A Toothpick Method for Mating *Leptosphaeria maculans*, the Causal Agent of Blackleg of Crucifers

Alemu Mengistu, Department of Plant Pathology, University of Wisconsin-Madison 53706; **Curt B. Hill,** 1190 A Rt. 19, Leeburg, GA 31763; and **Paul H. Williams,** Department of Plant Pathology, University of Wisconsin-Madison 53706

As production of canola and other oilseed brassicas increases worldwide, there is concern over the growing incidence of blackleg disease caused by the fungus *Leptosphaeria maculans* (Desmaz.) Ces. & de Not. *L. maculans* is a loculoascomycete (6) with heterothallic, bipolar mating control (1,9) and is the teleomorph of *Phoma lingam* (Tode:Fr.) Desmaz. (8). Primary infection of oilseed brassicas results from ascospores discharged from pseudothecia produced on dead, colonized brassica stubble (10). Pycnidia of the Phoma stage are found in leaf, stem, and pod lesions.

Variation in pathogenicity of *L. maculans* has been reported in numerous countries, including Australia, Canada, England, and the United States. The extent of pathogenic variation may be influenced by cultivars used, cropping practices, and climatic conditions (7). Sexual recombination appears to be one means by which variation is generated (2,5). However, there is a general lack of experimental evidence supporting this assumption.

Mating between two compatible strains of *L. maculans* and production of ascospores can be achieved in the laboratory by following a regimen of media, varying temperatures, UV-irradiation, and overlaying vegetative mycelium with water agar (WA). Asci produced with this method usually dissolve within 48 h of maturity within the pseudothecia, whereas under natural conditions on brassica stubble asci persist for weeks. The method reported here is an improvement over the mating procedures described previously (4) because it produces ascospores that remain viable for long periods.

MATERIALS AND METHODS

Mating-type isolates PHW 1245 (PG2, mating type +) and PHW 1223 (PG4, mating type –) (3) were placed 1 cm apart in a petri plate on 2% water agar (WA) and on agars containing 5, 10, 15, and 20% V8-juice. V8-juice at 50, 100, 150, and 200 ml volumes per liter were amended with 0.75, 1.5, 2.25, and 3.00 g of $CaCO_3$, respectively. The mating technique described earlier, in which isolates were grown first on V8 agar and then induced to mate by overlaying mycelium with WA and irradiating them with UV-irradiation (4), was used as a control. The seven treatments were replicated four times, and all treatments, except for the control and a set of 10% V8 agar, had toothpicks.

Autoclaved wooden toothpicks made from

Table 1. Mean number of pseudothecia of *Leptosphaeria maculans* on three types of toothpicks placed on various concentrations of V8 agar[a]

	No. of pseudothecia/toothpick			No. of pseudothecia in agar (mean/plate)		
Treatment	Round	Flat	Round with square center	Round	Flat	Round with square center
5% V8	17	81	51	24	25	24
10% V8	68	151	89	61	94	81
15% V8	0	6	0.3	0	1	0.3
20% V8	0	4	0	0	0	0.3
Water agar	0.3	8	4	0	0	0
$FLSD_{0.05}$[b]			23			

[a] V8 agar overlaid with water agar and 10% V8 agar with no toothpicks had mean pseudothecia numbers of 86 and 73, respectively.

[b] Fisher's least significant difference test ($P = 0.05$) within treatment means for number of pseudothecia.

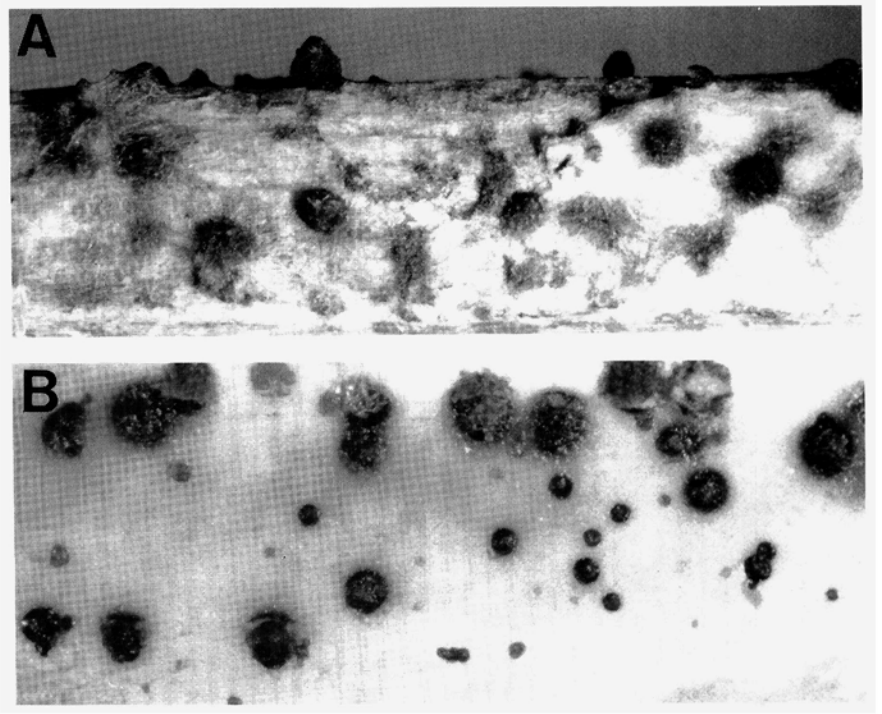

Fig. 1. (A) Toothpick with pseudothecia of *Leptosphaeria maculans*; **(B)** pseudothecia of *L. maculans* in V8 agar.

