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A Competitive Polymerase Chain Reaction to Quantify DNA of *Leptosphaeria maculans* During Blackleg Development in Oilseed Rape

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An assay based on the competitive polymerase chain reaction technique was developed to quantify *Leptosphaeria maculans* during blackleg disease development in oilseed rape leaves. By means of primers specific to the highly virulent type of *L. maculans,* a heterologous internal control template was prepared by amplifying and cloning DNA from *Leptosphaeria korrae* under low-stringency annealing conditions. Coamplification of *L. maculans* with the internal control DNA provided accurate quantification of 1 to 10^9 copies of target DNA. The assay was applied to a comparative study of *L. maculans* colonization of resistant and susceptible rape cultivars. The assay revealed that lesion size was associated with the quantity of *L. maculans* DNA during the first 12 days after inoculation of the susceptible cultivar Westar and the moderately resistant cultivar Legend. In these cultivars, the quantity of DNA per lesion increased during the first 12 days after inoculation and then declined. This decline in detectable fungal DNA coincided with abundant sporulation, rapid necrosis, and the onset of leaf senescence. Trace amounts of *L. maculans* DNA were detected in the resistant cultivar Glacier, in which lesion size was similar to that in the wounded, uninoculated check. The assay is rapid, accurate, and very sensitive and can be incorporated into conventional disease screening programs.

The polymerase chain reaction (PCR) is a powerful tool for amplifying and detecting specific nucleic acid molecules present in limited amounts (Erlich and Arnheim 1992; Steffan and Atlas 1991). During PCR, an initial number of target sequences are used as a template from which a large quantity of a specific product can be obtained based on the primers chosen. Although the amount of PCR product can be readily determined, it is difficult to deduce the initial copy number of the target molecule, because the efficiency of PCR can vary widely (Gilliland et al. 1990; Wang et al. 1989). The problem of quantifying target DNA from the amount of PCR product is a result of the exponential nature of PCR, in which small variations affecting amplification efficiency can result in dramatically different PCR product yields (Fox et al. 1992; Gilliland et al. 1990; Virdi et al. 1992; Wang et al. 1989).

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One approach to creating a quantitative PCR is to coamplify the molecule of interest with a competitor template bearing identical sites for attachment of primers (Fox et al. 1992; Gilliland et al. 1990; Hu et al. 1993; Piatak et al. 1993a; Stieger et al. 1991; Telenti et al. 1992). Because the competitor template has the same primer sites as the target, it acts as a competitor for the primers, and quantification is based on the relative yield of the PCR products amplified from the target and the competitor (Piatak et al. 1993a,b; Porcher et al. 1992; Wang et al. 1989). In order to quantify the PCR products from both the target and the competitor DNA, they must first be separated, and then the relative amounts of the PCR products is determined by densitometry or the incorporation of radioactivity (Hu et al. 1993; Liu et al. 1994; Überla et al. 1991; Virdi et al. 1992).

Several approaches have been taken to distinguish the PCR products amplified from target and competitor DNA. The competitor DNA can be obtained by site-directed mutagenesis of the target DNA, so that a restriction site is either added or deleted (Gilliland et al. 1990; Siebert and Larrick 1993). The PCR products are digested with a restriction enzyme, and the target and competitor PCR products are then separated by gel electrophoresis (Fox et al. 1992; Peten et al. 1993; Stieger et al. 1991; Telenti et al. 1992). Alternatively, a completely foreign DNA fragment can be engineered to contain primer templates (Wang et al. 1989) or obtained by PCR amplifications using the target primers and nontarget DNA under reduced annealing stringency (Förster 1994a; Liu et al. 1994; Telenti et al. 1992; Überla et al. 1991). These competitors are designed to be a different size from the target, so that they can be separated and then quantified following gel electrophoresis.

One application of quantitative PCR is in the identification and quantification of plant-pathogenic microorganisms (Hu et al. 1993; Liu et al. 1994). It would be useful to develop a method to specifically quantify *Leptosphaeria maculans,* a heterothallic ascomycete that causes blackleg disease of many crucifers (Gabrielson 1983; Venn 1979). *L. maculans* isolates from oilseed rape have been divided into two virulence types: highly virulent (HV) and weakly virulent (WV) (Gabrielson 1983; McGee and Petrie 1978). The WV type causes mild disease symptoms with no subsequent yield loss. However, it is very similar in appearance to the HV type, which is destructive and economically important (Taylor 1993). Therefore, a simple, accurate, and nonisotopic quantitative PCR

assay should also be specific for the HV type of *L. maculans*. This assay could find wide application in quantifying levels of the HV type in oilseed rape seed to ensure the distribution of pathogen-free seed and in breeding materials to more accurately assess levels of resistance.

