Bot. Bull. Acad. Sin. (1999) **40:** 101–106

Infection of alfalfa pollen by *Botrytis cinerea*

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(Received May 6, 1998; Accepted July 24, 1998)

Abstract. A microscopic study proved that pollen grains of alfalfa are susceptible to infection by *Botrytis cinerea*, an important pathogen for blossom blight of alfalfa in western Canada. Pollen grains were inoculated by mixing pollen with drops of a *B. cinerea* spore suspension on glass slides. Infection occurred by direct hyphal penetration of the pollen cell walls without the formation of appressoria. Infection through the germ pores was observed more frequently than through other parts of the pollen walls. Plasmolysis of the plasma membrane and destruction of the cytoplasm occurred as a result of intensive growth of hyphae in the cell lumen of infected pollen grains. The impact of pollen infection by *B. cinerea* on the epidemiology of blossom blight of alfalfa is discussed.

Keywords: Alfalfa; Blossom blight; *Botrytis cinerea*; Epidemiology; Infection of pollen.

Introduction

Botrytis cinerea Pers.:Fr. is an important pathogen causing blossom blight, pod rots, and fruit rots on many crops, including alfalfa (*Medicago sativa* L.) (Gossen et al., 1996; 1997). Numerous reports indicate that high disease incidence and severity are associated with the colonization of senescent petals by *B. cinerea* (Bachelder and Orton, 1962; Hartill and Campbell, 1974; Hunter et al., 1972; Jarvis and Borecka, 1968; Kunimoto et al., 1996; Ogawa and English, 1960). Some reports indicate that pollen stimulates spore germination (Hartill, 1975) and infection by *B. cinerea* (Bachelder and Orton, 1962; Chou and Preece, 1968; Ogawa and English, 1960).

Recent studies have shown that alfalfa pollen is susceptible to infection by plant pathogens such as *Verticillium albo-atrum* Reinke & Berthold (Huang and Kokko, 1985) and *Sclerotinia sclerotiorum* (Lib.) de Bary (Huang et al., 1997). The objective of this study was to determine the susceptibility of alfalfa pollen to *B. cinerea*, and to describe the mode of infection using light microscopy (LM) and electron microscopy.

Materials and Methods

Inoculation of Alfalfa Pollen

Seeds of alfalfa, cultivar Barrier, were sown in Cornell peat-lite mix (Boodley and Sheldrake, 1977) in plastic pots (15 cm diam.), and plants were grown to maturity in a greenhouse. Alfalfa flowers were excised and tripped to collect fresh pollen grains on glass slides. An aqueous spore suspension containing approximately 10^3 spores/ml of *B. cinerea* was made from 3-week-old cultures grown on potato dextrose agar in petri dishes. Pollen grains were inoculated with *B. cinerea* by mixing the pollen with five drops of the suspension on each slide. The slides were placed in a moist chamber and kept for five days at room temperature (20–22°C) before being prepared for microscopic examinations. Two controls, pollen grains in sterile water and *B. cinerea* spores in sterile water, were prepared and processed for microscopic examinations.

Light Microscopy

For light microscopy, each 5-day-old sample (slide) of pollen-spore mixture was mounted in lactophenol and covered with a cover slip. The mounted specimens were examined for infection of pollen by *B. cinerea* using a Zeiss Photomicroscope III. Control samples were also mounted and examined microscopically. The important features observed in the specimens were documented by phase contrast photomicrographs.

Scanning Electron Microscopy (SEM)

For SEM, the 5-day-old inoculated (*B. cinerea*) and uninoculated (control) pollen samples were immersed in 2% glutaraldehyde fixative in 0.05 M sodium phosphate buffer, pH 7.0, at 4°C overnight (16 h) and then brought to room temperature. The samples were washed (3 × 10 min) with the sodium phosphate buffer solution. The specimens were dehydrated in a graded series of ethanol and critical point dried (Polaron E3100) with liquid carbon dioxide as the transitional fluid. The material was adhered onto aluminum specimen mounts with colloidal silver paste, air-dried overnight and sputter-coated (Denton Vacuum Desk-1) with gold (approximately 15 nm thickness). The specimens were examined and photographed on a Hitachi S-570 SEM.

