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Distribution of *Leptosphaeria maculans* in two fields in southern Ontario as determined by the polymerase chain reaction

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Abstract

The distribution of the highly virulent and weakly virulent types of *Leptosphaeria maculans*, causal agent of blackleg of oilseed rape, was studied in two fields in southern Ontario. Using a polymerase chain reaction-based assay with primers specific for these virulence types, plant tissues were directly examined for the occurrence of the pathogen. The highly virulent type was detected in leaf, stem and crown tissue at most of the sampling sites. The weakly virulent type was detected only in leaf lesions at 50% of the sites in field 1 and 30% of the sites in field 2. Of 96 leaf lesions examined, 48 contained the highly virulent type, 12 contained the weakly virulent type and 16 contained both the highly virulent and weakly virulent types. *Sclerotinia sclerotiorum* was isolated from all leaf lesion that did not react with *L. maculans* virulence type-specific primers. Coinfection of single blackleg leaf lesions by both *L. maculans* virulence types thus occurred in oilseed rape plants in the field. Only the highly virulent type was detected in pseudothecia on stubble. Approximately 1% of the seed collected from these two fields contained *L. maculans*, and both the highly virulent and weakly virulent types were detected. The highly virulent type was more prevalent and non-specific in the kind of plant tissue it infected, whereas the weakly virulent type appeared to be limited to infecting leaves and seed. This study illustrates an application of polymerase chain reaction with virulence type-specific oligonucleotide primers to study the epidemiology of blackleg of oilseed rape.

Introduction

Leptosphaeria maculans (Desm.) Ces & de Not. [anamorph *Phoma lingam* (Tode: Fr) Desm.] is a heterothallic Ascomycete that causes blackleg disease of oilseed rape (*Brassica napus* L. and *B. rapa* L.) and many other crucifer species (Gabrielson, 1983). The fungus can affect all parts of susceptible oilseed rape plants, but infections of the stem and crown are associated with the largest yield losses. Seed yields have been reduced by more than 50% in some severely infested fields (Gugel and Petrie, 1992; Hall, 1992).

L. maculans isolates have been divided into two types based on virulence: highly virulent (HV) and weakly virulent (WV) (Gabrielson, 1983; McGee and Petrie, 1978). The HV type is the most economically important and is associated with destructive crown cankers that girdle the stem and cause the plant to lodge. Although widespread, the WV type is considered a minor problem because it does not cause major yield losses, and consequently has been less studied. However, systemic infection leading to pith colonization by this virulence type has been noted (Johnson and Lewis, 1994), and because damage to the pith is less noticeable than cortical damage, the importance of the WV type may have been underestimated in most field studies. The WV type is rarely isolated from infected plant tissues from the field but can constitute up to 30% of *L. maculans* isolates recovered from contaminated seed (Chigogora and Hall, 1995). The source of the WV type is unclear, as are interactions between the WV and HV virulence types. Observations in England (Humpherson-Jones, 1986) and Saskatchewan (Petrie, 1993b; 1994) show large geographical differences in the prevalence of the virulence types, with a decline or absence of the WV type in areas where the HV type

has become prevalent. There is little information about the survival and spread of the WV type in the field, and the co-distribution and interaction of the WV and HV virulence types have not been determined under natural field conditions.

Several methods have been developed for identifying *L. maculans* virulence types. These include cultural and pathogenic characteristics, isozymes, monoclonal antibodies, restriction fragment length polymorphisms, random amplified polymorphic DNA, electrophoretic karyotyping and virulence type-specific DNA probes (McGee and Petrie, 1978; Plummer et al., 1994; Stace-Smith et al., 1993; Taylor, 1993; Xue et al., 1992). Recent advances in specific diagnostic tests based on the nucleotide sequence of internal transcribed spacer regions of ribosomal DNA have provided accurate diagnosis of *L. maculans* virulence types (Mahuku et al., 1995; Taylor, 1993; Xue et al., 1992), and permit rapid detection of the WV and HV types. A particularly valuable method to detect and differentiate closely-related species of plant pathogens utilizes the polymerase chain reaction (PCR) (Henson and French, 1993). The PCR approach not only requires less time to perform but is also more sensitive than other molecular detection methods. To identify a pathogen by PCR, diagnostic fragments of DNA are amplified with specific primers and then directly viewed in stained agarose gels.

