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Infection of Canola Pollen by *Sclerotinia sclerotiorum*

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ABSTRACT

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The shape of mature pollen grains of canola (*Brassica napus* L.), cv. Westar, is ellipsoidal when air-dried but is spheroidal when moistened. Data from image analysis showed that the water-imbibed spheroidal pollen grains had a small (5.2%) increase in volume compared to the air-dried, ellipsoidal pollen. Ascospores of *Sclerotinia sclerotiorum* germinated in sterile water with or without canola pollen grains. The germ tubes of ascospores grew slowly and remained short in water in the absence of pollen but they grew rapidly and developed into branched mycelia in 48 h in the presence of pollen. Examination of 5-day-old ascospore-pollen mixtures revealed that pollen grains were susceptible to infection by *S. sclerotiorum*. Infection occurred by direct hyphal penetration of the cell walls without the formation of appressoria or infection cushions. Hyphal penetration was more commonly observed through the germinative pores than other parts of the pollen walls. Plasmolysis and disintegration of the pollen cytoplasm occurred as a result of hyphal ramifications within the infected pollen grains.

Key words: Canola pollen, *Sclerotinia sclerotiorum*, *Brassica napus*.

INTRODUCTION

In the absence of an exogenous nutrient source, relatively few ascospores of *Sclerotinia sclerotiorum* (Lib.) de Bary germinated (7) and the germ tubes were short (2). Pollen and pollen diffusates are an excellent source of nutrients for promoting spore germination and mycelial growth of fungi including *S. sclerotiorum* (7, 30) and *Botrytis cinerea* Pers. ex Fr. (3, 22). Infection of healthy plant tissues by ascospores of *S. sclerotiorum* occurred only in the presence of exogenous nutrients (1, 2, 5, 25). Pollen has also been found to increase infection by *Phoma betae* Frank (32), *Helminthosporium sativum* Pamm., King and Bakke (6) and *Septoria nodorum* (Berk.) Berk. (6).

Sclerotinia sclerotiorum is an important pathogen for blossom blight of alfalfa (*Medicago sativa* L.) (8), pod rot of dry pea (*Pisum sativum* L.) (11), white mold of dry bean (*Phaseolus vulgaris* L.) (14), and stem and pod rot of canola/rapeseed (*Brassica* spp.) (15). Previous reports indicate that healthy pollen grains of host crops such as alfalfa (16) and dry pea (12) are susceptible to infection by *S. sclerotiorum*. Stelfox *et al*. (29) reported contamination of pollen grains of rapeseeds with ascospores of *S. sclerotiorum* under growth room and field conditions. They found that transportation of the *S. sclerotiorum*-contaminated pollen to rapeseed flowers by honeybees (*Apis mellifera* L.) resulted in the development of head blight under greenhouse conditions. The objective of this paper is to describe the infection of canola pollen by *S. sclerotiorum*.

MATERIALS AND METHODS

Morphology of canola pollen

Mature canola blossoms were collected from greenhouse-grown and field-grown plants, cultivar Westar, and pollen grains were showered onto a glass slide by gently tapping the anthers. After photographic documentation of the

dry mount pollen grains using a compound microscope, a few drops of sterile distilled water were added to the pollen and the slides were kept at room temperature (20-24 C) in a moist chamber for 24 hr. Representative pollen grains were photographed. A Tracor Northern 8502 image analyzer (Noran Inc., Middleton, WI) with a Dage 81 B/W video camera (DAGE-MTI, Inc., Michigan City, IN) was used to analyze the size and volume of individual pollen grains from photographs of identical magnification. The volume (*V*) of the pollen grains was calculated using the equation, $V=4/3\,\pi$ (maximum caliper projection $\div$ 2) $\times$ (minimum caliper projection $\div$ 2)^2 (26).