Recent advances in developing specific diagnostic tests based on the nucleotide sequences of internal transcribed spacer regions (ITS) of ribosomal genes have provided accurate diagnosis of the *L. maculans* virulence types (Plummer et al. 1994; Taylor 1993; Xue et al. 1992). We have developed a molecular assay for quantifying *L. maculans* DNA of the HV type by employing a competitor and target sequence from the ribosomal ITS, and we have used the assay to monitor fungal development in infected oilseed rape plants.

Table 1. Sources of DNA tested with PCR using primers HV17S and HV26C

Species	Isolate	400-bp Fragment
Brassica napus cv. Westar	...	–
Cephalosporium sp.	Csp-40	–
Magnaporthe poae	Mp73-1	–
Gaeumannomyces graminis	Gg-1	–
	Gg-3	–
Cladosporium sp.	Clad-40	–
Drechslera poae	Dp-1	–
Pythium sp.	Psp-20	–
Fusarium sp.	Fsp-02	–
Sclerotinia homoeocarpa	SH-01	–
Rhizoctonia solani	RS1/T (AG1)	–
Trichothecium roseum	Tr-01	–
Phoma sp.	PT1	–
Leptosphaeria korrae	LK-10	–
Alternaria sp.	Alt-1	–
Leptosphaeria maculans		
Weakly virulent isolates	Unity	–
	LM3	–
	LM7	–
	LM63	–
	2350	–
	2376	–
	2384	–
	2379	–
Highly virulent isolates	LM48	+
	1988	+
	PG2A	+
	PG3A	+
	PG4A	+
	PG4C	+
	Leroy	+
	2373	+

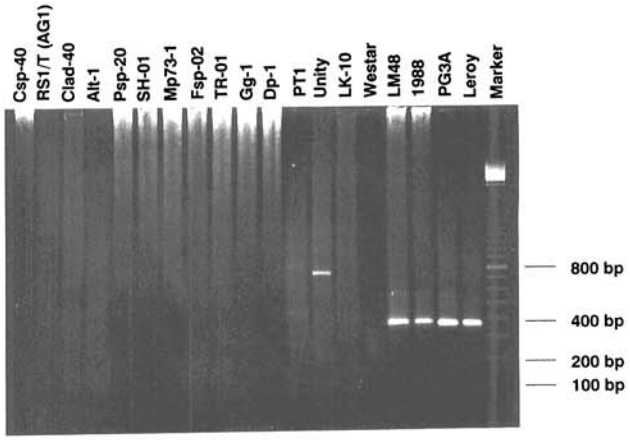

Fig. 1. Specific detection of highly virulent isolates of *Leptosphaeria maculans* by polymerase chain reaction with primers HV17S and HV26C. The marker is a 100-bp ladder. All isolates are listed in Table 1. Amplification of a 400-bp fragment occurred with DNA from highly virulent isolates but not with DNA from weakly virulent isolates of *L. maculans*, other fungi, or canola.

RESULTS

Primer specificity and sensitivity.

Amplifications with the primers HV17S and HV26C yielded a single 400-bp PCR product from all of the HV isolates of *L. maculans* (Table 1 and Fig. 1). There were no amplification products with DNA from the WV isolates of *L. maculans*, nontarget fungi including saprophytes and other pathogens, or uninfected *Brassica napus* cv. Westar (Table 1 and Fig. 1). The primer pair was tested for sensitivity to cloned competitor DNA. When the cloned competitor DNA was diluted in Tris-EDTA (TE) buffer, only 10^7 ag of competitor DNA could be reliably detected (Fig. 2A). A very faint band was observed for 10^6 ag of cloned competitor DNA, but this was not considered reliable and was not visible in the figure. However, our assay could detect 10^1 ag of the cloned competitor DNA if it had been diluted in the presence of 5 ng/μl of healthy *B. napus* DNA in TE buffer (Fig. 2B). The presence of heterologous DNA stabilizes the target DNA and makes it available for amplification. However, low detection levels were observed when 10^3, 10^2, and 10^1 ag of competitor DNA were amplified. There was no amplification product in control reactions lacking template DNA.

Accuracy of quantitative PCR.

