A polymerase chain reaction for the detection of the take-all fungus, Gaeumannomyces graminis, in infected wheat seedlings
by Kurt Robert Schesser

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Microbiology
Montana State University
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Abstract:
Gaeumannomyces araminis var. tritici, a filamentous ascomycete, causes the wheat and barley disease take-all. Symptoms of take-all include blackened roots, sterile heads, and eventual death of the plant, all of which can be caused by other plant pathogens or environmental conditions. Currently diagnosis of G. araminis var. tritici as the etiologic agent of take-all involves culturing the fungus on a highly selective medium which is variable in its ability to recover the fungus from infected tissue. In this project I used the polymerase chain reaction (PCR) to detect the presence of G. araminis var. tritici DNA in infected wheat seedlings.

PCR is a method of DNA amplification which is both highly specific and extremely sensitive. The specificity arises from the use of two oligonucleotide primers which are made complementary to sequences of the DNA fragment of interest. Sensitivity is a result of the amplification process which proceeds exponentially. Here I describe the use of PCR to amplify a G. araminis-specific DNA fragment which was present in wheat seedlings inoculated with the fungus. The fragment was not produced in uninfected seedlings or seedlings infected with other phytopathogenic fungi. This method of detection has potential use in soil assays for the take-all fungus which would facilitate disease forecasting.
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A thesis submitted in partial fulfillment
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Microbiology

MONTANA STATE UNIVERSITY
Bozeman, Montana
September 1990
APPROVAL

of a thesis submitted by

Kurt Robert Schesser

This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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Head, Major Department

Approved for the College of Graduate Studies

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Date 4 Sept 1990
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Gaeumannomyces graminis var. tritici, a filamentous ascomycete, causes the wheat and barley disease take-all. Symptoms of take-all include blackened roots, sterile heads, and eventual death of the plant, all of which can be caused by other plant pathogens or environmental conditions. Currently diagnosis of G. graminis var. tritici as the etiologic agent of take-all involves culturing the fungus on a highly selective medium which is variable in its ability to recover the fungus from infected tissue. In this project I used the polymerase chain reaction (PCR) to detect the presence of G. graminis var. tritici DNA in infected wheat seedlings.

PCR is a method of DNA amplification which is both highly specific and extremely sensitive. The specificity arises from the use of two oligonucleotide primers which are made complementary to sequences of the DNA fragment of interest. Sensitivity is a result of the amplification process which proceeds exponentially. Here I describe the use of PCR to amplify a G. graminis-specific DNA fragment which was present in wheat seedlings inoculated with the fungus. The fragment was not produced in uninfected seedlings or seedlings infected with other phytopathogenic fungi. This method of detection has potential use in soil assays for the take-all fungus which would facilitate disease forecasting.
INTRODUCTION

Gaeumannomyces graminis is a soilborne, filamentous ascomycete of worldwide distribution in the north and south temperate zones (22). In the field the fungus is associated almost exclusively with root and crown tissue of many members of the Gramineae plant family. Colonization of roots and crowns of susceptible plants by G. graminis can lead to either a pathogenic or nonpathogenic condition for the plant, depending on both the variety of the fungus and the species of the host plant (73). G. graminis var. tritici is the etiologic agent of take-all disease of wheat and barley, which economically is the most important root disease for these cereals worldwide. It is characterized by penetration of the root cortical layers and colonization of the stele by the pathogen which leads to blackened roots, stunted growth, sterile heads, and eventual death of the plant. G. graminis var. avenae causes take-all in oats as well as in wheat and barley. G. graminis var. graminis, which causes crown sheath rot disease of rice and spring dead spot of bermuda, is considered a nonpathogenic parasite of wheat and barley since it does not extend into the stele or cause symptoms characteristic of take-all. This project concerns the take-all fungus, G. graminis var. tritici, and its detection in infected wheat seedlings.
Take-All and its Control

The disease take-all was first recognized in South Australia in 1852 and the first recorded assignment of *G. graminis* var. *tritici* as the etiologic agent of the disease was made in France in 1890 (22). In 1913 take-all was first recognized in England and by the late 1940’s it had become a significant yield-limiting disease in England as well as the rest of Europe, Australia, and the United States. This dramatic increase in take-all’s destructiveness was for the most part due to a change in farming practices, as a shift towards monoculturing and away from rotational farming techniques had occurred (30). The reason monoculturing has increased the relative success of *G. graminis* var. *tritici* can best be understood in terms of its disease cycle.

Disease cycle

The pathology of take-all can be divided into three somewhat overlapping phases; parasitism and infection, spread of the fungus through a stand of wheat or barley within a growing season, and saprophytic survival of the fungus providing inoculum for the next growing season (31). Under laboratory conditions 2-3 germ tubes emerge from each ascospore. These pigmented germ tubes, or runner hyphae, give rise to infection hyphae anywhere along the length of the former in response to the presence of susceptible plant roots. Infection hyphae penetrate the epidermal or root
hair cells by forming an appressorium-like swelling termed an infection peg (77). It is believed that this penetration is mostly an enzymatic process since cell wall material is observed to dissolve just ahead of the infection peg (65). In response to this insult the host plant thickens its secondary cell wall with lignin or ligneous material, forming structures called lignitubers in an effort to contain the invading fungus. Infection hyphae eventually are able to penetrate the lignitubers which subsequently leads to the cell's death. When the infection hyphae reach the epidermis, blockage of the xylem occurs both by hyphae and lignitubers which results in the breakdown of stelar tissue and extensive host cell death. The fungus grows freely in the dead tissue. How far the fungus is able to proceed through the cell layers of the host determines the extent of pathogenicity of the disease. Weakly pathogenic isolates of G. graminis var. tritici are more often found in the cortical layer while more pathogenic strains are isolated from the stele (14). The overall effects of infection on the plant include blackened roots, stunted growth, and whiteheads (65).

