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A conventional PCR technique to detect *Septoria tritici* in wheat seeds

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Abstract. A PCR assay was developed for detection of wheat seed naturally contaminated with *Septoria tritici. S. tritici* specific primers were derived from strict alignment of ITS and α-tubulin sequences of the pathogen. The specificity of four sets of synthesised oligonucleotide pairs (A, B, C and D) were tested using isolates from *S. tritici*, other selected fungi and wheat seeds. A single DNA fragment was amplified from *S. tritici* isolates with all primer pairs, whereas no product was generated from other DNA sources. *S. tritici* was also detected in wheat seed lots collected from plants with variable pycnidial coverage on the upper two leaves. PCR detection of as little as 0.5 pg of *S. tritici* genomic DNA was possible. This is the first report on the detection of *S. tritici* DNA in naturally infested wheat seeds. This PCR based assay is simple, rapid, specific, sensitive and suitable for routine detection of the wheat pathogen in infested wheat seeds.

Additional keywords: *Mycosphaerella graminicola*, seedborne pathogen, Septoria leaf blotch, wheat.

Introduction

Mycosphaerella graminicola (anamorph *Septoria tritici*) is an economically important pathogen causing leaf blotch of bread and durum wheat. The pathogen is distributed worldwide and causes significant losses in many wheat-growing areas (Van Ginkel and Rajaram 1993). The disease occurs particularly in countries with a humid, temperate to cool climate (Wiese 1987) and the symptoms are characterised by necrotic blotches that contain varying densities of pycnidia, the asexual fructifications (Kema *et al*. 1996).

Wheat seedlings may become infested soon after emergence and under favourable weather conditions, e.g. frequent rains together with temperatures of 12−25°C. The disease progresses vertically to the upper plant parts. Severe yield losses occur if the fungus infests the upper two leaves (Ziv and Eyal 1974; Robert *et al*. 2005) that are the main source of carbohydrates for grain filling (Zilberstein *et al*. 1985).

Breeding for leaf blotch resistance is the most economical approach to controlling the disease (Eyal *et al*. 1985). Alternatively, foliar fungicides or protection of seedlings by seed treatments with systemic fungicides (e.g. benzimidazoles) are used as chemical control (Rohel *et al*. 1998). The no-tillage system harbours more inoculum of *S. tritici* than the conventional system and fungicidal seed treatment alone may not be effective for disease control (Eyal *et al*. 1987).

Mycosphaerella graminicola is thought to have originated in the Middle East and been globally dispersed on wheat seed as a result of the expansion of wheat cultivation (McDonald *et al*. 1999; Banke *et al*. 2004). It has been proposed that movements of infested wheat seeds are likely to have influenced the population genetics of *S. tritici* (Banke and McDonald 2005).

Although several foliar wheat diseases (tan spot by *Drechslera tritici-repentis*, spot blotch by *Bipolaris sorokiniana*, Septoria glume blotch by *Stagonospora nodorum* and *Alternaria infectoria* complex) are associated with seed transmission (Mathur and Cunfer 1993), it is unknown how *S. tritici* grain infestation occurs. Experiments using an agar plate technique to determine the presence of the pathogen inside the seeds were first reported by Brokenshire (1975). This method is not practical for routine seed testing because it is time consuming and *S. tritici* is easily overgrown by fast-growing saprophytes of wheat.

Advances in molecular biology such as the polymerase chain reaction (PCR) have provided exciting opportunities for development of specific and sensitive assays that have been extensively used for identification and detection of several fungal plant pathogens (Vandemark *et al*. 2000; Konstantinova *et al*. 2002; Lee *et al*. 2002; Kulik *et al*. 2004; Fountaine *et al*. 2007; Gayoso *et al*. 2007). In this sense, molecular approaches have gained special attention as they are useful for detecting seed infestations that are of low infestation levels or asymptomatic (Lee *et al*. 2001; Tegli *et al*. 2002; Chadha and Gopalakrishna 2006).

Several PCR based assays for *S. tritici* detection have been reported so far. Beck and Ligon (1995) reported specific primers to distinguish *M. graminicola* from *S. nodorum* and Fraaije *et al*. (1999, 2001) developed two specific primer pairs for quantitative detection of the pathogen. However, Guo *et al*. (2006) found that the above-mentioned primers were not specific,

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yielding multiple bands from some other wheat pathogens. So they developed four new specific primer pairs and developed a sensitive real-time PCR assay for monitoring *M. graminicola* development in inoculated and naturally infested leaves of wheat plants.

