Pathogenicity Grouping of Isolates of *Leptosphaeria maculans* on *Brassica napus* Cultivars and Their Disease Reaction Profiles on Rapid-Cycling Brassicas

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ABSTRACT

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The pathogenic variability among isolates of *Leptosphaeria maculans* from different geographic regions was tested using 39 isolates from North America, Europe, and Australia against *Brassica napus* cultivars Westar, Quinta, and Glacier. An additional 62 single-ascospore isolates from oilseed rape debris from Saskatchewan and Manitoba, Canada, and from Western Australia and New South Wales, Australia, were also tested to study pathogen variability within locations. Based on the reaction on cotyledons of Westar, Quinta, and Glacier, isolates were categorized into four pathogenicity groups (PG). PG1 isolates were distinguished by the lack of virulence on Westar, Glacier, and Quinta. PG2 isolates were virulent only on Westar but tended to give slightly more susceptible interaction phenotypes on Quinta than on Glacier. PG3 isolates were virulent on both Westar and Glacier and intermediate on Quinta. PG4 isolates were virulent on all three cultivars. All single-ascospore isolates from Canada were classified as PG2, whereas isolates from Australia were more varied in virulence on the differentials and could be classed in groups PG2, PG3, or PG4. When rapid-cycling populations of *B. rapa*, *B. nigra*, *B. oleracea*, *B. juncea*, *B. napus*, *B. carinata*, and *Raphanus sativus* were tested with isolates characteristic of PG1, PG2, PG3, and PG4, further discrimination among PG groups was not observed. The virulence profiles within each of the rapid-cycling cruciferous species indicated plant-to-plant variation for interaction phenotype within populations of *B. rapa*, *B. oleracea*, *B. napus*, and *R. sativus*. Through selection within the rapid-cycling base populations of these species, it may be possible to obtain inbreds with more precisely defined interaction phenotypes to specific pathogenicity groups of *L. maculans*.

The introduction of *B. napus* L. var. *oleifera* (Metzger) Sink (oilseed rape) cultivars resistant to *Leptosphaeria maculans* (Desmaz.) Ces. & De Not. (anamorph = *Phoma lingam* (Tode:Fr.) Desmaz.), the cause of blackleg disease of crucifers, has reduced disease incidence, thus allowing the continued production of intensively grown rape in several European countries. As crucifer cultivars have been grown more intensively and more widely, differences in the pathogenic and ecological adaptation of the blackleg fungus have become apparent. An understanding of the virulence structure of the pathogen population will be of value in developing effective strategies for breeding for resistance to this disease.

Variation in pathogenicity of *L. maculans* has been reported from Australia (15), Canada (9), England (4), and the United States (1,11), but the practical implications of these observations are not clear. In Australia, Thurling and Venn (15) found significant interactions among three populations of *L. maculans* from rape stubble and cultivars of *B. napus* (rape) and *B. rapa* L. (syn. *B. campestris* L.) (turnip rape) and concluded that isolates of *L. maculans* from various sites differed in pathogenicity. Those isolates that were virulent were considered to be responsible for extensive crop losses in Australia.

Prior to 1975, only less virulent isolates of *L. maculans* producing superficial stem lesions had been isolated from rape and turnip rape in Canada (9,14). However, virulent isolates that cause severe stem cankers have since become widely established (6,14,16). In the United States, virulent isolates of *L. maculans* have been isolated from cabbage (1) and canola, *B. napus* (11). In England, virulent and avirulent isolates have been isolated from seed crops of various *Brassica* species (4). Differences in cultural characteristics (3), sirodesmin production (7), mating behavior (10), and restriction fragment length polymorphisms (8) have shown that avirulent isolates are distinctly different.

Although the existence of virulent and avirulent isolates of *L. maculans* has been extensively documented (2,5,7,13,14), knowledge regarding variability among aggressive isolates is fragmentary because differential hosts were not available. Koch et al (8) evaluated 20 oilseed rape cultivars representing winter and spring types against 39 *L. maculans* isolates and found Westar, Quinta, and Glacier to be differential hosts for these isolates. The use of these cultivars becomes useful for a better understanding of the host-pathogen interaction between cruciferous species and *L. maculans*.

The purpose of this study, therefore, was to evaluate the range of variability in virulence among isolates of *L. maculans* using three differential cultivars of *B. napus* and to evaluate the interaction phenotype profiles within populations of the model rapid-cycling *Brassica* species and *Raphanus sativus* L. (17).