To determine whether the ratio of the PCR products had a direct quantitative relationship with the initial level of template, known amounts of competitor DNA ranging from 10^4 to 10^{10} ag were used as standards. By varying the amounts of competitor DNA while the target DNA amount was constant, a dilution was reached at which the starting amounts of *L. maculans* and competitor DNA were equivalent, so that equal amounts of their respective PCR products accumulated (Fig. 3). The relationship between the log of the concentration of the competitor DNA and the ratio of the PCR products was linear between 10^4 and 10^{10} ag, and the coefficient of determination was very high ($r^2 = 0.99$) (Fig. 4).

The accuracy of the assay was assessed by the relationship between the amount of *L. maculans* DNA added to the assay and the quantity of *L. maculans* DNA estimated by quantitative PCR (Fig. 5). A high coefficient of determination ($r^2 = 0.99$) was obtained, indicating that the PCR assay is very accurate in estimating the quantity of *L. maculans* DNA over a wide range (Fig. 5).

Quantification of *L. maculans* DNA in infected oilseed rape plants.

The quantitative assay was used to monitor the colonization of three oilseed rape cultivars by *L. maculans* isolate Leroy (Fig. 6). Lesion diameters increased to a maximum at day 15 in the cultivar Westar, day 12 in the cultivar Legend, and day 9 in the cultivar Glacier. Lesions were largest in Westar, intermediate in Legend, and smallest in Glacier. The quantity of

L. maculans DNA paralleled the lesion size in the susceptible cultivar Westar for the first 12 days following inoculation (Fig. 7). A similar trend was observed for the moderately resistant cultivar Legend, but the quantity of *L. maculans* was significantly lower than in Westar. The quantity of DNA in the resistant cultivar Glacier was never significantly greater than that of the initial inoculum, showing that fungal colonization of this cultivar was extremely limited. The results show that fungal colonization and DNA quantity were directly related to susceptibility. Lesion diameter (in millimeters) was positively correlated with the amount of *L. maculans* DNA up to day 12 in Westar ($r = 0.99$) and Legend ($r = 0.90$). After day 12, the quantity of *L. maculans* dropped sharply in Westar and Legend, and this drop in detected DNA coincided with pycnidia formation, chlorosis, necrosis, and eventually the onset of leaf senescence. DNA of *L. maculans* in inoculated leaves continued to decline up to 18 days after inoculation, when it could still be detected in Westar but not in Legend and Glacier. No *L. maculans* DNA could be detected in any uninoculated plants.

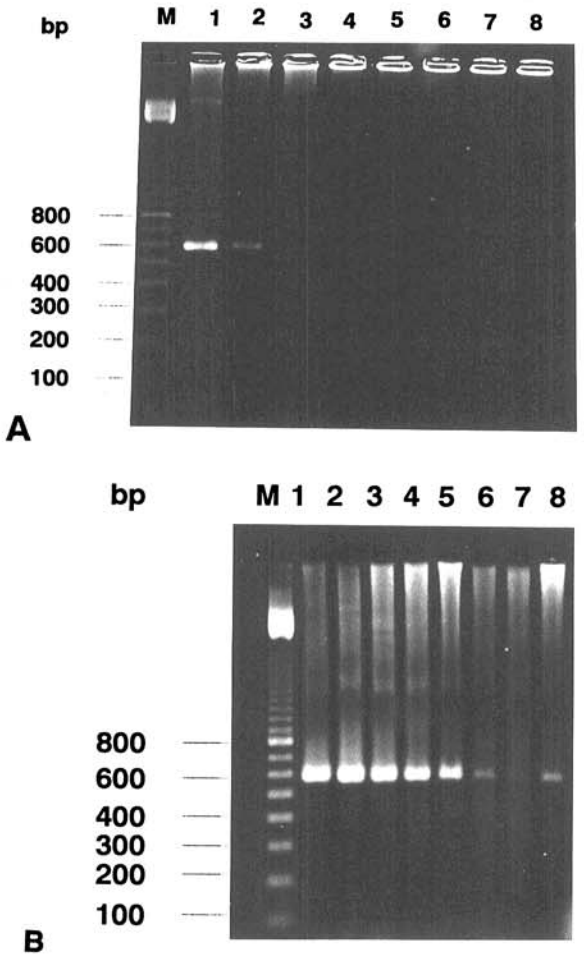

Fig. 2. Sensitivity of detection of cloned competitor DNA by polymerase chain reaction using primers HV17S and HV26C. **A,** Lane M is a 100-bp ladder. Lanes 1–8 are 10-fold serial dilutions of the competitor DNA from 10^8 to 10^1 ag. Amplifications were conducted in dilutions made in 0.5× Tris-EDTA buffer only. **B,** Amplifications were conducted in dilutions made in 0.5× Tris-EDTA buffer containing 5 ng of DNA from healthy *Brassica napus* per µl.

