Selection and characterization of genomic DNA clones of Pyrenophora teres and their application for disease diagnosis via the polymerase chain reaction (PCR)
by Baltazar Montes Baltazar

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Plant Pathology
Montana State University

Abstract:
Polymerase Chain Reaction (PCR) protocols were developed for the diagnosis of net and spot forms of Pvrenophora teres. Low copy number sequences selected from a P. teres f. sp. maculata random genomic library were used as a source of probes. Emphasis was placed on those sequences identifying DNA polymorphisms between net and spot isolates and with little or no sequence similarity with barley, wheat, or triticale genomes.

Sequences identifying a large deletion in genomic DNAs of net and spot isolates were preferred over sequences detecting small DNA changes. Sequence data of two informative clones, pPtm-290, and pPtm-60, were used to construct primer sets to amplify the corresponding sequence in genomic DNAs of net and spot isolates present in barley plants infected with these pathogens.

PCR results demonstrated the potential of the PCR as a diagnostic tool for P. teres. All the PCR experiments conducted with primers designated as Pt-1 and Pt-2 constructed using the sequence data from pPtm-290, showed a strict correlation between the presence of a 430 bp band and the presence of the pathogen in genomic DNAs of barley infected with the net form, spot form or both pathogens.

PCR experiments with primers Pt-3 and Pt-4 constructed using sequence data from pPtm-60, indicated that it is possible to detect polymorphic bands between net and spot isolates as evidenced by the PCR products analyzed in an ethidium bromide agarose gel.

PCR analysis offers a sensitive, rapid, inexpensive, and non-radioactive technique for the diagnosis of P. teres infection in field-grown barley plants. Future experiments should focus on the ability of the PCR to detect P. teres and P. graminea in infected barley seeds. Additionally, PCR-based protocols for P. teres diagnosis could possibly be incorporated in seed certification programs to avoid the distribution of infected seed in farmer fields.

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A thesis submitted in partial fulfillment
of the requirements for the degree

of

Doctor of Philosophy

in

Plant Pathology

MONTANA STATE UNIVERSITY
Bozeman, Montana

December 1990

APPROVAL

of a thesis submitted by

Baltazar Montes Baltazar

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.

Nov. 27, 1990
Date

Albert S. Shapiro
Chairperson, Graduate Committee

Approved for the Major Department

Nov. 27, 1990
Date

[signature]
Head, Major Department

Approved for the College of Graduate Studies

Nov. 27, 1990
Date

[signature]
Dean, Graduate Studies

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Date NOV. 17, 1990

To my parents, Lorenzo and Sabina,
my wife, Maria del Carmen,
and my son Alberto

ACKNOWLEDGEMENTS

I would like to thank the wheat program authorities, M. Prescott, S. Rajaram, B. Curtis and A. Klatt of CIMMYT for giving me the chance of further pursuing my education.

I wish to thank my major advisor, Dr. A. L. Scharen, for his guidance, encouragement, and positive comments throughout the course of my graduate study and preparation of this thesis.

Special thanks are extended to Dr. Victor Raboy, my co-advisor, for his technical guidance, laboratory facilities and for providing me with a positive environment for the realization of these experiments. His ideas and ability to synthesize the data obtained were helpful for the completion of this thesis. I further want to thank Drs. D. E. Mathre, L. Talbert, D. Sands and R. Johnson for serving on my graduate committee and giving their time and expertise. Suewiya Pickett and Maria del Mar Sanchez deserve special thanks for their kindness and patience in teaching me most of the molecular biology techniques. Thanks are also extended to the USDA-ARS, MSU-ICARDA joint project and CIMMYT for providing me with financial support.

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ABSTRACT

Polymerase Chain Reaction (PCR) protocols were developed for the diagnosis of net and spot forms of Pyrenophora teres. Low copy number sequences selected from a P. teres f. sp. maculata random genomic library were used as a source of probes. Emphasis was placed on those sequences identifying DNA polymorphisms between net and spot isolates and with little or no sequence similarity with barley, wheat, or triticale genomes.

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PCR analysis offers a sensitive, rapid, inexpensive, and non-radioactive technique for the diagnosis of P. teres infection in field-grown barley plants. Future experiments should focus on the ability of the PCR to detect P. teres and P. graminea in infected barley seeds. Additionally, PCR-based protocols for P. teres diagnosis could possibly be incorporated in seed certification programs to avoid the distribution of infected seed in farmer fields.