How the spread of G. graminis var. tritici occurs within the soil is not well understood due to the almost complete lack of a reliable soil isolation method (discussed below). Samual and Garrett (56) demonstrated that moistened perithecia, when present in whiteheads, eject ascospores at
a rate of several hundred per min from a single diseased wheat culm. This observation agreed with epidemiological data which suggested that production of whiteheads is influenced by precipitation and wind. These dispersed ascospores survived 3-4 days in dry conditions and about a week in wet conditions. For longer term survival G. graminis mycelia appears to remain associated with wheat residue by which inoculum for the next growing season is provided. Soil within planted wheat and barley rows are more infectious than the soil between rows suggesting that the fungus survives better as a parasite than as a saprophyte (29). Brown and Hornby (7) showed that G. graminis var. tritici grew very little, if at all, in soil without wheat seedlings being present and other studies demonstrated a strong relationship between pathogenicity of the fungus and its survival on wheat stubble (11). These studies suggest how monoculturing increases the severity of take-all by increasing the amount of plant residue harboring the fungus. The fungus survives poorly in soil conditions which favor microbial growth, such as highly aerated and moist soils, due to its low competitive saprophytic ability (21).

**Disease control**

Presently the only completely effective control strategy for take-all involves crop rotation of susceptible
hosts away from the infested fields for a period of one or two years. The success of this control measure is due to the poor saprophytic survival of the fungus, as outlined above, and the activity of both self-inhibitory strains of *G. graminis* var. *tritici* and other fungi and bacteria. The microbiological basis of the latter was demonstrated by showing that the suppressive properties of inhibitory soils were lost upon heat treatment or irradiation (62,64). Romanos, et al., (52) described a factor released by certain cultures of *G. graminis* var. *tritici* grown at pH 3.5-4.5 which was both self-inhibitory for the take-all fungus and inhibitory for the growth of other fungi. Highly pathogenic strains, when grown at pH 4, produced very little of this inhibitory compound while weaker pathogenic strains produced relatively more and as a result grew slower (45). The pH dependency of the production of this compound suggests the basis for work which showed that field treatments which decreased the soil pH reduced the severity of the disease and increased the pathogen-suppressiveness of the soil (12,24,63). *G. graminis* var. *graminis*, a nonpathogenic parasite of cereals, to some extent confers cross-protection to host plants against take-all attack (79). The grassland fungus, *Phialophora radicicola*, has an infection habit almost indistinguishable to *G. graminis* var. *tritici* infection, covering the roots with dark, branching hyphae, but penetrating mostly senescent or dead cells and thus
causing little lignituber formation or vascular
discoloration (28,60,66). *P. radicicola* is considered an
avirulent parasite of wheat which provides some protection
against take-all when cross-inoculated with *G. graminis* var.
tritici (60). *P. radicicola* was once believed to be the
main microbial agent responsible for the phenomenon of take-
all decline (T.A.D.) (30) which is currently believed to be
caused by fluorescent pseudomonads (76). T.A.D. follows the
maximum development of the disease in soils which have grown
cereals continuously for 7-8 years. The bacterium
responsible for T.A.D., *Pseudomonas fluorescens*, produces a
phenazine antibiotic which is active *in vitro* against the
take-all fungus and other root rotting fungal pathogens
(69). Weller et al. (76) were able to show that isolates of
*P. fluorescens* from roots grown in suppressive soils were
more inhibitory for *G. graminis* var. tritici growth *in vitro*
than those isolated from roots growing in noninhibitory
soils. Further work showed that the presence of antibiotic-
producing strains of *P. fluorescens* could be correlated with
the lack of take-all disease symptoms displayed by field
grown plants (70). Treatment of wheat seeds with antibiotic
producing stains of *P. fluorescens* resulted in plants with
fewer disease symptoms when challenged with *G. graminis* var.
tritici in both greenhouse and field tests (75). Yields
were increased 147% in fumigated soil and 27% in natural
soil over nontreated seeds.
Take-all diagnosis

The traditional approach of diagnosis of plant diseases has been based on identification of the pathogen either by its isolation or by symptoms displayed by the host (10,32). *G. graminis* var. *tritici* is difficult to isolate from diseased tissue and causes disease symptoms similar to those caused by other fungal plant pathogens or environmental conditions. Traditionally, take-all has been diagnosed when plants display blackened roots, stunted growth, prematurely ripened heads (whiteheads), and ears containing shriveled grain (15). Whiteheads, which may not occur on all *G. graminis* infected plants, may be caused by poor soil conditions, insects, or other microbial pathogens. Black roots may be caused by *Cochliobolus sativus* or mycorrhizae and stem base blackening by *Fusarium* spp. or *Leptosphaeria narmair* (30). As previously discussed, *Phialophora radicicola* covers roots with dark, branching hyphae which penetrate the plant cell wall inducing the plant to form lignitubers to a limited extent (60). Therefore, to confirm a field diagnosis, isolation of the fungus is required.

Pathogen isolation and disease forecasting

Direct isolation of *G. graminis* var. *tritici* from infected plants is difficult due to its slow growth rate relative to other soil and plant tissue microorganisms (16). In addition, the fungus in culture does not normally produce
characteristic asexual or sexual spores which aid in identification. One isolation technique takes advantage of *G. graminis* penetration of host tissue and involves the surface sterilization of a small section of infected root tissue followed by plating on potato dextrose agar (PDA) containing streptomycin (2). Juhnke, et al., (33) developed a medium which is able to isolate the fungus directly from plant material. The medium includes a variety of antibiotics and synthetic antimicrobics specific for other soil and plant microorganisms. Also included in this medium is L-beta-3,4-dihydroxyphenylalanine (L-DOPA) which *G. graminis* uses as a substrate for production of a melanin pigment. On such a highly selective medium the growth of the fungus is relatively slower than on a nonselective medium and there is variability in recovering viable *G. graminis* from infected plant tissue (Henson, unpub. observations).

Establishing that *G. graminis* is the causative agent of take-all-like symptoms is important for crop-loss appraisal and forecasting of the disease. The forecasting of take-all is very difficult due to the fact that an epidemic is a process that involves several growing seasons and can be discontinuous in time (30). Additionally very little is known about the biology of the saprophytic phase of the fungus (discussed above) and our ability to detect *G. graminis* in soil either directly or using host response
assays is very limited (discussed below). Environmental factors such as the past weather have been somewhat successful in the USSR and East Germany for disease prediction (30). Take-all epidemics appear to be associated with warm dry periods in winter, probably because competing microorganisms are somewhat inhibited under these conditions.

