Polymerase Chain Reaction Assays for the Detection of *Stagonospora nodorum* and *Septoria tritici* in Wheat

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ABSTRACT

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Polymerase chain reaction (PCR) primers have been developed that can distinguish important wheat pathogenic fungal species and detect their DNA in infected wheat leaf tissues. *Septoria tritici* (teleomorph *Mycosphaerella graminicola*) and *Stagonospora nodorum* = *Septoria nodorum* (teleomorph *Phaeosphaeria nodorum*), are agronomically significant pathogens of wheat, causing wheat leaf and glume blotch, respectively. Primers made to conserved sequences of the ribosomal DNA were used to PCR amplify and clone the internal transcribed spacer (ITS) regions from *S. nodorum* and *S. tritici*. The ITS regions were sequenced, the sequences were aligned, and species-specific PCR primers were designed to the polymorphic regions. These ITS-derived primers were used to amplify specific fragments from the fungi from which they were designed, thus allowing the detection of the pathogens. The primers also successfully amplified similar-sized fragments from wheat tissues infected with the respective fungi.

Additional keyword: diagnostics, Septoria glume blotch, Septoria leaf blotch.

The major pathogens of small grains include *Septoria tritici* Roberge in Desmaz. (teleomorph *Mycosphaerella graminicola* (Fuckel) J. Schröt in Cohn) and *Stagonospora nodorum* (Berk.) Castellani & E. G. Germano = *Septoria nodorum* (Berk.) Berk. (teleomorph *Phaeosphaeria nodorum* (E. Muller) Hedjaroude). *Septoria tritici* causes Septoria leaf blotch in wheat and also infects triticale and rye (6). *Stagonospora nodorum* is the causative agent of Septoria glume blotch in wheat and can also infect triticale, rye, and barley (6). Substantial wheat yield losses can be attributed to these pathogens, especially during growing seasons with high rainfall (7). *Stagonospora avenae* Bissett f. sp. *triticea* T. Johnson is parasitic on oats, wheat, and triticale (6) and *Septoria passerinii* Sacc. is restricted to barley (6).

These diseases, collectively known as Septoria complex, occur in all wheat-growing areas at important levels, and different Septoria complex pathogens frequently occur concurrently within fields and on individual plants (16). Typically the most commonly found species are *S. tritici* and *S. nodorum*. It is often difficult to visually detect or distinguish between the different Septoria complex pathogens at the onset of infection. This makes the decision of whether or not to treat the crop with fungicides, and which fungicides to use, difficult because different species of Septoria complex pathogens can be more, or less, sensitive to different fungicides. In particular, the differential symptomology caused by different isolates and species of these fungi makes the accurate predictive determination of potential disease loss difficult. Consequently, the availability of improved diagnostic techniques for the rapid and accurate identification of specific pathogens would be of considerable use to growers and field pathologists.

Ribosomal genes are suitable for use as molecular probes because of their high copy number. Despite the high conservation between functional ribosomal DNA (rDNA) coding sequences, the nontranscribed and transcribed spacer sequences are usually less well conserved and are thus suitable as target sequences for the detection of recent evolutionary divergence. The polymerase chain reaction (PCR) is a highly sensitive and specific technique for the amplification of nucleic acids (4). This technique has previously been applied in the detection of plant pathogens, including fungi (2,5,11) and viruses (12). The presence of *Gaeumannomyces graminis* in different grass hosts has been detected using PCR (2), and random amplified polymorphic DNA markers were able to distinguish numerous races of *Gremmeniella abietina*, the causal agent of scleroderris canker in conifers (3). We have developed species-specific PCR primers based on sequence analysis of the internal transcribed spacer (ITS) regions of *S. nodorum* and *S. tritici*. These primers are specific for *S. tritici* and *S. nodorum* and use of them in PCR reactions enables the detection and differentiation of these pathogens in infected wheat tissues.

MATERIALS AND METHODS

Fungal isolates and genomic DNA extraction. The fungal strains used in this study and their sources are listed in Table 1. Fungi were grown on potato-dextrose agar (Difco) and mycelial fragments from these cultures were used to inoculate 150 ml of potato-dextrose broth in 500-ml flasks. Cultures were incubated on an orbital shaker at 28 C for 7–11 days. Mycelia were collected by centrifugation and ground in liquid nitrogen to facilitate the isolation of total genomic DNA by the protocol of Lee and Taylor (8).