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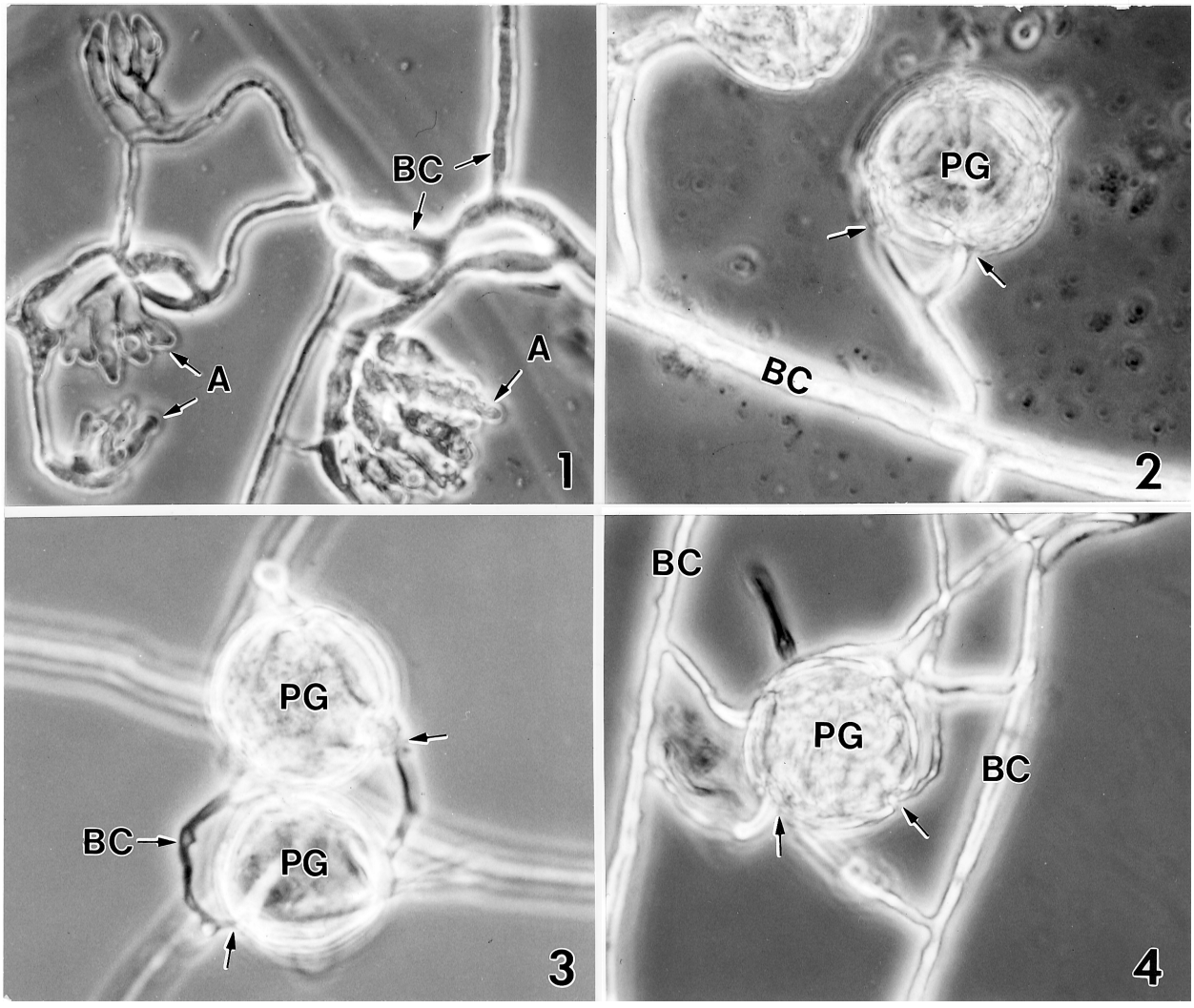
Transmission Electron Microscopy (TEM)

