Special Report

Protocols for in Vitro Sporulation, Ascospore Release, Sexual Mating, and Fertility in Crosses of *Leptosphaeria maculans*

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ABSTRACT

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Leptosphaeria maculans ascospores were produced in vitro by pairing single ascospore isolates on V8 juice agar. Incubation for 7 days at 24 C with continuous fluorescent lighting provided for intermingling of the developing fungal colonies. Production of pseudothecia and ascospores was stimulated by pouring a 20-ml layer of 2% water agar cooled to 55 C over a culture grown on V8 juice agar, then incubating the dishes for 4 wk at 16 C under black light with a 12-hr photoperiod. Ascospores were recoverable only within 48 hr after they matured. Release of ascospores was facilitated by placing a drop of 5% β-glucuronidase over the asci, followed in 30–60 sec by two drops of sterile water. The number of pseudothecia varied significantly among the isolates tested. Crosses between isolates WA11 × WA30, WA22 × WA13, WA22 × WA30, WA32 × WA30, WA43 × WA40, and WA74 × WA30 had the highest number of pseudothecia and were the most fertile of the isolates tested.

Additional keywords: black leg, brassica disease, *Phoma lingam*, spore storage

Leptosphaeria maculans (Desmaz.) Ces. & De Not., the causal organism of the blackleg disease of oilseed rape, cultivated cole crops, and weedy crucifers, causes significant crop losses in many rapeseed-producing countries of the world (3,6). *L. maculans* is the teliomorph of *Phoma lingam* (Tode:Fr.) Desmaz. (9,11). It produces pseudothecia prolifically on oilseed rape residues and abundant pycnidia of the anamorphic stage on succulent leaf, stem, and pod tissues. *L. maculans* is a loculoascomycete (8) with heterothallic, bipolar (one gene, two alleles) mating control (1). The major source of inoculum for primary infection is ascospores discharged from infected rape residues (3). Although pseudothecia are produced abundantly in the field, the production of ascospores in culture through controlled mating has been variable, slow, and often unreliable. Others have successfully induced the formation of pseudothecia in vitro (7,10), but there is a need for more reliable and repeatable methods of ascospore production for controlled mating and genetic analysis of *L. maculans*. Xu et al (12) developed an agar layering method for inducing formation of pseudothecia. The method reported herein is an improvement on the layering method; it enables routine mating of the fungus and studies on its genetics. It also provides a protocol for inducing sporulation, ascospore release, and culture storage techniques.

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MATERIALS AND METHODS

Ascospore release from rape residues. Stem pieces of oileed rape with pseudothecia were obtained from a field in Western Australia and stored under dry conditions at room temperature. The pieces were immersed in tap water for 30–60 sec to stimulate ascospore discharge and attached to the interior base of Pyrex preparation dishes (diameter 100 mm, height 80 mm) with petroleum jelly. The dishes were inverted, placed over petri dish bases containing 2% water agar, and incubated at 24 C under continuous fluorescent light. After 48 hr of incubation, ascospores had discharged and germinated on the water agar. Under a stereomicroscope (50×), germinating ascospores were transferred singly with a needle to plates containing V8 juice agar (5).

Storage of cultures and pycnidiospores. Single ascospore transfers on V8 juice agar were incubated (Percival Model I-35LLVL plant growth chamber) at 24 C with continuous cool-white fluorescent light (100–150 $\mu E \cdot s^{-1} \cdot m^{-2}$). Profuse production of pycnidia occurred under these conditions in 5–6 days. Pycnidiospore suspensions were prepared by cutting a 1 × 3 cm agar strip with pycnidia from an actively growing culture, transferring the strip to a test tube containing 10 ml of sterile distilled water, and agitating the tube until a cloudy suspension of spores was observed. The suspension was then spread over the surface of V8 juice agar, and plates were incubated as before.