Abramova *et al*. (2008) described a PCR system using the fluorescent amplification-based specific hybridisation (FLASH) for detection of *S. tritici* and *S. nodorum* in infested soil, leaf, and seed samples. However, both techniques have a high cost and are not available in many laboratories.

We hypothesised that, as is common in other foliar wheat pathogens, the transport of *S. tritici* into the wheat seed could be one of the mechanisms to complete the disease cycle, and therefore, there are two possibilities for *S. tritici* transmission through the seed: (a) the pathogen is transported as vegetative mycelium inside internal tissues of the seed, e.g. *S. nodorum*, *D. tritici repentis*, *Alternaria triticina* and *B. sorokiniana*, or (b) the pathogen is latent in the embryo, e.g. *Ustilago* spp. (Mathur and Cunfer 1993). For this reason, it is desirable to develop a reliable and sensitive method for detecting seedborne infestation by *S. tritici.*

Ribosomal genes and the spacers between them provide attractive targets for detection and phylogenetic studies of microorganisms. They possess conserved and variable sequences that allow their easy amplification and sequencing. Genes for 5.8 S, 18 S and 28 S fungal nuclear rRNA genes are usually organised in identical conserved repeats with 60–200 copies per haploid genome (Bruns *et al*. 1991; Stackebrandt *et al*. 1992). Considerably greater sequence variation is found in the internal transcribed spacer (ITS) region between the rRNA genes within a rRNA repeat unit, and this region has been widely used for developing species-specific primers and probes for detection of fungal plant pathogens (Johanson and Jeger 1993; Beck and Ligon 1995; Goodwin *et al*. 1995; Lee *et al*. 2001; Zur *et al*. 2002; Balmas *et al*. 2005).

Other specifically conserved sequences among fungi are ideal candidates as targets for detection assays. Microtubules in eukaryotes contain three related tubulin proteins (α, β and γ) that are conserved, although they have shown small differences. Tubulins from a wide variety of fungi have been characterised. In particular, α-tubulin sequence analysis was used to group *S. tritici* isolates (Rohel *et al*. 1998).

The availability of improved diagnostic techniques for the rapid and accurate identification of *S. tritici* would be useful to growers and field pathologists. We report here a conventional PCR-based assay approach using ITS and α-tubulin-derived primers that can be used for specific detection of *S. tritici* in naturally infested wheat seeds.

Methods

Fungal isolates and wheat seed lots

The *S. tritici* isolates FALP 92067 and FALP 20090 were obtained from wheat plants collected at INTA Pergamino and MAG Barrow Experimental Stations, respectively. Isolates of *Alternaria radicina*, *Aspergillus nidulans*, *Epicoccum* sp., *Fusarium graminearum*, *Bipolaris cynodontis*, *B. infectoria*, *B. nodulosa*, *B. papendorfii*, *B. sorokiniana*, *B. specifera*, *D. tritici-repentis*, *Saccharomyces cerevisiae*, *Schizosacharomyces pombe* and *Ustilago maydis* were obtained from the Laboratorio de Fitopatología, Facultad de Ciencias Agrarias-UNLP and Spegazzini Institute (La Plata, Argentina) The *S. nodorum* and *Rhynchosporium secalis* isolates were supply by Dr Bruce McDonald (Institute for Plant Sciences, Phytopathology Group, Federal Institute of Technology, ETH Zentrum, LFW, Zürich, Switzerland). All isolates mentioned above were cultured on potato dextrose agar plates at 22°C and stored at 4°C.

Wheat seeds from plants with variable pycnidial coverage in the upper two leaves were collected from five commercial varieties and three experimental lines (Table 1). They were collected from naturally infested fields from an endemic region of the Great Wheat Argentine Zone.