INTRODUCTION

Net blotch is an economically significant disease of barley (Hordeum vulgare L.) and is caused by the fungus Pyrenophora teres f. sp. teres Smedeg. Yield losses as high as 40 % have been reported when susceptible barley cultivars are infected with a virulent biotype of P. teres. A decrease in grain quality, particularly with regard to brewing characteristics, has also been associated with barley plants infected with P. teres. The appearance of a new biotype described as the spot form of P. teres has been a major problem to plant breeders and pathologists. The origin of the spot form of P. teres is not known at present.

Resistant germplasm and early diagnosis of P. teres are essential components in managing and preventing losses in barley plants infected with these pathogens. While most research has focused on the development of resistant germplasm, few studies have concentrated on the fungus itself.

Disease diagnosis of P. teres is currently made on the basis of symptom expression. However, it is often difficult to make an accurate identification based on the appearance of symptoms. The spot form of P. teres closely resembles the spot blotch disease caused by Cochliobolus sativus (Ito

and Kurib.) Drechsl. ex Dastur., which attacks wheat, barley and other grasses, complicating still more its identification.

Detection tests based on the use of specific and sensitive nucleic acid probes have been used effectively in a number of plant pathogen systems. The use of radioisotopes has limited the use of molecular probes for disease diagnosis.

The utility of the Polymerase Chain Reaction (PCR) as a tool for the molecular identification of plant pathogens is becoming increasingly important. It is highly sensitive, rapid, and does not require the use of radioactive labelled probes.

This study was undertaken to evaluate the potential of the Polymerase Chain Reaction (PCR) for the diagnosis of P. teres infection in field-grown barley plants.

REVIEW OF LITERATURE

The Disease

Pyrenophora teres is the causal organism of net blotch of barley (*Hordeum vulgare* L.). Symptoms induced by *P. teres* occur on the blades and sheaths of the leaves and may extend to the flowers and grains. Initial lesions appear as minute spots or streaks which increase in size to form narrow, dark brown, longitudinal and transverse streaks, producing a net-like pattern. This netting appearance is characteristic of *P. teres* and it is from this appearance that the disease gets its common name of net blotch (Drechsler, 1923; Dickson, 1956).

Isolates of *P. teres* have been detected that produce spot-like lesions rather than netting on barley (McDonald, 1967; Smedegard-Petersen, 1971; Bockelman *et al.*, 1983; Karki and Sharp, 1986). The symptoms induced by the spot form of *P. teres* consist of dark brown, elliptical or fusiform lesions measuring up to 3 x 6 mm in size and surrounded by a chlorotic zone of varying width (Smedegard-Petersen, 1971). Isolates producing a spot phenotype in barley plants have been considered to be *P. teres* because of their ability to cross with isolates of *P. teres* that produce the netting symptoms (McDonald, 1967);

Smedegard-Petersen, 1971). Based on their ability to intercross with each other, the designation Pyrenophora teres Drechs. forma maculata for the spot type of P. teres, and Pyrenophora teres Drechs. forma teres for the net type was suggested (Smedegard-Petersen, 1971).

The spot form of P. teres has been found to be more prevalent than the net form wherever both forms are present (Smedegard-Petersen, 1971; Karki and Sharp, 1986; Tekauz and Buchannon, 1977). The origin of the spot form of P. teres in the U.S.A. and Canada is not known with certainty. Nevertheless, it is believed that this biotype may have been introduced into these regions on contaminated barley seed (Tekauz and Buchannon, 1977).

Resistance in barley to the net form of P. teres has not been associated with resistance to the spot form of P. teres or vice versa (McDonald, 1967; Smedegard-Petersen, 1976; Karki and Sharp, 1986; Tekauz and Buchannon, 1977). However, further investigation is required to elucidate more information regarding the genetics and inheritance of resistance in barley to both forms of P. teres.

The Pathogen

The anamorph of this fungus Drechslera Ito., syn. Helminthosporium teres Sacc., is placed in the class Hyphomycetes, based on the fact that the conidia are not borne in pycnidia or acervuli (Talbot, 1971). The binomial

Helminthosporium teres was first applied by Saccardo to a fungus collected on leaves of barley in 1881 (Drechsler, 1923). The fungus was described as having 3-septate conidiophores arising in a group of five from a green substratum and bearing at the tip a single conidium. The conidia were described as dark green structures, thick walled, 4-5 septate, ellipsoidal or subcylindrical and tapering toward the rounded ends (Drechsler, 1923).

The telomorph of this organism produces asci and ascospores, placing it in the subdivision Ascomycotina. The asci are bitunicate, placing it in the class Loculoascomycetes and the presence of pseudoparaphyses places it in the order Pleosporales (Talbot, 1971). Drechsler (1923), was the first to describe the ascigerous stage of Pyrenophora teres. He placed it in the genus Pyrenophora, based on the presence of setae on the perithecial surface.