Bruce McDonald from Texas A&M University supplied genomic DNA from eight isolates of *S. tritici*. Chris Caten from the University of Birmingham supplied purified fungal DNAs from six European isolates of *S. nodorum*. Gary Bergstrom and Peter Ueng supplied the isolate of *S. avenae* f. sp. *triticea* (Table 1).

PCR amplification. PCRs were performed with the GeneAmp Kit from Perkin-Elmer/Cetus (Norwalk, CT) using 50 mM KCl, 2.5 mM $MgCl_2$, 10 mM Tris-HCl, pH8.3, containing 200 mM of each dTTP, dATP, dCTP, and dGTP, 50 pmol primer, 2.5 units of *Taq* polymerase and 25 ng of genomic DNA in a final volume of 50 μl. Reactions were run for 30 cycles, each consisting of 15 s at 94 C, 15 s at 60 C, and 45 s at 72 C in a Perkin-Elmer Model 9600 thermal cycler. The products were analyzed by electrophoresis of a 20-μl aliquot of each PCR sample on a 1.1–1.2% horizontal agarose gel.

Southern analysis. DNA from agarose gels was transferred to MagnaGraph nylon transfer membrane (Micron Separations Inc., Westboro, MA) by Southern transfer (14). DNA probes were radioactively labeled by random priming according to the manufacturer's procedure (Pharmacia, Uppsala, Sweden). Prehybridization and hybridization reactions were performed in bottles at

37 C in a Robbins Scientific (Sunnyvale, CA) oven with 50% formamide. Membranes were washed twice at room temperature in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) with 0.1% sodium dodecyl sulfate for 15 min each followed by two washings at 37 C in 0.1× SSC plus 0.1% sodium dodecyl sulfate for 15 min each. Membranes were exposed on X-ray film (Eastman Kodak, Rochester, NY) with intensifying screens for 5 min to 24 h.

Isolation of the ITS regions. The approximately 550-bp ITS region fragments were PCR amplified from 25 ng of genomic DNA isolated from *S. nodorum* (ATCC #24425) and *S. tritici* (ATCC #26517) using 50 pmol of primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) (15) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (15). PCR was performed as described above except 100 μl reactions were used and the annealing temperature was 50 C. The ITS fragments were purified by isopropanol precipitation according to Maniatis (10). The DNA was resuspended in 50 μl of distilled H_2O and the amplified ITS fragments were cloned using the TA Cloning Kit and pCR II cloning vector (Invitrogen Corporation, San Diego, CA). The DNA sequences of the ITS regions were determined by the dideoxynucleotide chain termination method (13) using an automated sequencer (Applied Biosystems model 373A, Foster City, CA) and the primers ITS1, ITS2 (5′-GCTGCGTTCTTCATCGATGC-3′) (15),

TABLE 1. Source of fungal DNA test isolates

Isolate	Fungus	Origin	Source
ATCC #24425	*Stagonospora nodorum* (teleomorph *Phaeosphaeria nodorum*)	Montana	ATCC[a]
BS3	*Stagonospora nodorum* (teleomorph *Phaeosphaeria nodorum*)	Ireland	C. Caten[b]
BS6	*Stagonospora nodorum* (teleomorph *Phaeosphaeria nodorum*)	Ireland	C. Caten
BS175	*Stagonospora nodorum* (teleomorph *Phaeosphaeria nodorum*)	England	C. Caten
BS425	*Stagonospora nodorum* (teleomorph *Phaeosphaeria nodorum*)	England	C. Caten
α′5	*Stagonospora nodorum* (teleomorph *Phaeosphaeria nodorum*)	France	C. Caten
m300	*Stagonospora nodorum* (teleomorph *Phaeosphaeria nodorum*)	England	C. Caten
ATCC #26517	*Septoria tritici* (teleomorph *Mycosphaerella graminicola*)	Minnesota	ATCC
TKV2a	*Septoria tritici* (teleomorph *Mycosphaerella graminicola*)	Turkey	B. McDonald[c]
SYK2	*Septoria tritici* (teleomorph *Mycosphaerella graminicola*)	Syria	B. McDonald
ISZC36.2	*Septoria tritici* (teleomorph *Mycosphaerella graminicola*)	Israel	B. McDonald
CNRC4a.1	*Septoria tritici* (teleomorph *Mycosphaerella graminicola*)	Canada	B. McDonald
ALA1a	*Septoria tritici* (teleomorph *Mycosphaerella graminicola*)	Algeria	B. McDonald
GEB2a.1	*Septoria tritici* (teleomorph *Mycosphaerella graminicola*)	Germany	B. McDonald
UK92D2	*Septoria tritici* (teleomorph *Mycosphaerella graminicola*)	UK	B. McDonald
DNB1a	*Septoria tritici* (teleomorph *Mycosphaerella graminicola*)	Denmark	B. McDonald
ATCC #38699	*Septoria glycines* Hemmi	Illinois	ATCC
ATCC #22585	*Septoria passerinii* Saccardo	Minnesota	ATCC
ATCC #60972	*Pseudocercosporella herpotrichoides* Nirenberg var. *herpotrichoides*	Germany	ATCC
ATCC #62012	*Pseudocercosporella aestiva* Nirenberg	Germany	ATCC
ATCC #44234	*Ceratobasidium cereale* Murray *et* Burpee (anamorph = *Rhizoctonia cerealis*)	Netherlands	ATCC
ATCC #11404	*Drechslera sorokiniana* (Saccardo) Subramanian *et* Jain	Minnesota	ATCC
ATCC #22116	*Mycosphaerella fijiensis* Meredith *et* Lawrence (anamorph = *Paracercospora fijiensis*)	Philippines	ATCC
ATCC #22115	*Mycosphaerella musicola* Leach (anamorph = *Pseudocercospora musae*)	Philippines	ATCC
ATCC #26380	*Stagonospora avenae* f. sp. *triticea* T. Johnson (teleomorph = *Phaeosphaeria*)	Minnesota	Bergstrom/Ueng[d]