Corresponding author: A. Mengistu
E-mail address: alm@plantpath.wisc.edu

Accepted for publication 20 March 1995.

birch, *Betula* sp., were placed on the agar surface between the two mating types. Round, flat, and round toothpicks with square centers were compared with media without toothpicks for their ability to support pseudothecia. The cultures were incubated at 24°C with 24 h of cool-white fluorescent light (100 to 150 $\mu E\ s^{-1}m^{-2}$) for 7 days or until the mycelia of the mating types had intermingled. The plates then were placed in a 16°C incubator with alternating periods of 12 h of near UV-light and darkness (4). At 4, 5, 6, 7, 8, and 9 weeks after placing the toothpicks on the media, toothpicks and media were examined under 40× magnification and counted for mature pseudothecia. Pseudothecia produced on toothpicks were stored in vials containing silica gel after air-drying. Ascospore discharge and viability were checked. Fisher's least significant difference test was used to compare all the treatments.

RESULTS AND DISCUSSION

Pseudothecia formed on the media and on the toothpicks in four V8 concentrations, WA, 10% V8 with no toothpick, and the control. The number of pseudothecia varied according to treatment. Significantly more mature pseudothecia were produced on toothpicks placed in 5 and 10% V8 agar than in 15 or 20% V8 agar or WA (Table 1).

After 7 weeks of incubation, pseudothecia containing mature ascospores were produced on the surface of V8 agar and on the exposed surfaces of the toothpicks. The highest number of pseudothecia developed on the flat toothpicks, with more on the sides than on the upper surface (Fig. 1). Ten percent V8 agar without toothpicks produced a mean number of 73 pseudothecia per plate, whereas the overlaying method, which had twenty percent V8 agar overlaid with two percent WA (4), had a mean number of 86 pseudothecia per plate. Significantly fewer pseudothecia were produced on WA plates than on the 20% V8 agar overlaid with WA or the 10% V8 agar without toothpicks. Pseudothecia also were formed on 15 and 20% V8 agar; however, no asci developed in these agars even after 9 weeks of incubation. A few pseudothecia developed on toothpicks on WA (r = 0 to 14 per plate), and none developed on the agar.

Despite the longer time for pseudothecia development and ascospore maturation, 7 weeks, on toothpicks compared with 4 weeks to produce ascospores by the V8-WA overlaying method, production on toothpicks has the important advantage of producing mature ascospores that remain viable after 2 years of storage at 4°C. Pseudothecia produced on toothpicks and stored in vials containing silica gel after being air-dried discharged ascospores that were viable after 2 years, providing a convenient source of ascospores for genetic and pathogenicity studies.

LITERATURE CITED

1. Boudart, G. 1981. Modalites de l'attaque parasitaire des crucifers par *Leptosphaeria maculans* (Desm.) Ces et de Not. (f. c. *Phoma lingam*) agent de la necrose du par voir pathogen. These de Docteur. Universite Science et Technologie de Lille, Lille, France.
2. McGee, D. C., and Petrie, G. A. 1979. Variability of *Leptosphaeria maculans* in relation to blackleg of oilseed rape. Phytopathology 68:625-630.
3. Mengistu, A., Rimmer, S. R., Koch, E., and Williams, P. H. 1991. Pathogenicity grouping of isolates of *Leptosphaeria maculans* on *Brassica napus* cultivars and their disease reaction profiles on rapid-cycling brassicas. Plant Dis. 75:1279-1282.
4. Mengistu, A., Rimmer, S. R., and Williams, P. H. 1993. Protocols for in vitro sporulation, ascospore release, sexual mating, and fertility in crosses of *Leptosphaeria maculans*. Plant Dis. 77:538-540.
5. Petrie, G. A., and Lewis, P. A. 1985. Sexual compatibility of isolates of the rapeseed blackleg fungus *Leptosphaeria maculans* from Canada, Australia and England. Can. J. Plant Pathol. 7:113-133.
6. Punithalingham, E., and Holiday, P. 1972. *Leptosphaeria maculans*. No. 331, Descriptions of Pathogenic Fungi and Bacteria. Commonwealth Mycological Institute and the Association of Applied Biologists, Kew, England.
7. Rimmer, S. R., and Van den Berg, C. G. J. 1992. Resistance of oilseed *Brassica* spp. to blackleg caused by *Leptosphaeria maculans*. Can. J. Plant Pathol. 14:56-66.
8. Smith, H. C., and Sutton, B. C. 1964. *Leptosphaeria maculans*, the ascogenous state of *Phoma Lingam*. Trans. Br. Mycol. Soc. 90:415-420.
9. Venn, L. A. 1979. The genetic control of sexual compatibility in *Leptosphaeria maculans*. Aust. J. Plant Pathol. 8:5-6.
10. Williams, P. H. 1992. Biology of *Leptosphaeria maculans*. Can. J. Plant Pathol. 14:30-35.
11. Wood, P. M., and Barbetti, M. J. 1977. A study on the inoculation of rape seedlings with ascospores and pycnidiospores of the blackleg disease causal agent *Leptosphaeria maculans*. J. Aust. Inst. Agric. Sci. 43:79-80.

