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A Simple, Sensitive, and Rapid Method for Detecting Seed Contaminated with Highly Virulent *Leptosphaeria maculans*

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A primer-directed DNA amplification polymerase chain reaction assay for detection of seed contaminated with highly virulent *Leptosphaeria maculans* was developed. The primers were derived from a 5,238-bp repetitive sequence present only in the highly virulent isolates of the fungus. A procedure for isolating DNA from organisms infesting germinating seed was also developed. Seeds were added to liquid fungal minimal medium, and the culture was incubated for 3 days at room temperature with shaking. The organisms were collected from the cultures by centrifugation and lysed with a combination of sodium dodecyl sulfate and proteinase K. The DNA was extracted with organic solvents and with a high-salt–cetyltrimethylammonium bromide solution. It was also precipitated with a low-salt–cetyltrimethylammonium bromide solution. The extensive treatments used for minimizing polysaccharide contamination greatly improved the reliability of the assay. The minimum contamination level (2 of 1,000 seeds) that was tested was successfully detected with this DNA isolation procedure. The reliability of the assay was 96% at the 1 to 2% level of seed contamination. The described method is less laborious and requires only 4 to 5 days for completion in comparison to the 11 to 22 days required for the currently employed methods. In addition, large sample sizes can be easily handled, thus reducing the probability of contaminated seed escaping detection.

The introduction of primer-directed amplification of DNA with a thermostable DNA polymerase facilitated the analysis of genomes from limited quantities of material (16). Since that time, a large number of research groups have combined the use of specific oligonucleotide primers and DNA amplification to detect the presence of particular DNA sequences in a background of unrelated sequences. These diagnostic tests are useful not only in the medical and forensic areas but also in the food and agricultural industries. For example, unique primers that amplify a portion of the toxin genes of *Staphylococcus aureus* are used to detect this organism in food samples (24). The presence of the phytopathogenic fungi *Gaeumannomyces graminis* and *Phoma tracheiphila* can be detected in infected plants by amplification with primers derived from mitochondrial and repetitive DNAs, respectively (15, 19).

Blackleg of oilseed rape and other crucifers causes extensive yield losses worldwide (2). The disease is caused by *Leptosphaeria maculans* (Desm.) Ces. et de Not. [anamorph, *Phoma lingam* (Tode: Fr.) Desm.], a heterothallic ascomycete. The fungus can be spread by infested brassica crop residue, brassica seed, and other cruciferous plants and their residues (4). Wood and Barbetti proposed that contaminated seed is responsible for the introduction of *L. maculans* into an area and could produce an epidemic under favorable environmental conditions (25). The *L. maculans* isolates from brassica plants are divided into two pathogenicity groups, highly virulent (aggressive) and weakly virulent (nonaggressive) (9). The weakly virulent isolates cause only mild disease symptoms with no subsequent yield loss, and their presence in seed is not a matter of serious concern (22). The highly virulent strain of *L. maculans* was first detected in western Canada in three widely separated fields in 1975 (2). This wide spatial separation indicated that the diseased plants arose from contaminated seed. Since that time, the disease has spread over virtually all of western Canada. The Peace River region of Alberta and British Columbia is one of the few remaining blackleg-free, rapeseed-growing areas. It is important to prevent the introduction of the disease into this area since rapeseed varieties with good field resistance to blackleg and suitability to Canadian growing conditions are not yet available.

The International Seed Testing Association recommends the 2,4-dichlorophenoxyacetic acid blotter method for detecting *L. maculans* contamination in crucifer seed (8). This method can be used to distinguish *L. maculans* from saprophytic species of *Phoma*. However, it does not differentiate between the highly and weakly virulent isolates. The methods currently employed for differentiating between these groups require individual plating of seed onto fungal medium, induction of fungal sporulation, and isolation of pycnidia from *L. maculans* mycelia. The isolates are subsequently tested for (i) growth rate on potato dextrose agar (weakly virulent isolates grow faster), (ii) pigment production on modified Czapek medium (weakly virulent isolates produce pigment; highly virulent ones do not), (iii) conidial germination (weakly virulent isolates produce longer germ tubes in the first 40 to 44 h of growth) (14), and (iv) pathogenicity on cotyledons (6). Only small sample sizes can be practically handled in these tests, which are both time-consuming and laborious. I have used a repetitive sequence specific to highly virulent isolates of *L. maculans* (21) to develop a polymerase chain reaction (PCR)-based test for seed contamination that surmounts these difficulties (20).