[a] American Type Culture Collection, Rockville, MD.
[b] Chris Caten, University of Birmingham, UK.
[c] Bruce McDonald, Texas A&M University, College Station.
[d] Gary Bergstrom, Cornell University, and Peter Ueng, USDA-ARS, Beltsville, MD.

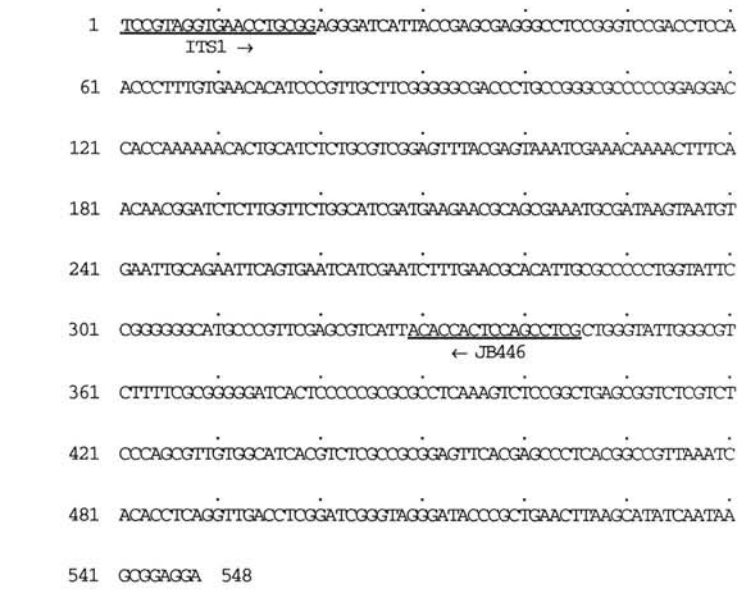

Fig. 1. Nucleotide sequence of the internal transcribed spacer region derived from the *Septoria tritici* isolate ATCC #26517. The sequence includes the ITS1 primer sequence, the ITS4 primer sequence, and the 5.8S ribosomal DNA sequence. The ITS1 and JB446 primers are underlined and indicated.

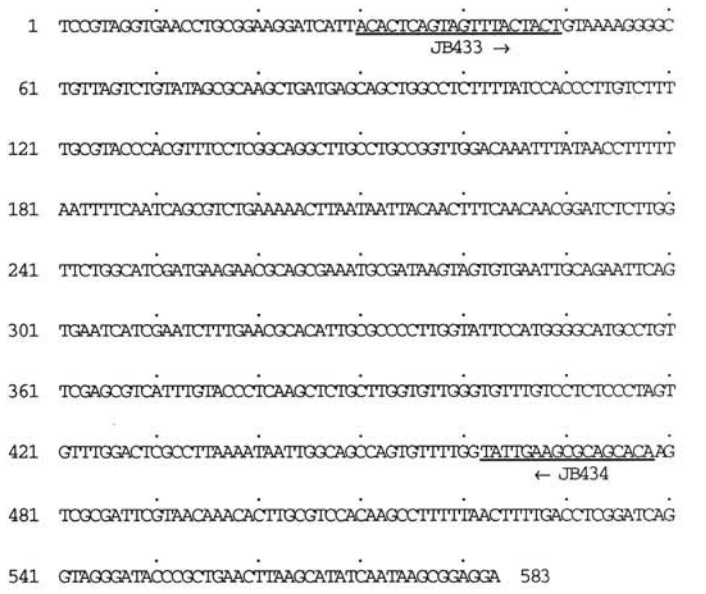

Fig. 2. Nucleotide sequence of the internal transcribed spacer region derived from the *Stagonospora nodorum* isolate ATCC #24425. The sequence includes the ITS1 primer sequence, the ITS4 primer sequence, and the 5.8S ribosomal DNA sequence. The JB433 and JB434 primers are underlined and indicated.