MATERIALS AND METHODS

Pathogen collection and storage. Isolates of *L. maculans* from different geographic regions reported to vary in pathogenicity were obtained either from the *L. maculans* collection of the Crucifer Genetics Cooperative (CrGC) (Department of Plant Pathology, University of Wisconsin-Madison, Madison, WI) or from a number of individuals in Australia and North America. Single-ascospore isolates from two sites in Australia (Mt. Barker, Western Australia [WA] and Wagga Wagga, New South Wales [NSW]) and two in Canada (Melfort, Saskatchewan [SA] and Elgin, Manitoba [MA]) were obtained from infected oilseed rape residues collected in 1987.

Small pieces of diseased stems were immersed in water for 30–60 s to stimulate ascospore discharge onto water agar. The inside of the petri dish lids were covered with petroleum jelly and the pieces of debris were attached. After spore discharge onto the water agar, single ascospores were transferred with the aid of a stereo microscope (×50) to plates containing V8 juice agar (12). After 6 days of incubation at 24 C under continuous cool-white fluorescent light (100–150 $\mu E \cdot m^{-2} \cdot s^{-1}$), pathogen identity was confirmed by pycnidium morphology and pycnidiospore size and shape. Inoculum of *L. maculans* was produced on V8 agar under similar conditions. A 1 × 3 cm rectangle, aseptically cut from an actively sporulating culture on V8 agar, was transferred to 10 ml of sterile distilled water in a test tube and the tube

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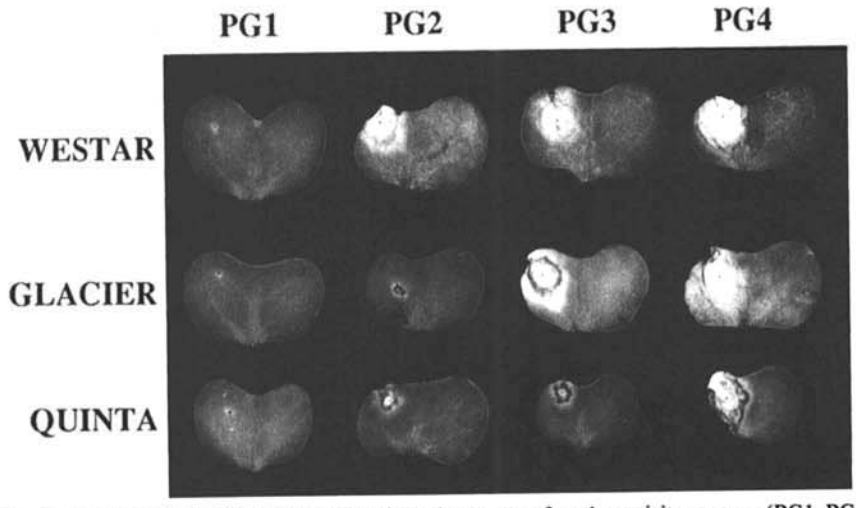

Fig. 1. Symptoms of differential interaction phenotype of pathogenicity groups (PG1–PG4) of *Leptosphaeria maculans* on cotyledons of three *Brassica napus* cultivars. Wester had an IP = 0, 7–9, 7–9, and 7–9; Glacier had an IP = 0, 0–2, 7–9, and 7-9; and Quinta had an IP = 0, 3–6, 3–6, and 7–9 with PG1, PG2, PG3, and PG4, respectively.

was agitated vigorously until the resulting suspension of pycnidiospores became turbid. The spore suspension (0.5 ml) was spread over the agar surface and plates were incubated at 24 C under continuous fluorescent light. In 4–5 days, they developed a dense lawn of mature pycnidia with very little aerial mycelium.

To collect the pycnidiospores for inoculum, the cultures were flooded with 10 ml of sterile distilled water, the surface was gently rubbed with a bent glass rod, and the pycnidiospores were removed with a pipette (17). The spore concentration was adjusted to 1.5×10^7 spores ml^{-1}, transferred to 1.5-ml polyethylene microcentrifuge capsules, frozen, and kept at −20 C until required. Spores have been stored frozen for 6 mo without loss in viability. To reduce variation among experiments and eliminate subculturing, the pathogen was inoculated directly from frozen stock each time it was used.