Molecular Diagnosis

Recently some of the techniques of molecular biology have been used in detecting plant pathogens in diseased plants. These methods involve the detection of pathogen-specific molecules, either unique antigens or DNA sequences, in standardized assays. The detection of pathogen-specific antigens or DNA sequences is not dependent on an observable host response or the development of disease symptoms. Antigens specific for a given phytopathogen are detected immunologically with either polyclonal or monoclonal antibodies (PAb's and MAb's, respectively). Both viral and bacterial plant pathogens have been detected directly in infected tissue using enzyme-linked immunosorbant assays (ELISA's). A MAb raised against citrus tristeza virus was able to differentiate viral pathovars (48). Using ELISA's and immunogold silver stain dot-immunobinding assays Xanthomonas campestris can be detected in infected geranium plants with a PAb although there was some degree of cross-
reactivity with other *X. campestris* pathovars and phytopathogenic bacteria and fungi (1).

**DNA Probes**

The detection of pathogen-specific DNA sequences potentially has a greater degree of specificity, and when used in conjunction with the polymerase chain reaction, sensitivity. Deoxyribonucleic acid (DNA) serves as the genetic material and exists in two complementary strands hydrogen-bonded together. Under appropriate conditions such as high temperature or low salt concentrations the two complementary strands denature and exist as single-stranded molecules. When the temperature is lowered or the salt concentration raised, each DNA single-stranded molecule will rehybridize with its complementary strand. Cloned or chemically synthesized DNA may hybridize to genomic DNA if the two sequences are complementary and if the conditions are appropriate. If DNA is extracted from diseased plant tissue and immobilized on a solid support such as nitrocellulose filter paper, then the blotted DNA can be probed with radioactively-labeled cloned DNA which will hybridize to strands with sufficient homology. After washing off unhybridized DNA, the blot is exposed to X-ray film and any radioactive DNA bound to the filter paper can be detected. Cloned DNA derived from a pathogen with little homology to DNA sequences found in the host plant or other
microorganisms can be used diagnostically for plant disease detection. DNA probes have been developed for the detection of bacterial, fungal, nematodal, and mycoplasma-like plant pathogens. Thompson, et al. (71), cloned a 5 kilobase (kb) probe from the bacterium Clavibacter michiganense subsp. michiganense which did not hybridize, under the conditions tested, to DNA from a closely related avirulent C. michiganense strain. The virulent strain was detected directly in sap of infected tomato plants. The degree of hybridization can also be used to distinguish between closely related pathogens. A DNA probe directed against Pseudomonas syringae pv. tomato could differentiate, based on the strength of hybridization, this pathogen from P. syringae pv. syringae (18). An internal probe which hybridized to both strains equally well was used to differentiate between weakly hybridizing P. syringae pv. syringae and small amounts of P. syringae pv. tomato DNA. The detection limit of this assay was 2 ng of purified P. syringae pv. tomato DNA which corresponds to the in situ DNA released from $10^5$ bacterial cells. Besal et al., (5) was able to detect single nematode cysts with a mitochondrial-derived probe when cyst homogenates were applied directly to nitrocellulose filters. The direct detection of pathogens using DNA probes make it possible to detect microbes which are difficult or impossible to culture in vitro such as mycoplasma-like organisms (MLO’s). In this case the
development of the probe is difficult since purified DNA from the pathogen is unobtainable. Kirkpatrick et al., (34) cloned random fragments of DNA derived from infected tissue and was able to isolate a fragment which hybridized only to DNA from infected tissue and not to DNA from healthy tissues.

DNA probes have been used successfully for detection of fungal plant pathogens and for epidemiological studies. Probes for the latter purpose are able to genetically separate closely related populations. Restriction fragment length polymorphic (RFLP’s) probes hybridize to fragments of different sizes generated after the fungal DNA is digested with a restriction enzyme and subjected to electrophoresis through an agarose gel. Hybridization patterns can be compared between closely related fungi and in some cases a specific pattern can be correlated to some other characteristic of the pathogen such as host range. The molecular basis for the differential hybridization pattern is probably due to a single base change which either creates or abolishes a restriction enzyme site. Manicom, et al., (41) cloned a 3.4 kb probe from *Fusarium oxysporium* which produced hybridization patterns that correlated to vegetative compatibility groups (VCG’s) and therefore could be used to assign *Fusarium* forma specialis. Currently the VCG assignment of a *Fusarium* isolate is determined by pathogenicity tests on a number of possible hosts requiring
a considerable amount of time and labor.

A number of DNA probes have been developed for use in the diagnosis of fungal plant pathogens. A probe directed against a very highly repeated DNA sequence (approximately 4% of the genome) of Phoma tracheiphila, a fungal pathogen of lemon, was able to detect the pathogen directly in infected seedlings (50). The detection of lower-copy pathogen-specific DNA directly in infected tissue may require amplification of the target DNA sequence. A probe specific for Phytophthora citrophthora, which causes a citrus root disease, could not detect the pathogen-specific DNA sequence in hybridization assays with total DNA extracted from infected roots (25). The probe could detect P. citrophthora after the infected root was plated on a semiselective medium which allowed a limited amount of growth of the fungus.

Recently a 4.3 kb DNA fragment was cloned from the mitochondrial genome of G. graminis var. tritici (26). Although this probe specifically hybridized to purified Gaeumannomyces DNA, it was unable to detect fungal DNA in diseased tissue by direct hybridization of the probe with DNA extracted from infected wheat seedlings. In order to detect G. graminis infection it was necessary to subculture the fungus from infected seedlings and extract DNA from the subculture for hybridization with the probe. Thus, amplification of the target sequence for the detection of
the pathogen directly in infected tissue can be accomplished either by culturing diseased tissue on a medium selective for the pathogen (25,26) or as described here by using the polymerase chain reaction (PCR).

**The Polymerase Chain Reaction**

PCR involves the denaturing of the target DNA followed by hybridization of two oligonucleotide primers which are homologous to opposite strand sequences that flank the DNA to be amplified. Primed template DNA is then extended with DNA polymerase and deoxynucleotides to produce two copies of the target DNA. Completed strands of one primer extension become a template for the other primer. Repetition of denaturation, primer annealing, and primer extension result in exponential amplification of the fragment of interest which can be visualized on a stained agarose gel. The specificity of PCR is a function of the primers, which are usually 20-30 base pairs in length, and the annealing temperature, which usually ranges from 37 C to 65 C. Pairs of chemically synthesized primers that hybridize to many regions of template DNA will result in a large number of PCR products. A low annealing temperature will allow the primers to hybridize to sequences with less homology which likewise will result in a number of nonspecific PCR products.