DISCUSSION

Several alternative approaches to quantifying fungi have been described. These methods include measurement of chitin, a cell wall constituent (Manibhushanrao and Mani 1993; Roche et al. 1993); ergosterol, the predominant sterol component of many fungi (Gardner et al. 1993; Gretenkort and Ingram 1993); and antigens, by enzyme-linked immunosorbent assay (ELISA) (Harrison et al. 1990; Newton and Reglinski 1993). Chitin and ergosterol measurements are both non-

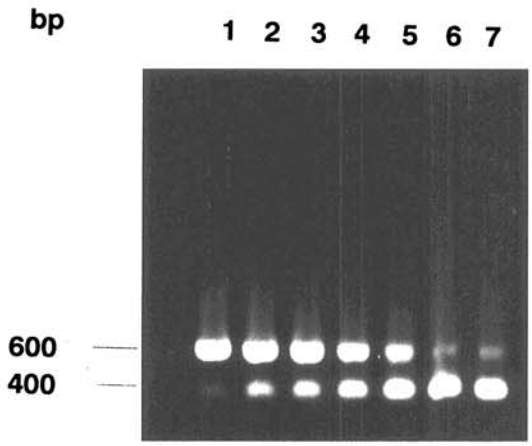

Fig. 3. Competitive polymerase chain reaction with purified DNA of *Leptosphaeria maculans* isolate Leroy. The gel shows coamplification with a constant 10^6 ag of *L. maculans* DNA and varying amounts of competitor DNA. The amount of competitor DNA added to each reaction was as follows: lane 1, 10^9 ag; lane 2, 10^8 ag; lane 3, 10^7 ag; lane 4, 10^6 ag; lane 5, 10^5 ag; lane 6, 10^4 ag; and lane 7, 10^3 ag.

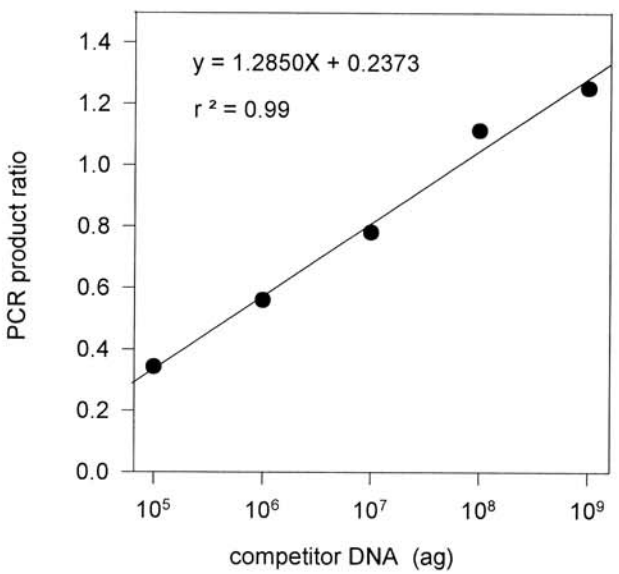

Fig. 4. Competitive polymerase chain reaction (PCR) quantification of *Leptosphaeria maculans* DNA. Regression analysis of the ratio of PCR product yield from competitor and target DNA versus the concentration of the competitor DNA. Analysis was based on integrated band intensity after correction for the length differences of the PCR products from target and competitor DNA. A constant 10^6 ag of *L. maculans* DNA was added, and the amount of competitor DNA ranged from 10^5 to 10^9 ag.

species-specific, since the compounds are components of many fungi. Furthermore, the chitin assay is based on the colorimetric detection of the chitin subunits glucosamine and *N*-acetylglucosamine, both of which have been detected in uninfected plant tissues, and Schnürer (1993) showed that there was no correlation between fungal biomass and ergosterol level once the fungus starts to sporulate. Serological assays, such as ELISA, can be highly specific, but the specificity usually requires the production of monoclonal antibodies, which can be tedious and time-consuming.