The fungus overwinters as mycelium in plant debris as well as in infected seed. It also overwinters as perithecia on infested straw. Early in the growing season, when environmental conditions are favorable, conidia are produced on infested plant debris. In addition, perithecia release asci and ascospores which represent a source of primary inoculum which will then produce primary infection on barley plants.

Genetics of Pyrenophora teres

Studies were conducted to determine the inheritance of factors controlling the expression of symptoms and pathogenicity in crosses between P. teres and P. graminea, cause of the stripe disease of barley (Smedegard-Petersen, 1971, 1976, 1977, and 1983). Three loci designated as Ss, for the spot form of P. teres, Nn, for the net form of P. teres and Gg, for P. graminea were identified which determine symptoms produced on barley plants. The loci for spot (Ss) and net (Nn) segregated independently. The loci for spot (Ss) and barley stripe (Gg) were closely linked.

The inheritance of morphological characters of P. teres in culture has also been determined (McDonald, 1967). The ability to form reproductive structures in culture was dependent on a single gene whereas other morphological characters under study, such as color and vertical tufts of mycelium, were multi-genic.

The heterothallic nature of P. teres has also been demonstrated (McDonald, 1963; Smedegard-Petersen, 1978). Monoconidial or monoascosporic isolates did not produce fertile perithecia when paired with other than their opposite mating types. In contrast, mature perithecia of P. teres were produced by growing mixed suspensions of two compatible single-spore isolates.

Cytogenetic studies indicated that somatic cells of P. teres were multinucleate, and 5 chromosomes were observed in the nuclei of this fungus (Magnus, 1969).

Restriction Fragment Length Polymorphisms (RFLPs) have been useful as genetic markers for some plant pathogens (Michelmore and Hulbert, 1987). These polymorphisms result from specific differences in DNA sequence that alter the size of the fragment obtained after digestion of genomic DNA with type II restriction endonucleases.

Using RFLPs as genetic markers, the worldwide variation and linkage analysis of Bremia lactucae has been investigated (Hulbert et al., 1988). RFLPs have also been useful to determine the genetics of virulence of the obligately parasitic barley powdery mildew fungus (Christiansen and Giese, 1990). Bulat and Mironenko (1989) used RFLP analysis to determine the genetic variation in isolates of P. teres and P. graminea. Ribosomal DNA of Saccharomyces cerevisiae and total genomic DNA of a P. graminea isolate were used as hybridization probes. According to this study isolates of P. teres and P. graminea could be distinguished when these probes were hybridized with DNAs of these two pathogens. It was also found that P. teres and P. graminea contain a high-repeat ribosomal DNA from S. cerevisiae that presumably is absent in other closely related fungi. Based on the DNA polymorphisms observed, Bulat and Mironenko (1989) concluded that P. teres and P. graminea should be united as the same

species of P. teres and ascribed them to different forma specialis.

Recent Developments in Plant Disease Diagnosis

Recent advances in molecular biology are being applied to the development of rapid, specific, and sensitive tools for the detection of plant pathogens. Immuno-assays and DNA base probes are among the more convenient techniques suggested for the detection of plant pathogens (Miller and Martin, 1988).

In recent years a new method was devised called "Polymerase Chain Reaction" (PCR) whereby a nucleic acid sequence can be exponentially amplified in vitro (Mullis and Faloona, 1987). PCR involves repeated cycles of DNA strand synthesis directed by sequence-specific synthetic oligo-nucleotide primers, permitting exponential amplification of that specific sequence out of a crude genomic DNA preparation (Mullis and Faloona, 1987; Cherfas, 1990).

A modification of this approach, termed "Inverse PCR" (IPCR), allowing amplification of regions flanking segments of known sequence, has been described (Ochman et al., 1988 and 1990; Triglia et al., 1988). This technique is based on the digestion of source DNA with restriction enzymes and circularization of cleavage products before amplification using primers synthesized in the opposite orientation to

those normally employed for PCR. IPCR has been used to clone genomic sequences flanking transposable elements from the maize genome (Earp et al., 1990). PCR products can then be visualized in an agarose gel stained with ethidium bromide, avoiding the use of radioactive material. This, along with the specificity, sensitivity, and rapidity, have made the PCR a powerful tool for disease diagnosis in humans (Banghman et al., 1989; Li et al., 1988), animals (Belak et al., 1989), insects (Moser et al., 1989) and food-borne bacterial pathogens (Lampel et al., 1990).