Infection of canola pollen by *S. sclerotiorum*

Mature apothecia of *S. sclerotiorum*, isolate sun-87, were produced using the method described by Huang and Kozub (10). Ascospore suspensions containing 2×10^4 spores/ml were made by washing apothecia in sterile distilled water. Half of the spore suspensions were mixed with pollen grains to make pollen-ascospore mixtures containing 1×10^3 grains/ml. Subsamples from the suspensions of ascospores, pollen, and ascospore-pollen mixture were pipetted onto glass slides, incubated at room temperature for 48 hr and examined under a compound microscope for germination and germ tube growth of ascospores. There were 10 replicates for each treatment. For the treatments of spore-pollen mixture or spore alone, 100 randomly selected spores of each replicate were examined for germination and germ-tube growth.

For infection of pollen, samples of the pollen suspension and the pollen-ascospore suspension were prepared by the method described previously. Subsamples from the suspensions were pipetted onto glass slides, incubated at room temperature for 5 days, and used for light and electron microscopic studies. For light microscopy, the specimens were mounted in lactophenol, covered with cover slips and examined using a Zeiss Photomicroscope III. For electron microscopy, samples of pollen or ascospore-pollen mixtures were fixed in a 2% solution of glutaraldehyde in 0.05M sodium phosphate buffer, pH 7.2. Samples were left to fix overnight (16 hr) at 4 C and then brought to room temperature after the initial fixation. 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 3 hr. Samples were again washed in the buffer solution and dehydrated using a graded series of ethanol. At this point samples were divided in half, one half for scanning electron microscopy (SEM) and the other half for transmission electron microscopy (TEM).

For SEM, the samples were critical point dried (Polaron E3100, Watford, England) with liquid carbon dioxide as a transitional fluid. The material was adhered onto aluminum specimen mounts with colloidal silver paste, air dried overnight and then sputter-coated (Denton Vacuum Desk-1, Cherry Hill, New Jersey) with gold (approx. 15 nm thickness). The specimens were examined and photographed on a Hitachi S-570 scanning electron microscope at 20 kV. For TEM, the specimens were stained in 5% uranyl acetate in 50% ethanol (1 hr), infiltrated with Spurr's low-viscosity embedding medium (27) and polymerized for 8 hr at 70 C. Serial sections were cut with a diamond knife using a Reichert OM-U3 ultramicrotome (Wien, Austria). Sections were mounted on slotted, formvar-coated grids, stained with 5% aqueous lead citrate and 5% uranyl acetate and examined and photographed using a Hitachi H-7100 transmission electron microscope at 75 kV.

RESULTS

Examinations of dry mount specimens revealed that freshly collected pollen grains of canola were ellipsoidal in shape, with the germinative clefts (colpate) visible as deep, longitudinal furrows. The average volume of the ellipsoid pollen was $3.36\times10^5\,\mu m^3$ (Table 1). After the pollen was immersed in water 24 hr, the shape of pollen grains had transformed from ellipsoidal to spheroidal. The average volume of the spherical pollen was $3.54\times10^5\,\mu m^3$,

TABLE 1. Size and shape of air dried and hydrated canola pollen grains

Condition[1]	Pollen Shape	N[2]	Projection Maximum (μm)	Projection Minimum (μm)	Aspect Ratio	Volume (μm^3)
Air-dried	Ellipsoidal	42	133.32±9.32	69.17±7.10	1.97± 0.24	336,352
Hydrated	Spheroidal	41	92.73±12.37	83.13±11.62	1.09±0.05	354,617

[1.] Air-dried = pollen grains dusted on glass slides; Hydrated = pollen grains submerged in sterile water for 24 hr.
[2.] Number of pollen grains measured.

representing a 5.2% increase compared to the air-dried pollen (Table 1). This small increase in volume of the hydrated, spheroidal pollen is due to a 30.4% reduction in the maximum projection and a 16.8% increase in the minimum projection (Table 1).