The goal of our work was to use virulence type-specific oligonucleotide primers to study the occurrence and distribution of HV and WV virulence types of *L. maculans* in infected oilseed rape plant tissues under field conditions.

Materials and methods

Collection of tissues of oilseed rape

Two commercial fields of winter oilseed rape (*B. napus* cv. Falcon), approximately 20 km apart near Ripley, Ontario, were examined in 1993. Ten sampling sites, each 2 m square, were located 50 m apart along a transect through each field. In early June, five plants with leaf lesions were collected from each site. Sixty leaf lesions from field 1 and 36 from field 2 were examined. At harvest in August, five plants were obtained from each site and one crown and one stem segment were removed from each plant. Lesions were associated with 29 and 40 stem segments and with 28 and 8 crown segments in fields 1 and 2, respectively. In October, five pieces of stubble bearing pseudothecia were collected from each site. Samples were returned to the laboratory within 24 h and kept at 4 °C until processed. *L. maculans* was isolated from randomly selected lesions on leaf, stem and crown tissues from each site and identified by morphology and PCR as described later (Mahuku et al., 1995; Xue et al., 1992).

DNA extraction

DNA was extracted directly from infected plant material using the method described by O'Gorman et al. (1994) with the following modifications. Plant tissue was frozen in liquid nitrogen and ground to a fine powder in a mortar. Approximately 300 mg of ground tissue was suspended in 500 μl of 2% CTAB extraction buffer (2% CTAB (w/v) (hexadecyltrimethylammonium bromide, Sigma Chemical Co.), 100 mM Tris-HCl pH 8.0, 20 mM EDTA pH 8.0, 1.4 M NaCl, and 1% polyvinyl-pyrrolidone), and incubated at 60 °C for 30 min. The suspension was then extracted once with one volume of phenol:chloroform:isoamyl alcohol (25:24:1) followed by an equal volume of chloroform:isoamyl alcohol (24:1) until the interface was clear. The aqueous phase was transferred to a new tube, and DNA precipitated by adding 1/10 volume 3 M sodium acetate and 2 volumes 95% ethanol. The DNA pellet was resuspended in 30 μl TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0) and stored at −20 °C until use. To extract DNA from pseudothecia, stubble pieces with abundant pseudothecia were surface sterilized in 1% NaOCl for 2 min, rinsed in sterile distilled water, and air dried. Pseudothecia were removed and individually suspended in 200 μl of 10 mM Tris pH 8.0. DNA was extracted from a pseudothecium by boiling for 20 min, as reported by Henson et al. (1993). Samples were kept at 4 °C when in use or at −20 °C for storage.

Two thousand seeds collected from each field were evaluated for infection by *L. maculans* using the filter paper/freeze method (Chigogora and Hall, 1995). Seeds bearing pycnidia were individually suspended in 500 μl of 10 mM Tris pH 8.0, and DNA was extracted by the boiling technique (Henson et al., 1993).

Detection and virulence typing of Leptosphaeria maculans *by PCR*

PCR for specific detection of the HV type using primers HV17S, 5′-CCCATTTTCAAAGCACTGCC-3′ and HV26C, 5′-GAGTCCCAAGTGGAACAAACA-3′ was done as described by Mahuku et al. (1995).