Recently, work has been focused on the feasibility of using PCR for disease diagnosis in infected plants. "Mal seco", a serious wilt disease in lemon orchards and caused by the fungus Phoma tracheiphila has been detected using PCR as a diagnostic tool (Rollo et al., 1990). Detection of mycoplasma like organisms of aster yellows (Schaff et al., 1990), and apple scar skin viroid, dapple apple viroid, and/or pear rusty skin viroid-infected tissue has also been possible using PCR approaches (Hadidi and Yang, 1990). Currently, PCR is being used for diagnosis of the Take-all disease of wheat caused by Gaeumannomyces graminis var. tritici (J. Henson, personal communication).

MATERIALS AND METHODS

Isolate Selection and Designation

P. teres isolates collected in Mexico correspond to four locations used by the International Maize and Wheat Improvement center (CIMMYT) to select barley cultivars resistant to net blotch (Hugo Vivar, personal communication). Other isolates were from the U.S.A. and Colombia. Isolates were collected so that their virulence could be determined and compared. Isolates were selected based on differential reactions on some specific cultivars previously investigated (Karki and Sharp, 1986; Bjarko, 1979). Subsequently, 12 isolates of net form and 8 isolates of spot form of P. teres were selected for evaluation with 32 differential cultivars. Isolates were named on the basis of state or country, collector, and place of collection (Table 1). Other Pyrenophora and Cochliobolus species used are listed in Appendix C.

Isolate Maintenance and Pathogen Isolation

Leaf tissue showing characteristic symptoms of net or spot forms of P. teres and leaf spots or blights associated with Pyrenophora and Cochliobolus species listed in Appendix C were cut into pieces 10 mm long and surface-sterilized in

Table 1. Net and spot forms of P. teres representing several geographical regions.

Isolate Designation	Origin	Name of Collector	Type of Reaction
Mex-2 (Anahuac-1)	Mexico	H. Vivar	net
Mex-3 (Anahuac-2)	Mexico	H. Vivar	net
Mex-4 (Cd. Sahagun)	Mexico	H. Vivar	net
Mex-5 (La Lagunilla)	Mexico	H. Vivar	net
Mex-6 (E. Zapata)	Mexico	H. Vivar	net
Calif-1 (Tulare County)	California	B. Steffenson	net
Calif-3 (Fresno County)	California	B. Steffenson	net
Calif-5 (Solano County)	California	B. Steffenson	net
Pt-R	Montana	M. Bjarko	net
Sco-B	Montana	M. Bjarko	net
86-Hfi	Montana	M. Bjarko	net
MN-1	Minnesota	B. Steffenson	net
Col-1 (Iraka County)	Colombia	H. Vivar	spot
Col-2 (Tundama County)	Colombia	H. Vivar	spot
Col-3 (Boyaca County)	Colombia	H. Vivar	spot
Pt 6-2	Wyoming	M. Bjarko	spot
Fc-1	Montana	M. Bjarko	spot
Con-S	Montana	M. Bjarko	spot
Hort Farm	Montana	B. Baltazar	spot
Post Farm	Montana	B. Baltazar	spot

1% sodium hypochlorite solution (NaOHCl) for about 1 minute. Three or four leaf pieces of each sample were transferred to petri plates containing 2% agar and incubated at 16°C with alternating 12 h light and 12 hr of darkness. Single conidia emerging from the leaf tissue were transferred onto the centers of petri plates containing V-8 juice agar. Maintenance of monoculture isolates in V-8 juice agar medium, subsequent subculture and inoculum preparation were conducted as previously described (Karki and Sharp, 1986).

Cultivar Selection and Planting

The 32 barley cultivars used as differential varieties are listed in Table 2. Selection was based on differential reaction to isolates of net and spot forms of P. teres. These included current commercial varieties grown in Montana and neighboring states.

Table 2. Barley cultivars used in determining pathogenic variation of net and spot forms of P. teres.

Cultivar	CI Number
Klages	CI 15478
Moravian III	CI 15812
Hector	CI 15514
Gallatin	PI 491534
Beecher	CI 11292
Morex	CI 15773
Robust	PI 476976
Piroline	CI 9558
Clark	CI 15857
Harrison	CI 10667
Menuet	PI 428490
Bowman	PI 483237
Betzes	CI 6398
Freja	CI 11303
Tifang	CI 4407
Herta	CI 12325
Compana	CI 5438
Erbet	CI 13826
Manker	CI 15549
Summit	CI 1136
Vireo	CI 7584
	CI 9776
	CI 5791
	CI 9819
	CI 2750
	CI 1615
	CI 5845
	CI 12821
	CI 14023
	CI 1197

Inoculation

Ten day old barley seedlings were inoculated by spraying 100 ml of inoculum suspension with an atomizer driven by compressed air. Inoculated plants were incubated in a saturated dark dew chamber at 16°C for 24 h, then for 8 days at 21-24°C.