ITS4 and the universal -20 (5′-GTAAAACGACGGCCAGT-3′) and Reverse (5′-AACAGCTATGACCATG-3′) primers. DNA sequences were analyzed using software from DNASTAR (Madison, WI).

DNA extraction from wheat leaves. DNA was extracted from wheat leaves using the Rapid DNA Extraction protocol from the IsoQuick Nucleic Acid Extraction Kit (MicroProbe Corporation, Garden Grove, CA). Typical yields were 5–10 μg of total DNA from 0.2 g of leaf tissue. Approximately 100 ng of total DNA were used in each PCR assay.

Synthesis of species-specificific primers. PCR primers specific for the *S. tritici* ITS sequence (Fig. 1) and *S. nodorum* ITS sequence (Fig. 2) were identified based on sequence analysis of these regions. In addition, the rDNA gene-specific primers ITS1, ITS2, ITS3, and ITS4 (15) were synthesized for testing in combination with the primers specific for the ITS region. Oligonucleotides were synthesized using an Applied Biosystems (Foster City, CA) model 394 automated DNA synthesizer. PCRs were performed as described above using different primer combinations in an attempt to amplify a unique species-specific fragment from purified fungal DNA.

RESULTS

Determination of primer specificity to purified fungal genomic DNA. The *S. tritici*–specific primer JB446 (5′-CGAGGCTGG-AGTGGTGT-3′) and ITS1 resulted in the amplification of a 345-bp fragment from all nine of the *S. tritici* isolates listed in Table 1 (Fig. 3). There was no cross-reactivity with purified genomic DNA from any strains of the following cereal pathogens that were tested: *S. nodorum, S. glycines, S. passerinii, P. herpotrichoides, P. aestiva, C. cereale,* and *D. sorokiniana* (Figs. 3 and 4). DNA from the other fungal species did not produce amplification products with the *S. tritici*–specific primers. Similarly, a 448-bp fragment was amplified from all seven of the *S. nodorum* isolates listed in Table 1 using the *S. nodorum*–specific primers, JB433 (5′-ACACTCAGTAGTTTACTACT-3′) and JB434 (5′-TGTGC-TGCGCTTCAATA-3′) (Fig. 5). DNA from other cereal pathogens, including those listed above for the *S. tritici*–specific primers, were also tested. DNA from these fungi produced no PCR products when amplified with primers JB433 and JB434 (Figs. 5 and 6).

In order to investigate the specificity of the *S. nodorum*– and *S. tritici*–specific primers, we examined whether they would produce similar-sized PCR products from closely related species. *Mycosphaerella fijiensis* and *M. musicola* are pathogens of banana and are closely related to *S. tritici,* while *Septoria avenae* f. sp. *triticae* (*Stagonospora avenae*) is parasitic on wheat, oats, and triticale, and is a close relative of *S. nodorum* (1). The *S.*