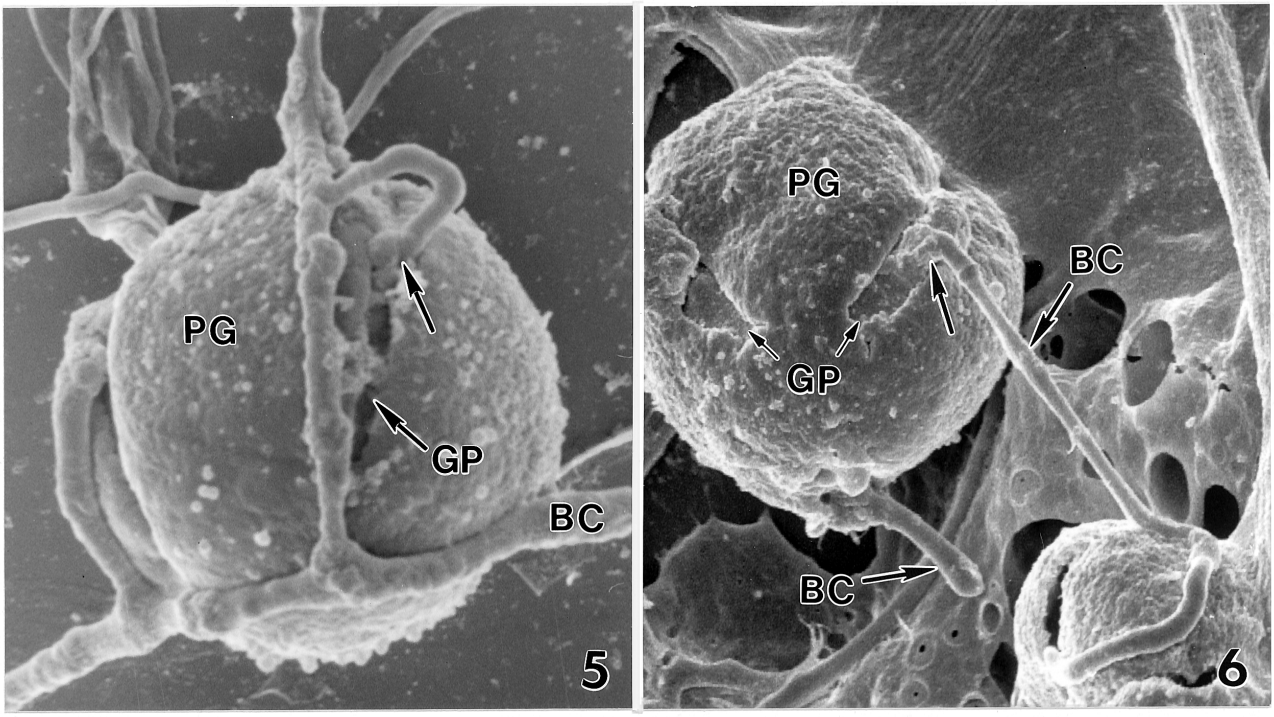
For TEM, the 5-day-old inoculated (*B. cinerea*) and uninoculated (control) pollen samples were immersed in 2% glutaraldehyde in 0.05 M sodium cacodylate buffer, pH 7.0, at 4°C overnight (16 h) and then brought to room temperature. The samples were washed (3 × 10 min) with the sodium phosphate buffer solution. The material was post-fixed in 2% osmium tetroxide, in the same buffer for 2 h, stained in 5% uranyl acetate in 50% ethanol (1 h), dehydrated in a graded series of ethanol, infiltrated with Spurr's low-viscosity embedding medium (Spurr, 1969) and polymerized 8 h at 70°C. Serial sections were cut (Kokko et al., 1990) with a diamond knife using a Reichert OM-U3 ultramicrotome. Sections were mounted on slotted, formvar-coated grids, stained with 5% aqueous lead citrate and 5% uranyl acetate, and examined with a Hitachi H-7100 transmission electron microscope at 75 Kv.