Table 1. Pseudothecia formation among 12 single ascospore isolates[a] of different pathogenicity groups (PG) of *Leptosphaeria maculans* from Western Australia paired in a half diallel cross

Mating type	(+)	(+)	(+)	(+)	(+)	(+)
PG[b]	3	3	3	2	4	2
Isolate no.	WA13	WA17	WA30	WA40	WA51	WA52
WA11(PG3)(−)	++[c]	++	++	+−	+−	+−
	1[d]	1	5	1	1	1
WA16(PG3)(−)	+−	+−	++	++	+−	+−
	2	1	2	2	1	2
WA22(PG3)(−)	++	++	++	++	++	++
	5	1	4	1	1	1
WA32(PG4)(−)	++	++	++	++	++	++
	2	2	5	2	1	3
WA43(PG2)(−)	++	+−	+−	++	+−	++
	1	2	2	4	1	3
WA74(PG2)(−)	++	++	++	++	++	++
	2	3	5	2	3	2

[a] Isolates were recovered from oilseed rape stubble obtained from Western Australia.
[b] Based on differential interaction phenotype of isolates on cotyledons of *Brassica napus* cvs. Westar, Quinta, and Glacier (4).
[c] Presence (+) or absence (−) of pseudothecia formation of paired isolates (two replicates).
[d] Values beneath a compatible mating indicate degree of fertility of paired isolates on a 0–5 scale, where 0 = 0, 1 = 1–10, 2 = 11–30, 3 = 31–60, 4 = 61–100, and 5 = >100 pseudothecia per plate.

Cultures of *L. maculans* can be stored as mycelium and pycnidia dried on filter paper disks or as a frozen pycnidiospore suspension. A 1-ml pycnidiospore suspension was spread over V8 juice agar, and sterile cellulose filter paper disks (6 mm in diameter) were placed on the agar surface with the conidial layer. Plates were incubated at 24 C under fluorescent light. In 5–7 days, abundant pycnidia developed on the filter paper disks. Disks were removed with forceps and dried for 3–5 days at room temperature over indicator silica gel desiccant in a sealed chamber. Disks then were stored at 4 to −20 C in vials containing silica gel. Cultures of *L. maculans* on filter paper disks remained viable and free of contaminants after 2 yr of storage. The filter paper discs have been used directly for mating *L. maculans* isolates. They are easily transported and a convenient source of viable pycnidiospores for culturing or testing virulence of isolates. *L. maculans* stored at −20 C as frozen pycnidiospores remained viable and pathogenic after 2 yr. Frozen pycnidiospores were used as inoculum after thawing for 15 min at room temperature.

Sexual mating. Eighty single ascospore isolates obtained from stem pieces collected in Australia were used to investigate techniques for mating, ascospore recovery, and fertility of isolates. V8 juice agar was determined to be the best medium for sexual mating (2,12). Two actively growing cultures originating from separate single ascospores were paired per plate by random. The pairs were placed 1 cm apart on V8 juice agar in a plastic or glass petri dish and sealed with Parafilm. Plates were placed 18 cm from continuous fluorescent light (40W) and incubated at 24 C. After 7 days, when developing fungal isolates had intermingled (Fig. 1A), 20 ml of 2% water agar cooled to 55 C was poured gently over the culture, starting from the edge. This allowed the agar to cool further as it covered the plates. Plates were resealed and placed in an unopened lightproof incubator for 4 wk at 16 C under black lights (Sylvania 40W, BLB with UV screen) with a 12-hr photoperiod. Intensity and wavelength of the black light were measured with a portable research spectroradiometer (LICOR LI 1800). The irradiance over the culture was 8.6 $\mu E \cdot m^{-2} \cdot s^{-1}$, with a peak of 368 μmol and a half-band width of 25 μmol.