Disease Rating and Statistical Analysis

Disease ratings were made 10 days after inoculation on a scale 1-10 as previously described (Tekauz, 1985). Disease ratings were based on percentage of leaf area infected and lesion size. Cultivars rated lower than 4.3 were considered resistant and others were considered susceptible. This separation point for resistance and susceptible reaction has been described as the most appropriate based on a cluster analysis by taking the average disease ratings and adding the standard errors of the means (Karki and Sharp, 1986). This experimental arrangement corresponds to a single replication of two-factor factorial with 32 and 20 levels, respectively, for variety and isolate. Statistical analysis for the main effects of variety and isolate employed the conventional calculation of the mean squared for the interaction between the factors as the denominator for the F-ratios (and when developing the standard error for making LSD multiple comparisons). Such statistical tests are valid when factor

levels are considered random, i.e., the cultivars and isolates studied represent a sample from a larger set of cultivars and isolates toward which statistical inference is directed. These statistical tests are conservative when inference is directed only toward the specific set of isolates and cultivars studied in this research. It is not possible by this analysis to consider interaction itself statistically (Snedecor and Cochran, 1989).

Fungus DNA Extraction

DNA of P. teres f. sp. teres and P. teres f. sp. maculata isolates was extracted by using a modification (Appendix A) of a method previously described (Murray and Thompson, 1980). Twenty day old monocultures of net and/or spot form of P. teres kept at 16°C were used as a source of material for subculture. Agar blocks containing mycelia and conidia were transferred to 1000 ml flasks containing 50 ml of V-8 juice, 0.75 g of $CaCO_3$ and 200 ml of distilled water and kept at room temperature for 10-15 days. The mycelium was collected, rinsed with distilled water and kept at -70°C until used for DNA extraction.

Library Construction and Selection of Clones

A P. teres f. sp. maculata genomic library was constructed in the plasmid vector pUC12 according to the pUC cloning kit manufacturer's recommendations (Boehringer

Mannheim). Total genomic DNA isolated from the spot form of P. teres isolate identified as "Hort-Farm" was used for library construction. This isolate was collected at the Horticultural Research Farm of the Agricultural Experiment Station located 1 mile west of Montana State University. The "Hort-Farm" isolate was also found to be one of the most virulent.

Plasmid and genomic DNA were digested with Acc I and Tag I restriction enzymes respectively and ligated using T4 ligase. Recombinant plasmids containing DNA inserts of P. teres f. sp. maculata were evaluated using two approaches. The first approach consisted of cleaving the inserts from the plasmid vector with Eco RI and Pst I restriction endonucleases, Southern blotted, and probed with nick-translated total genomic DNA of P. teres f. sp. maculata. The second approach was the dot blot method as previously described (Landry and Michelmore, 1985). To detect RFLPs, selected inserts preliminarily characterized as low copy number by the gel fractionation and dot blot methods were used as probes and hybridized to Southern blots containing restriction endonuclease-digested total genomic DNAs of P. teres f. sp. maculata and P. teres f. sp. teres isolates representing different geographical areas (Table 1).

Restriction Endonuclease Digestion and Gel Electrophoresis

Two to five ug aliquots of genomic DNA of net and/or spot form of P. teres were digested to completion with 2 to 5 units of a given restriction enzyme per ug of genomic DNA. Restriction fragments were size-fractionated by gel electrophoresis on a 24 x 20.5 cm 0.8% agarose gel in TBE (8.9 mM Tris-HCl, 89 mM Boric acid, 2 mM EDTA pH 8.0) at 1.2 V/cm overnight. Lanes of molecular size standards (Hind III digested phage lambda digested DNA) were included in each gel.

Blot Hybridization Techniques

Restricted DNAs were transferred to Zeta-Probe nylon membranes according to modified methods of Southern (Southern, 1985). Agarose gel with restricted DNAs was soaked twice with denaturing buffer (1.5 M NaCl, 0.5 M NaOH) 20 min each. The denatured gel was then soaked twice in neutralizing buffer (1 M Tris-HCl pH=8, 1.5 M NaCl) and blotted overnight in 10x SSC as described in Appendix B.

Plasmids carrying desired fragments were isolated from E. coli hosts using the miniprep procedure described by Birnboim and Doly (1979). Approximately 0.1-1 ug of miniprep was labelled by nick-translation (Rigby et al., 1977). The labeled probes were then passed through a G50 sephadex column to separate the labeled DNA from unincorporated nucleotides. Prior to hybridization, the

labelled probes were denatured by heating to 95-100°C for 10 minutes.