DNA extraction

Plugs of all fungal isolates grown in solid culture medium were transferred to liquid minimal medium (sucrose 10 g, yeast extract 10 g, distilled water 1 L) and grown for 1 week at room temperature with shaking on a rotary shaker, collected by filtration, lyophilised and ground into a powder with a mortar and pestle. DNA was extracted using a modified version of the CTAB (cetyltrimethylammonium bromide) method as described below (Murray and Thompson 1980). Seven hundred and fifty microlitres of extraction buffer (100 mM Tris-HCl pH 8,

Table 1. Wheat seeds lots tested in this study for the presence of *S. tritici*

Lot	Cultivar	Geographic origin	Year of collection	Pycnidial covering (%)	Susceptibility to *S. tritici*
1	CROC1/AE·SQUARROSA (205)//K.AUZ[A]	Barrow, Tres Arroyos	December 2004	35	High
2	CMH76A.977/SERV//CMH76A.977/ CMH79A.307[A]	Barrow, Tres Arroyos	December 2004	40	High
3	Buck Arrayan[B]	Barrow, Tres Arroyos	December 2004	45–50	High
4	SHA3/SER1//PSN/BOW[A]	Barrow, Tres Arroyos	December 2004	50	High
5	Klein Sagitario[B]	Barrow, Tres Arroyos	December 2004	50	High
6	Buck Sorzal[B]	Barrow, Tres Arroyos	December 2004	50	High
7	Buck Sorzal[B]	Barrow, Tres Arroyos	December 2004	60	High
8	Klein Chaja[B]	Barrow, Tres Arroyos	December 2004	70	High
9	Pro INTA Gaucho[B]	Buck, Necochea	January 2005	no data	High

[A]Experimental lines.
[B]Commercial varieties.

100 mM EDTA, 250 mM NaCl, 2% CTAB) and 15 µL of 2-mercaptoethanol were added to the ground tissue and incubated at 65°C for 30 min. Cellular proteins were precipitated with 300 µL of potassium acetate 300 mM, pH 4.8. After centrifugation the supernatant was transferred to a new tube, purified with 500 µL of phenol : chloroform : isoamyl alcohol (25 : 24 : 1) and chloroform : isoamyl alcohol (24 : 1) until the interphase became transparent. Nucleic acids were precipitated by adding 750 µL of cold isopropanol followed by incubation at 4°C for 30 min. After centrifugation, the pellet was rinsed twice with 500 µL of 70% ethanol, air-dried and dissolved in 100 µL of Tris-EDTA buffer containing 10 mM Tris-HCl, (pH 8) and 1 mM EDTA.

DNA from infested wheat seeds was isolated as follows: a minimum of 100 seeds of each wheat cultivar described in Table 1 and healthy plants of the Pincén cultivar were surface sterilised by rinsing once with $HgCl_2$ 0.01% in 1% ethanol for 2 min and twice with sterile deionised water. To favour *S. tritici* mycelium proliferation within the seeds, they were incubated in a moist chamber for 48 h then immediately frozen at −20°C, lyophilised and ground into a powder with a pestle. For each extraction of total genomic DNA, 0.1 g (20 seeds) of lyophilised seeds was used. DNA was extracted using the DNeasy PLANT miniKit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA quality and concentration were determined with a spectrophotometer and electrophoresis on 1% agarose gels.

Primer design

Forward and reverse *S. tritici* specific primers were designed by aligning and comparing the nucleotide sequence of *S. tritici* α-tubulin (EMBL accession No Y14509) and ITS sequences of *S. tritici* (EMBL accession No AY152603) with those of other fungi (*Alternaria triticina*, *A. alternata*, *A. nidulans*, *B. sorokiniana*, *F. graminearum*, *Mycosphaerella fijiensis*, *M. musicola*, *N. crassa*, *Penicillium* sp., *R. secalis*, *S. cerevisiae*, *S. pombe*, *Septoria glycines*, *S. nodorum* and *U. maydis*) commonly associated with wheat and wheat relatives, and with those of the plant species *Triticum aestivum*, *Avena sativa* and *Hordeum vulgare*. The CLUSTAL X method of the MegAlign program of the DNAStar software package (Jeanmougin *et al*. 1998) was used for the alignments. Four oligonucleotides within the α-tubulin region and two oligonucleotides within the ITS region were designed based on sequence differences between *S. tritici* and the other fungi and plants, with an average G + C content ranging from 45 to 72% (Table 2). The OligoTech program version 1.0 was used to check the properties of the designed oligonucleotides.

