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Infection of cotton pollen by *Verticillium dahliae*

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ABSTRACT

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A light- and electron-microscopic study revealed that the presence of cotton pollen grains or cotton pollen diffusates enhanced the growth of germ tubes of *Verticillium dahliae*, the causal agent of Verticillium wilt of cotton. Microscopic examinations of 4-day-old samples of pollen- spore mixtures revealed that cotton pollen grains were susceptible to infection by *V. dahliae*. Infection of pollen grains occurred by direct hyphal tip penetration of the pollen cell walls, ramification of invaded hyphae in the cell lumen, and disintegration of pollen cytoplasm. Sharp etching and cleavage of both exine and intine layers of pollen cell walls were evident at the penetration sites. The potential impact of *V. dahliae*-infected pollen on the epidemiology of Verticillium wilt of cotton was also discussed.

Key words: cotton, infection of pollen, *Gossypium hirsutum*, *Verticillium dahliae*, verticillium wilt.

INTRODUCTION

Verticillium wilt, caused by *Verticillium dahliae* Kleb., is an important disease of cotton (*Gossypium hirsutum* L.) [1] and it has caused severe yield losses in China, especially during the 1990's [18,21]. Symptoms of Verticillium wilt of cotton appeared most frequently from early flowering stage to maturity [17]. The flowering period of cotton is long-lasting due to indeterminate flowering habit.

Previous reports indicate that pollen diffusates are an excellent source of nutrients for promoting spore germination and mycelial growth of fungi, such as *Sclerotinia sclerotiorum* (Lib.) de Bary [7,25]. Other reports indicate that pollen grains are susceptible to infection by fungal pathogens. For example, pollen grains of alfalfa (*Medicago sativa* L.) are susceptible to infection by *Verticillium albo-atrum* Reinke & Berth. [9], *S. sclerotiorum* [12] and *Botrytis cinerea* Pers. : Fr. [13], and pollen grains of pea (*Pisum sativum* L.) [10], and canola (*Brassica napus* L.) [14] are susceptible to infection by *S. sclerotiorum*. Infection of cotton pollen by *V. dahliae* has not been reported. The objectives of this study were to determine the effect of cotton pollen and pollen diffusate on germination of spores of *V. dahliae* and to demonstrate the infection process of cotton pollen by *V. dahliae*.

MATERIALS AND METHODS

Effect of cotton pollen and pollen diffusates on spore germination and germ-tube growth of *V. dahliae*

Seeds of cotton, cultivar 868, were sown in Cornell peat-lite mix [3] in plastic pots (15 cm diam.) and plants were kept in a greenhouse and watered as required. Cotton pollen grains, collected from newly-opened flowers, were added to sterile water to prepare a suspension, containing 10^3 pollen grains ml^{-1}. After incubation for 24 h, the suspension was filtered through a sterile filter (0.2 μm) (NALGENE Brand Products, Nalge Company, Rochester, New York 14602-0365 USA) to collect pollen diffusates.

An aqueous spore suspension, containing approximately 10^3 spores ml^{-1} of *V. dahliae*, was made from 15-day-old cultures that were grown at room temperature (20-22℃) in potato dextrose broth, in bottles, agitated on a shaker (160 rpm). The spore suspension was mixed with pollen diffusate at 1:1 ratio (v/v), or it was mixed with a suspension containing 10^3 pollen grains ml^{-1} at 1:1 ratio (v/v). The mixtures were flooded on the surface of water agar in Petri dishes, 0.5 ml/dish. After incubation at room temperature for 9, 13, and 24 h, the cultures were examined for the germination of conidia and the growth of germ tubes using a Zeiss Photomicroscope III. For controls, the spore suspension, 10^3 spores ml^{-1}, and the pollen suspension, 10^3 pollen grains ml^{-1}, were flooded on water agar separately at 0.5 ml/dish and examined microscopically following the same method described above. The growth of germ tubes of individual conidia was scored using a 1 to 5 scale: 1, germ tube is indiscernible; 2, germ tube is shorter than the length of the conidium; 3, germ tube is longer than the length of the conidium or has one lateral branch; 4, germ tube has 2-3 lateral branches; and 5, germ tube has more than 3 lateral branches. A growth index (GI) was calculated for each

treatment using the formula: GI = Σ (N x G) / T, where N = number of conidia, G = germ tube growth rating, and T = total number of conidia examined. Data on growth index of germ tubes were collected based on 50 germinated conidia in each replicate (petri dish) at 3 replicates per treatment.