PCR can provide extraordinary sensitivity and specificity in the detection of fungi, but inherent features of the reaction prevent its use for reliable quantification (Fox et al. 1992; Piatak et al. 1993a; Yang et al. 1993). We have developed a method for quantifying *L. maculans* by employing a nonhomologous competitor template in a competitive PCR assay. The assay is highly specific to the HV type of *L. maculans* and could accurately detect and quantify 1 to 10^9 ag of target *L. maculans* DNA. The utility of the competitive PCR assay was demonstrated by monitoring the colonization of resistant and susceptible oilseed rape plants by the HV type of *L. maculans*.

A competitor template was incorporated into the quantitative assay to compete with the target sequence for primers. We took the approach to create a competitor template by cross-species amplification using the target primers and reduced annealing stringency. The advantage of using a heterologous competitor is that it reduces the chances of heteroduplex formation between target and competitor PCR strands, since the competitor has no homology to the target except for the short primer template (Hu et al. 1993; Yang et al. 1993). The potential disadvantage of such a heterologous competitor DNA is the possibility that it may not be amplified with the same efficiency as the target sequences (Pannetier et al. 1993). However, it has been shown in a number of cases that the target and heterologous competitor templates share similar amplification kinetics (Förster 1994a,b; Liu et al. 1994; Piatak et al. 1993a,b; Siebert and Larrick 1993; Telenti et al. 1992).

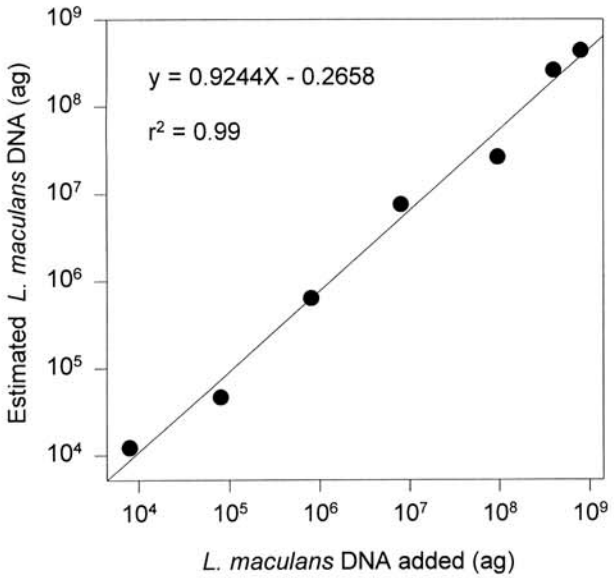

Fig. 5. Accuracy of polymerase chain reaction (PCR) quantification of *Leptosphaeria maculans* DNA as demonstrated by the relationship between the amount of *L. maculans* DNA added to the PCR reaction mixture and the amount of *L. maculans* DNA estimated by competitive PCR. Serial dilutions of purified *L. maculans* DNA were made in 0.5× TE containing 5 ng of DNA from healthy *Brassica napus* per µl and then coamplified with competitor DNA in concentrations ranging from 10^4 to 10^9 ag.

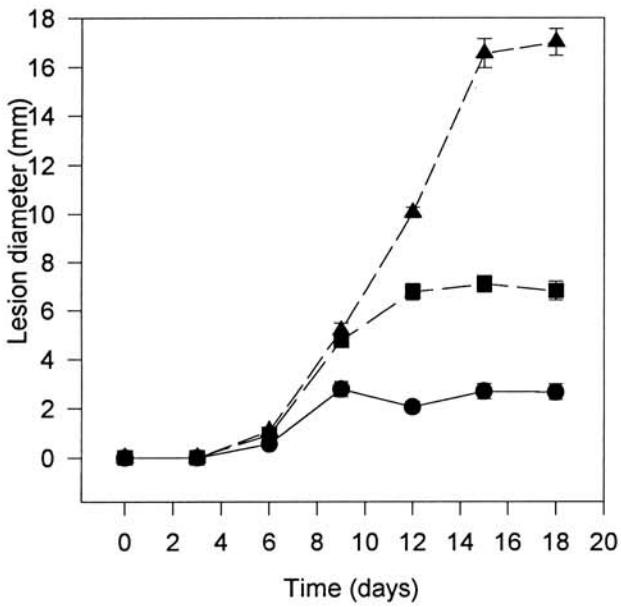

Fig. 6. Diameter of lesions in susceptible and resistant oilseed rape cultivars caused by a highly virulent isolate of *Leptosphaeria maculans*. The first fully expanded true leaf was inoculated with 10^7 spores of *L. maculans* isolate Leroy per ml, and lesion diameter was measured 12 h after inoculation and then at 3-day intervals. The three cultivars were the susceptible Westar (triangles), the moderately resistant Legend (squares), and the resistant Glacier (circles).