Salute to APS Sustaining Associates

This section is designed to help APS members understand more about APS Sustaining Associates. Information is supplied by company representatives. Each month features different companies. A complete listing appears in each issue of *Phytopathology.*

University of Swaziland, Faculty of Agriculture, Luyengo, Swaziland.

The Tryon Group. Contact: Timothy Tryon, 4134 Mandrake Road, Madison, WI 53704-1625.

Uniroyal Chemical Company. Contact: Allyn R. Bell, 74 Amity Road, Bethany, CT 06524-3402; 203/393-2163, Fax: 203/393-2290. Uniroyal established an agricultural chemical company more than 45 years ago as a developer and supplier of fungicides, herbicides, miticides, and plant growth regulants. Emphasis was directed toward providing unique products in each of these areas. With the introduction of systemic fungicides for cereal/cotton disease control, the company began a solid commitment to seed treatment technology worldwide. Gustafson, Inc., an associate, has strengthened its efforts in this technology. Uniroyal also markets several soil fungicides for row crops, turf, and ornamentals. Its current spectrum of fungicide products consists of carboxin (Vitavax), etridiazole (Terrazole), oxycarboxin (Plantvax), PCNB (Terraclor), triflumizole (Terraguard), and Thiram. Efforts are directed at foliar fungicides for fruit and field crops, including both systemic and nonsystemic active ingredients. The company has active programs with various universities, USDA pathologists, and extension people in the United States to evaluate these candidates in disease management programs.

Universiteitsbibliotheek SZ 302. Contact: F. Huijser, Postbus 19185, Amsterdam, GD 1000 Netherlands; 020-5259111, Fax: 31-2521-15888.

Valent USA Corporation. Contact: R. Holm, P.O. Box 8025, Walnut Creek, CA 94596; 510/256-2718, Fax: 510/256-2776. Valent is a U.S. agricultural chemical development and sales company owned by Sumitomo Chemical Company. The national office is in Walnut Creek. Research farms are in California, Illinois, and Mississippi. Valent develops compounds synthesized by Sumitomo or acquired from other sources.

Wilbur-Ellis Company. Contact: Larry Butler, Product Development Manager, P.O. Box 437, 215 N. Summer St., West Burlington, IA 52655-0437; 800/677-6324, Fax: 319/753-0404; or David Schulteis, R&D Manager, P.O. Box 1286, Fresno, CA 93715; 209/442-1220, Fax: 319/753-0404. Wilbur-Ellis Company is a major manufacturer and distributor of seed-treating chemicals, equipment, seed colorants, seed-coating equipment, and polymer seed coatings. We are currently researching the use of new or improved seed-treatment fungicides, insecticides, and biologicals for disease control and application methods with conventional and coating technology. Our company annually supports seed-treatment research with plant pathologists throughout the United States. Wilbur-Ellis is also one of the largest distributors of agricultural pesticides in the United States.

ZENECA Ag Products. Contact: James A. Frank, Western Research Center, P.O. Box 4023, 1200 So. 47th St., Richmond, CA 94804-0023; 510/231-1295, Fax: 510/231-1255. ZENECA, Inc., is a wholly owned subsidiary of Imperial Chemical Industries, PLC, based in the United Kingdom. The Ag Products Group of ZENECA, Inc., (formerly ICI Agricultural Products) was formed in 1970. ZENECA Agrochemicals in England is a major supplier of commercial fungicides for cereal, vines, and top fruit throughout the world. In the United States, ZENECA Ag Products manufactures Captan and Vapam fungicides, which are sold through key distributors. Insecticides, herbicides, rodenticides, and plant growth regulators are also manufactured and marketed. Research and development efforts are underway to identify and characterize proprietary fungicides suitable for the U.S. market in such crops as apples, grapes, potatoes, peanuts, pecans, and turf. ZENECA Ag Products has three research facilities located in North Carolina, Illinois, and Mississippi, and a greenhouse/laboratory complex at the Western Research Center in Richmond. In addition, ZENECA is involved in biocontrol and a biotechnology program in the United Kingdom and has a biotechnology facility in Iowa, site of ICI Seeds, Inc. ZENECA supports plant pathologists worldwide. Its goal is to achieve maximum plant health compatible with sustainable agroecosystems.