The theoretical basis of primer directed DNA
amplification was first proposed a number of years ago (35) and only recently shown to be possible. The first published research using PCR involved using a pair of primers which flanked the single base polymorphic site which causes Sickle Cell Anemia (54). The single base change alters a restriction site and therefore its presence can be detected by the hybridization pattern of the amplified DNA in southern blots. The use of PCR increased the level of sensitivity by two orders of magnitude by reducing the amount of starting DNA required for the detection of the base change. The specificity of PCR increased dramatically with the use of the thermostable DNA polymerase of *Thermus aquaticus* (Taq) (53). Previously the *Escherichia coli* Klenow fragment polymerase I was used and had to be added fresh after each denaturing step and had a maximum activity at 37 C. The Taq polymerase can withstand extended and repeated incubations at 95 C and has maximum activity at 72 C. In addition to simplifying the procedure and increasing the specificity of the products, the use of Taq polymerase also was reported to increase both the yield of PCR products and the maximum length product which could be obtained, 2-3 kb (53).

**PCR for diagnosis**

In a relatively short time PCR has become a widely used tool in molecular biology research and the basic
amplification process has given rise to a large array of related techniques, some of which are outlined in Table 1.

Table 1. Related PCR techniques.

<table>
<thead>
<tr>
<th>Technique</th>
<th>Purpose</th>
<th>Ref.</th>
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<tbody>
<tr>
<td>Inverse PCR</td>
<td>Chromosome crawling</td>
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</tr>
<tr>
<td>Competitive nucleotide priming</td>
<td>Mutation analysis</td>
<td>23</td>
</tr>
<tr>
<td>MOPAC</td>
<td>cDNA amplification</td>
<td>37</td>
</tr>
<tr>
<td>Anchored PCR</td>
<td>cDNA amplification</td>
<td>39</td>
</tr>
<tr>
<td>RACE</td>
<td>cDNA synthesis</td>
<td>20</td>
</tr>
<tr>
<td>Asymmetric PCR</td>
<td>Single-strand synthesis</td>
<td>55</td>
</tr>
<tr>
<td>Multiplex PCR</td>
<td>Genetic screening</td>
<td>9</td>
</tr>
</tbody>
</table>

In the area of diagnostics of infectious and genetic diseases the technology has spawned a growth industry. Plant and animal pathogens which were before too few in number to detect in the infected host have become assessable in situ. This detection is not dependent on isolating or culturing the pathogen and requires only a minute amount of DNA to be present in the diseased tissue or sample. PCR has been used successfully for detecting a wide range of microbial pathogens in a variety of sample types (Table 2).
Table 2. Detection of pathogens using PCR.

<table>
<thead>
<tr>
<th>Microbe</th>
<th>Sample</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Viral</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pseudorabies</td>
<td>Cell culture</td>
<td>4</td>
</tr>
<tr>
<td>HTLV-I</td>
<td>Spinal fluid</td>
<td>3</td>
</tr>
<tr>
<td>HIV-I</td>
<td>Blood</td>
<td>47</td>
</tr>
<tr>
<td>Hepatitis B</td>
<td>Serum</td>
<td>40</td>
</tr>
<tr>
<td>Cytomegalovirus</td>
<td>Urine</td>
<td>17</td>
</tr>
<tr>
<td><strong>Bacterial</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Shigella flexneri</em></td>
<td>Lettuce</td>
<td>36</td>
</tr>
<tr>
<td><strong>Protozoan</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Trypanosoma brucei</em></td>
<td>Blood</td>
<td>44</td>
</tr>
<tr>
<td><strong>Fungal</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Phoma tracheiphila</em></td>
<td>Lignified tissue</td>
<td>51</td>
</tr>
</tbody>
</table>

To date there has been only one published account of using PCR for the detection of a phytopathogen in infected plant material. This involved the amplification of the highly repeated sequence of *Phoma tracheiphila* discussed above (51). Although the probe containing a portion of the repeated sequence could detect the fungus in the soft tissue of infected lemon trees it performed poorly with lignified branch samples. The use of PCR allowed for the direct detection of the pathogen in the woody tissue as well as the infected soft tissues without hybridizations and radioactivity. Fungal DNA has been amplified for phylogenetic studies in which PCR was used to facilitate sequencing of a portion of the 16S-like rDNA gene of a
variety of fungal species (78). Also PCR has been successfully used to amplify DNA from dried fungal herbarium specimens which had been stored for 1-50 years under a variety of conditions (8). Here I describe the use of the polymerase chain reaction for the amplification of G. graminis-specific DNA. This method was successful in detecting G. graminis var. tritici DNA in infected wheat seedlings directly without subculturing the fungus. The PCR products were visible on stained agarose gels and they were shown to be G. graminis-specific by southern blot analysis. The method described here has potential use for diagnosis of diseases caused by Gaeumannomyces and the detection of the fungus in soil which would aid in take-all forecasting.
Fungi, DNA Isolation, and Plant Infections

Fungi used in this study are listed in Table 3. Cultures were grown on L medium (43) at 24°C and DNA was isolated as previously described (26). DNA from infected and uninfected control plants (six seedlings, approximately 0.6 g/DNA extract) was isolated by a fungal DNA protocol (38) except that whole roots were used as starting material. Ponderosa spring wheat seeds were surface sterilized in a solution of bleach (10%) and ethanol (10%) for 20 min, washed with distilled water, and placed in 200 ml volume glass jars containing 25 ml 1.2% agar. Seeds were inoculated at the time of planting with a single agar plug taken from a fungal culture with a pasteur pipet. Seedlings usually displayed disease symptoms within two weeks of planting and DNA extracted either three weeks or one month after planting.
Table 3. Fungi used in this study.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Source</th>
</tr>
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<tbody>
<tr>
<td>Cochliobolus sativus</td>
<td>D. Mathre</td>
</tr>
<tr>
<td>Fusarium culmorum</td>
<td>D. Mathre</td>
</tr>
<tr>
<td>Gaeumannomyces graminis</td>
<td></td>
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<tr>
<td>var. avenae</td>
<td>S. Wong via D. Mathre</td>
</tr>
<tr>
<td>var. graminis</td>
<td>C. Rothrock via D. Mathre</td>
</tr>
<tr>
<td>var. tritici</td>
<td>D. Mathre</td>
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<tr>
<td>Rhizoctonia</td>
<td>D. Mathre</td>
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<tr>
<td>Trichoderma</td>
<td>D. Mathre</td>
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Restriction Mapping and Subcloning

Restriction enzymes were obtained from Stratagene (La Jolla, CA) and USB (Cleveland, OH) and were used according to the directions of the manufacturers. For large scale plasmid preparations the alkali/SDS isolation method was used (6) followed by Sephacryl S-1000 chromatography (Pharmacia, Uppsala) using a high salt column buffer (10 mM Tris [pH 8], 1 mM EDTA, 1 M NaCl). The DNA concentration of the collected fractions was determined by measuring the absorbance at 260 nm. The second A260 peak fractions were pooled and precipitated with one volume isopropanol and resuspended in TE buffer (10 mM Tris [pH 8], 1 mM EDTA) at a concentration of 70-1185 μg/ml. Plasmid minipreps for transformant screenings also utilized alkali/SDS starting
with 2 ml overnight bacterial cultures.