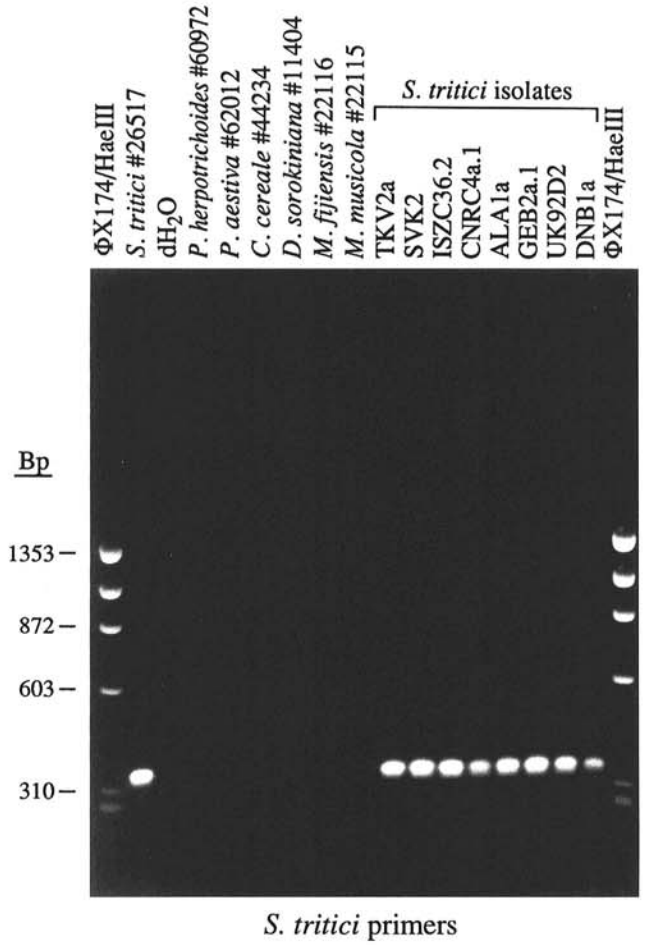

Fig. 3. Ethidium bromide–stained agarose gel of polymerase chain reaction amplification products using *Septoria tritici*–specific primer JB446 and ITS1 with fungal DNA of cereal and banana pathogens and *S. tritici* isolates.

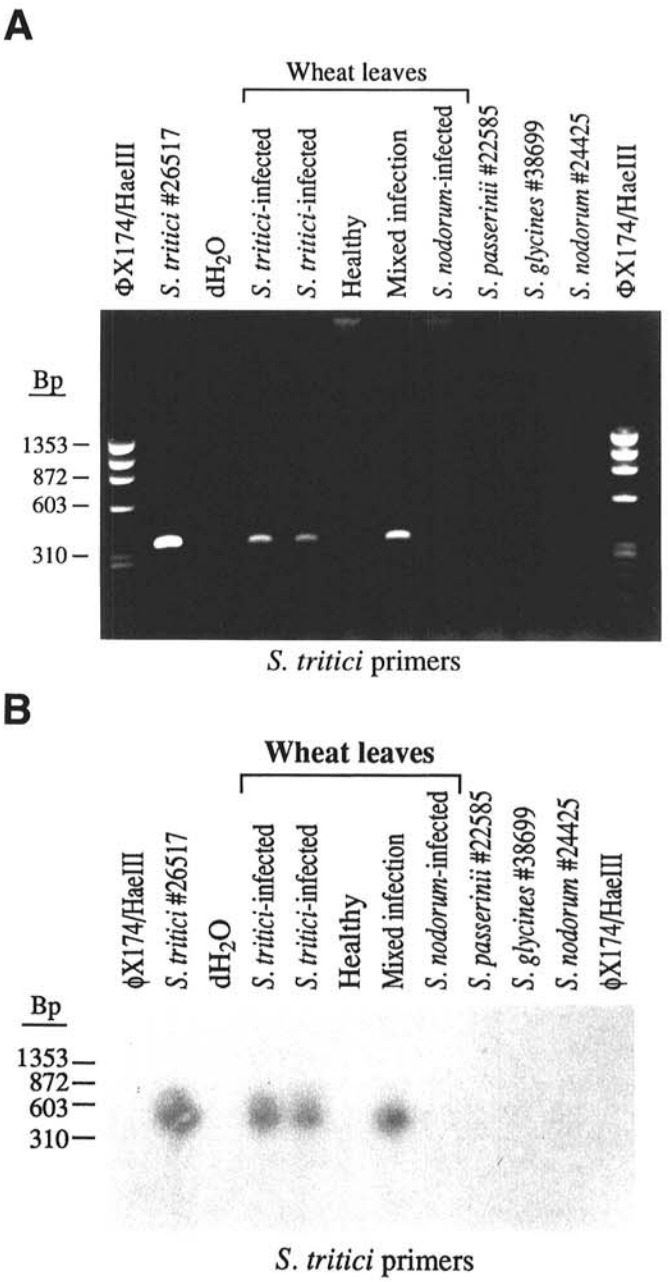

Fig. 4. Ethidium bromide–stained agarose gel of polymerase chain reaction (PCR) products (**A**) probed with a radiolabeled PCR fragment from *Septoria tritici* (**B**). Amplifications were done using *S. tritici*–specific primer JB446 and ITS1 with fungal DNA from other *Septoria* spp. and infested wheat leaves.

tritici-specific primers did not produce PCR products from *M. fijiensis* or *M. musicola* template DNA (Fig. 3). However, the *S. nodorum*-specific primers amplified a DNA fragment from *S. avenae* f. sp. *triticae* genomic DNA that was the same size as that amplified from *S. nodorum* isolates (*data not shown*).

