

## Infection of Alfalfa Pollen by *Sclerotinia sclerotiorum*

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Blossom blight, caused by *Sclerotinia sclerotiorum*, has become an important disease of alfalfa (*Medicago sativa* L.) in seed production areas of western Canada. Studies using light microscopy and scanning and transmission electron microscopy revealed that pollen grains of alfalfa are susceptible to infection by *S. sclerotiorum*. Ascospores of *S. sclerotiorum* germinated readily in water with or without pollen grains. Examinations of ascospore-pollen mixtures incubated at room temperature (20–22°C) for 5 days revealed that numerous pollen grains were infected by *S. sclerotiorum* by direct hyphal penetration through the equatorial germinative pores or through the exine and intine layers of the pollen wall without the formation of infection cushions or appressoria. After penetration, hyphae ramified within the pollen grains, causing plasmolysis of the cytoplasmic membrane and eventual disintegration of the pollen cytoplasm. The study suggests that alfalfa pollen may play a role in the epidemiology of blossom blight in alfalfa.

**KEY WORDS:** Alfalfa (*Medicago sativa*) pollen; infection by *Sclerotinia sclerotiorum*; ascospores; hyphae; blossom blight epidemiology.

### INTRODUCTION

Invasion of pollen by plant pathogens has been recorded for numerous viruses (18) but rarely for fungi (1), despite numerous examples of fungal pathogens attacking blossoms of higher plants. Recently, evidence of invasion of pollen grains by fungal pathogens has been documented in several reports. These include infection of alfalfa (*Medicago sativa* L.) pollen by *Verticillium albo-atrum* Reinke & Berth. (10,14) and of pea (*Pisum sativum* L.) pollen by *Sclerotinia sclerotiorum* (Lib.) de Bary (12).

*Sclerotinia sclerotiorum* is an important pathogen causing serious losses to many crops including alfalfa (23). Both *S. sclerotiorum* (9) and *Botrytis cinerea* Pers. ex Fr. (6) are the causal agents of blossom blight of alfalfa in seed production fields in western Canada. Although infection of alfalfa pollen by alfalfa mosaic virus (22,28) or *V. albo-atrum* (10,14) has been reported, there is no record of infection of alfalfa pollen by *S. sclerotiorum*. The objectives of this study were to determine whether pollen grains of alfalfa are susceptible to infection by *S. sclerotiorum* and to demonstrate the process of any infection.

### MATERIALS AND METHODS

#### *Inoculation of alfalfa pollen with S. sclerotiorum*

Mature apothecia of *S. sclerotiorum*, isolate sun-87, were produced by the method described by Huang and Kozub (13). Ascospores were placed on glass slides by gently

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touching the hymenium surface against the slides. Florets were excised from greenhouse-grown plants of alfalfa, cvs. 'Anchor' and 'Barrier'. Pollen grains were showered onto ascospores on the glass slide by tripping, using a wooden toothpick to trip the keel petal of each floret. Approximately 10 drops of sterile distilled water were added to the spore-pollen mixture. The slides were kept at room temperature (20–22°C) in a moist chamber (100% r.h.) for 2 days and the spore-pollen mixtures were examined for germination of ascospores and pollen grains by light microscopy. Samples of the spore-pollen mixture were incubated for 5 days and used for studying infection of pollen by light and electron microscopy. For controls, pollen grains or ascospores alone were prepared by the same glass slide procedure described above.

#### *Light Microscopy*

Two- and 5-day-old samples, ascospore-pollen mixtures, pollen, or ascospores, on glass slides were mounted by adding a few drops of lactophenol to the sample and covering with cover slips. The 2-day-old materials were examined for spore and pollen germination under brightfield using a Zeiss Photomicroscope III. The 5-day-old materials were examined for infection of pollen by *S. sclerotiorum* under brightfield or phase contrast.

#### *Scanning Electron Microscopy*

Samples of 5-day-old, ascospore-pollen mixtures were removed from the glass slides and 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 then washed (3 x 10 min) with the buffer solution and 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 with a Hitachi S-570 scanning electron microscope.

#### *Transmission Electron Microscopy*

Samples of 5-day-old, ascospore-pollen mixtures 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 x 10 min) with the same sodium cacodylate 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 (24) and polymerized 8 h at 70°C. Serial sections were cut (16) 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

Ascospores of *S. sclerotiorum* germinated in sterile distilled water in the presence, or not, of alfalfa pollen. In random examinations of 1,000 spores from the 2-day-old cultures in each treatment, the germination rate was 62.3% and 82.0% for the treatments of

ascospores alone and ascospore–pollen mixture, respectively. After 2 days of incubation, the germ tube of ascospores in the control remained short, 1–2 septae, but the germ tube of the ascospores in the presence of pollen grains developed into long, multi-branched hyphae. Germination of the pollen grains of alfalfa occurred rarely, either in water alone or in ascospore–pollen mixtures.