Primer selection and specificity to S. tritici

All designed primers were tested for the ability to amplify fragments of the expected size (Table 2) and for specificity to *S. tritici* at different annealing temperatures using purified DNA preparations from the pathogen and other fungi. PCR amplifications were performed in a total volume of 20 µL containing 5 ng of purified fungal DNA, 1.5 to 2.0 mM $MgCl_2$, each deoxynucleoside triphosphate at a concentration of 0.25 µM, 0.5 µM of each primer and 1 U of Go-Taq DNA polymerase (Promega Corporation, Madison, USA) in the corresponding reaction buffer. Amplifications were performed with a thermocycler Mastercycler gradient (Eppendorf AG, Hamburg, Germany). The number of cycles and annealing temperature for every PCR reaction was adjusted for each primer combination. PCR products were analysed by electrophoresis in 1% (w/v) agarose gels in 1× TAE buffer containing 40 mM Tris/NaAcO (pH 8) and 1 mM EDTA, at 100 V for 40 min, stained with ethidium bromide and photographed with a FOTO/Analyst Express system (Fotodyne Inc., Hartland, WI, USA).

Detection of S. tritici on wheat seeds

Primers were also tested for the detection of *S. tritici* from wheat seeds. Twenty seeds per cultivar were ground to a fine powder and DNA was extracted as described above. Amplifications were performed using each primer set listed in Table 2 and PCR conditions were adjusted for each primer pair. There were five replicates per experiment.

Cloning and sequencing of PCR products

PCR amplified products were ligated to the cloning vector pGEM-T Easy (Promega, Madison, Wisconsin), which was used for transforming *E. coli* DH5α by standard protocols

Table 2. Description of primer sets and PCR conditions used for amplification of *S. tritici* DNA

Set	Sequence	Position (bp)[C]	Expected fragment size (bp)	PCR cycles (number)	PCR annealing temperature (°C)
A[A](d)	5′-CCAAAAAACACTGCATCTCTGCG-3′	97	336	25	65
(r)	5′-CGTGAACTCCGCGGCGAGACGTG-3′	433			
B[B] (d)	5′-GGACGACGACGGCTTTTCCACC-3′	410	1263	30	58
(r)	5′-CCGCCCTCATCACCCTCG-3′	1673			
C[B](d)	5′-GGAAATCAGCATGTCCTGCTTCGAGC CAAACAG-3′	1203	470	30	62
(r)	5′-CCGCCCTCATCACCCTCG-3′	1673			
D[B](d)	5′-GCAGGAAATCAGCATGTCC-3′	1200	473	30	58
(r)	5′-CCGCCCTCATCACCCTCG-3′	1673			

[A]Primer set derived from the internal ITS region of *S. tritici*.
[B]Primer sets derived from the α-tubulin gene of *S. tritici*. d, direct primer; r, reverse primer.
[C]Position starting from the first base of the ITS sequence (NO AY152603) and from the start codon sequence of α-tubulin gene (No Y14509) of *S. tritici.*

(Sambrook and Russell 2001). DNA sequencing was carried out by MACROGEN services (Korea). BLASTX of the National Center for Biotechnology Information (http:// www.ncbi.nlm.nih.gov, version 2.2.6) was used for DNA sequence analysis.

Sensitivity and specificity assay

To determine the sensivity of the α-tubulin and ITS primers, a serial dilution of *S. tritici* DNA was prepared in Tris-EDTA buffer to concentrations ranging from 5 ng/μL to 1 fg μL. The serial dilution was repeated twice.

To establish the limit of detection of the PCR seed assay, wheat seeds harvested from plants with different levels of severity on the flag leaf [highly infested (>50%), middle infested (40–50%), low infested (<40%) and non-infested (0%)] were selected (Table 1).

Results

Evaluation and specificity of α-tubulin and ITS primers

Amplification of *S. tritici* DNA with ITS and α-tubulin primer sets using the indicated annealing temperatures resulted in fragments of expected sizes (Table 2). The identity of the amplified DNA fragments was confirmed after cloning and sequencing.

No amplification was observed with purified genomic DNA of *A. alternata*, *A. triticina*, *B. cynodontis*, *B. infectoria*, *B. nodulosa*, *B. papendorfi*, *B. sorokiniana*, *D. tritici-repentis*, *Epicoccum* sp., *F. graminearum*, *R. secalis*, *S. nodorum*, and *U. maydis* (pathogens of cereals), or other fungal species *A. nidulans*, *N. crassa*, *Penicillium* sp., *S. cerevisiae*, and *S. pombe*, or wheat, barley and oat (Fig. 1, and not shown).

Optimisation of conditions and evaluation of plant infestation level to detect S. tritici *on seeds*

In the initial PCR assays with the four primer sets using DNA from *S. tritici*-contaminated wheat seeds, the target DNA sequence was not amplified. However, the amplification was successful when we used DNA from disinfested seeds incubated in humid chamber for 48 h. DNA fragments of expected sizes were obtained with the four primer pairs (Fig. 1, lanes 12–16, and not shown).