Results

Examination of 5-day-old samples revealed that the germination rate of conidia of *B. cinerea* was high (90%) in the pollen-spore mixture but was low (20%) in the control. The germ tubes from germinated spores in the controls remained short. In contrast, the germ tubes in the pollen-spore mixture grew rapidly and developed into long, multi-branched hyphae. Numerous multicellular, lobate appressoria, which developed into infection cushions, were formed in the glass slide preparations (Figure 1). However, infection of a pollen grain was always achieved by direct hyphal penetration (Figures 2–5) without the involvement of appressoria. Penetration of pollen grains occurred most frequently by a side branch of a hypha (Figures 2, 4, 5). Hyphal penetration occurred more readily through the germ pores (Figures 5, 10) than through other parts of the cell wall (Figures 8, 10). Hyphae in the in-



Figures 1–4. Photomicrographs showing formation of appressoria of *Botrytis cinerea* (Figure 1) and infection of alfalfa pollen by the pathogen (Figures 2–4). Note multicellular, lobate appressoria (A), which developed into infection cushions (Figure 1). Note also the infection of pollen grains (PG) by direct hyphal penetration of *B. cinerea* (BC) without the formation of appressoria (arrows). Figure 1, ×520; Figure 2, ×1,000; Figure 3, ×1,000; Figure 4, ×1,000.



Figures 5 and 6. Scanning electron micrographs showing hyphae of *Botrytis cinerea* (BC) penetrating the alfalfa pollen (PG) (Figure 5, arrow) or emerging from the infected pollen (PG) (Figure 6, arrow) through a germ pore (GP). Figure 5, ×2,800; Figure 6, ×2,500.

fected pollen grain re-emerged through the germ pores (Figure 6) or other parts of the cell wall. A positive chemotropism was evident as the lateral branches often grew toward pollen grains (Figures 2, 4, 5).