Table 1. Classification of isolates of *Leptosphaeria maculans* from different continents into pathogenicity groups based on virulence on *Brassica napus* cultivars Westar, Quinta, and Glacier

Origin	Total number of isolates	Number of isolates in pathogenicity group (PG)[a]			
		1	2	3	4
Australia	10	5	1	1	3
Europe	18	2	3	7	6
North America	9	4	3	1	1
Africa	2	1	1	0	0

[a]PG1 isolates were avirulent on Westar, Quinta, and Glacier; PG2 isolates were virulent on Westar (IP 7–9), intermediate on Quinta (IP 3–6), and less virulent on Glacier (IP 0–2); PG3 isolates were virulent on Westar and Glacier (IP 7–9) and had intermediate virulence on Quinta (IP 3–6); and PG4 isolates were virulent on Westar, Quinta, and Glacier (IP 7–9).

Inoculation and evaluation. Seeds of *B. n. oleifera* cvs. Westar (spring rape), Glacier (winter rape), and Quinta (winter rape); rapid-cycling *Brassica* spp. *B. rapa* (CrGC 1-1), *B. nigra* (L.) W. Koch (CrGC 2-1), *B. oleracea* L. (CrGC 3-1), *B. juncea* (L.) Czernj. & Coss. (CrGC 4-1), *B. napus* (CrGC 5-1 and CrGC 5-2), and *B. carinata* A. Braun (CrGC 6-1); and rapid-cycling *R. sativus* (CrGC 7-1), obtained from the Crucifer Genetics Cooperative, were sown in plastic flats fitted with 96 celled packs filled with peat lite, Jiffy-mix. Seedlings were maintained in a growth chamber at 24 C and 90% RH, with continuous illumination at a photon flux density of 250 $\mu E \cdot m^{-2} \cdot s^{-1}$ from Sylvania cool-white fluorescent bulbs. Seven days after sowing, seedlings were wounded in the center of each cotyledon by puncturing with a coverslip forceps and inoculated with a 10-μl droplet of inoculum (1.5×10^7 spores ml^{-1}), which was placed over the wound on each cotyledon of each seedling.

Inoculated seedlings were returned to the growth chamber. Developing primary leaves were removed every 2 or 3 days by pinching the growing tip of the seedlings. This ensured that cotyledons continued to expand and remain green until evaluation for interaction phenotypes between *Brassica* and *L. maculans* were made. Unpinched plants reacted in the same way except that the cotyledons turned yellow and dropped 10 days after seeding when the first true leaves appeared. Interaction phenotypes (IP) were scored 10 days after inoculation using a scale of 0–9 where 0 = no visible symptoms and 9 = collapse of tissue with abundant pycnidial formation around the inoculated area (17). An IP of 0–2 was considered resistant and an IP of 7–9 was considered susceptible.

RESULTS

Pathogenicity studies. Evaluation of a number of isolates of *L. maculans* revealed that most isolates were capable of causing severe infection and symptoms on the cultivar Westar. Avirulent isolates were distinguished from virulent isolates on Westar, which was susceptible to all virulent isolates (Fig. 1). Glacier and Quinta were susceptible (IP 7-9) to some virulent isolates but resistant (IP 0–2) to others. Some of the isolates that were virulent on Glacier were less virulent on Quinta (IP 3–6). Some isolates were virulent on both Quinta and Glacier. Thus, the *L. maculans* isolates could be classified into four groups using these three *B. napus* cultivars (Fig. 1). We propose these groups be designated as pathogenicity groups PG1–PG4.

PG1 isolates were equivalent to avirulent isolates as previously reported (3) and were avirulent on Westar. Isolates of *L. maculans* reported in the literature as aggressive or virulent (5,7,9) can be subdivided into three groups—PG2, PG3, and PG4. PG2 isolates were virulent on Westar and avirulent (IP 0–2) on Glacier. PG3 isolates were virulent

Table 2. Classification of single-ascospore isolates[a] of *Leptosphaeria maculans* from Australia and Canada into pathogenicity groups based on virulence on *Brassica napus* cultivars Westar, Quinta, and Glacier

Origin	Total number of isolates	Number of isolates in pathogenicity group (PG)[b]			
		1	2	3	4
Australia					
Western Australia	19	0	4	13	2
New South Wales	15	0	0	2	13
Canada					
Manitoba	15	0	15	0	0
Saskatchewan	13	0	13	0	0

[a]Isolates derived from oilseed rape debris from four locations—Mount Barker, Western Australia; Wagga Wagga, New South Wales; Elgin, Manitoba; and Melfort, Saskatchewan. Isolates tested for virulence were selected at random from larger samples obtained from various pieces of debris.

[b]PG1 isolates were avirulent on Westar, Quinta, and Glacier; PG2 isolates were virulent on Westar (IP 7–9), intermediate on Quinta (IP 3–6), and less virulent on Glacier (IP 0–2); PG3 isolates were virulent on Westar and Glacier (IP 7–9) and had intermediate virulence on Quinta (IP 3–6); and PG4 isolates were virulent on Westar, Quinta, and Glacier (IP 7–9).