Determination of primer specificity to plant tissue infected with fungi. PCR using the *S. tritici*-specific primer JB446 and ITS1 and template DNA derived from *S. tritici*-infected, and *S. tritici* and *S. nodorum*-infected wheat leaf tissues resulted in the amplification of a 345-bp fragment (Fig. 4A). This primer pair did not amplify a fragment of similar size from healthy wheat leaf tissue nor from *S. nodorum*-infected wheat tissue. Furthermore, after Southern blotting the PCR fragment from *S. tritici* was found to hybridize to the PCR product from infected wheat DNA (Fig. 4B). Similar results were obtained with the *S. nodorum*-specific primers JB433 and JB434. These primers amplified a 448-bp fragment from *S. nodorum*-infected wheat tissue, from a wheat leaf sample infested with both *S. nodorum* and *S. tritici,* as well as from purified genomic DNA of *S. nodorum* (Fig. 6A). However, the *S. nodorum*-specific primers JB433 and JB434 did not amplify fragments from healthy wheat tissue or from purified genomic DNA of *S. tritici.* Wheat leaves that were visually assessed and determined to be infected with *S. tritici,* but not with *S. nodorum,* produced the 345-bp fragment that is diagnostic for *S. tritici* infections after PCR with the *S. tritici*-specific primers. However, PCR amplification from this DNA using the *S. nodorum*-specific primer pair (JB433 and JB434) produced a faint 448-bp fragment that is indicative of the presence of *S. nodorum,* suggesting that these tissues also had a low level of infection by *S. nodorum* that was not evident upon visual inspection of the tissue. Southern hybridization analysis showed that the ITS fragment cloned from *S. nodorum* hybridized to the PCR product from the DNA derived from this tissue (Fig. 6B).

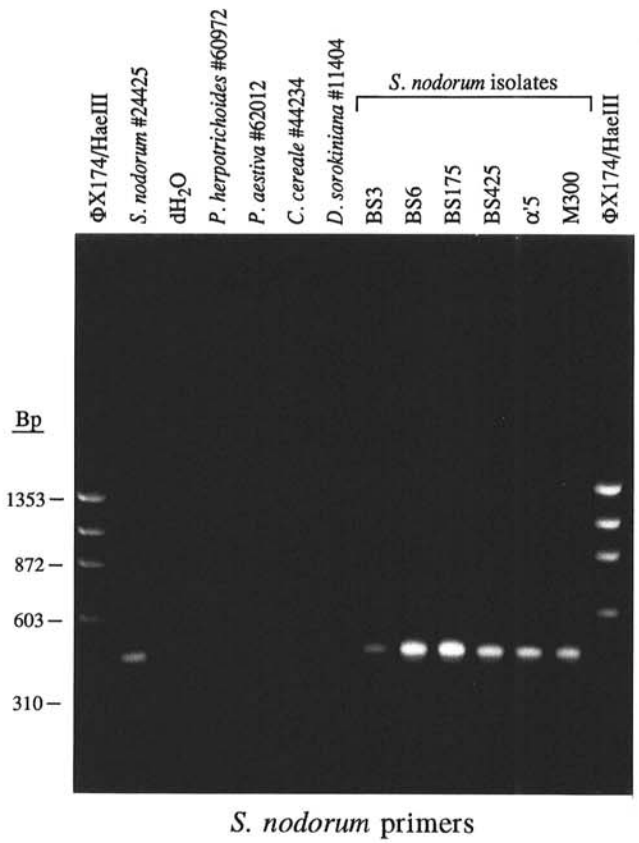

Fig. 5. Ethidium bromide–stained agarose gel of polymerase chain reaction amplification products using *Stagonospora nodorum*-specific primers JB433 and JB434 with fungal DNA from cereal pathogens and *S. nodorum* isolates.