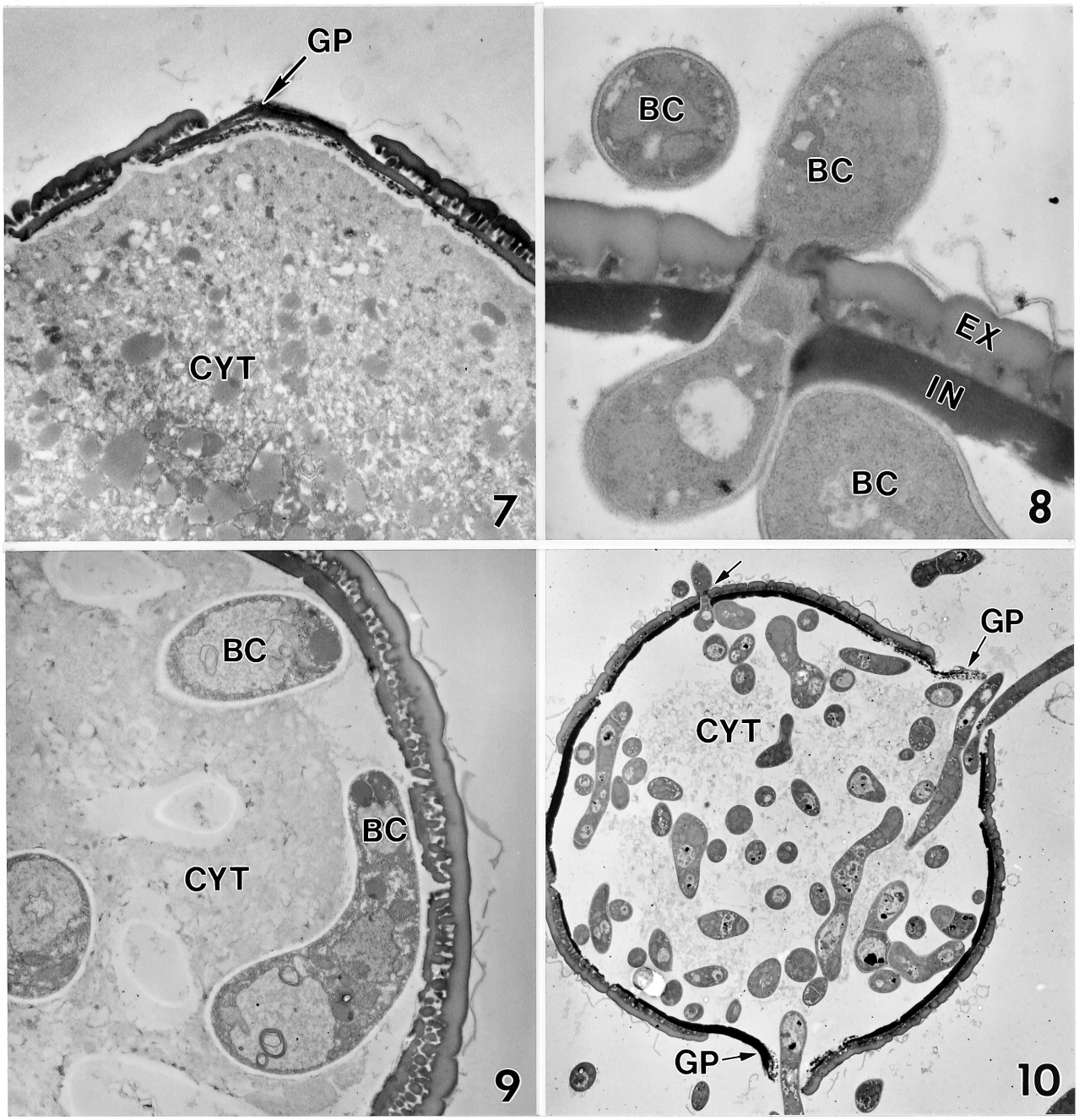
Each alfalfa pollen grain has a tricolpate structure with three long furrows that are equidistant from each other (Figure 6). The ultrastructure of a healthy pollen grain showed dense cytoplasm with numerous small vacuoles (Figure 7). The cytoplasm was surrounded by the thin plasma membrane and two well-defined cell wall layers, exine and intine (Figure 7). The cell wall of the germ pore region was thinner than the cell wall of the non-germ pore region (Figure 7). Both cell wall etching (Figure 8) and the formation of cone-shaped cell wall openings (Figure 10) were observed at infection sites. Plasmolysis of the plasma membrane occurred as the result of fungal invasion of the pollen grain (Figure 9). The intensive growth and multiplication of invading hyphae caused the eventual destruction and disintegration of the pollen cytoplasm (Figure 10).

Discussion

The present study together with previous studies, reveals that alfalfa pollen is susceptible to infection by plant pathogens such as *V. albo-atrum* (Huang and Kokko, 1985), *S. sclerotiorum* (Huang et al., 1997), and *B. cinerea.* The mode of infection for all of these pathogens is by direct hyphal penetration of the pollen cell wall without the formation of appressoria. Penetration of the cell wall is likely achieved by chemical force as evidenced by cell wall dissolution (Figure 8) as well as by physical force as evidenced by the cone-shaped wall rupture (Figure 10) at the site of infection. In studying the infection of pollen grains of Monterey pine (*Pinus radiata* D. Don) by *Retiarius superficiaris* Olivier, Olivier (1978) reported that a swelling of the hyphal tip before penetration of the pollen walls is sometimes noticeable but no true appressorium is formed. Olivier and Williams (1975) observed a slight depression of the cell walls at the infection site, and concluded that penetration of pine pollen by *Retiarius* spp. is achieved by mechanical force.