(IP 7–9) on both Westar and Glacier, but when inoculated on Quinta, nonsporulating lesions on the cotyledons with an intermediate IP, 3–6, were produced 10 days after inoculation. The IPs of both PG2 and PG3 isolates on Quinta were intermediate (IP 3–6), but high IPs were obtained when PG4 was inoculated on this host. Thus, PG4 isolates were virulent on all three cultivars.

The geographic distribution of the isolates classified into pathogenicity groups is given in Table 1. Isolates representing each PG could be found in Australia, Europe, and North America. When single-ascospore isolates from Canada and Australia were tested for pathogenic variability on cotyledons of the three differential *B. napus* cultivars, only slight variations in PGs were observed within three of the four sites tested (Table 2). Ascospore isolates from Manitoba and Saskatchewan, Canada, were all PG2 and isolates from New South Wales, Australia, were all PG4, except two isolates that were PG3. Western Australian isolates were the most variable, with four PG2 isolates, 13 PG3 isolates, and two PG4 isolates. No avirulent (PG1) ascospore isolates were obtained.

When isolates of each of the four PGs of *L. maculans* were inoculated on the cotyledons of rapid-cycling crucifers, *B. nigra* (CrGC 2-1), *B. juncea* (CrGC 4-1), and *B. carinata* (CrGC 6-1) were resistant, whereas the rapid-cycling *B. rapa* (CrGC 1-1), *B. oleracea* (CrGC 3-1), *B. napus* (CrGC 5-1 and CrGC 5-2), and *R. sativus* (CrGC 7-1) had high frequencies of plants susceptible to PG2, PG3, and PG4 (Fig. 2A–G). The PG1 isolate was avirulent on all of the species tested. There were, however, 10–20% of plants with IPs in the range of 3–5 for PG2 and PG3 but not for PG4. The greatest variation in IP to PG2 and PG3 isolates occurred in the *B. napus* (CrGC 5-1 and CrGC 5-2) populations. The rapid-cycling population of *B. napus* (CrGC 5-2) had 2–3% of the plants with an IP of 3–5 for PG4. *B. nigra* (CrGC 2-1), *B. juncea* (CrGC 4-1), and *B. carinata* (CrGC 6-1) had a high percentage of resistant plants. The inoculation was performed twice with similar results.

DISCUSSION

Isolates of *L. maculans* from the United States, Europe, Australia, and Canada were differentiated into four pathogenicity groups based on the IPs obtained on cotyledons of *B. napus* cvs. Westar, Quinta, and Glacier. Because of the small sample size and the nonrandom collection of these isolates, no conclusions about relative frequencies of PG types can be drawn. One PG4 isolate was obtained from Canada, but none were obtained from collections of oilseed rape residues. It seems likely, then, that PG4 isolates are infrequent in the Canadian populations, the dominating pathotype being PG2. The large percentage of the oilseed rape hectarage in Canada planted to the susceptible Westar in recent years may account for the predominance of PG2 isolates. In Australia, where little oilseed rape is currently grown because of the severity of blackleg in the 1970s, most of the isolates were PG4. The collections of oilseed rape debris were taken from plant breeders' disease nurseries where the major emphasis in plant breeding has been to select resistance to blackleg disease. It may be that after years of resistance breeding, selection for more virulent pathotypes has occurred. Alternatively, there may be more natural variation in virulence in Australian populations than in Canadian populations of *L. maculans*, as it is only in recent years that more aggressive isolates of *L. maculans* have been observed in Canada (13).

The range of IPs elicited within each of the rapid-cycling species base populations for each PG group suggested that within the rapid-cycling crucifers, there is considerable potential for the development of lines capable of differentiating a wide range of virulence in *L. maculans*. Although the isolates studied were all originally isolated from *B. napus* stubble, PG2, PG3, and PG4 were capable of