Inoculation of Cotton Pollen with *V. dahliae*

To inoculate pollen grains with *V. dahliae*, a spore suspension, containing10^3 conidia/ml^{-1} was flooded on water agar in Petri dishes, 0.5 ml/dish, and pollen grains from freshly-excised cotton flowers were dusted on the agar surface. The Petri dish cultures were incubated for 4 days, at room temperature, and examined for colonization and sporulation of *V. dahliae* on pollen grains using a Zeiss Photomicroscope III. For controls, pollen grains and *V. dahliae* spores in sterile water, respectively, were prepared for microscopic examination by the same method described above. For each treatment, there were 6 replicates (petri dishes).

Scanning Electron Microscopy (SEM)

For SEM, the 4-day-old pollen samples inoculated with *V. dahliae*, and uninoculated control, were immersed in 2% glutaraldehyde fixative in 0.05 M sodium phosphate buffer, pH 7.0, at 4 ℃ overnight (16 h) and then moved to room temperature. The samples were washed 3 times, 10 min. each, with the sodium phosphate buffer solution. The specimens were dehydrated in a graded ethanol series 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.

Transmission Electron Microscopy (TEM)

For TEM, the 4-day-old pollen samples, inoculated with *V. dahliae* and uninoculated control, were immersed in 2% glutaraldehyde in 0.05 M sodium cacodylate buffer, pH 7.0, at 4℃ overnight (16 h) and then moved to room temperature. The samples were washed 3 times, 10 min. each, with the 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 ethanol series, infiltrated with Spurr's low-viscosity embedding medium [22] and polymerized 8 h at 70℃. 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

Conidia of *V. dahliae* germinated readily with or without the presence of pollen grains or pollen diffusates, and after incubation for 24 h, the germination rate reached 100% for all treatments including control (Table 1). However, conidia germinated more rapidly in the presence of pollen or pollen diffusates. For example, after incubation for 9 h, 68.7% and 64.0% of conidia germinated for the treatments of pollen and pollen diffusates, respectively, whereas only 44.0% of conidia germinated for the control. Moreover, pollen and pollen diffusates stimulated the growth and development of germ tubes of conidia. After incubation for 24 h, the growth index of germ tubes was 3.25 for the untreated control, which was significantly (P<0.05) lower than 4.63 and 4.76 for the treatments of pollen grains and pollen diffusates, respectively (Table 1). While the germ tubes in the untreated control remained short and mostly unbranched, the germ tubes in samples treated with pollen grains or pollen diffusates grew rapidly and developed into long, multi-branched hyphae.

A healthy pollen grain of cotton has numerous spines (Fig. 1) and the surface of the pollen grain is covered with a mucilaginous substance (Fig. 2). Examination of 4-day-old samples of pollen-conidia mixtures revealed that pollen grains were colonized by mycelia of *V. dahliae* resulting in the formation of verticillate conidiophores on the pollen grain (Fig. 3). No mucilaginous substance was observed on the surface of pollen grains colonized by *V. dahliae* (Fig. 4). Infection of pollen grains by *V. dahliae* was frequently observed in the 4-day-old samples. Infection occurred by