Restriction mapping of pMSU315, a pUC18 plasmid with a 4.3 kb Gaeumannomyces-specific insert (26), was done by digesting 2 ug of the plasmid with a variety of restriction enzymes in separate 20 ul reactions. Digests were analyzed by electrophoresis using 0.75% agarose gels with a running voltage of 4.4 V/cm. A lambda HindIII digest was used as a molecular weight marker and restriction fragment sizes were calculated with the GEL computer program written by Fristensky and based on the method of Schaffer and Sederoff (59).

Subtraction subcloning was accomplished by digesting 20 ug of pMSU315 with restriction enzymes in separate reactions that cut within the 4.3 kb insert as well as the multiple cloning site. The plasmid was then religated and approximately 1 ug used to transform Escherichia coli strain DH-alpha (Bethesda Research Laboratories) (13). Transformants were screened and those with plasmids of the appropriate size (i.e. pMSU315 minus the fragment which was removed by the restriction enzyme digestion as predicted by the restriction map) were cultured for large-scale plasmid preparations. Restriction enzymes used for deletion plasmid constructions were EcoRI, HindIII, PstI, SacI, and XbaI, and their corresponding plasmids were named pMSU542, pMSU543, pMSU544, pMSU541, and pMSU540, respectively.
**Sequencing**

DNA sequencing was performed using the Sanger chain-termination method (58). The plasmids constructed above, plus the parent plasmid pMSU315, were prepared for sequencing by the alkaline-denaturation method (72) which involved denaturing the plasmid in 0.2 M NaOH, 0.2 mM EDTA for 5 min at room temperature. The solution was then neutralized with 0.1 volume of 3 M sodium acetate (pH 5) and the DNA was precipitated with 2 volumes 95% ethanol. After incubation on ice for 10 min, a 5 min spin in a microfuge was used to pellet the DNA. The supernatant was decanted and the DNA pellet was washed with 160 ul cold 70% ethanol and allowed to dry. The DNA pellet was resuspended in 7 ul of distilled water. Sequencing was performed using a Sequenase version 2.0 kit (USB, Cleveland) following the instructions of the manufacturer. Labeling reactions were done at 18 C for 10 min and the termination reactions were performed at 40 C for 5 min. Three oligonucleotide sequencing primers were used; M13 -20 forward, M13 Reverse (Stratagene), and KS2R (described below). Electrophoresis was done on a BRL model 50 sequencing apparatus with 6% polyacrylamide gels as described by Mann (42,57). The gels were fixed in 5% acetic acid, 5% methanol overnight, bonded to 3MM Whatman paper, and vacuum dried at 65 C for one hour. XO-MAT AR film (Kodak) was exposed 4 days and processed with GBX developer and fixer (Kodak) according to the
manufacturer's instructions. Sequencing ladders were read manually and sequence comparison was made by searching GENEMBL and GENE BANK (19).

PCR

Oligodeoxyribonucleotide primers were chosen from the pMSU540 and pMSU541 sequencing data. Primers KS1F and KS2R flanked a 407 bp fragment containing nested primer sequences KS4F and KS5R which encompassed a 138 bp fragment (see Table 4 for sequences). Primers were prepared on an ABI 381A DNA synthesizer (Applied Biosystems, Foster City, CA) followed by cleavage of the trityl group. The primers were resuspended in 150 ul distilled water and the concentration was determined by reading the A260 (1 A260 unit equals 20 ug/ml of single-stranded oligonucleotides).

Master PCR reaction mixes were prepared in 1.5 ml microcentrifuge tubes placed on ice. The mixtures contained distilled water, primers (100 pmol each), all four nucleotides (0.125 mM each) (USB), Tag polymerase (3)

Table 4. Sequences of oligodeoxyribonucleic PCR primers used in this study.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
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<tr>
<td>KS1F</td>
<td>5'-TACGGCTGTACCGCATGATCTACTA-3'</td>
</tr>
<tr>
<td>KS2R</td>
<td>5'-ATGAGGCCAGAGGTCCCGTCAAAA-3'</td>
</tr>
<tr>
<td>KS4F</td>
<td>5'-CTGTCGGAAGCGAAGAAGTAC-3'</td>
</tr>
<tr>
<td>KS5R</td>
<td>5'-GTAAGTCTACGCGTAGTTGGC-3'</td>
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units/100 ul) (Perkin-Elmer-Cetus, Norwalk, CT), and PCR buffer (50 mM KCl, 10 mM Tris[pH 8.3], 15 mM MgCl$_2$, and 0.1% gelatin, final concentration). The mixtures were gently mixed and 100 ul were aliquoted into 0.5 ml microcentrifuge tubes containing the template DNA. Two drops of light mineral oil (Sigma, St. Louis) were added to the reaction tubes which were kept on ice until placed in the thermal cycler. Template DNA, in 1 ul volumes, used per reaction was 1 ng pMSU315, 0.2 ug purified total fungal, or 0.02-0.1 ug from infected or uninfected plants. PCR reactions, unless otherwise specified, were started with the outside primers, KS1F and KS2R, and after 20 cycles (1 min at 92 C, 2 min at 42 C, and 4 min at 72 C) reaction products were diluted 50-fold, and 1 ul was amplified another 30 cycles (1 min at 92 C, 2 min at 52 C, and 4 min at 72 C) with the inside primers, KS4F and KS4R. Automated PCR was performed with a Perkin-Elmer-Cetus thermal cycler (Norwalk, CT) and manual temperature cycling with three heat blocks (55).