Pollen grains of canola stimulated germination of ascospores of S. sclerotiorum. After incubation at room temperature 48 hr, the average frequency of germination was 76.6%, ranging from 59 to 90%, in the ascospore-pollen mixture compared to 49.9%, ranging from 35 to 63% in the control. While the germ tubes from ascospores in water remained short with 0-2 septa, the germ tubes of the ascospores in the pollen suspension all developed into long, multi-branched hyphae. No germination of pollen grains was observed in the 48 hr samples with or without ascospores.

Each pollen grain of canola is tricolpate, with three elongated furrows that are equidistant from each other. Light and scanning electron microscopic examinations of 5-day- old ascospore-pollen mixtures showed that numerous pollen grains were infected by hyphae of *S. sclerotiorum* penetrating into the pollen (Figs. 1, 2, 3, 4) or re-emerged from the pollen (Fig. 5). Infection of a pollen grain occurred by direct hyphal penetration (Figs. 1, 2, 3, 4) without the mation of appressoria or infection cushions. Hyphal penetration or re-emergence through the furrows and germ pores (Figs. 4, 5) was more commonly observed than through other parts of the cell walls.

The ultrastructure of a healthy pollen grain of canola revealed well-defined cell walls, exine and intine layers, plasma membrane, dense cytoplasm and vacuoles (Fig. 6). Hyphal penetration (Fig. 7) or re-emergence (Fig. 8) through the cell walls was evident by the depression of the cell walls at the infection site. Extensive etching of the pollen wall associated with the infecting hyphae was observed (Fig. 9). Plasmolysis of the plasma membrane and disintegration of the cytoplasm occurred as a result of hyphal growth in the cell lumen of infected pollen grains (Fig. 9).

DISCUSSION

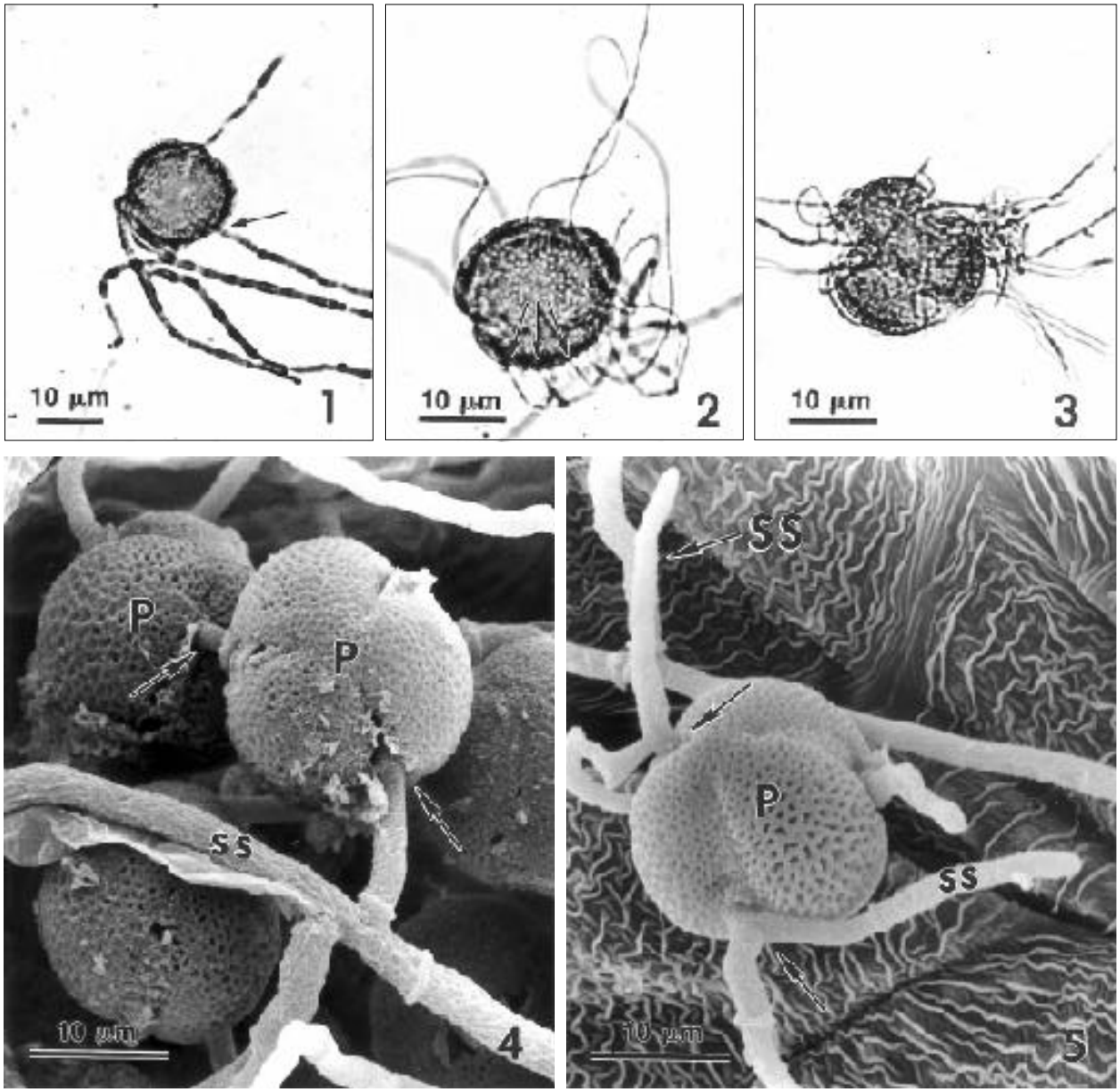
This study reveals that the shape of canola pollen grains is influenced by the level of hydration, ellipsoid in dry conditions and spheroid in moist conditions. The drastic changes in shape observed in the laboratory are likely to occur in nature because of environmental variations due to rain and dew. Iwanami *et al*. (17) reported that the thin exine layer of the pollen walls at the germinative pores or furrows permitted volume changes pollen grains. The 5.2% increase in cell volume of canola pollen when moistened may be due to absorption of water through the three germinative pores.