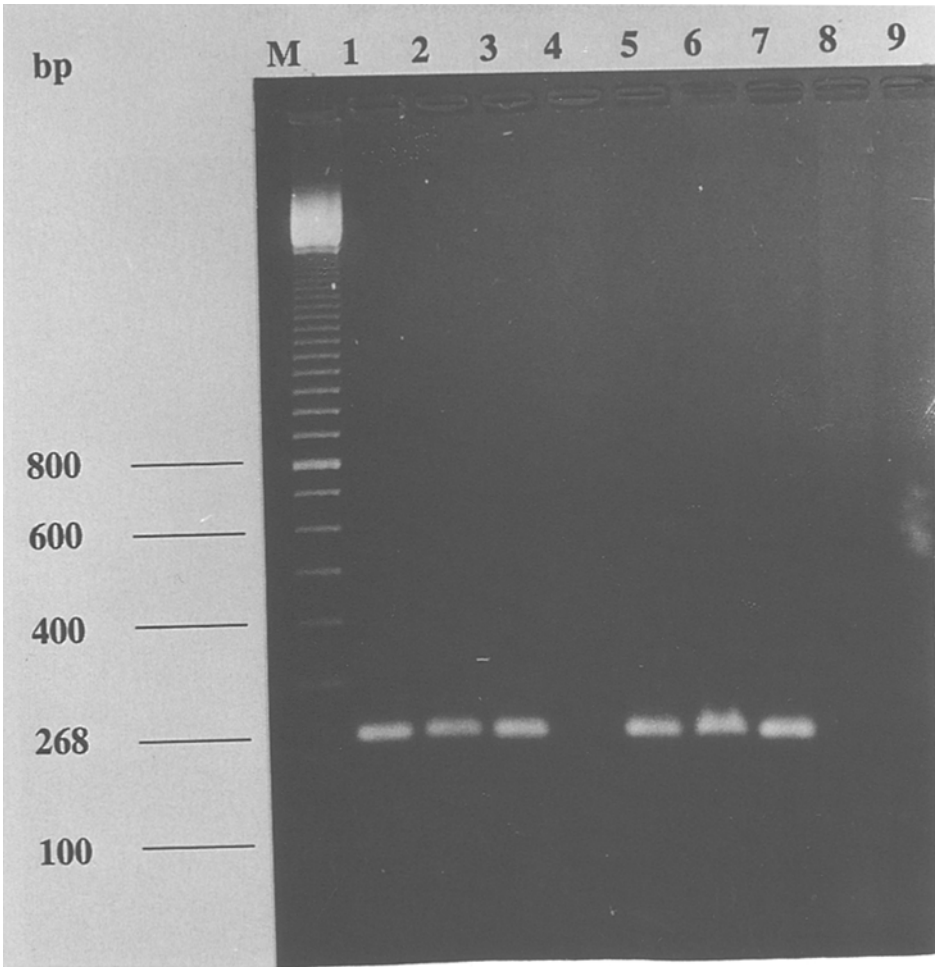

Figure 1. Specific detection of the highly virulent type of *Leptosphaeria maculans* directly in infected plant tissues from the field by the polymerase chain reaction. DNA used in lanes 1–3, and 5–7 was extracted from *L. maculans* infected leaf lesions, lanes 4 and 8 contained DNA extracted from symptomless oilseed rape leaves, no DNA was added to lane 9 and lane M was the 100-bp ladder marker. DNA used in lanes 1–4 was extracted using the boiling method and in lanes 5–8 was extracted by the modified CTAB method.

Specific detection of the WV type was done using primers WV17S, 5′-CCCTTCTATCAGAGGATTGG-3′ and 5.8C, 5′-GCATCGATGAAGAACGCAGC-3′, described by Xue et al. (1992).

Results

Extraction of Leptosphaeria maculans *DNA from lesions*

PCR amplification products of DNA extracted from blackleg lesions by the boiling method described by Henson et al. (1993) were similar to those using DNA extracted by the CTAB method described by O'Gorman et al. (1994) (Figures 1 and 2), showing that the boiling method is an efficient DNA extraction method that can be used for the rapid processing of large numbers of samples.