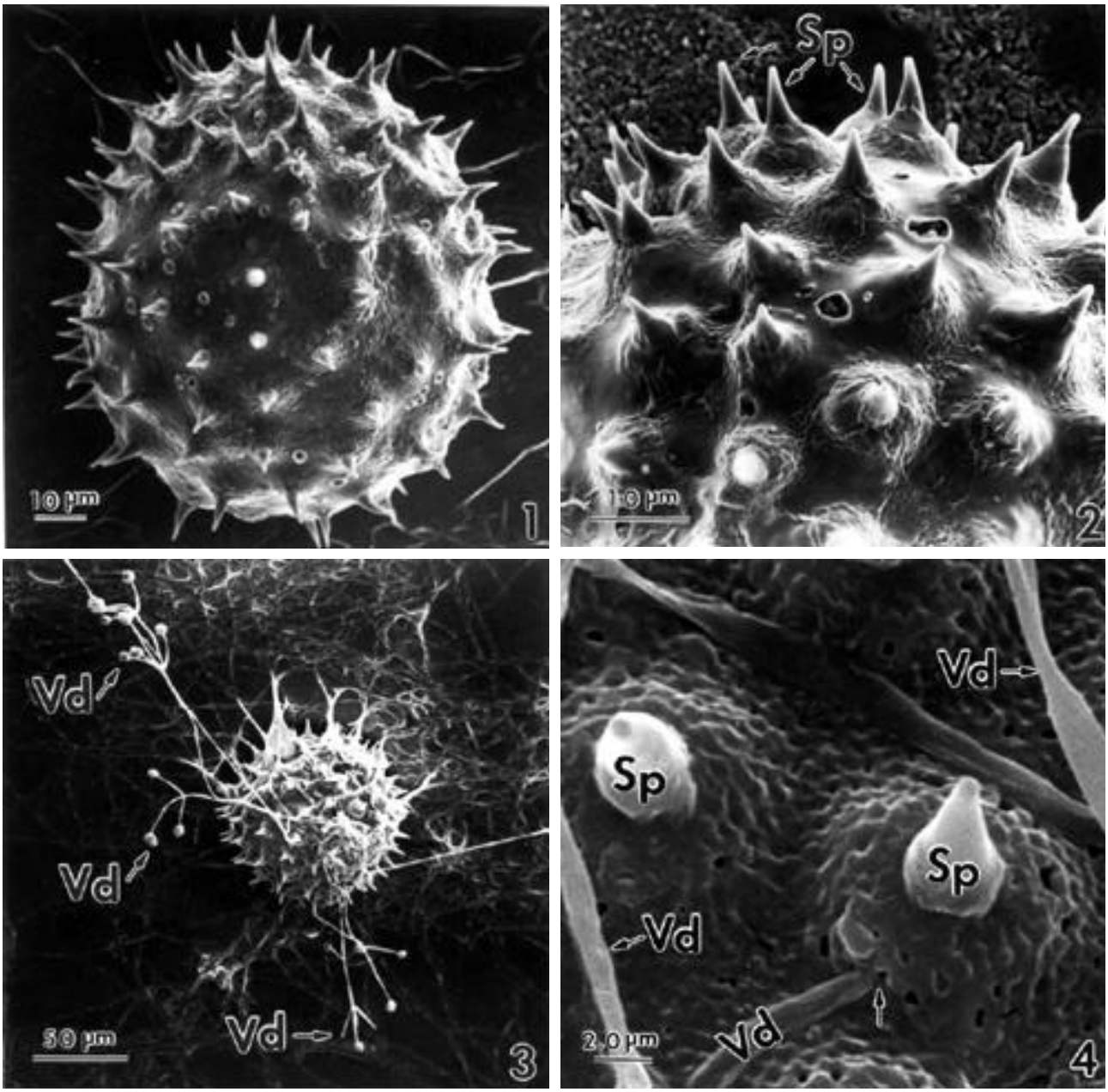
Table 1. Effects of pollen grains and pollen diffusates of cotton on germination of conidia and growth of germ tubes of *Verticillium dahliae*.

Treatment	Incubation time[1]					
	9 h		13 h		24 h	
	Germination (%)	Growth Index[2] (1-5)	Germination (%)	Growth Index[2] (1-5)	Germination (%)	Growth Index[2] (1-5)
Pollen grains	68.7 a [3]	1.69 a	98.0	2.68 a	100	4.63 a
Pollen diffusate	64.0 a	1.64 a	99.3	2.51 a	100	4.76 a
Control	44.0 b	1.44 b	89.3	2.19 b	100	3.25 b