The results of the present study confirm other reports (7, 16, 30) that pollen stimulates ascospore germination and subsequent mycelial growth of *S. sclerotiorum*. Pollen grains are rich in protein, amino acids and sugars (33). Stanley and Search (28) reported that nutrients were released in water rapidly when dry pollen was moistened. The observed rapid development of germ tubes into multi-branched hyphae in the ascospore-pollen mixture may be due to nutrients released from canola pollen. In studying infection of pine pollen by *Retiarius superficiaris* sp. nov. and *Retiarius bovicornutus* sp. nov., Olivier (23) suggested that pollen diffusates serve as attractants the growth of hyphae toward the pollen.

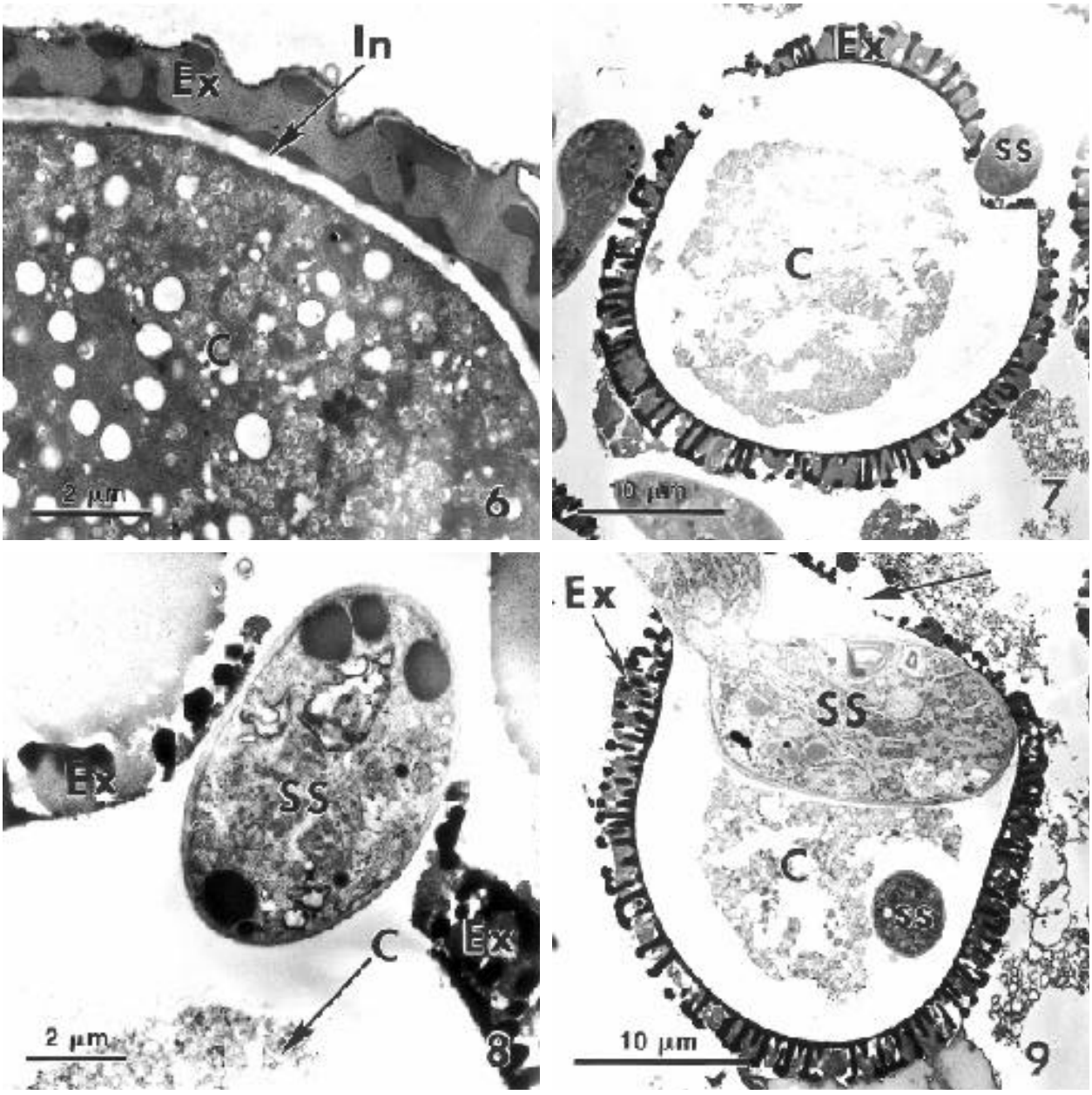
Infection of canola pollen by *S. sclerotiorum* occurs more readily through the germination pores than through other parts of the pollen walls. The weak resistance of germination pores was also observed in infection of pea pollen by *S. sclerotiorum* (12) and alfalfa pollen by *Verticillium albo-atrum* Reinke and Berthold (9), *S. sclerotiorum* (16), and pine (*Pinus radiata*) pollen by *R. superficiaris* and *R. bovicornutus* (23). The exine wall contains sporopollenins which are remarkably stable and resistant against various chemicals such as sulfuric acid (17). The ultrastructural evidence of the thin exine layer at the colpial regions in contrast to the thick exine layer in other parts of pollen walls, was observed in pollen grains of canola, dry pea (12) and alfalfa (9, 16). Knox and Heslop-Harrison (19) reported that cell wall protein was detected most abundantly in the intine layer at the colpial regions of tricolpate pollen grains. Therefore, the weak resistance of pollen germinative pores to fungal invasion may be related to their specific thin exine wall structure and rich intine-bound protein.

Previous reports suggest that infection of pollen coats is mechanical because of the evidence of slight depression of the cell wall caused by the penetrating hypha (23, 24). However, the evidence of cell wall depression and cell wall degradation at penetration sites suggests that both physical and chemical forces are involved in the infection of canola pollen by *S. sclerotiorum*. This process is identical to the infection of *S. sclerotiorum* on pea (12) and alfalfa (16) pollen as well as the infection of *V. albo-atrum* on alfalfa pollen (9).