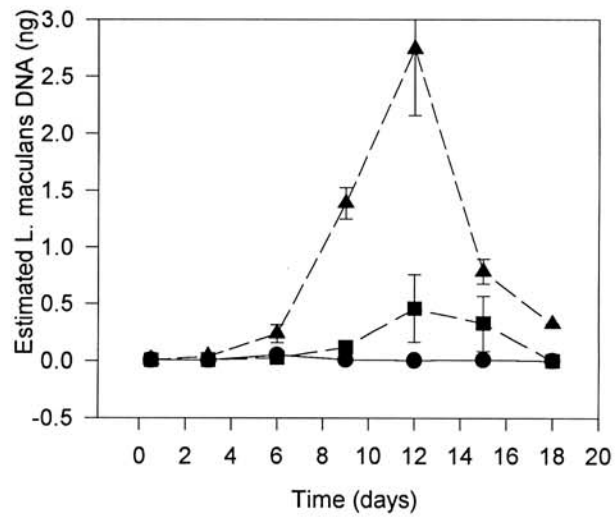

Fig. 7. Specific detection and quantification of DNA from a highly virulent isolate of *Leptosphaeria maculans* directly in infected plant tissue by the competitive polymerase chain reaction. The first fully expanded true leaf was inoculated with 10^7 spores of *L. maculans* isolate Leroy per ml, and DNA was measured 12 h after inoculation and then at 3-day intervals. DNA was extracted from a 20-mm leaf disk centered on the site of inoculation, and the amount of *L. maculans* DNA was determined by the competitive polymerase chain reaction. The three cultivars were the susceptible Westar (triangles), the moderately resistant Legend (squares), and the resistant Glacier (circles).

The heterologous control template used in this study appeared to have the same amplification kinetics as the target sequences, and accurate quantification was possible after adjustments were made for differences in size of the PCR products.

Our assay revealed that rapid tissue colonization is associated with an increase in the quantity of target DNA. *L. maculans* is a hemibiotroph (Hammond et al. 1985), and during its biotrophic phase, successful systemic infection is established. In compatible interactions, pioneer hyphae advance continually through the living tissue, and colonization always precedes necrosis. Our results show a direct correlation between lesion size and DNA quantity during the biotrophic phase. This relationship was most evident in the susceptible cultivar Westar but also occurred in the moderately resistant cultivar Legend. In the necrotrophic phase *L. maculans* causes tissue collapse and disintegration followed by necrosis of infected areas. In the susceptible cultivar Westar, necrotic lesions were associated with abundant spore production. However, there was a marked decrease in fungal DNA quantity, which could have resulted from spore release and hyphal autolysis. It is highly unlikely that the decline in *L. maculans* DNA was due to spore release, because all the pycnidia analyzed still contained mature spores. Hyphal lysis and disintegration in infection zones has been reported in the interaction of other hosts and parasites, such as the interaction of orchids and mycorrhizal *Rhizoctonia* (Hadley and Williamson 1972) and the interaction of the tuberous rhizome of *Gastodia elata* and mycelial strands of *Armillaria mellea* (Akai 1959). The most likely explanation is that the decrease in the amount of *L. maculans* DNA extracted and detected is related to hyphal lysis. Hyphal disintegration, the formation of globular vesicles that stained dense with trypan blue, and an accumulation of brown pigments have been observed in interactions of *L. maculans* and resistant oilseed rape cultivars (Hammond and Lewis 1987; Hammond et al. 1985).

Necrosis was also associated with brown pigments in DNA extracts from older lesions, and PCR amplifications were inhibited with these extracts. Brown pigments in necrotic tissue infected by *L. maculans* have been reported (Hammond and Lewis 1987; Xi and Morrall 1993). These pigments are associated with the host defense response and are probably polyphenolics, which could bind to DNA and interfere with the DNA extraction efficiency and amplification. Successful amplification of DNA extracted from lesions on day 15 and day 18 after inoculation required a 10-fold dilution of the lesion DNA extract. Even the competitor did not amplify prior to dilution, showing the utility of competitor DNA as an indicator of inhibition and as a control against the occasional reaction failure. Despite the presence of PCR inhibitors, the quantification remains accurate, because the assay is based on the quantification of relative, not absolute, amounts of PCR product derived from the target sequence and the competitor. If the competitor is able to amplify, then quantification is possible regardless of PCR inhibitors. Therefore, we conclude that the decline in *L. maculans* DNA is not an artifact of increasing levels of PCR inhibitors.