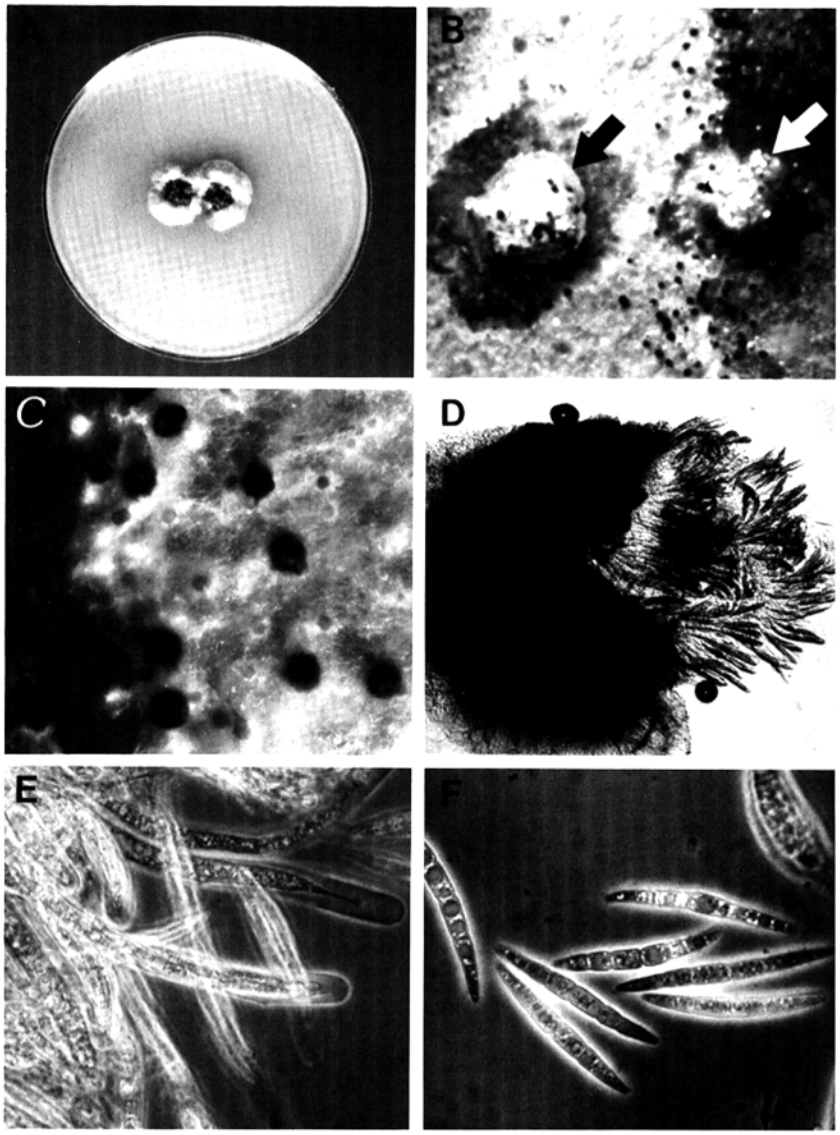

Fig. 1. Mating between isolates of *Leptosphaeria maculans:* **(A)** Pairing of isolates for mating, **(B)** pseudothecia in a band between two mating isolates (arrows), **(C)** pseudothecia in culture, **(D)** crushed pseudothecium with asci, **(E)** asci containing ascospores, and **(F)** released ascospores.

RESULTS AND DISCUSSION

Sexual mating. Among 40 single ascospore pairs, only 12 produced mature pseudothecia within 28 days after overlaying the 2% water agar. Pseudothecia formed in a band where the two mating types had interfaced (Fig. 1B) and also in scattered sites through the entire culture. Pseudothecia were differentiated from pycnidia on the basis of morphology and color. Pseudothecia were dark and globose (Fig. 1C), whereas pycnidia were brown to black and had longer necks with amethyst to pink cirrhi extruding from their ostioles.

Ascospores were recovered within 48 hr of pseudothecial maturation. Since no discharge or oozing of ascospores was observed after 48 hr, with or without refrigration, it is possible that asci may have dissolved within pseudothecia because of the in vitro environment. Under a dissecting microscope, mature pseudothecia were isolated with a fine needle and crushed on a 5% water agar block with fine-tipped forceps to release the asci (Fig. 1D). The walls of the asci (Fig. 1E) were ruptured within 30–60 sec of exposure to a drop of 5% β-glucuronidase (Sigma type H-2). Then the enzyme was diluted with two drops of sterile distilled water and a bent glass rod was used to spread the ascospores over 5% water agar (Fig. 1F). The plates were examined for ascospore germination after 48 hr. Well-separated germinating ascospores were transferred singly to V8 juice agar plates. This technique enables genetic characterization of *L. maculans* isolates.

Differences in fertility among isolates. Each of the paired isolates produced varying numbers of pseudothecia, indicating varying levels of fertility. Among 33 pairings, the following six pairs produced a high number of pseudothecia: WA11 × WA30, WA22 × WA13, WA22 × WA30, WA32 × WA30, WA43 × WA40, and WA74 × WA30 (Table 1). The fact that these combinations produced a high number of pseudothecia suggests that it should be possible to select isolates of *L. maculans* with increased capacity for pseudothecia and ascospore production.

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