Figures 1-4. Brightfield (Fig. 1) and phase contrast (Figs. 2-4) of photomicrographs showing infection of alfalfa pollen grains by *Sclerotinia sclerotiorum*. Pollen grains were mixed with ascospores in sterile water for 5 days. Fig. 1. Formation of appressoria (A) of *S. sclerotiorum* by dichotomously branched hyphae. Fig. 2. A germinated ascospore (SP) with one of the germ tubes (SS) penetrated and emerged (arrow) through the same germ pore of the pollen grain (PG). Figs. 3 and 4. Side branch hyphae of *S. sclerotiorum* (SS) penetrating the PGs through one germ pore (arrows) and emerging through another pore (Fig. 4, arrowhead). Note that hyphal penetration occurred without the formation of appressoria (Figs. 2-4). Magnifications: Fig. 1, x650; Fig. 2, x1,400; Fig. 3, x2,000; Fig. 4, x2,200.

Results of examinations of 5-day-old ascospore–pollen mixtures showed that alfalfa pollen grains are susceptible to infection by *S. sclerotiorum*. Although appressoria of *S. sclerotiorum* with characteristic dichotomous branching were frequently observed in the glass slide preparations (Fig. 1), infection of a pollen grain was always achieved by direct hyphal penetration (Figs. 2-6) without the involvement of appressoria or infection cushions. Penetration of pollen grains occurred either by a germ tube of the ascospore (Fig. 2) or, more commonly, by a side branch of a hypha (Figs. 3,4). Hyphal penetration through the germ pores (Figs. 2-4,5) was observed more frequently than through other parts of the cell wall (Fig. 6). Hyphae in the infected pollen could emerge through the germ pores (Figs. 2,4) or other parts of the cell wall (Figs. 8,9).

Figures 5 and 6. Scanning electron micrographs of alfalfa pollen grains infected by hyphae of *Sclerotinia sclerotiorum* (SS). Note that hyphal penetration occurred either through germ pores (GP) (Fig. 5 arrows) or through the cell wall beyond the GP regions (Fig. 6, arrow). Magnifications: Fig. 5, x2,400; Fig. 6, x3,300.

Transmission electron micrographs showed that a healthy pollen grain of alfalfa had well-defined wall layers, exine and intine (Figs. 7,8,10) and dense cytoplasm (Fig. 7). The cell wall at the germ pore region (Figs. 7,9,10) was thinner than that of other regions (Fig. 8). Etching of the pollen wall at the hyphae penetration site was evident (Fig. 8). Ramification of invaded hyphae of *S. sclerotiorum* in the pollen grain resulted in plasmolysis of the pollen cytoplasmic membrane (Figs. 9,10) and disintegration of the cytoplasm (Fig. 10).

Figures 7-10. Transmission electron micrographs of uninfected (Fig. 7) and *Sclerotinia sclerotiorum*-infected (Figs. 8-10) pollen grains of alfalfa. Fig. 7. A healthy pollen grain showing thick cell walls (arrow) and dense cytoplasm (Cyt). Note the thin cell wall at the germ pore (GP) region. Fig. 8. A hypha penetrating through the cell wall with signs of enzymatic dissolution of the wall (arrow). Fig. 9. Cross-section of an infected pollen grain with hyphae ramifying throughout the disintegrating cytoplasm (Cyt) and two points of hyphal penetration (arrows) not associated with a germ pore (GP). Fig. 10. An infected pollen grain showing plasmolysis as a result of hyphal ramification. EX=exine layer of the pollen wall, IN=intine layer of the pollen wall, Cyt=pollen cytoplasm, GP=pollen germ pore, SS=*Sclerotinia sclerotiorum*. Magnifications: Fig. 7, x4,800; Fig. 8, x11,500; Fig. 9, x2,300; Fig. 10, x6,400.

## DISCUSSION

This study showed that fresh, living alfalfa pollen grains from mature flowers are susceptible to infection by *S. sclerotiorum*. The mode of hyphal penetration of the cell wall of alfalfa pollen is similar to that observed in the infection of pea pollen by *S. sclerotiorum* (12) and alfalfa pollen by *V. albo-atrum* (10). This direct hyphal infection process of pollen by *S. sclerotiorum* is in contrast to the infection by this pathogen of other host tissues such as bean hypocotyls (17) and pea pods (11), which involves the formation of appressoria or infection cushions. Despite the frequent presence of appressoria in the pollen–ascospore mixtures in this study, the reason for the difference in the mode of infection of *S. sclerotiorum* on pollen versus other host tissues remains unknown.