We also investigated the relationship between the plant leaf infestation level and the transmission of the pathogen to the seeds. *S. tritici* target DNA sequence was detected in seeds from plants with a minimum of 40% of pycnidial coverage in the upper two leaves.

Limit of detection (sensitivity) of the S. tritici *assay*

In sensitivity tests, the amplification reactions were performed with decreasing amounts of *S. tritici* genomic DNA to determine the minimum amount of input DNA required to yield a detectable product with the four primer sets. The threshold of *S. tritici* detection by PCR differed depending on the primer pair used. Set A successfully amplified the target DNA sequence from as little as 0.5 pg. Sets B, C, and D amplified the target DNA from 50, 50 and 25 pg, respectively (Fig. 2, and not shown).

Discussion

In our preliminary studies conducted on several lots of *S. tritici* infested wheat seeds, fungal mycelium was not detected by applying seed washing technique, plating seeds on PDA medium or microscope examination of the intact or cut infested seeds, as was demonstrated by Brokenshire (1975). These results prompted us to investigate molecular-based detection techniques.

PCR-based assays are very important tools that have facilitated the identification of many plant pathogens (Ward *et al*. 2004). In this study, a conventional PCR based test was developed to detect *S. tritici* in wheat seed using pathogen-specific primers derived from the ITS region of rDNA and α-tubulin gene of the fungus. This approach is much more rapid than conventional methods for *S. tritici* detection such as the cultural method, which takes ~10 days for incubation

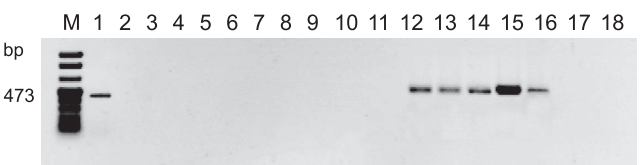

Fig. 1. Agarose gel electrophoresis of the PCR amplification products obtained with the primer set D showing its specificity to detect *S. tritici*. Amplification products from fungus DNA (lane 1: *S. tritici*; lane 2: *A. radicina*; lane 3: *Epiccocum* sp.; lane 4: *F. graminearum*; lane 5: *B. specifera*; lane 6: *B. papendorfii*; lane 7: *B. infectoria*; lane 8: *B. sorokiniana*; lane 9: *D. tritici-repentis*; lane 10: *B. cynodontis*; and lane 11: *B. nodulosa*) or wheat DNA (lane 12: Buck Arrayán cv.; lane 13: Klein Chajá cv.; lane 14: CMH76A.977/SERV//CMH76A.977/CMH79A.307 line; lane 15: Buck Sorzal cv.; lane 16: SHA3/SER1//PSN/BOW line; lane 17: Pincén cv. and lane 18: control without DNA). M: Molecular marker 100 bp ladder (Promega Corporation, Madison, USA). Similar results were obtained with the primer sets A, B and C (data not shown).

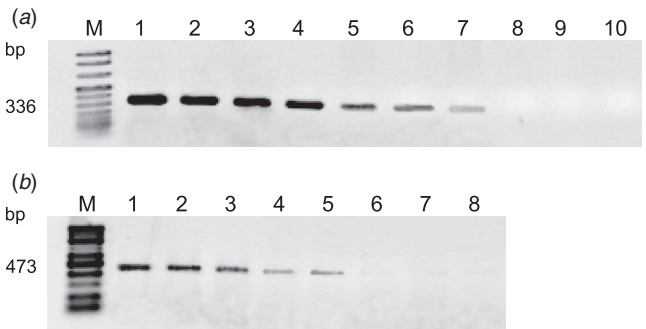

Fig. 2. Agarose gel electrophoresis of the PCR products obtained to establish the limit of detection of *S. tritici.* (a) Primer pair A was used in the PCR reaction with serial dilutions of *S. tritici* genomic DNA. Lane 1: 10 ng; lane 2: 5 ng; lane 3: 50 pg; lane 4: 10 pg; lane 5: 5 pg, lane 6: 1 pg; lane 7: 0·5 pg, lane 8: 25 fg, lane 9: 10 fg, lane 10: 1 fg. M: molecular size standard VIII-ladder (Boheringher). (b) Primer pair D was used in the PCR reaction with serial dilutions of *S. tritici* genomic DNA. Lane 1: 10 ng; lane 2: 5 ng; lane 3: 1 ng; lane 4: 05 ng; lane 5: 25 pg, lane 6: 1 pg; lane 7: 500 fg; lane 8: 50 fg. M: molecular size standard V-ladder (Boheringher).