Formation of appressoria and infection cushions are important features in the infection of plant tissues such as rapeseed leaves (18), pea pods (11) and bean hypocotyls (20) by *S. sclerotiorum*. However, evidence from present and



Figs. 1-5. Photomicrographs (Figs. 1-3) and scanning electron micrographs (Figs. 4, 5) showing infection of canola pollen by *Sclerotinia sclerotiorum*. Note the direct hyphal penetration of a pollen grain (Fig. 1, arrow), multiple infection sites on a pollen grain (Fig. 2, arrows) and destruction of a pollen grain (Fig. 3). Note also hyphae penetrating (Fig. 4, arrows) or emerging (Fig. 5, arrows) through germ pores of pollen grains. Fig. 1, ca x540; Figs. 2 and 3, ca x900; Figs. 4 and 5, x1760. SS = *Sclerotinia sclerotiorum*, P = pollen grain.



Figs. 6-9. Transmission electron micrographs showing healthy (Fig. 6) and *S. sclerotiorum*-infected (Figs. 7-9) pollen grains of canola. Note a hypha penetrating (Fig. 7) or emerging (Fig. 8) through the exine layer of the cell walls, partial collapse of exine wall (Fig. 9, arrow) and cytoplasm disintegration of the infected pollen grains. Fig. 6, x10,400; Figs. 7 and 9, x3,250; Fig. 8. x9,600. SS = *Sclerotinia sclerotiorum*, Ex = exine wall, In = intine wall, C = cytoplasm.Document No. 9798

previous studies (9) indicates that infection of pollen by fungal pathogens including *S. sclerotiorum* (12, 16) is achieved by direct hyphal penetration without the formation of appressoria or infection cushions. Olivier (23) reported that a swelling of the hyphal tip of *R. superficiaris* before penetration of the pine pollen coats was sometimes noticeable, but no true appressorium was formed. The reason for the difference in the mode of infection between pollen and other plant tissues by the same pathogen remains unknown.

In addition to senescent petals (21, 31), and stamens (11), pollen may be another important source of substrate for primary infection of host plants by ascospores of *S. sclerotiorum.* Pollen is an important food for bees, and the sole source of nitrogen for young bees (4). Huang *et al*. (13) observed that alfalfa leafcutter bees (*Megachile rotundata* (Fab.)) foraging in an alfalfa field with high incidence of Verticillium wilt often carried *V. albo-atrum*-infected pollen grains on their bodies. Stelfox *et al*. (29) reported that *S. sclerotiorum*-contaminated pollen grains were transmitted to rapeseed flowers by honeybees and caused pod rot of rapeseed. The danger of transmission of the pathogen to pods and seeds of canola by *S. sclerotiorum* would be further increased if the pathogen-infected pollen grains were transported to stigmas, anthers or petals by honeybees during their flower visiting and foraging activities.

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摘　要

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由影像分析研究顯示新鮮風乾之油菜 (Conola) 花粉粒多呈橢圓形，經吸水後則轉變為圓形而且容積增大約 5.2%。花粉粒置於無菌水中可以促進菌核病菌 (*Sclerotinia sclerotiorum*) 的子囊孢子 (ascospores) 之發芽與菌絲之生長。用顯微鏡檢查孢子與花粉混合培養 5 天的材料，可以觀察到很多圓形花粉粒受子囊孢子侵染，所有為害方式都是由菌絲尖端直接穿透花粉細胞壁，而未有經由特殊入侵器 (Infection cushion) 或附著器 (appressoria) 才造成入侵的現象。一般菌絲經由花粉粒之發芽孔入侵較為常見，而由其他花粉部位入侵則較為罕見。菌絲入侵後可導致花粉粒細胞原生質分離，細胞質融解以至於細胞壁破碎等現象。

關鍵詞：油菜、Canola, *Brassica napus*, 花粉、菌核病菌、*Sclerotinia Sclerotiorum*。