MATERIALS AND METHODS

Fungal isolate and *Brassica napus* seed. The highly virulent *L. maculans* isolate Leroy was obtained from R. K. Gugel (Agriculture Canada Research Station, Saskatoon, Saskatchewan, Canada). The isolate was cultured on V8 juice agar (3). For DNA isolation, plugs from plates were transferred to liquid minimal medium and grown for 1 week at room temperature with shaking. The minimal medium con-

sisted of the following, per liter: 3.12 g of KNO_3, 0.75 g of K_2HPO_4, 0.75 g of KH_2PO_4, 0.1 g of NaCl, 0.28 g of asparagine, 0.1 g of $CaCl_2 \cdot 2H_2O$, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.4 mg of $ZnSO_4 \cdot 7H_2O$, 79 μg of $CuSO_4 \cdot 5H_2O$, 41 μg of $MnSO_4 \cdot 4H_2O$, 18 μg of MoO_3 (85%), 0.5 mg of $FeC_6H_5O_7$, 38 μg of $Na_2B_4O_7 \cdot 10H_2O$, 0.1 mg of thiamine, and 15 g of glucose; pH adjusted to 6.55 (23).

Two different seed lots of the *B. napus* variety Tristar were obtained from R. K. Gugel. Both seed lots were tested for blackleg contamination by the currently available methods (6). One lot grown in California was found to be free of blackleg contamination, and the second lot grown in Saskatchewan was found to contain 1 to 2% contaminated seed.

DNA isolation. The mycelium of the fungal isolate was collected by filtration, freeze-dried, and vortexed in the presence of glass beads. The DNA was extracted from the resulting powder by using a method described by Murray and Thompson (13).

DNA from organisms infesting the *B. napus* seed was isolated in the following manner. The seed was surface disinfested by soaking in 1.0% (vol/vol) NaOCl (6.0% available chlorine) for 15 min and rinsed in sterile deionized H_2O. This surface disinfestation duplicates the results of seed treatments administered before planting. Two grams of seed was added to 50 ml of minimal medium (23), and the mixture was cultured for 72 h at 26 ± 2°C with shaking. The culture was poured through two layers of sterile cheesecloth into tubes and centrifuged at 2,500 × *g* for 10 min. The pellets were washed twice with sterile H_2O, transferred to microcentrifuge tubes, and dried overnight in a speed-vac (Savant Instruments, Farmingdale, N.Y.). These pellets were resuspended in a buffer containing 10 mM Tris-HCl (pH 7.8), 5 mM EDTA, and 0.5% (wt/vol) sodium dodecyl sulfate. Proteinase K (20 mg/ml; Sigma) was added to a final concentration of 100 μg/ml, and the samples were incubated at 56°C for 4 h. The samples were then extracted sequentially with phenol, phenol-chloroform, and chloroform. The nucleic acids were precipitated by adding an equal volume of 50 mM Tris-HCl (pH 8.0)–10 mM EDTA–1.0% (wt/vol) cetyltrimethylammonium bromide (CTAB) and then immediately centrifuged at 12,000 × *g* for 30 min. The samples were dried and dissolved in 1.2 M NaCl, and CTAB (10%, wt/vol) was added to a final concentration of 1.0%. This solution was incubated at 65°C for 1 h, extracted once with chloroform, and ethanol precipitated. The pellets were dissolved in Tris-EDTA and treated with RNase A (50 μg/ml) and RNase T1 (200 U/ml) at 37°C for 1 h. A more accurate determination of the DNA content of the samples could be made following the RNase treatment. The DNA was extracted once with phenol-chloroform and precipitated with ethanol. The pellet was dissolved in Tris-EDTA, and the DNA concentration was measured by A_{260}.

DNA amplification conditions. Five nanograms of purified fungal DNA (except as stated in Results) or 150 ng of seed culture DNA was added to each amplification reaction. The reactions contained 200 μM deoxynucleoside triphosphates, 165 nM each primer, 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 5 mM $MgSO_4$, 0.1% (vol/vol) Triton X-100, 27.5 μM tetramethylammonium chloride, and 2.5 U of *Taq* polymerase (Life Technologies). The amplifications were performed in a Barnstead Thermolyne thermal cycler, using the following program: (i) 96°C dwell for 2 min; (ii) 35 ramped cycles consisting of 94°C for 30 s, 71°C for 30 s, and 72°C for 4 min; and (iii) final extension at 72°C for 7 min.