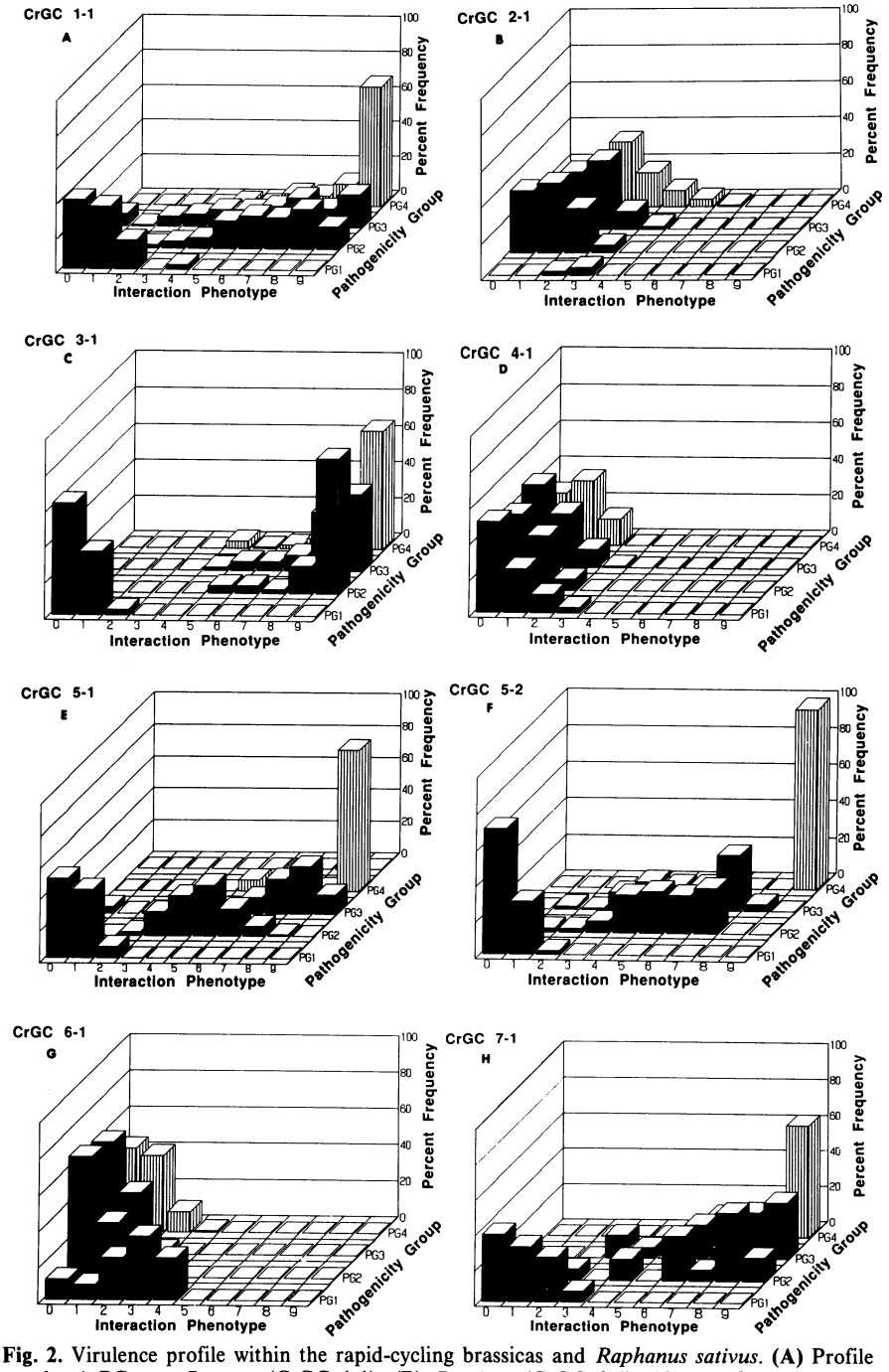

Fig. 2. Virulence profile within the rapid-cycling brassicas and *Raphanus sativus*. **(A)** Profile on the 4 PGs on *B. rapa* (CrGC 1-1); **(B)** *B. nigra* (CrGC 2-1); **(C)** *B. oleracea* (CrGC 3-1); **(D)** *B. juncea* (CrGC 4-1); **(E)** *B. napus* (CrGC 5-1); **(F)** *B. napus* (CrGC 5-2, dwarf); **(G)** *B. carinata* (CrGC 6-1); and **(H)** *R. sativus* (CrGC 7-1).

causing severe symptoms on some plants of *B. rapa*, *B. oleracea*, and *R. sativus*. As is characteristic of most *L. maculans* isolates from *Brassica* species, disease severity was substantially reduced on *B. nigra*, *B. juncea*, and *B. carinata*. As might be expected, resistance to PG4 isolates was expressed only within the three *Brassica* species having the *B* genome (*B. nigra*, *B. juncea*, and *B. carinata*). In this study, we have chosen deliberately not to use the term race, preferring the broader term of pathogenicity group, PG, until a better understanding of the genetic basis for pathogenicity, pathogenic variability, and host-pathogen interaction is understood.

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