The study confirms a previous report (10) that the germ pores of alfalfa pollen are more vulnerable to invasion by fungal pathogens than are other parts of the cell wall. The pollen grain of alfalfa is tricolporate, having three elongated furrows with a pore or aperture in each furrow (3,10). The exine layer of the pollen wall contains sporopollenins which are remarkably stable and resistant to various chemicals such as sulfuric acid (15). The present study indicated that the pollen walls are susceptible to invasion by *S. sclerotiorum* despite their resistant nature to chemicals. Ultrastructure studies in the present and previous (10) work indicated that the cell wall of alfalfa pollen is thin at the germ pore regions, compared with the other regions. The thin cell wall at the germination pore region may be a weak barrier against invasion by fungal pathogens.

Previous reports indicated that pollen grains stimulate the growth of fungal pathogens such as *S. sclerotiorum* (7), *B. cinerea* (2,7), *Helminthosporium sativum* Pamm., King & Bakke (4) and *Phoma betae* Frank (27). The intense hyphal proliferation of fungal pathogens in infected pollen grains of host plants observed in the present and previous (10,12) studies suggests that, in addition to senescent petals (8,19,21,26) and stamens (11), pollen is a potential substrate for ascospores of *S. sclerotiorum* in initiating blossom blight of alfalfa.

Successful production of alfalfa seed depends upon cross-pollination by insects. Several pollinating insects have been reported as important vectors of fungal pathogens, including honeybees (*Apis mellifera* L.) for *S. sclerotiorum* (25), alfalfa leafcutter bees (*Megachile rotundata* (Fabricius) for *V. albo-atrum* (14), and fig wasp (*Blastophaga psenes* L.) for *Fusarium moniliforme* J. Sheld. (20). Stelfox *et al.* (25) reported that rapeseed (*Brassica* spp.) pollen contaminated with ascospores of *S. sclerotiorum* can be transported by honeybees, causing head blight of rapeseed plants. The evidence of infection of alfalfa pollen by *S. sclerotiorum* in the present study suggests that transported alfalfa pollen grains can serve as a source of the pathogen. Since the alfalfa leafcutter bee is the most important pollinator used in commercial alfalfa seed production in Canada (5), it is likely that leafcutter bees disseminate *S. sclerotiorum*-infected pollen grains in alfalfa fields. Therefore, the finding of infection of alfalfa pollen by *S. sclerotiorum* may have significant implications for the alfalfa seed and leafcutter bee industries in western Canada.