Determination of primer sensitivity. PCR amplification, using the *S. nodorum*-specific primers JB433 and JB434 and a series of *S. nodorum* genomic DNA samples ranging in concentration from 0.25 pg to 250 ng, demonstrated the ability to detect the fungus with as little as 2.5 pg of DNA (Fig. 7). A similar level of sensitivity was seen with the *S. tritici*-specific primers JB446 and ITS1 (*data not shown*). In addition, PCR analysis of this

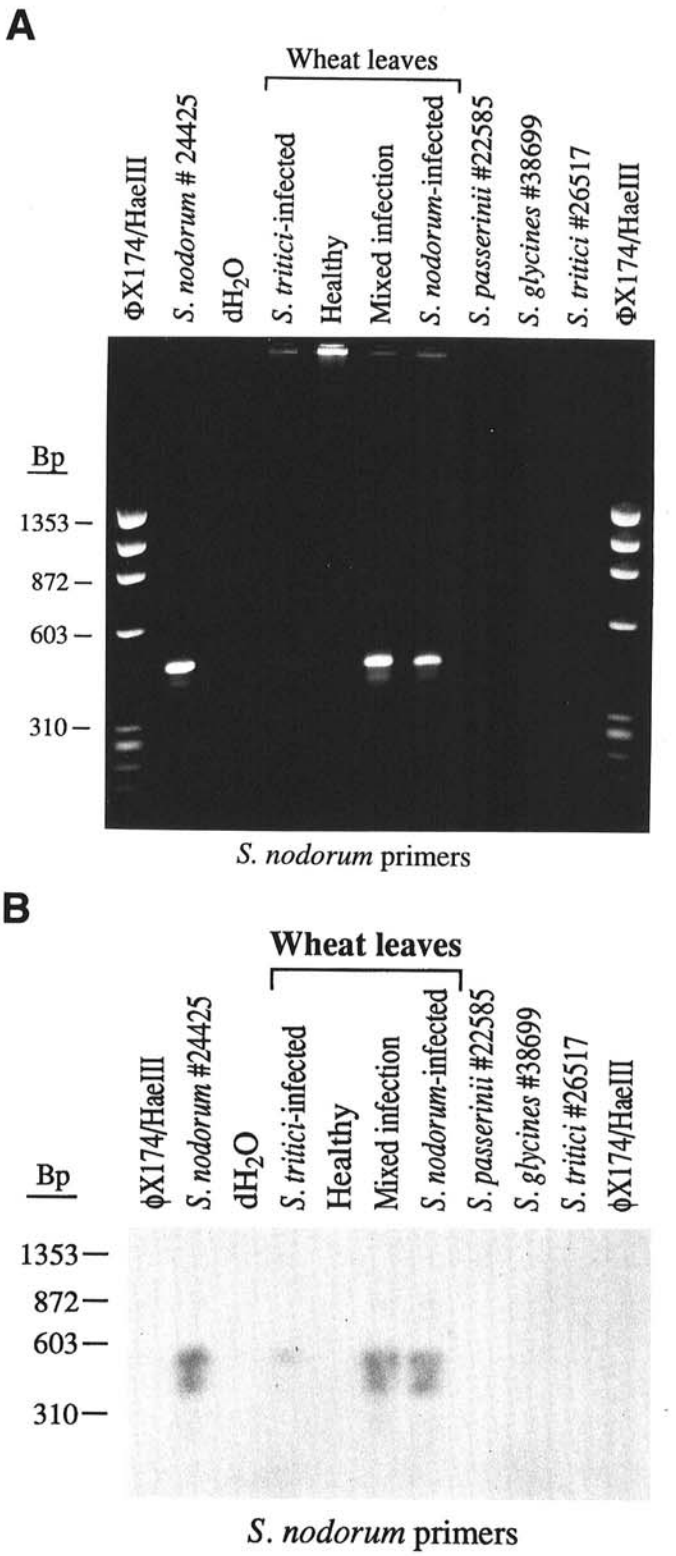

Fig. 6. Ethidium bromide–stained agarose gel of polymerase chain reaction (PCR) products (**A**) probed with a radiolabeled PCR fragment from *Stagonospora nodorum* (**B**). Amplifications were performed using *S. nodorum*-specific primers JB433 and JB434 with fungal DNA from other *Septoria* spp. and from infested wheat leaves.

dilution series demonstrated that the response was generally relative to the amount of template DNA in the reaction. Lower amounts of template DNA generated smaller amounts of PCR product than samples containing more template DNA, as judged from the intensity of the ethidium bromide–stained fragments on agarose gels (Fig. 7).