Distribution and incidence of Leptosphaeria maculans *virulence types in leaf tissue*

In the 20 sampling sites in fields 1 and 2, leaf lesions contained only the HV type at 9 and 7 sites, only the WV type at 3 and 2 sites, and both types at 5 and 1 sites, respectively (Table 1). The HV type alone was detected in 27 lesions in field 1 and in 21 lesions in field 2. The WV type alone was detected in 7 lesions in field 1 and in 5 lesions in field 2, and the HV and WV types occurred together in 13 lesions in field 1 and in 3 lesions in field 2. Over the two fields, the HV type was 4 times more abundant than the WV type in leaf lesions, and slightly more leaf lesions contained both virulence

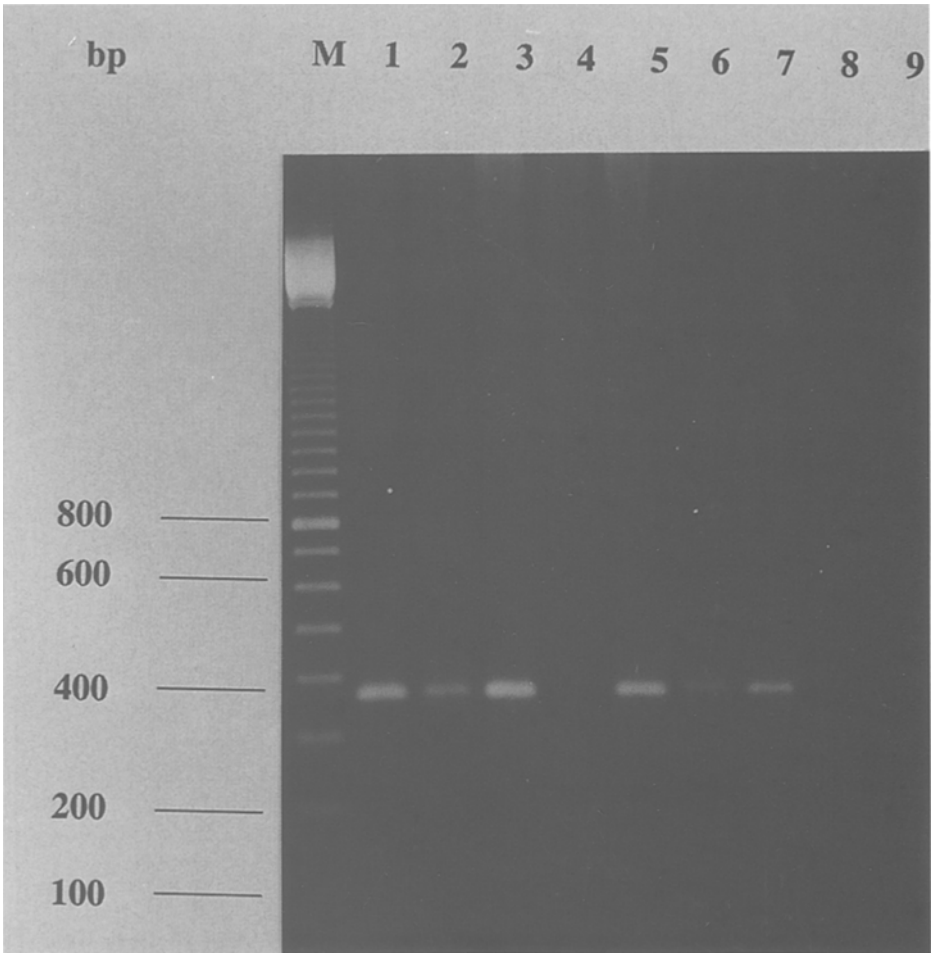

Figure 2. Specific detection of the weakly virulent type of *Leptosphaeria maculans* directly in infected plant tissue from the field by the polymerase chain reaction. DNA used in lanes 1–3, and 5–7 was extracted from *L. maculans* infected leaf lesions, lanes 4 and 8 contained DNA extracted from symptomless oilseed rape leaves, no DNA was added to lane 9 and lane M was the 100-bp ladder marker. DNA used in lanes 1–4 was extracted using the boiling method and in lanes 5–8 was extracted by the modified CTAB method.

types than contained the WV type alone. Both types were distributed throughout both fields. Mean diameters of lesions containing HV, WV and both HV and WV types were not significantly different ($p < 0.05$) (Table 1), indicating that HV and WV types can cause lesions of similar sizes under field conditions. *Sclerotinia sclerotiorum* (Lib). De Bary was isolated from all leaf lesions that did not react with *L. maculans* virulence type-specific primers.

