. tritici* DNA per reaction, whereas the sensitivity of gel electrophoresis of about 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 is now 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. Although more samples have to be tested, the method is robust, rapid and appears to work well in practice. It might not be as accurate as the competitive PCR approach, whereby an amount of internal DNA standard is added which can be measured independently from the

amount of the target amplicon (see Nicholson *et al.* 1996). However, even the latter method is not ideal, because it cannot check the efficiency of the DNA extraction and the quality of the target DNA template.

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, such as 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 hybridize 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 the formation of necrotic lesions, the biomass increased only slightly or even decreased, but then increased rapidly during necrosis and formation of pycnidia. 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 (Payne *et al.* unpublished) but cannot be used in the field.

Our data show that the PCR/PicoGreen assay can be used for this purpose. For field samples, no aspecific amplification products were 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). For some samples, e.g. 11–13 and 14–16, originating from two different localities (Table 2), the effect of the fungicide application could also be measured (Fig. 4). Infection levels for the treated samples were lower than those in untreated samples, indicating that fungicide efficacy can be measured with the PCR/PicoGreen assay.

Further research with the PCR/PicoGreen assay will be carried out in order to improve our understanding of *S. tritici* epidemics in the field and to enhance current risk-assessment models through better measurement of cultivar resistance and the effects of fungicides.

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