DISCUSSION

Although the rDNA genes of related organisms are generally highly conserved, the nontranscribed and ITS regions are usually less well conserved (15). The more extensive sequence divergence in the ITS region has been the basis for the development of PCR assays that are able to detect plant pathogens, including those of the genera *Mycosphaerella* (5) and *Verticillium* (11). For other genera, whether sufficient sequence divergence exists in the ITS region to develop similar PCR assays for relevant plant pathogens is uncertain and must be determined empirically. We describe here the development of a PCR-based assay that is capable of detecting, and differentiating, *S. tritici* and *S. nodorum,* two important pathogens of wheat. This assay effectively detected the presence of pathogens in wheat tissues that were visually determined to be infected with these pathogens. The PCR primers derived from *S. nodorum* were, however, unable to differentiate purified DNA derived from *S. nodorum* and a single isolate tested of *S. avenae* f. sp. *triticea.* No PCR DNA products were produced with either the *S. nodorum*– or *S. tritici*–specific primers from DNA derived from healthy wheat tisssues. One wheat sample that was thought to be infected only with *S. tritici* based on visual assessment produced a faint *S. nodorum* PCR amplification product of 448 bp with the *S. nodorum* primers. This suggests that the level of sensitivity of PCR detection is greater than that of visual inspection, and that PCR is able to detect the presence of the pathogen before the development of obvious disease symptoms.

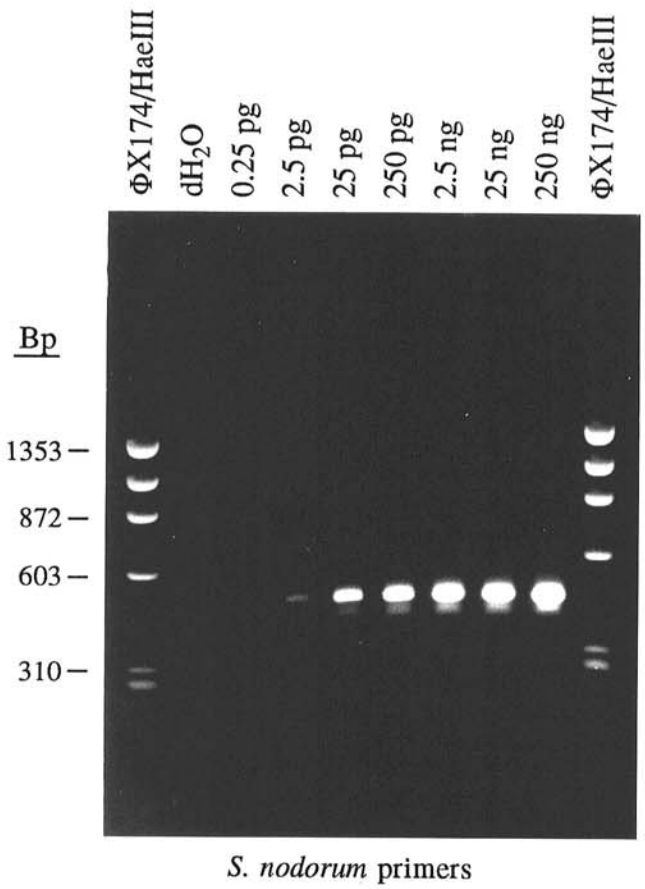

Fig. 7. Ethidium bromide–stained agarose gel of polymerase chain reaction products from amplification of 0.25 pg to 250 ng of genomic *Stagonospora nodorum* DNA using *S. nodorum*–specific primers JB433 and JB434.

PCR has been shown to be extremely sensitive, and capable of detecting a single molecule of template DNA (9). Lee and Taylor (8) reported PCR amplification of rDNA sequences from a single spore of *Neurospora tetrasperma.* Indeed, in this study, *S. nodorum*– and *S. tritici*–specific PCR products were generated from the respective template DNAs that contained picogram levels of purified genomic DNA. Furthermore, the PCR assay demonstrated a quantitative response since the intensity of the PCR products were relative to the amount of fungal template DNA used (Fig. 7). The high sensitivity of PCR enables quick and accurate detection of only a few fungal cells in infested plant tissues, often well before visible disease symptoms are apparent. Thus, pathogen diagnostics based on PCR technology are likely to become an important aspect of modern integrated pest management practices in agriculture. The early detection of plant disease allows the judicious use of agricultural fungicides and early treatment is often more effective as the pathogen is less well established in its host.

PCR-based diagnostic methods have several advantages over serological methods. The use of species-specific primers appears to produce few, if any, of the false positive reactions that can be encountered with serological assays due to cross-reactivity of antibodies with antigens derived from the plant or other microbes that may be present on it. Since only very small amounts of plant DNA are required for the PCR assay, the problems associated with low antigen titer with enzyme-linked immunosorbent assays could be resolved. Most significantly, the cost and time required for PCR-assay development are substantially less than the cost and time required to develop a similar serological assay. The production and purification of antibodies is more expensive and time consuming than is the development of new PCR primers. For the above reasons, PCR diagnosis of plant disease is likely to become a commonly used technology for the benefit of modern agriculture.

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