Distribution and incidence of Leptosphaeria maculans *in tissue, stubble and seed samples*

At harvest, only the HV type was detected in stem and crown samples, and detection of the HV type was always associated with a visible lesion (Table 2). The HV type was detected at 7 and 8 sites in stem samples and at 9 and 3 sites in crown samples, in fields 1 and 2 respectively; it was detected in 20 of 29 stem lesions and 18 of 28 crown lesions in field 1 and in 30 of 44 stem lesions and 7 of 8 crown lesions in field 2. The HV type was equally abundant in stem and crown samples (40 and 36% respectively) in field 1 but was more common in stem (60%) than crown (14%) samples in field 2.

In early fall, the HV type was generally distributed on stubble through both fields, occurring at 9 sites in field 1 and at all sites in field 2 (Table 3). The WV type was not detected on stubble. Of the 50 pseudothecia examined per field, 30 from field 1 and 38 from field 2 reacted positively to the probe specific to the HV type.

Table 1. Number and diameter of leaf lesions containing highly virulent, weakly virulent or both virulence types of *Leptosphaeria maculans* in two oilseed rape fields in southern Ontario as determined by the polymerase chain reaction

Sampling site	Field 1[a]				Field 2			
	HV	WV	HV & WV	Other[b]	HV	WV	HV & WV	Other
1	0	0	3	0	0	0	0	0
2	2	2	2	1	1	3	0	1
3	2	3	2	1	1	0	0	3
4	5	0	0	2	0	0	0	0
5	5	0	0	0	2	0	3	0
6	1	0	2	0	4	0	0	1
7	5	0	0	2	4	2	0	1
8	3	0	0	3	4	0	0	1
9	3	0	0	3	5	0	0	0
10	1	2	4	1	0	0	0	0
Total	27	7	13	13	21	5	3	7
Incidence (%)	45	12	22	22	58	14	8	19
Lesion[c] diameter (mm)	12.66	14.40	11.85		14.60	10.48	12.27	

[a] Forty-two of 50 plants sampled in field 1 had leaf lesions, and 60 lesions were examined for infection by *L. maculans*. In field 2, 29 of 50 plants had leaf lesions and 36 lesions were examined. The number of lesions examined is the sum of HV, WV, HV&WV and other.

[b] Other are lesions which did not react with *L. maculans*-specific primers. *S. sclerotiorum* was isolated from these lesions.

[c] Mean diameter of lesions containing the HV, WV and both HV and WV types were not significantly different (LSD = 0.05).

Twenty pseudothecia from field 1 and 12 from field 2 did not react with primers specific to *L. maculans*; these pseudothecia may have been from other fungi or from forms of *L. maculans* which could not react with our primers. *L. maculans* was detected in 0.9% and 1.3% of the seed collected from fields 1 and 2, respectively. Most infected seed (78% and 69% in fields 1 and 2) tested positive for the HV type; the remainder contained the WV type.

Discussion

Our goal was to use specific oligonucleotide primers and PCR to study the occurrence and distribution of *L. maculans* virulence types in two oilseed rape fields. The results show that both virulence types were present and widely distributed throughout each field, but the HV type was much more prevalent. Similar dominance of the HV type has been reported in southern Ontario (Hall et al., 1993), Saskatchewan (Petrie, 1993a) and Australia (Plummer et al., 1994). The HV type colonized leaves, stems, crowns and seed, whereas the WV type was detected only in leaves and seed; this confirms previous reports in Ontario of the presence of only the HV type in stems and crowns (Hall et al., 1993) and the HV and WV types in seed (Chigogora and Hall, 1995). In other studies, the WV type has been isolated from a wide range of sources including leaves (Hammond and Lewis, 1986), stubble (Bonman and Gabrielson, 1981), ascospores (McGee and Petrie, 1979) and seed (Bonman and Gabrielson, 1981). Johnson and Lewis (1994), found minor differences in the appearance of leaf infections by the HV and WV types in the field, but leaf lesions caused by the WV type were usually smaller with less sporulation. We did not find a relationship between lesion size and the virulence type suggesting that the two types cause similar leaf symptoms under the conditions of southern Ontario.