The primers used in the amplification reactions were derived from the sequence of LMR1 (GenBank accession M77515), a repetitive element found only in highly virulent isolates of *L. maculans* (21). The Primer Designer, version 1.0, program from Scientific & Educational Software was used to select the optimal primers from the sequence. The primers were each 24 nucleotides in length, had a minimum G+C content of 45% and a minimum melting temperature of 67°C, and were chosen to amplify fragments of approximately 1.0 kb or less. The sequence of the primers, their initial positions (in base pairs) in the LMR1 sequence, and the size of the expected amplification products are given in Table 1. The PCR products were separated by gel electrophoresis in 0.8% agarose–1× TAE gels (17) and stained with ethidium bromide for photography.

TABLE 1. Description of primer sets used for PCR amplification of *L. maculans* DNA

Set	Sequence	LMR1 position (bp)	Expected fragment size (bp)
A	5′-GCGCTATTACACATGCCTAACAGG-3′	881	1,145
	5′-TCCTCTATGCTAAGCTAGCTGTGC-3′	2026C	
B	5′-TACTAGGAGGCTCTATAAGTGCGG-3′	2382	1,168
	5′-AAGGTATTAGGAGAGCTAGGAGGC-3′	3550C	
C	5′-GCCTCCTAGCTCTCCTAATACCTT-3′	3527	1,010
	5′-CTAGCAAGGAAGTAGGCAGGTAAG-3′	4537C	
D	5′-GCGTAAGAAGCGTGCCTTAGAGTC-3′	4259	580
	5′-TCCTGCTCCTACTCCTTCTCTAGC-3′	4839C	
E	5′-GGTAGAGCTAGAGGAGGTAGATAA-3′	1917	486
	5′-GCACTTATAGAGCCTCCTAGTAGT-3′	2403C	

RESULTS AND DISCUSSION

In recent years, several groups have demonstrated that highly and weakly virulent isolates of *L. maculans* can be distinguished on the bases of DNA polymorphisms (5, 7), amplification products generated by random amplified polymorphic DNA primers (1, 18), fingerprinting with oligonucleotide probes (10), rDNA sequence (11), and the presence of a particular repetitive DNA sequence (12, 21). These accumulated data indicated that it should be possible to develop a PCR-based assay for seed contaminated with the highly virulent isolates of the fungus.

The LMR1 element is a 5,238-bp, moderately repetitive (80 copies per genome) DNA fragment cloned from the *L. maculans* highly virulent isolate Leroy. The element hybridizes, under stringent conditions, to the DNA of every highly virulent isolate examined but not to DNA of any weakly virulent isolates (21). Its sequence provided a large resource for specific primer selection, and its comparatively high copy number per genome increased the probability of obtaining a detectable amplification product from a small amount of input DNA.

Amplification of Leroy DNA. The five sets of primers derived from the LMR1 sequence were tested for the ability to amplify fragments of the expected size (Table 1) from DNA of the Leroy isolate. The amplification products, shown in Fig. 1, were the expected size. However, the products from primer sets B and C were only faintly visible on gels and are not readily apparent in Fig. 1. These results may be due to sequence polymorphisms in the copies of the

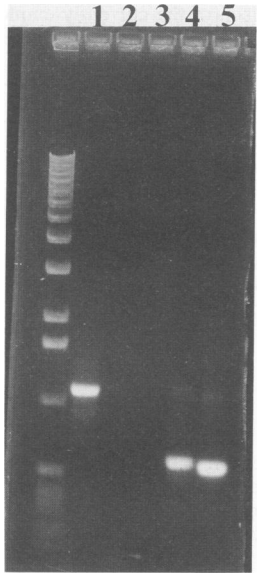

FIG. 1. PCR products from reactions containing 5.0 ng of DNA from the virulent isolate Leroy amplified with five different sets of primers derived from the sequence of the *L. maculans* repetitive element LMR1. The sequence of the primer sets, their base pair positions within the LMR1 sequence, and the expected sizes of the products are given in Table 1. The size of the major amplification product is stated beside the set designation (lanes, left to right): 1-kb ladder; (1) set A, 1,145 bp; (2) set B, 1,168 bp; (3) set C, 1,010 bp; (4) set D, 580 bp; (5) set E, 486 bp.

element or an unusually recalcitrant secondary structure which prevented primer binding.