(Brokenshire 1975). An additional advantage of the PCR-based assay is that it does not rely on visual identification of *S. tritici*, which is complicated by the frequent occurrence of other fast-growing fungi.

Nuclear rDNA sequences have been an alternative source for designing species-specific primers, and particularly for detecting *S. tritici* from DNA extracted from infested leaves. A set of specific *S. tritici* ITS primers was designed by Beck and Ligon (1995). However, Fraaije *et al*. (1999) demonstrated that when using those primers at an annealing temperature of 60°C, two amplification products were obtained: a fragment specific for *S. tritici* and a second product which was also amplified from healthy wheat leaf samples.

Guo *et al*. (2006) designed four specific primers for *S. tritici* detection based on rRNA and actin gene sequences of the pathogen, or random amplified polymorphic DNA (RAPD) for developing a real-time assay for monitoring fungal development on wheat plants. We have used a different strategy for primer design to specifically detect *S. tritici* on naturally infested wheat seeds, being mindful that other genetically similar fungi are also present in the wheat environment and on taxonomically-related wheat species. Our results show that the four set of primers designed based on ITS rDNA or α-tubulin sequences are specific for *S. tritici* detection. Since no other fragments were detected from healthy plants or other microorganisms living in the wheat seed or soil environment, our assay could be used for *S. tritici* tests on field samples.

As reported, the detection of phytopathogenic fungi in plant material and seeds can be difficult, especially at low infestation levels (Konstantinova *et al*. 2002). Particularly, DNA extracts prepared from seeds usually contain low levels of the target DNA sequence. This can be easily avoided by including an enrichment step to increase the amount of target fungus in the seed sample. This approach has been adopted in other PCR-based seed assays such as the detection of *Leptosphaeria* spp. from rapeseed (Taylor 1993) and *Alternaria radicina* from carrot seed (Pryor and Gilbertson 2001). To overcome the low pathogen levels in our work, a pre-assay incubation step was used to increase the fungal biomass of *S. tritici* in wheat seeds. This approach was not applied in experiments carried out for *S. tritici* detection on wheat seeds by Abramova *et al*. (2008), which could not detect *S. tritici* using a fluorescent method.

Results presented here demonstrate that the PCR-based method developed in this study provides an efficient strategy for the detection of *S. tritici* in wheat seeds, even when they look healthy. This type of test has the potential to be used for early identification and routine testing of the leaf blotch pathogen on wheat seeds and could contribute to more effective disease management practices. In practical terms, this research forms the basis of the development of an ideal quantitative methodology using a real-time PCR procedure. Future research will be directed towards understanding the infestation pathway and localisation of *S. tritici* in the seed.

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References

Abramova SL, Ryazantsev DY, Voinova TM, Zavriev SK (2008) Diagnostics of Phytopathogen Fungi *Septoria tritici* and *Stagonospora nodorum* by Fluorescent Amplification–Based Specific Hybridization (FLASH) PCR. *Russian Journal of Bioorganic Chemistry* **34**, 97–102.

Balmas V, Schern B, Ghignone S, Salem AOM, Cacciola SO, Migheli O (2005) Characterisation of *Phoma tracheiphila* by RAPD-PCR, microsatellite-primed PCR and ITS rDNA sequencing and development of specific primers for *in planta* PCR detection. *European Journal of Plant Pathology* **111**, 235–247. doi: 10.1007/s10658-004-4173-x

Banke S, McDonald BA (2005) Migration patterns among global populations of the pathogenic fungus *Mycosphaerella graminicola. Molecular Ecology* **14**, 1881–1896. doi: 10.1111/j.1365-294X.2005.02536.x

Banke S, Peschon A, McDonald BA (2004) Phylogenetic analysis of globally distributed *Mycosphaerella graminicola* populations based on three DNA sequence loci. *Fungal Genetics and Biology* **41**, 226–238. doi: 10.1016/j.fgb.2003.09.006

Beck JJ, Ligon JM (1995) PCR assays for the detection of *Stagonospora nodorum* and *Septoria tritici* in wheat. *Phytopathology* **85**, 319–324. doi: 10.1094/Phyto-85-319

Brokenshire T (1975) Wheat seed infection by *Septoria tritici. Transactions of the British Mycological Society* **62**, 331–334.