Zeta-probe membranes were prehybridized in 20 ml 100% formamide, 10 ml 20xSSC, 0.8 ml 50x Denhardts', 1 ml 1M NaH_2PO_4 pH=6.5, and 1 ml of herring sperm DNA at 42°C in a air shaker for 6 to 24 hours. Denatured probes were added to the bagged filters and prehybridization mix and bags resealed and incubated at 42°C for 6 to 24 hours.

The hybridized Zeta-Probe membranes were washed with three different solutions. The first (2x SSC, 0.1% SDS) and the second (0.2x SSC, 0.1% SDS) washes were conducted at room temperature in a platform shaker changing the solutions 3 times every 5 minutes. The last wash (0.1 SSC, 1% SDS) was carried out at 65°C in a shaking water bath changing the solution 2 times every 30 minutes. Washed filters were wrapped in plastic wrap and placed adjacent to a sheet of X-ray film in an exposure cassette with two intensifying screens.

Sequence Analysis

Desired DNA fragments cloned into pUC12 were sequenced or partially sequenced according to the modified Sequenase sequencing kit manufacturer's recommendations (United States Biochemical Corporation).

Supercoiled plasmid DNA was isolated according to the "maxi" preparation method and purified through a Sephadex

column or in "minipreps" as previously described (Hattori and Sakaki, 1986). Plasmid DNA isolated by minipreps were extracted with phenol/chloroform 3 to 4 times, incubated with ribonuclease at 37°C for 30 min, and then mixed well with 20% polyethylene glycol 6000-2.5 M NaCl to provide DNA pure enough for sequencing. The sequence of the fragments was confirmed by sequencing them several times in both orientations. The forward (17-mer, 3'TGACCGGCAGCAAAATG-5'), and reverse (17-mer, 5'-CAGGAAACAGCTATGAC-3'), M13/pUC universal primers were used for annealing. Primer concentration of 1.5 pmol and 2 ul of 500 Ci/mmol equal 20 uC of [^{35}S] dATP were used per reaction.

Primer Design

Sequencing data were used to design a set of primers flanking DNA regions to be amplified via PCR. Primers consisted of 20 nucleotides each and located at the 5' and 3'ends of opposite strands of the amplified product. Primers were selected based on GC content (60%) and a lack of internal and interprimer homology.

Polymerase Chain Reaction (PCR) Protocols

PCR protocols and reactions were conducted by using a modified Perkin Elmer Cetus GeneAmp PCR Corporation kit. Because of the risk of cross contamination from handling of many identical PCR reactions, it was necessary to employ

strict precautions as recommended (Kwok and Higuchi, 1989). Many of these precautions simply require good sterile techniques and awareness of potential contamination sources. Dedicated pipets and reagents for handling PCR products were assigned prior to performing the experiments. Negative controls (omitting DNA template) were also included in each PCR experiment.

A 50 ul volume reaction was conducted as follows: 32 ul of sterile ddH2O, 5 ul 10x PCR buffer (10mM $MgCl_2$, 100mM Tris-Cl pH=8.3, and 500 mM KCl) (1x final concentration), 8 ul of 1.25 mM/nucleotide dNTP's mixture (200 uM/nucleotide final concentration), a final concentration of 200 pmol/primer and 100 ng of genomic DNA or 20ng of plasmid. The samples were subjected to an initial melt to inactivate any proteases present in the DNA samples before adding the AmpliTaq DNA Polymerase. The protocol most commonly used in this study for plasmid and genomic fungal DNA amplification was as follows:

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1) Template melting at 95°C for 5 minutes (initial melt).

2) Add 1.25 units/50 ul total volume of AmpliTaq DNA Polymerase.

3) Start cycling at 94°C for 1 min (melt), 55°C for 2 min (anneal), and 72°C (extension) for four minutes.

4) After the last cycle was completed the samples were kept at 72°C for 10 min to complete the strands.

Other PCR protocols consisted in varying the annealing temperature to 60 and 65°C. Other parameters were maintained constant.

Confirmation of PCR Products Identity by Southern Blot and Sequence Analysis

PCR products were identified by Southern blot and DNA sequence. Southern transfers were conducted by using a modified method of Southern (Southern, 1975). Details of the Southern transfer used are given in Appendix B. Prior to sequencing, amplified PCR products were passed through a Centricon 30 filter, phenol/chloroform extracted, ethanol precipitated and cloned in the plasmids pUC18 and pUC19. DNA sequencing was conducted according to the modified Sequenase sequencing kit manufacturer's recommendations (United States Biochemical Corporation). Plasmid templates were prepared according to the "mini-prep" method (Hattori and Sakaki, 1986). Primer concentration of 1.5 pmol and 2 ul of 500 Ci/mmol equal 20 uC of [^{35}S] dATP were used per reaction.