Primer set D was chosen for use in the development of the seed contamination test. The use of this primer set in the reactions consistently produced the largest amount of product from DNA from the highly virulent isolates tested (data not shown).

Minimum detection level of target DNA. Amplification reactions were carried out with decreasing amounts of Leroy DNA to determine the minimum amount of input DNA required to produce a detectable product. The smallest amount of input DNA that led to a visible product was 10^{-13} g (Fig. 2). This represents the amount of LMR1 DNA present in approximately four nuclei on the basis of an

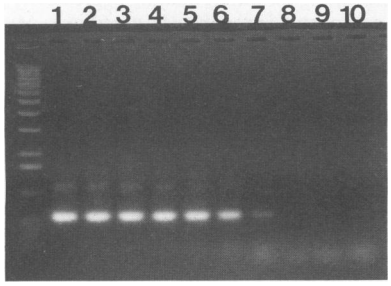

FIG. 2. Determination of the minimum amount of DNA from the virulent isolate Leroy that produces a visible product when amplified with primer set D. The amount of DNA added to each reaction was as follows (lanes, left to right): 1-kb ladder; (1) 5.0 ng; (2) 2.5 ng; (3) 1.0 ng; (4) 100 pg; (5) 10 pg; (6) 1.0 pg; (7) 100 fg; (8) 10 fg; (9) 1.0 fg; (10) 0.

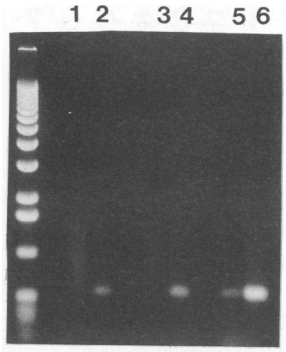

FIG. 3. Effects of seed culture time, culture medium, and DNA isolation procedure on amount of amplification product. Details are given in the text. Lanes, left to right: 1-kb ladder; (1) 48 h; (2) 72 h; (3) potato dextrose broth; (4) minimal medium; (5) ethanol used in the initial DNA precipitation; (6) CTAB used in the initial DNA precipitation.

average genome size of 27.6 megabases for a highly virulent isolate of *L. maculans* (12).

Factors affecting detection of contaminated seed. The following three components of sample preparation for amplification reactions were consistently found to affect the outcome: seed incubation time, culture medium, and DNA isolation procedure. Examples of the effects of these various factors on amplification are shown in Fig. 3. All cultures contained 2.0 g of seed from the 1 to 2% contaminated lot. The DNA added to the reactions shown in the first two lanes was isolated from cultures after either 48 or 72 h of incubation. A product was observed only in the reaction that contained DNA isolated after 72 h of incubation. The DNA added to the reactions shown in the next two lanes was isolated from either a potato dextrose broth or minimal medium culture. Only the DNA from the minimal medium culture gave a visible product. Inspection of the potato dextrose broth culture by light microscopy indicated that bacteria were the predominant organisms present in this medium, whereas in the minimal medium, which contained primarily nitrate as nitrogen source, the bacterial growth rate was considerably slowed. The inclusion of 100 μg of streptomycin sulfate per ml in this medium did not affect the results significantly. The target fungus was always detected in the minimal medium-grown cultures of contaminated seed. The DNA added to the reactions shown in the last two lanes of Fig. 3 was initially precipitated with either ethanol or CTAB. A greater amount of the amplification product resulted from the use of CTAB-precipitated DNA in the reaction.