**Product Analyses**

PCR reaction samples (20 ul per lane) were run on a 2% NuSieve (FMC, Rockland, ME), 1% agarose gel at 4.4 V/cm for 3.5 h. A BstNI digest of pBR322 purchased from New England Biolabs (Beverly, MA) was used as a molecular weight standard, yielding fragments of 1857, 1060, 929, 383, and 121 base pairs. Gels were stained with ethidium bromide and
photographed with type 57 high speed film (Polaroid). PCR products were transferred directly without depurination to a nylon membrane (Zeta-Probe, Bio-Rad, Richmond, CA) by alkaline capillary transfer (49). Nick translations were done with a kit purchased from Bethesda Research Laboratories (Gaithersburg, MD). Overnight, 65°C hybridizations were performed with labeled pMSU315 (10^8 cpm) (67). Membranes were washed according to the instructions of the manufacturer (Bio-Rad) and X-ray film (X-Omat AR, Kodak) was exposed for 8 h before development.
RESULTS

Restriction Mapping And Subcloning

Plasmid pMSU315, which contains a 4.3 kb fragment cloned from *G. graminis* var. *tritici*, was mapped with a variety of restriction endonucleases which have sites within the pUC18 multiple cloning site. This simplified the analysis by making it possible to quickly assign the polarity of the restriction sites. For example, digesting pMSU315 with SacI, which has a restriction site in the multiple cloning site 3' of the insert, generated fragments of approximately 5.2 kb and 2.0 kb. Since pUC18 is 2.8 kb in length, the SacI site within the insert must be located 2.0 kb from the multiple cloning restriction site (Fig. 1). When restriction enzyme digestion resulted in more than two fragments or in two closely sized fragments it was necessary to digest the plasmid with two restriction enzymes simultaneously. For example, KpnI digestion generated a 3.8 kb and a 3.2 kb fragment, and it was impossible to distinguish the fragments since either one could contain the vector. Performing a KpnI, SacI double digest generated fragments of 3.2 kb, 2.0 kb, and 1.9 kb, which indicated that the 3.2 kb fragment generated in the KpnI digestion contained the vector and about 500 base pairs of the insert. The KpnI site is therefore located 3.8 kb 5' of the right
multiple cloning site. Other pMSU315 restriction sites are diagrammed in Fig. 1.

**Fig. 1 Partial restriction map of pMSU315.** Numbers in parenthesis refer to distance, in kilobase pairs, of the restriction site from the lefthand border of the insert. Multiple cloning restriction sites are shown in bold. Double bar portion is sequenced.

In order to obtain DNA sequence data of regions within the cloned fragment with one set of primers, subtraction subcloning was performed. This involved digesting pMSU315 with a restriction enzyme that cuts within the insert as well as in the multiple cloning site. The cut plasmid is then religated and used to transform *E. coli*. Religation of the plasmid minus the cut out fragment is a first-order reaction which is favored over the second-order reaction of pMSU315 reforming intact after digestion (55). For example, pMSU315 digested with SacI generates 5.2 kb and a 2.0 kb fragments and when plasmids were recovered from ampicillin-resistant bacteria transformed with the religated DNA, they were of the predicted size of 5.2 kb. The newly constructed
plasmid, in this case pMSU541, could be used in sequencing reactions using a universal primer (below) which hybridizes to the flanking region of the right multiple cloning site and is extended 250-350 base pairs into the insert during the sequencing reactions. Therefore, the sequencing of pMSU541 generates approximately 330 base pairs of sequencing data in a region, as predicted from the restriction map, 2.0 kb 5' of the right multiple cloning site of the parent plasmid, pMSU315. In addition to SacI, PstI, EcoRI, HindIII, and XbaI, were used to generate the derivative plasmids pMSU544, pMSU542, pMSU543, and pMSU540, respectively.

**Sequencing And Primer Selection**

The target DNA fragment must be partially sequenced before selection and subsequent synthesis of primers for amplification of PCR. The plasmids, pMSU540, pMSU541, pMSU544, pMSU543, and pMSU542, were sequenced with either the M13 forward or reverse oligonucleotide primer depending on where the corresponding restriction site used to construct the derivative plasmid appears. The parent plasmid, pMSU315, was sequenced with both the forward and reverse primers to obtain sequence information at both ends of the cloned fragment. Plasmid pMSU544, when sequenced with the forward primer, yielded 48 bp of insert sequence which defined the 3' end of the cloned fragment (Fig. 1).
Priming pMSU315 with the forward oligonucleotide resulted in a sequence ladder which corresponded to 265 bp at the 5’ end of the cloned fragment. When the reverse primer was used, pMSU315 yielded 232 bp of sequence information 5’ of the *Pst*I restriction site. Plasmid pMSU543 primed with the forward oligonucleotide generated 221 bp of sequence located approximately 2.7 kb downstream of the 5’ end of the insert. For unexplained reasons, sequencing pMSU542 with the reverse primer did not result in a readable sequencing ladder.

Plasmids pMSU540 and pMSU541 were partially sequenced with the reverse primer. As already mentioned, 330 bp of sequence, located approximately 2.0 kb 5’ of the right multiple cloning site, was obtained when pMSU541 was sequenced. Plasmid pMSU540 generated 246 bp, including the *Sac*I site near the 3’ end, of sequence information located approximately 1.7 kb upstream of the right multiple cloning site (Fig. 2). Restriction mapping predicted the *Xba*I and *Sac*I sites to be approximately 300 bp apart which closely agrees with the sequencing data which suggests that these sites are about 275 bp apart. Ten unsequenced bases, as was determined later by sequencing with the primer KS2R (below), separated the 3’ of the pMSU540 and the 5’ end of the pMSU541 sequencing data. The beginning of the pMSU540 sequence to the end of the pMSU541 sequence encompassed a 587 bp region which was judged to be appropriate for the synthesis of PCR oligonucleotide primers.
Fig. 2. Sequence of the 587 bp located within pMSU315. Primer locations are indicated by brackets. The SacI restriction site is underlined.