Resistance to blackleg appears to be fungistatic. The fungus colonized the moderately resistant cultivar Legend, but host cell necrosis occurred ahead of the hyphal front, and fungal growth was contained. These lesions were clearly defined, and few or no pycnidia were observed. Lesion diameter was not contained in the susceptible cultivar Westar, and colonization preceded necrosis. The fungus was colonizing uninfected tissue ahead of the necrotic parts of the lesion. However, from day 15 onward, the lesions did not significantly increase in size, because infected leaves were senescing, and they abscised within 20 days after inoculation. The fungus was observed to infect the resistant cultivar Glacier and caused small lesions, but the amount of fungal DNA did not increase sufficiently to be statistically significant, showing that the resistance was fungistatic.

Our assay revealed that disease susceptibility was directly associated with the maximum quantity of fungal target DNA. Therefore, the incorporation of a competitive PCR assay into conventional disease resistance screening programs would be a valuable asset. Potential resistant plant lines could initially be selected on the basis of disease scores, and the most promising of these could be tested with the competitive PCR assay for a quantitative measure of resistance. Furthermore, significant colonization of stems has been shown to occur without the production of visible symptoms (Nathaniels and Taylor 1983). This assay would ensure accurate assessment of resistance to the pathogen in cases of latent infection. The competitive PCR assay can also be incorporated into routine seed health testing programs. This assay not only provides sensitive detection of the HV type against a background of other seed-infecting microorganisms but can also determine the level of inoculum within a seed lot. This could ensure the distribution of crucifer seed which meets acceptable standards according to international seed health testing regulations.

This is the first report of the application of competitive PCR to quantify DNA in a leaf-infecting pathogen. It would be interesting to determine if the quantity of DNA of other foliar plant-pathogenic fungi changes in the same manner, particularly if the drop in DNA quantity after sporulation is unique to the *L. maculans*–oilseed rape interaction or is common.

MATERIALS AND METHODS

Fungal isolates.

Isolates used in this study are listed in Table 1. *L. maculans* isolates were cultured for 5 to 7 days at 21°C on V8 juice agar covered with a sterile transparent cellophane film (Flexel, Atlanta, GA), and DNA was extracted following the CTAB method of Rogers and Bendich (1985) as modified by O'Gorman et al. (1994). *L. maculans* isolates PG2A, PG3A, PG4A, and PG4C were provided by D. E. Heshman, Princeton, Kentucky. Isolates Csp-40, Mp73-1, Gg-1, Gg-3, Clad-40, Dp-1, Psp-20, Fsp-02, SH-01, RS1/T (AG1), Tr-01, PT1, LK-10, and Alt-1 were obtained from T. Hsiang, University of Guelph, Guelph, Ontario, Canada. *L. maculans* isolates Leroy, Unity, LM3, LM7, LM63, 2350, 2376, 2384, 2379, LM48, 1988, and 2373 were obtained from our laboratory.

PCR.

PCR amplifications were carried out in 12.5-µl reaction mixtures containing 200 µM deoxynucleoside triphosphate, 0.5 µM primers, 0.15 units of $Vent_R$ DNA polymerase (New England Biolabs, Beverly, MA), 100 µg of bovine serum albumin per ml, 1× DNA polymerase buffer (10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl [pH 8.0], 1.5 mM $MgSO_4$,

and 0.1% Triton X-100), and 1 µl of DNA. Primers HV17S, 5′-CCATTTTCAAAGCACTGCG, and HV26C, 5′-GAGTCCCAAGTGGAACAAACA, were synthesized based on differences in sequence data from the ITS 1 and 2 of the HV and WV isolates of *L. maculans* (Xue et al. 1992; Morales et al. 1993). The reactions were overlaid with light mineral oil, and amplifications were performed in a BioOven Thermal Cycler (Biotherm, Arlington, VA). The reaction was performed with one cycle at 94°C for 5 min, 62°C for 2 min, and 72°C for 2 min. This was followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 62°C for 50 sec, extension at 72°C for 60 sec, and a final extension at 72°C for 10 min. The most rapid transition between temperatures was chosen (maximum of 1°C s^{-1}).

The PCR products were separated by electrophoresis in 1.2% agarose gels (Agarose MP, Boehringer, Mannheim, Germany) in TBE buffer (89 mM Tris-borate and 2 mM EDTA). The gels were then stained with ethidium bromide and viewed under 300-nm UV light.