Sensitivity of the assay for seed contamination. Various amounts of seeds from the contaminated lot were mixed with uncontaminated seed to determine the sensitivity of the assay. A total of 2.0 g of seeds was cultured for each sample; this sample size is roughly equivalent to 1,000 seeds. The approximate maximum numbers of contaminated seeds present in each culture were the following: 0, 2, 4, 6, 8, 10, 16, and 20. The results of the amplification of DNA isolated from a typical set of these cultures are shown in Fig. 4. A band of the expected size was present in all reactions that contained contaminated seeds. However, the reactions that contained DNA isolated from the 4/1,000 and the 8/1,000 contaminated seed cultures had only faintly visible bands that are not readily apparent in Fig. 4. In addition, the bands

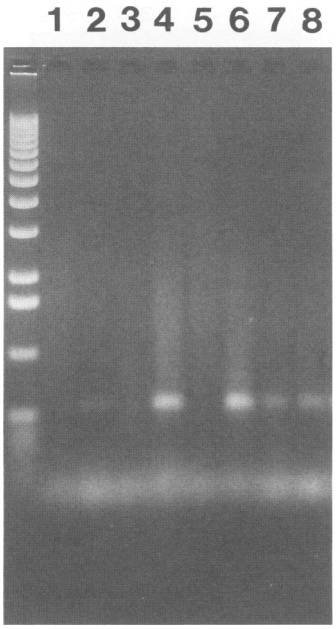

FIG. 4. Assessment of the detection levels of the PCR-based seed contamination test. The amount of the contaminated seed lot added to the uncontaminated lot and the estimated maximum number of contaminated seed present were as follows (lanes, left to right): 1-kb ladder; (1) 0 g, 0; (2) 0.1 g, 2; (3) 0.25 g, 4; (4) 0.5 g, 6; (5) 0.75 g, 8; (6) 1.0 g, 10; (7) 1.5 g, 16; (8) 2.0 g, 20.

seen in the reactions containing DNA from the 16/1,000 and 20/1,000 contaminated cultures were less intense than those containing DNA from the 6/1,000 and 10/1,000 cultures.

This sample-to-sample variation was consistently found in different sets of sample preparations. There was no apparent correlation between the amount of amplification product and the number of contaminated seeds present in the sample. A decrease in band intensity was also observed when the same DNA sample was used in amplifications over several successive days with interim storage at −20°C. Subsequently, it was found that an extraction of the DNA with a high-salt–CTAB solution alleviated the sample variation to a large extent (data not shown). Therefore, the probable explanation for the variation in product amounts is the presence of polysaccharides that complexed with the DNA and inhibited amplification. The inclusion of the CTAB extraction step raised the reliability of the assay from 73 to 96% at the 1 to 2% level of seed contamination. The sampling errors introduced by mixing seed from the contaminated lot with noncontaminated seed precluded determining the reliability of the assay at the lower contamination levels.

Advantages of the PCR seed contamination test. The detection method that was developed requires less than one-half the time of the current methods (Table 2) and does not require the plating of individual seed (20). Thus, it can be used to screen both greater numbers and larger sample sizes with high reliability. An additional advantage of this assay is that the outcome is determined by the presence, not the size, of an amplification product. Therefore, a fluorescein-antifluorescein-based enzyme-linked oligonucleotide sorbent assay can be developed to detect the amplification product in microtiter plates, thus alleviating the need for gel electrophoresis.

TABLE 2. Comparison of the currently employed plating method and the described PCR method for detection of seed contaminated with highly virulent *L. maculans*

Plating method	PCR method
Surface disinfest seed	Yes
Plate individual seed	No
Allow fungi to grow from seed, 72 h	Yes, 72 h
Transfer and induce fungal sporulation, 72–96 h	No
Isolate spores and culture, 48–72 h	No
Determine virulence (high or weak) of *L. maculans* isolates, 2–10 days	1.5 days
Total time = 11–22 days	4.5 days