The ultrastructural evidence in this study indicates that the colpate regions of alfalfa pollen grains are the most common sites of infection by *B. cinerea*. The weak resistance of the colpate regions of alfalfa pollen to invasion by *V. albo-atrum* (Huang and Kokko, 1985), *S. sclerotiorum* (Huang et al., 1997), and *B. cinerea* may be due to thin cell walls at the furrows and germ pores. Olivier (1978) observed infection of pine pollen by *Retiarius* spp. occurred most frequently at the germ pore region. Knox and Heslop-Harrison (1970) found that intine- bound protein is rich in the cell walls of the germ pore region. Therefore, the susceptibility of alfalfa pollen germ pores to invasion by pathogenic fungi may be due to the thin, but protein-rich, cell walls at the colpate regions.

Previous reports indicate that pollen stimulates spore germination and enhances lesion development by *B. cinerea* (Bachelder and Orton, 1962; Bekesi, 1979; 1982;



Figures 7–10. Transmission electron micrographs of healthy (Figure 7) and *Botrytis cinerea*-infected (Figures 8–10) pollen grains of alfalfa. Figure 7. A healthy pollen grain showing dense cytoplasm (CYT). Note the thin wall at the germ pore (GP) region and the thick wall outside the germ pore region. Figure 8. A hypha of *Botrytis cinerea* penetrating through the cell wall outside the germ pore region. Note etching of the exine (EX) and intine (IN) layers of the pollen grain wall at the penetration site. Figure 9. An infected pollen grain showing the presence of hyphae of *B. cinerea* (BC) inside the pollen grain and plasmolysis of the pollen cytoplasm (CYT). Figure 10. An infected pollen grain showing intense hyphal ramification and disintegration of the pollen cytoplasm (CYT). Note hyphae penetrating through the cell wall at the germ pore (GP) and outside the germ pore (arrow) regions. Figure 7, ×5,400; Figure 8, ×18,000; Figure 9, ×7,000; Figure 10, ×3,000.

Chou and Preece, 1968; Hartill, 1975; Hartill and Campbell, 1974; Ogawa and English, 1960) and *S. sclerotiorum* (Hartill, 1975; Dillard and Hunter, 1986). The dense growth of mycelia of fungal pathogens in infected pollen grains observed in the present (Figure 10) and previous studies (Huang and Kokko, 1985; Huang et al., 1997) suggests that the cytoplasm of alfalfa pollen is a good growth substrate for plant pathogens. Numerous reports indicate that pollen releases nutrients—including sugars, amino acids, and proteins—when it is moistened (Fokkema, 1971; Stanley and Search, 1971; Yamakawa, 1984). Thus, diffusates from

alfalfa pollen may be responsible for the positive chemotropism of hyphal growth of *B. cinerea* (Figure 4) observed in the present study.

Pollinating insects such as honeybees (*Apis mellifera* L.) (Stelfox et al., 1978), alfalfa leafcutter bees *Megachile rotundata* (Fabricius) (Huang et al., 1986), and fig wasps (*Blastophaga psenes* L.) (Michailides and Morgan, 1994) are important vectors for plant pathogenic fungi. Huang et al. (1986) observed *V. albo-atrum*-infected pollen grains were carried by leafcutter bees foraging in a field, the alfalfa plants of which suffered a high incidence of verticillium wilt. Stelfox et al. (1978) reported that the transmission of head blight of rapeseed (*Brassica* spp.) was attributed to the spread of *S. sclerotiorum*-infested pollen grains by honeybees. Alfalfa is a cross-pollinated crop which relies upon the use of leafcutter bees for commercial seed production (Goplen et al., 1980). Since both *B. cinerea* and *S. sclerotiorum* are important pathogens causing blossom blight of alfalfa (Gossen et al., 1996; 1997), the infection of pollen grains by *B. cinerea* observed in this study and by *S. sclerotiorum* observed in a previous study (Huang et al., 1997) suggests that pathogen-contaminated pollen grains may play a significant role in the epidemiology of blossom blight and thus have a negative impact on the alfalfa seed and leafcutter bee industries.

Acknowledgement. The authors thank B. Lee for technical assistance in electron microscopy. This is Lethbridge Research Centre Contribution No. 3879814.

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