Johnson and Lewis (1994), detected only the HV type in basal stem cankers; however they found internal lesions in the pith from which they commonly isolated the WV type, suggesting that the WV type is able to cause systemic infection even though it is unable to infect the cortex. We did not detect the WV type in the cortical tissues and pith of stem lesions that were

Table 2. Number of stem and crown samples containing the highly virulent type of *Leptosphaeria maculans* in two oilseed rape fields in southern Ontario as determined by the polymerase chain reaction

Sampling[a] site	Field 1						Field 2					
	Stem			Crown			Stem			Crown		
	Lesions[b] tested	HV[c]	Other[d]	Lesions tested	HV	Other	Lesions tested	HV	Other	Lesions tested	HV	Other
1	1	0	5	5	5	0	4	2	3	0	0	5
2	2	2	3	2	2	3	2	0	5	3	3	2
3	3	1	4	3	3	2	5	5	0	3	2	3
4	4	2	3	1	1	4	5	5	0	0	0	5
5	2	0	5	3	0	5	0	0	5	0	0	5
6	1	0	5	3	2	3	5	3	2	0	0	5
7	3	2	3	3	3	2	5	4	1	0	0	5
8	5	5	0	3	3	2	3	3	2	2	2	3
9	5	5	0	2	1	4	5	5	0	0	0	5
10	3	3	2	3	2	3	5	3	2	0	0	5
Total	29	20	30	28	18	32	44	30	20	8	7	43
Incidence (%)		40	60		36	64		60	40		14	86

[a] Five plants were collected from each sampling site and one stem and one crown segment were tested per plant.
[b] Total number of stem or crown lesions tested per sampling site.
[c] Number of lesions containing the highly virulent (HV) type of *L. maculans*.
[d] Stems and crowns which either had no visible lesions or contained lesions in which *L. maculans* was not detected. The WV type was not detected in any of the stems and crowns that were tested.

Table 3. Number of pseudothecia from stubble in two oilseed rape fields in southern Ontario identified as the highly virulent type of *Leptosphaeria maculans* by the polymerase chain reaction

Sampling site[a]	Field 1		Field 2	
	HV[b]	Other[c]	HV	Other
1	3	2	3	2
2	3	2	3	2
3	3	2	4	1
4	5	0	5	0
5	0	5	5	0
6	2	3	4	1
7	3	2	4	1
8	4	1	3	2
9	3	2	4	1
10	4	1	3	2
Total	30	20	38	12
Incidence (%)	60	40	76	24

[a] Five stubble pieces with pseudothecia were collected from each sampling site.
[b] Number of pseudothecia which reacted with *L. maculans* HV-specific primers.
[c] Number of pseudothecia that did not react with *L. maculans* HV and WV type-specific primers.

examined nor in the symptomless stems that we tested. If the WV type can systemically infect the pith, as has been suggested, then our results indicate that this may not be a common event in the field. The inability of the WV type to colonise the stem and crown may indicate a different infection process by this virulence type, further supporting the suggestion that the two types may be different species (Johnson and Lewis, 1994).