5'-ATTCAATGTCTAGAGACTCAAACGCTAGATAAAATAAACCAGACATTCTTTTTTTAT
ATTTATTAAAGGGAGTGCTATTTTGTGCTTGAACAAACTAAATAATTTAAAAAAAAACCAA
AAAATAT[KS1F->TACGGCTGTACCGCATGATCTACTA]TTACATCCTACCCNNNNNNC
CCCCNTCGCNTNTTGCCTGCGCAGCAACAYCTACCCCCCCCCCTT[KS4F->CTGTGCG
CAAGCGAAGAGTAC]TACCTCGTAATTCTAAATACATATTAGAAGTACGAAGTAAGCAAC
CAGGTTACATTTACGTATACCAGGGGTGAGGCTAAATTATATTTATATTAGTACGAGTAATATA
CGCTAGAGCTACTATAATATCATATAATTTTATA[GCCAACTACGGGTAGACTTAC<KS
5R]AATCTTTTTTTTTTATTTTTTTTTTTTTTTTTTTTTTTTTTACACTAAATNTTTTTCCATTAAAA
TCCTCAATAGACGCCATTCTACTCCTAAATATCCCATAGACCACCAACCAACATATTCACC
CAGATCCTTTAAAGACCATTATCCATCAATCTCATTTTTAGTTATA[TTTGACGGGACCTCT
GGCCTCAT<KS2R]ATTTAATCTA-3'

The dominant criterion used for the selection of a set of PCR primers is a GC content of about 50-60% in a 20-30 bp stretch of sequence. Sequencing data demonstrated (Fig. 2) many long tracts of A's, T's, and occasionally G's, imposing limitations on the sequences which could be used as primers. Another consideration for primer selection is the distance, measured in the number of nucleotides, between the two opposing primers. The polymerase chain reaction has been used successfully to amplify nucleotide sequences of less than 100 bp to approximately 3 kb (55). Two primers, KS1F
and KS2R, were chosen from the pMSU540 and pMSU541 sequencing data which demarcates a 456 bp fragment of the Gaeumannomyces-specific insert of pMSU315 (Fig. 3). This 456 bp had no open reading frame and did not show strong homology to any Genebank or Genembl sequence. A nested set of primers, KS4F and KS5R, which were contained within the 456 bp sequence were also synthesized. To facilitate PCR product analyses, primers which flanked the SacI restriction site were chosen.

Fig. 3 Partial restriction map of pMSU315 and location and direction of the oligodeoxyribonucleotide primers used in this study. Distances are in kilobase pairs.
PCR Generated Fragments

When the outside primers KS1F and KS2R were used in one round of amplification (25 cycles), two PCR products were generated when *G. graminis* var. *tritici* purified DNA was used as a template (Fig. 4, lane 13). When pMSU315 was used as a template, a single PCR product was produced which appeared to be identical to the larger fragment generated when total *G. graminis* DNA was amplified. The higher molecular weight products contained the *SacI* restriction site and both fragments hybridized to labeled pMSU315 in southern blots. The larger fragment was sized, using DNA molecular weight standards, as approximately 550 bp in length. The reason for the discrepancy between the actual product size and the predicted length of 456 bp was not determined. Under these same conditions a number of PCR products were generated when other purified fungal DNA were amplified. *Cochliobolus sativus* and *Fusarium culmorum* products are shown in Fig. 4, lanes 11 and 9, and although these products did not hybridize with labeled pMSU315 it was felt that this high level of nonspecific background products could lead to ambiguities when evaluating results from PCR reactions with DNA extracted from infected plants. In addition, the major amplification products using DNA extracted from *G. graminis* infected seedlings did not
appear, as judged from their size, to correspond to the amplification products of purified *G. graminis* or pMSU315 DNA.

![PCR products generated after first and second rounds of amplification of purified fungal DNA (lanes 8-13) and amplification of DNA from wheat seedlings infected with *G. graminis var. tritici* (*Ggt*) and other fungi (lanes 2-7). Lanes: 13,11,9, *Ggt*, *C. sativus*, and *F. culmorum*, after one round of amplification, respectively; 12,10,8, *Ggt*, *C. sativus* and *F. culmorum* after two rounds of amplification, respectively; amplification of wheat seedling DNA infected with *Ggt* (7), *C. sativus* (6), *F. culmorum* (5), *Rhizoctonia* (4), and *Trichoderma* (3); lane 2, uninfected; lane 1, pBR322 BstNI standard molecular weight markers.](image)

A second set of primers (KS4F and KS5R) were synthesized and tested for their ability to reduce the number of nonspecific amplification products. The inside nested primers were predicted to generate a 181 bp fragment containing the *SacI* restriction site (Fig. 2 and 3). Also,
they prime inside the long tracts of C's and T's in the 456 bp fragment which could possibly be involved in some of the discrepancies noted above. Sometimes when *G. graminis* DNA was amplified with the inside primers alone in one round of amplification there were multiple products detected on the agarose gel, only one of which contained the SacI restriction site. To reduce the number of nonspecific products generated during amplification, a second round of amplification with the nested set of primers was performed using the diluted products from the first round as the template.

Using two rounds of amplification, there were no *C. sativus* and *F. culmorum* PCR products detected on agarose gels following the second round (Fig. 4, lanes 10 and 8). When purified *G. graminis* var. *tritici* or pMSU315 DNA was subjected to two rounds of amplification, two fragments, approximately 188 and 280 bp in size, were visible on stained gels (Fig. 4, lane 12 and Fig. 5A, lanes 1,2). The smaller fragment was the predicted product from the inside primers but the origin of the 280 bp product was unclear. Both products contained a SacI restriction site and hybridized to labeled pMSU315 (Fig. 5B, lanes 1,2). Identical PCR products were obtained with DNA extracted from *G. graminis* var. *tritici*-infected wheat seedlings (Fig. 5A and 5B, lanes 3,4). Other related varieties of *Gaeumannomyces*, var. *graminis* and var. *avenae*, produced PCR
fragments identical in size to those derived from G. graminis var. tritici. No PCR products were detected in gels after amplification of DNA from uninfected seedling controls (Fig. 4, lane 2 and Fig. 5A, lane 5), purified wheat DNA (Fig. 5A, lane 6), or seedlings infected with other root colonizing fungi (Fig. 4, lanes 3-6). Labeled pMSU315 did not hybridize to any products which might be undetected in the ethidium bromide-stained agarose gels (Fig. 5A, lane 6; autoradiogram of the gel shown in Fig. 4, not shown). Automated and manual temperature cycling produced identical results (data not shown).
Fig 5. Agarose gel (A) and Southern blot probed with pMSU315 (B) of PCR products from the following templates; 1, pMSU315; 2, Ggt; 3, 4, DNA extracted from wheat seedlings infected with Ggt; 5, DNA from uninfected wheat seedlings; and 6, purified Chinese Spring wheat DNA; lane 7, pBR322 BstNI molecular weight marker.
DISCUSSION