Construction of competitor DNA.

To develop a competitor template, DNA from *L. korrae,* a pathogen of creeping bentgrass, was chosen (O'Gorman et al. 1994). Genomic DNA (4 ng) was amplified with the primers HV17S and HV26C under the conditions described above, except that the annealing temperature was lowered to 40°C, and 40 cycles were performed in a 25-µl reaction mixture. A 5-µl sample of the PCR mixture was analyzed on 1.5% agarose gel, and the remaining 20 µl was mixed with 30 µl of TE and extracted twice with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1). The aqueous layer was precipitated with 0.5 volume of 7.5 M ammonium acetate and 2 volumes of 95% ethanol. After centrifugation, the pellet was washed with 70% ethanol, dried under vacuum, resuspended in 4 µl of distilled water, and mixed with 0.5 µl of 10× Klenow buffer (0.5 M Tris-HCl, pH 7.6, and 0.1 M $MgCl_2$) and 2 units of DNA polymerase 1 (Klenow fragment). The mixture was incubated at 37°C for 1 h to generate blunt ends, and the DNA polymerase was inactivated at 75°C for 10 min. The blunt-end PCR product was ligated to pBluescript II SK^+ (Stratagene, La Jolla, CA), which had been digested with *Eco*RV. After 16 h at 15°C, the ligation mixture reaction was heated to 75°C for 10 min, incubated at 37°C for 2 h with 3 µl of 10× one-phor-all buffer (Pharmacia) and 5 units of *Eco*RV, and then used to transform competent cells of *E. coli* JM107. From one of the clones, a recombinant plasmid containing a 600-bp DNA fragment was purified and quantified by spectrophotometry for use as a competitor in the quantitative PCR assay.

Quantitative PCR.

PCR was performed under the conditions described above except that known concentrations of competitor DNA were added to the reaction. Following electrophoresis, the PCR products were photographed under a UV transilluminator with black and white film (Ilford EP4). The negatives were scanned by a BioImage Electrophoresis Analyzer (Millipore, Ann Arbor, MI) to determine the integrated band intensity. To compensate for differences in intensity due to the size of the PCR product, the band intensity of the competitor was multiplied by 0.67, which was the ratio of PCR product length of the target and the competitor. For linear regression analysis, the ratios of the adjusted band intensities of the competitor and target PCR product were plotted as a function of the log of the concentration of competitor DNA. To determine the accuracy and sensitivity of the quantitative PCR, serial dilutions of DNA purified from *L. maculans* isolate Leroy were made in TE buffer, pH 8.0, containing 5 ng of healthy plant DNA per µl. Following amplification, the quantity of *L. maculans* DNA in each dilution was estimated, and the relationship was determined between the quantity of *L. maculans* DNA added and estimated by the assay.

Colonization of oilseed rape by the HV type of *L. maculans*.

Leaves of 3-week-old oilseed rape (*Brassica napus*) cultivars Westar (susceptible), Legend (moderately resistant), and Glacier (resistant) were wound-inoculated at one site on each side of the midrib with 10 µl of a spore suspension (10^7 spores per ml) of *L. maculans* isolate Leroy, according to Hammond et al. (1985). Inoculated plants were sampled 0, 3, 6, 9, 12, 15, and 18 days after inoculation. Lesion diameter was measured at each sampling date. DNA was extracted from plants following the procedure described by Rogers and Bendich (1985) with the following modifications. A disk (20 mm in diameter) centered on the point of inoculation was removed from the leaf and ground with 0.1 g of sand in liquid nitrogen, 500 µl of extraction buffer was added, and the mixture was incubated at 65°C for 30 min, extracted twice with chloroform, and then precipitated with 2 volumes of 95% ethanol. The pellet was dissolved in 50 µl of 1× TE buffer, RNase A was added to a final concentration of 1 µg/ml, and the mixture was incubated for 1 h at 37°C, and extracted once with phenol/chloroform/isoamyl alcohol (25:24:1) and then with chloroform/isoamyl alcohol. The DNA was precipitated with 2 volumes of 95% ethanol, dried, and resuspended in 0.1× TE buffer. The amount of *L. maculans* DNA in the plant material was quantified by PCR assay. The concentration of internal DNA used for quantification was a serial dilution of plasmid DNA ranging from 10^3 to 10^9 ag (10^{-15} to 10^{-9} g).

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