RESULTS

Evaluation of Isolates and Germplasm

To begin this study, P. teres isolates and barley cultivars first needed to be classified according to their virulence and susceptibility respectively. Highly significant differences for virulence among both net and spot isolates were observed (Table 3). The range in virulence was greater among the net isolates as compared to the spot form isolates (Table 4). However, when the mean disease ratings of net and spot were compared, there were no differences in virulence between the two forms (Table 4).

Table 3. Analysis of variance of disease ratings of 12 net form and 8 spot form P. teres isolates on 32 barley cultivars.

Source	df	Mean square (a)
Cultivar	31	32.618 **
Isolate	19	80.661 **
Cultivar X Isolate	589	2.384

(a) = Significant at P = 0.01

Table 4. Comparative mean virulence of net and spot forms of P. teres from U.S.A., Mexico and Colombia using a set of 32 barley cultivars.

Isolate Designation	Origin	Type of Reaction	Virulence (Means)+
Calif-1 (Tulare County)	California	net	2.469 A
Mex-6 (E. Zapata)	Mexico	net	2.813 A
Mex-4 (Cd. Sahagun)	Mexico	net	2.875 A
Mex-2 (Anahuac-1)	Mexico	net	3.656 B
Mex-3 (Anahuac-2)	Mexico	net	4.250 CB
Pt-R	Montana	net	4.594 CD
Col-1 (Iraka County)	Colombia	spot	4.844 CED
Mex-5 (La Lagunilla)	Mexico	net	4.875 CED
Calif-5 (Solano County)	California	net	4.969 CEDF
Col-2 (Tundama County)	Colombia	spot	5.000 CEDF
Calif-3 (Fresno County)	California	net	5.188 EDF
Post Farm	Montana	spot	5.594 E F
Col-3 (Boyaca County)	Colombia	spot	5.719 G F
Con-S	Montana	spot	6.469 GH
Fc-1	Montana	spot	6.750 HI
MN-1	Minnesota	net	7.031 JHI
Pt 6-2	Wyoming	spot	7.125 JHI
Hort Farm	Montana	spot	7.219 JHI
Sco-B	Montana	net	7.313 J I
86-Hfi	Montana	net	7.563 J

+ Isolates followed by the same letter were not significantly different at .01 % as determined by the multiple mean comparisons based on LSD.

Isolates from Mexico were the least virulent along with a California isolate designated as Calif-1. Other isolates from the U.S.A. were the most virulent. Pt-R a Montana isolate, was not classified as highly virulent as previously described (Bjarko, 1979).

The barley cultivars also differed in their ability to react upon infection with net or spot forms of P. teres (Table 3). The CI 9776 appeared to be highly resistant to most of the isolates, CI 5791 and CI 7584 were resistant and CI 14023, CI 9819, CI 1197, and CI 5845 had moderate resistance. Compana, Erbet, Harrison, and Clark were the most susceptible, and Klages, Hector, and Moravian III moderately susceptible (Table 5). Based on these results the susceptible cultivar Compana, and the moderately resistant cultivar CI 9819, were selected for use in the disease diagnosis studies.

Library Construction and Selection of P.teres f.sp. maculata Genomic DNA Clones

A partial pUC genomic DNA library of P. teres f. sp. maculata was constructed. The total genomic DNA of "Hort-Farm" a virulent isolate (Table 4), was used for library construction. The P. teres f. sp. maculata library was first screened to identified clones containing low copy sequences.

Single or low copy sequences could be useful in inheritance and mapping studies as well as for disease

Table 5. Comparative mean resistance of 32 differential cultivars upon infection with 12 net form and 8 spot form P. teres isolates.