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## REFERENCES

1. Agrios, G.N. (1980) Vectors of Plant Pathogens. Academic Press, New York, NY. pp. 293-324.
2. Chou, M.C. and Preece, T.F. (1968) The effect of pollen grains on infections caused by *Botrytis cinerea* Pers. ex Fr. *Ann. Appl. Biol.* 62:11-22.
3. Crompton, C.W. and Wojtas, W.A. (1993) Pollen Grains of Canadian Honey Plants. Agric. and Agri-Food Can. Publ. #1892/E.
4. Fokkema, N.J. (1968) The influence of pollen in the phyllosphere of rye on colonization by saprophytic fungi and on infection by *Helminthosporium sativum* and other leaf pathogens. Diss. Amsterdam Meded. Phytopathol. Lab. "Willie Commelin Scholten" 87. in: *Neth. J. Pl. Pathol.* (1971) 77: Suppl. 1.
5. Goplen, B.P., Baenzler, H., Bailey, L.D., Gross, A.T.H., Hanna, M.R., Michaud, R., Richards, K.W., Waddington, J. (1980) Growing and Managing Alfalfa in Canada. Agric. and Agri-Food Can. Publ. #1705.
6. Gossen, B.D., Harrison, L.M., Holley, J. and Smith, S.R. (1996) Survey of blossom blight of alfalfa on the Canadian Prairies in 1995. *Can. Plant Dis. Surv.* 76: 123-125.
7. Hartill, W.F.T. (1975) Germination of *Botrytis* and *Sclerotinia* spores in the presence of pollen on tobacco leaves. *N.Z. J. Agric. Res.* 18: 405-407.
8. Hartill, W.F.T. and Campbell, J.M. (1974) Effects of flower removal on the development of the *Sclerotinia/Botrytis* complex of tobacco. *N.Z. J. Agric. Res.* 17: 147-152.
9. Holley, J., Linowski, R., Gossen, B. and Harrison, L. (1995) *Sclerotinia sclerotiorum* causes blossom blight of alfalfa. *Proc. Ann. Meeting of the Plant Pathology Society of Alberta* (Lethbridge, Alberta, Canada).
10. Huang, H.C. and Kokko, E.G. (1985) Infection of alfalfa pollen by *Verticillium albo-atrum*. *Phytopathology* 75: 859-865.
11. Huang, H.C. and Kokko, E.G. (1992) Pod rot of dry peas due to infection by ascospores from *Sclerotinia sclerotiorum*. *Plant Dis.* 76: 597-600.
12. Huang, H.C. and Kokko, E.G. (1993) Infection of pea pollen by *Sclerotinia sclerotiorum*. *Trends Agric. Sci.-Plant Pathol.* 1: 13-17.
13. Huang, H.C. and Kozub, G.C. (1989) A simple method for production of apothecia from sclerotia of *Sclerotinia sclerotiorum*. *Plant Prot. Bull. (Taiwan)* 31: 333-345.
14. Huang, H.C., Richards, K.W. and Kokko, E.G. (1986) Role of the leafcutter bee in dissemination of *Verticillium albo-atrum* in alfalfa. *Phytopathology* 76: 75-80.
15. Iwanami, Y., Sasakuma, T. and Yamada, Y. (1988) Pollen: Illustrations and Scanning Electronmicrographs. Kodansha Scientific Books, Tokyo, and Springer-Verlag, Berlin.
16. Kokko, E.G., Gowen, B. and Jahnke, C.R. (1990) An inexpensive water volume control device for ultramicrotomy. in: *Electron Microscopy 1990: Proc. 12th Int. Congr. for Electron Microscopy* (Seattle, WA, USA), pp. 732-733.
17. Lumsden, R.D. and Dow, R.L. (1973) Histopathology of *Sclerotinia sclerotiorum* infection of bean. *Phytopathology* 63: 708-715.
18. Mandahar, C.L. (1981) Virus transmission through seed and pollen. pp. 241-292 in: Maramorosch, K. and Harris, K.F. [Eds.] *Plant Diseases and Vectors, Ecology and Epidemiology*. Academic Press, New York, NY.
19. McLean, D.M. (1958) Role of dead flower parts in infection of certain crucifers by *Sclerotinia sclerotiorum* (Lib.) de Bary. *Plant Dis. Rep.* 42: 663-666.
20. Michailides, T.J. and Morgan, D.P. (1994) Dynamics of *Blastophaga psenes* populations, availability of caprifigs, and fig endosepsis caused by *Fusarium moniliforme*. *Phytopathology* 84: 1254-1263.
21. Natti, J.J. (1971) Epidemiology and control of bean white mold. *Phytopathology* 61: 669-674.
22. Pesic, Z., Hiruki, C. and Chen, M.H. (1988) Detection of viral antigen by immunogold cytochemistry in ovules, pollen, and anthers of alfalfa infected with alfalfa mosaic virus. *Phytopathology* 78: 1027-1032.

23. Purdy, L.H. (1979) *Sclerotinia sclerotiorum*: History, diseases, symptomatology, host range, geographic distribution, and impact. *Phytopathology* 69: 875-880.
24. Spurr, A.R. (1969) A low-viscosity epoxy embedding medium for electron microscopy. *J. Ultrastruct. Res.* 26: 31-43.
25. Stelfox, D., Williams, J.R., Soehngen, U. and Topping, R.C. (1978) Transport of *Sclerotinia sclerotiorum* ascospores by rapeseed pollen in Alberta. *Plant Dis. Rep.* 62: 576-579.
26. Sutton, D.C. and Deverall, B.J. (1983) Studies on infection of bean (*Phaseolus vulgaris*) and soybean (*Glycine max*) by ascospores of *Sclerotinia sclerotiorum*. *Plant Pathol.* 32: 251-261.
27. Warren, R.C. (1972) The effect of pollen on the fungal leaf microflora of *Beta vulgaris* L. and on infection of leaves by *Phoma betae*. *Neth. J. Pl. Pathol.* 78: 89-98.
28. Wilcoxson, R.D., Johnson, L.E.B., and Frosheiser, F.I. (1975) Variation in the aggregation forms of alfalfa mosaic virus strains in different alfalfa organs. *Phytopathology* 65: 1249-1254.