Declines in the incidence of the WV type in areas where the HV type has become prevalent have been reported (Humpherson-Jones, 1983; Petrie, 1994), suggesting that the two types are in direct competition. Ascospores are believed to be the major source of inoculum where the disease is already present (Gugel and Petrie, 1992; Hall, 1992), however we did not detect the WV type in any of the pseudothecia tested. Thus, the lack of ascospore production by the WV type may explain the preponderance of the HV type in southern Ontario. Johnson and Lewis (1994) noticed superficial chlorotic lesions on the inflorescence stem and mainly isolated the WV type from these lesions; they postulated that such lesions were capable of producing pseudothecia which may well be sources of

inoculum in natural epidemics. However, there have been no reports of WV-type pseudothecia on inflorescence stems. Conditions required by the two virulence types for sexual reproduction appear to be different (Gabrielson, 1983), and thus environmental conditions of southern Ontario may not be conducive for pseudothecia production by the WV type.

Incidence of seed infection in our study ranged from 0.9% to 1.3%, consistent with reports by Hall et al. (1993), for southern Ontario. The high incidence of both strains in seed may explain their distribution throughout the field. Bonman and Gabrielson (1981) did not observe blackleg lesions on cabbage siliques, yet they yielded infected seed. It is possible that the WV type is causing latent infection and then infecting the seed, but we did not detect either of the virulence types in non-infected plant tissue collected from these fields. However, systemic infection by the WV type has been demonstrated in other studies (Johnson and Lewis, 1994).

We detected both virulence types occurring together in 21% of the leaf lesions examined in the two fields. Coinfection in single lesions by variants of the same pathogen has been reported for other fungi (Adachi and Tsuge, 1994; McDonald and Martinez, 1990). McDonald and Martinez (1990) employed restriction fragment length polymorphism (RFLP) markers to analyze genetic variability of single-pycnidial isolates of *Mycosphaerella graminicola* (Fuckel) J. Schrot in Cohn that were obtained from single lesions of wheat leaves; their data suggested that there was coinfection in wheat lesions by different isolates. Adachi and Tsuge (1994) reported coinfection of 20% of the black spot lesions of pear by non-pathogenic and pathogenic isolates of *Alternaria alternata* (Fr.) Keissl. in the field. Our results with PCR and virulence type-specific primers show that coinfection by the HV and WV types of *L. maculans* might be occurring commonly in the field.

Coinfection of single lesions by different isolates could provide an opportunity for genetic interaction between different genotypes. Taylor and Borgmann (1994) reported the presence of a repeat element (LMR1) of the HV type of *L. maculans* in one WV isolate, and this WV isolate was more pathogenic to oilseed rape than other WV isolates lacking the element; they concluded that the presence of the LMR1 element in this WV isolate may indicate that a rare genetic transfer event had occurred. However, matings in vitro between WV and HV types have failed (Gabrielson, 1983; Williams, 1992), and there is yet no evidence that inter-type mating occurs in nature. The considerable evidence for genetic differences between the HV and WV type, provided by RFLP analysis, random amplified polymorphic DNA (RAPD) analysis, estimates of chromosome number, and nucleotide sequence of 5.8S and internal transcribed spacer regions of ribosomal DNA suggests that the HV and WV types are very distinct and may even be different species (Williams, 1992; Morales et al., 1993; Plummer and Howlett, 1993). If this is true, then transfer of genetic material between the HV and WV types is likely to be extremely rare.

Coinfection may also enable different virulence types to interact physiologically. It is not known whether the WV type establishes in the oilseed rape leaf before, during or after the process of infection by the HV type. We are exploring the coexistence and interaction of the two *L. maculans* types in coinoculated blackleg leaf lesions.

Our study has demonstrated the reliability and usefulness of PCR and virulence type-specific oligonucleotide primers to differentiate HV and WV types of *L. maculans* from the field. Absence of a reliable and rapid diagnostic technique to differentiate the two virulence types has limited studies of the epidemiology of *L. maculans* virulence types, and alternatives such as ELISA and the PCR-based assays have been developed (Johnson and Lewis, 1994; Stace-Smith et al., 1993; Xue et al., 1992). The PCR-based assays are rapid and reliable and can be used to analyze populations of HV and WV types independently and directly in plant tissue. Furthermore, we have shown that coinfection by the HV and WV type is common, raising the possibility that physiological and genetic interactions between the HV and WV types of *L. maculans* occur under field conditions.

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