Although the cereal disease take-all can be diagnosed visually, unambiguous assignment of *G. graminis* var. *tritici* as the etiological agent is complicated by the fact that many of the disease symptoms may be caused by other microorganisms or environmental conditions. Here is described the use of the polymerase chain reaction for detection of *Gaeumannomyces*-specific DNA in infected wheat seedlings. This method generated microgram quantities of DNA products which could be readily identified as to their specificity. Primer-directed DNA amplification has the potential to become a routine laboratory technique used in the field of diagnostics for both animal and plant diseases. The technique is not equipment intensive as was shown in this project with the use of three heating blocks for temperature cycling. Decreasing the temperature ramp time in manual temperature cycling (an automated temperature cycler has a ramp time of about 1°C/sec) appeared to have no effect on the results. If an automated temperature cycler is used, the technique requires little more labor than preparing a crude DNA extract of the sample tissue, pipetting the reaction components, and after the reactions are completed, product analysis. Care does have to be exercised in the handling of the reaction components,
including the preparation of the template DNA, due to the inherent sensitivity of this technique. Theoretically one molecule of the target DNA sequence is sufficient to produce a positive result. Also, another important consideration is the relative concentration of any PCR products, in terms of the number of molecules (molarity), which are visible on an ethidium bromide-stained gel. These products may serve as templates for subsequent reactions. In spite of the enormous threat of contamination no extra precautions were taken in this study since the negative controls (no added DNA template added or uninfected seedlings) never produced products visible on stained gels or which hybridized to labeled pMSU315. This may be a reflection of the relative efficiency of the amplification under the conditions used in this work rather than any skill possessed by the experimenter.

**Diagnostic PCR**

Two fragments of approximately 188 and 280 bp were generated with *G. graminis* total DNA, pMSU315, and DNA extracted with *G. graminis*-infected seedlings. Although only one 181 bp PCR product was predicted, two fragments were produced. Both fragments hybridized with labeled pMSU315, the plasmid bearing a 4.3 kb mitochondrial fragment cloned from *G. graminis* var. *tritici*. Initially it was thought that the larger fragment was a product of KS1F/KS5R
priming which would be predicted to generate a 272 bp PCR product. Outside primer KS1F, according to this explanation, was in sufficient concentration following dilution of the first round products to produce enough product to be visible on stained agarose gels. Calculations of the maximum yield of KS1F/KS5R priming products (i.e. how much 272 bp product could be generated from KS1F carried over from the first round of amplification) showed that only nanogram amounts of the 272 bp product could be produced. In addition, digestion of the 280 bp fragment with SacI did not produce restriction fragments which would be expected had either of the outside primers been used to generated the 280 bp fragment. Perhaps one or both of the inside primers anneals to a second sequence(s) on pMSU315, extension from which would generate the 280 bp fragment. Regardless of its origin, this fragment is consistently produced during amplification of G. graminis DNA and hence is diagnostically useful.

Identical PCR products were obtained with other varieties of Gaeumannomyces graminis indicating that this technique may be useful for diagnosis of other diseases such as spring dead spot of bermuda and crown rot of rice caused by G. graminis var. graminis and take-all of oats caused by G. graminis var. avenae. In addition, this technique would probably be useful for other G. graminis var. tritici
strains isolated throughout the world since they also contain the 4.3 kb DNA sequence (Henson, unpublished observations).

**Nested PCR**

To reduce the number of nonspecific PCR products generated during the amplification reactions, two sets of primers were used. The outside primers, KS1F and KS2R, were used in a first round of amplification which consisted of 20 cycles with a relatively low annealing temperature (42 C). The first round products were then diluted and added to a second reaction mixture containing the inside primers, KS4F and KS5R. The second round of amplification used a higher annealing temperature (52 C) and ran for 30 cycles. This approach increased both the specificity, as judged by the decrease in nonspecific products observed in the gels compared to products generated after one round of amplification, and the sensitivity. Theoretically the use of nested primer sets in two rounds of amplification would not be expected to produce a nonspecific product since the probability of both sets of primers hybridizing to a heterologous site, in the proper orientations, would have to be exceedingly small. Sensitivity is increased by two rounds of amplification simply by the increased number of cycles used and the addition of fresh *Taq* polymerase after 20 cycles. The polymerase is reported to lose a significant amount of its activity after 20-30 cycles (55). Sensitivity
is an important consideration when one is starting with a relatively small number of copies of the template. The 181 bp fragment that was amplified in this project is contained in a larger sequence which is nonrepeated (26), therefore only one template per mitochondrial genome is present. The relative number of template copies is greatly decreased when DNA is extracted from infected plant tissue compared to when purified fungal DNA is used.

Applications

The technique described in this project has possible applications for detecting the fungus in field-grown plants and in soil. Primer directed amplification of G. graminis DNA might possibly find use in early diagnosis of the take-all in seedlings which are not displaying obvious disease symptoms. Currently, diagnosis of take-all is done on the basis of the host response and definitive identification of G. graminis var. tritici as the etiological agent requires isolating the fungus from diseased plants. Since G. graminis is a relatively poor competitor, a highly selective medium which is also somewhat inhibitory for G. graminis is required for isolation of the fungus from plant samples and therefore may not recover all viable hyphae (33; Henson and Mathre, unpublished observations).

Use of the polymerase chain reaction may allow the development of a Gaeumannomyces soil detection assay. The
use of nested primer sets, with their increased specificity, may make it possible to detect the fungus in soil where many microorganisms exist. Currently one cannot reliably detect the presence of *G. graminis* var. *tritici* in known infested soils. Bioassays, in which seedlings are grown in soil from suspect plots, were shown to be unreliable and were discontinued by the late 1960's (30). Hyphae isolation methods only rarely detect the take-all fungus in soil (74) and the highly selective medium described above is inconsistent in its ability to isolate *G. graminis* from soil (33). PCR has been successful in detecting as few as one bacterial cell per gram soil sediment from environmental samples (68) which is over a four log increase in sensitivity compared to the number of bacterial cells detected when a DNA probe was used alone (27). A soil detection assay for *Gaeumannomyces* would greatly facilitate forecasting of take-all which currently has a reputation as being an unpredictable disease (33).
LITERATURE CITED