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REFERENCES

1. **Goodwin, P. H., and S. L. Annis.** 1991. Rapid identification of genetic variation and pathotype of *Leptosphaeria maculans* by random amplified polymorphic DNA assay. Appl. Environ. Microbiol. **57:**2482–2486.
2. **Gugel, R. K., and G. A. Petrie.** 1992. History, occurrence, impact, and control of blackleg of rapeseed. Can. J. Plant Pathol. **14:**36–45.
3. **Gugel, R. K., G. Séguin-Swartz, and G. A. Petrie.** 1990. Pathogenicity of three isolates of *Leptosphaeria maculans* on *Brassica* species and other crucifers. Can. J. Plant Pathol. **12:**75–82.
4. **Hall, R.** 1992. Epidemiology of blackleg of oilseed rape. Can. J. Plant Pathol. **14:**46–55.
5. **Johnson, R. D., and B. G. Lewis.** 1990. DNA polymorphism in *Leptosphaeria maculans*. Physiol. Mol. Plant Pathol. **37:**417–424.
6. **Kharbanda, P. D.** 1989. Seed testing for blackleg of canola. Alberta Environmental Centre, Vegreville, Alberta, Canada.
7. **Koch, E., K. Song, T. C. Osborn, and P. H. Williams.** 1991. Relationship between pathogenicity and phylogeny based on restriction fragment length polymorphism in *Leptosphaeria maculans*. Mol. Plant Microbe Interact. **4:**341–349.
8. **Maguire, J. D., R. L. Gabrielson, M. W. Mulanax, and T. S. Russell.** 1978. Factors affecting the sensitivity of 2,4-D assays of crucifer seed for *Phoma lingam*. Seed Sci. Technol. **6:**915–924.
9. **McGee, D. C., and G. A. Petrie.** 1978. Variability of *Leptosphaeria maculans* in relation to blackleg of oilseed rape. Phytopathology **68:**625–630.
10. **Meyer, W., E. Lieckfeldt, J. Wöstemeyer, and T. Börner.** 1992. DNA fingerprinting for differentiating aggressivity groups of the rape seed pathogen *Leptosphaeria maculans*. Mycol. Res. **96:**651–657.
11. **Morales, V. M., L. E. Pelcher, and J. L. Taylor.** 1993. Comparison of the 5.8s rDNA and internal transcribed spacer sequences of isolates of *Leptosphaeria maculans* from different pathogenicity groups. Curr. Genet. **23:**490–495.
12. **Morales, V. M., G. Séguin-Swartz, and J. L. Taylor.** 1993. Chromosome size polymorphism in *Leptosphaeria maculans*. Phytopathology **83:**503–509.
13. **Murray, M. G., and W. F. Thompson.** 1980. Rapid isolation of high molecular weight plant DNA. Nucleic Acids Res. **8:**4321–4325.
14. **Petrie, G. A.** 1988. The rapid differentiation of virulent and weakly virulent strains of *Leptosphaeria maculans* (blackleg or stem canker) and related pycnidial fungi from *Brassica* seeds and stems. Can. J. Plant Pathol. **10:**188–190.
15. **Rollo, F., R. Salvi, and P. Torchia.** 1990. Highly sensitive and fast detection of *Phoma tracheiphila* by polymerase chain

reaction. Appl. Microbiol. Biotechnol. **32:**572–576.
16. **Saiki, R. K., D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, and H. A. Erlich.** 1988. Primer-directed amplification of DNA with a thermostable DNA polymerase. Science **239:**487–491.
17. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
18. **Schäfer, C., and J. Wöstemeyer.** 1992. Random primer dependent PCR differentiates aggressive from non-aggressive isolates of the oilseed rape pathogen *Phoma lingam* (*Leptosphaeria maculans*). J. Phytopathol. **136:**124–136.
19. **Schesser, K., A. Luder, and J. M. Henson.** 1991. Use of polymerase chain reaction to detect the take-all fungus, *Gaeumannomyces graminis*, in infected wheat plants. Appl. Environ. Microbiol. **57:**553–556.
20. **Taylor, J. L.** March 1993. Canadian patent application 2,092,115.
21. **Taylor, J. L., and I. E. Borgmann.** Submitted for publication.
22. **Thomas, P. M.** 1984. Weeds, insects and diseases, p. 1045–1048. *In* Canola growers manual. Canola Council of Canada, Winnipeg, Manitoba, Canada.
23. **Tinline, R. D., J. R. Srauffer, and J. G. Dickson.** 1960. *Cochliobolus sativus*. III. Effects of ultraviolet irradiation. Can. J. Bot. **38:**275–282.
24. **Tsen, H. Y., and T. R. Chen.** 1992. Use of the polymerase chain reaction for specific detection of type-A, type-D, and type-E enterotoxigenic *Staphylococcus aureus* in foods. Appl. Microbiol. Biotechnol. **37:**685–690.
25. **Wood, P. M., and M. J. Barbetti.** 1977. The role of seed infection in the spread of blackleg of rape in Western Australia. Aust. J. Exp. Agric. Anim. Husb. **17:**1040–1044.