Cultivar	Mean (+)	Disease Rating
CI 9776	2.55 A	HR
CI 5791	2.90 AB	R
CI 7584	3.05 ABC	R
CI 14023	3.70 BCD	MR
CI 9819	3.80 BCD	MR
CI 1197	4.00 CD	MR
CI 5845	4.30 E D	MR
Robust	4.55 EF D	MS
CI 1615	4.55 EF D	MS
Beecher	4.55 EF D	MS
CI 2750	5.00 EFG	MS
CI 2330	5.00 EFG	MS
Tifang	5.05 EFGH	MS
CI 12821	5.20 EFGHI	MS
Vireo	5.20 EFGHI	MS
Summit	5.45 FGHI	MS
Menuet	5.55 J GHI	MS
Freja	5.65 JKGHI	MS
Morex	5.70 JKGHI	MS
Manker	5.70 JKGHI	MS
Bowman	6.00 JK HIL	MS
Gallatin	6.05 JK IL	MS
Betzes	6.10 JK IL	MS
Herta	6.10 JK IL	MS
Piroline	6.15 JK IL	MS
Moravian III	6.50 JKM L	S
Hector	6.50 JKM L	S
Klages	6.55 KM L	S
Clark	6.80 M L	VS
Harrison	6.85 N M L	VS
Erbet	7.25 N M	VS
Compana	7.80 N	VS

(+) = Mean of disease reading on 32 differential cultivars. Cultivars followed by the same letter are not significantly different at the .01% level as indicated by LSD mean comparison.

diagnosis using Southern blots or PCR. A single or few bands will be observed in Southern blots and a single amplification product will be expected upon fractionation of PCR products in agarose gels. Sequences representing repetitive DNA might produce more complex banding patterns making difficult the identification, and confirmation of the amplified PCR products.

Two approaches were used to prescreen random genomic clones of P. teres representing low copy sequences. The first approach utilized gel fractionation of inserts cleaved from the plasmid vector using Eco RI and Pst I restriction endonucleases (Fig. 1A). Products were then transferred onto Zeta-probe, and probed with nick-translated label "Hort Farm" total genomic DNA (Fig. 1B). This method permitted estimation of both copy number and size of the inserts. The size of the evaluated clones varied from 100 bp to 1700 bp. Inserts that had a low level of hybridization were considered to be single or low copy (Fig. 1B, lanes 3 and 5 to 10). Those fragments with high hybridization signals were considered to contain repetitive DNA (Fig. 1B, lanes 2 and 4).

The second approach was the dot blot which estimates copy number. An example of the selection strategy is illustrated in Fig. 2. As in the gel fractionation method, those clones with low hybridization signal were low copy (lanes 2f and 2g), and those fragments with high hybridization were

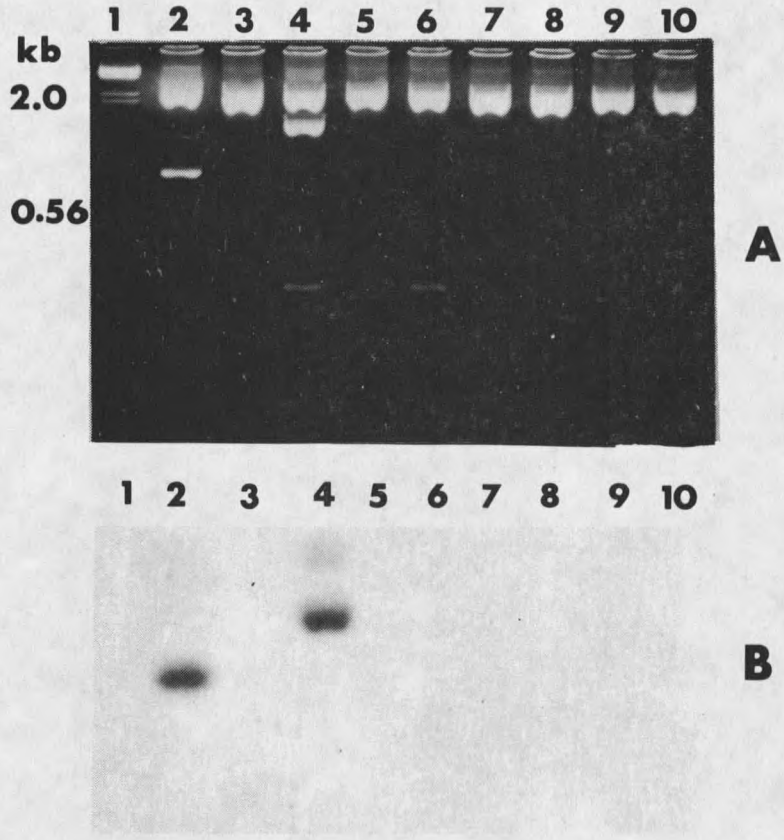

Fig. 1. Gel fractionation method to screen low copy inserts of P. teres f. sp. maculata cloned into the plasmid pUC12. A) ethidium bromide of fragments cleaved from the plasmid vector using Eco RI and Pst I restriction endonucleases; B) Southern blot of the same fragments hybridized to labelled P. teres f. sp. maculata total genomic DNA.