Bruns TD, White TJ, Taylor JW (1991) Fungal molecular systematics. *Annual Review of Ecology and Systematics* **22**, 525–564. doi: 10.1146/annurev.es.22.110191.002521

Chadha S, Gopalakrishna T (2006) Detection of *Magnaporthe grisea* in infested rice seeds using polymerase chain reaction. *Journal of Applied Microbiology* **100**, 1147–1153. doi: 10.1111/j.1365-2672.2006.02920.x

Eyal Z, Scharen AL, Huffman MD, Prescott JM (1985) Global insights into virulence frequencies of *Mycosphaerella graminicola. Phytopathology* **75**, 1456–1462. doi: 10.1094/Phyto-75-1456

Eyal Z, Scharen AL, Prescott JM, Van Ginkel M (1987) 'The Septoria Diseases of Wheat: Concepts and Methods of Disease Management.' p. 52. (CIMMYT: México, D.F, México)

Fountaine JM, Shaw MW, Napier B, Ward E, Fraaije BA (2007) Application of real-time and multiplex polymerase chain reaction assays to study leaf blotch epidemics in barley. *Phytopathology* **97**, 297–303. doi: 10.1094/PHYTO-97-3-0297

Fraaije BA, Lovell DJ, Rohel EA, 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. doi: 10.1046/j.1365-2672.1999.00716.x

Fraaije BA, Lovell DJ, Coelho JM, Baldwin S, Hollomon DW (2001) PCR-based assays to assess wheat varietal resistance to blotch (*Septoria tritici* and *Stagonospora nodorum*) and rust (*Puccinia striiformis* and *Puccinia recondita*) diseases. *European Journal of Plant Patholology* **107**, 905–917. doi: 10.1023/A:1013119206261

Gayoso C, Martínez O, Pomar F, Merino F (2007) Assessment of real-time PCR as a method for determining the presence of *Verticillium dahliae* in different Solanaceae cultivars. *European Journal of Plant Pathology* **118**, 199–209. doi: 10.1007/s10658-007-9134-8

Goodwin PH, Hsiang T, Xue BG, Liu HW (1995) Differentiation of *Gaeumannomyces graminis* from other turf-grass fungi by amplification with primers from ribosomal internal transcribed spacers. *Plant Pathology* **44**, 384–391. doi: 10.1111/j.1365-3059.1995.tb02791.x

Guo JR, Schneider F, Verreet JA (2006) Presymptomatic and quantitative detection of *Mycosphaerella graminicola* development in wheat using a real-time PCR assay. *FEMS Microbiology Letters* **262**, 223–229.

Jeanmougin F, Thompson JD, Gouy M, Higgins DG, Gibson TJ (1998) Multiple sequence alignment with CLUSTAL X. *Trends in Biochemical Sciences* **23**, 403–405. doi: 10.1016/S0968-0004(98)01285-7

Johanson LF, Jeger MJ (1993) Use of PCR for detection of *Mycosphaerella fijiensis* and *M. musicola*, the causal agents of Sigatoka leaf spots in banana and plantain. *Mycological Research* **97**, 670–674.

Kema GHJ, Yu D, Rijkenberg FHJ, Shaw MW, Baayen RP (1996) Histology of the pathogenesis of *Mycosphaerella graminicola. Phytopathology* **86**, 777–786. doi: 10.1094/Phyto-86-777

Konstantinova P, Bonants PJM, Pelzer VG, Zowen PVD, Bulk RVD (2002) Development of specific primers for detection and identification of *Alternaria* spp. in carrot material by PCR and comparison with blotter and plating assays. *Mycological Research* **106**, 23–33. doi: 10.1017/S0953756201005160

Kulik T, Fordonski G, Pzczolkowoska A, Plodxien K, Lapinski M (2004) Development of PCR assay based on ITS2 rDNA polymorphism for the detection and differentiation of *Fusarium sporotrichioides. FEMS Microbiology Letters* **239**, 181–186. doi: 10.1016/j.femsle.2004.08.037

Lee HK, Tewari JP, Turkington TK (2001) A PCR-based assay to detect *Rhynchosporium secalis* in barley seed. *Plant Disease* **85**, 220–225. doi: 10.1094/PDIS.2001.85.2.220

Lee HK, Tewari JP, Turknington TK (2002) Quantification of seedborne infection by *Rhynchosporium secalis* in barley using competitive PCR. *Plant Pathology* **51**, 217–224. doi: 10.1046/j.1365-3059.2002.00685.x

Mathur SB, Cunfer BM (1993) 'Seedborne diseases and seed health testing of wheat.' (Danish Government Institute of Seed Pathology for Developing Countries: Copenhagen)

McDonald BA, Zhan J, Yarden O, Hogan K, Garton J, Pettway RE (1999) The population genetics *of Mycosphaerella graminicola* and *Phaeosphaeria nodorum.* In 'Septoria on Cereals: A Study of Pathosystems'. (Eds JA Lucas, P Bowyer, HM Anderson) pp. 44–69. (CABI: Wallingford)

Murray MJ, Thompson WW (1980) Rapid isolation for high molecular-weight plant DNA. *Nucleic Acid Research* **8**, 4321–4325. doi: 10.1093/nar/8.19.4321

Pryor B, Gilbertson RL (2001) A PCR-based assay for detection of *Alternaria radicina* on carrot seed. *Plant Disease* **85**, 18–23. doi: 10.1094/PDIS.2001.85.1.18

Robert C, Bancal MO, Lannou C, Ney B (2005) Quantification of the effects of *Septoria tritici* blotch on wheat leaf gas exchange with respect to lesion age, leaf number and leaf nitrogen status. *Journal of Experimental Botany* **57**, 225–234. doi: 10.1093/jxb/eri153

Rohel EA, Payne AC, Hall L, Barker H, Butters J, Hollomon DW (1998) Isolation and characterization of α-tubulin genes from *Septoria tritici* and *Rhynchosporium secalis*, and comparative analysis of fungal α-tubulin sequences. *Cell Motility and the Cytoskeleton* **41**, 247–253. doi: 10.1002/(SICI)1097-0169(1998)41:3<247::AID-CM5>3.0.CO;2-7

Sambrook J, Russell DW (2001) 'Molecular Cloning: A Laboratory Manual'. (Cold Spring Harbor: Laboratory Press: Cold Spring Harbour)

Stackebrandt E, Liesack W, Witt D (1992) Ribosomal RNA and rDNA sequences analyses. *Gene* **115**, 255–260. doi: 10.1016/0378-1119(92)90567-9

Taylor JL (1993) A simple, sensitive and rapid method for detecting seed contaminated with highly virulent *Leptosphaeria maculans. Applied and Environmental Microbiology* **59**, 3681–3685.

Tegli S, Sereni A, Surico G (2002) PCR-based assay for the detection of *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* in bean seeds. *Letters in Applied Microbiology* **35**, 331–337. doi: 10.1046/j.1472-765X.2002.01187.x

Van Ginkel M, Rajaram S (1993) Breeding for durable resistance in wheat: An international perspective. In 'Durability of disease resistance'. (Eds TH Jacobs, JE Parlevliet) pp. 259–272. (Kluwer Academic Publishers: Dordrecht)

Vandemark GJ, Kraft JM, Larsen RC, Gritsenko MA, Boge WL (2000) A PCR-based assay by sequence characterised DNA markers for the identification and detection of *Aphanomyces euteiches. Phytopathology* **90**, 1137–1144. doi: 10.1094/PHYTO.2000.90.10.1137

Ward E, Foster SJ, Fraaije BA, Mc Cartney AH (2004) Plant pathogen diagnostics: immunological and nucleic acid-based approaches. *The Annals of Applied Biology* **145**, 1–16. doi: 10.1111/j.1744-7348.2004.tb00354.x

Wiese MV (1987) 'Compendium of wheat diseases.' 2nd edn. (APS Press: St. Paul)

Zilberstein M, Blum A, Eyal Z (1985) Chemical desiccation of wheat plants as a simulator of postanthesis speckled leaf blotch stress. *Phytopathology* **75**, 226–230. doi: 10.1094/Phyto-75-226

Ziv O, Eyal Z (1974) Assessment of yield component losses in plants of spring wheat cultivars caused by selected isolates of *Septoria tritici. Phytopathology* **8**, 791–796.

Zur G, Simón E, Hallerman E, Kasi Y (2002) Detection of Alternaria fungal contamination in cereal grains by a polymerase chain reaction-based assay. *Journal of Food Protection* **65**, 1433–1440.

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