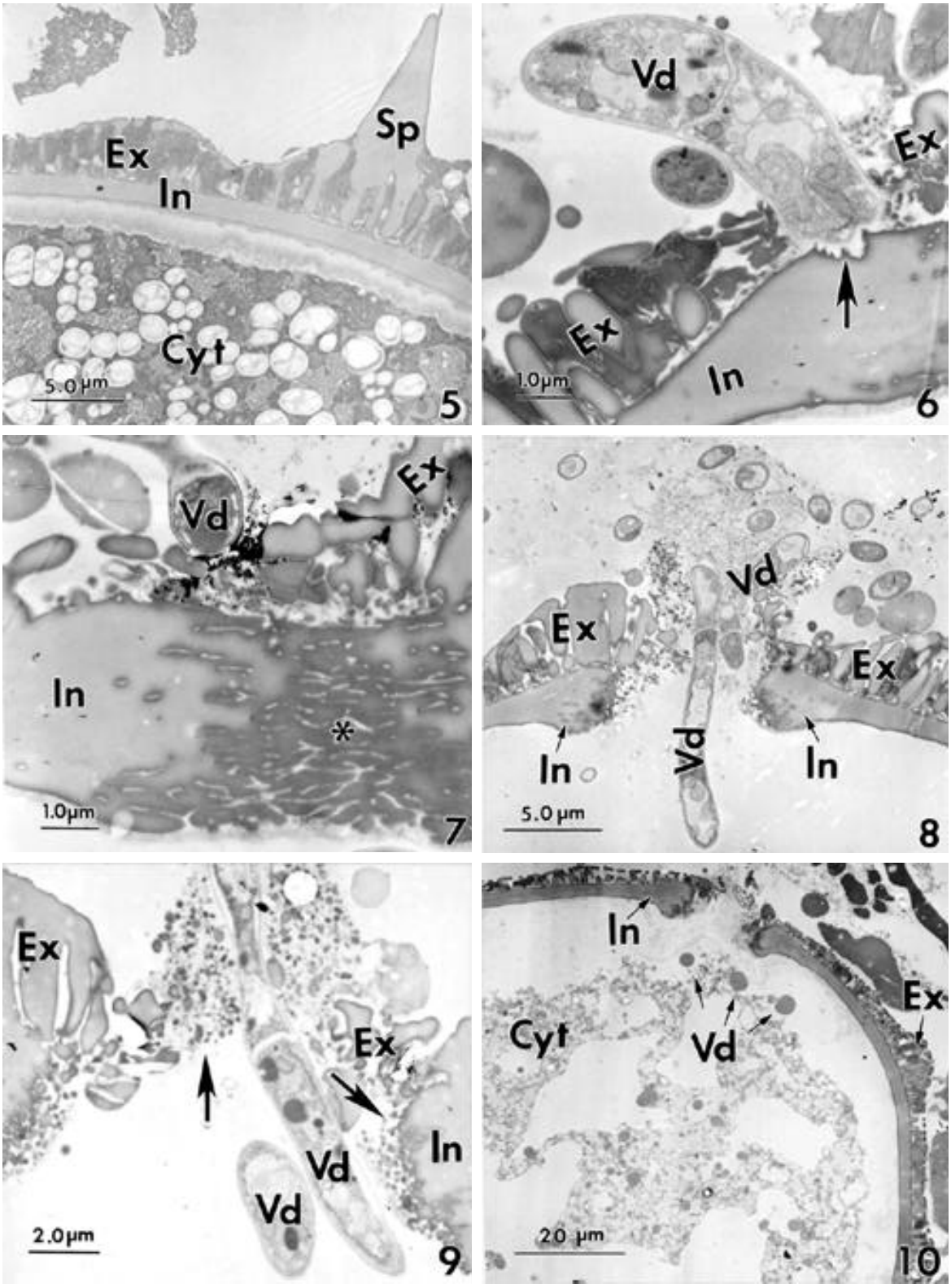
1. Data were collected after incubation for 9 h, 13 h and 24 h.
2. Growth Index: 1: germ tube is indiscernible; 2: germ tube is shorter than the length of the conidium; 3: germ tube is longer than the length of the conidium or has one lateral branch; 4: germ tube has 2-3 lateral branches; 5: germ tube has more than 3 lateral branches.
3. Means followed by same letters are not significantly different at P=0.05 level (Duncan's Multiple Range Test).

hyphal tips, which penetrated into the pollen cell walls without the formation of special structures, such as appressoria or infection cushions (Fig. 4).

Results of TEM studies showed that a healthy pollen grain of cotton contained two well- defined wall layers, exine and intine, a thin plasma membrane, and dense cytoplasm with numerous starch granules (Fig. 5). The sharp spines were formed by the protrusion of the exine wall. When a cotton pollen grain was infected by *V. dahliae*, the most prominent feature was the breakdown of the exine layer (Figs. 6-9) and the swelling of the intine layer (Figs. 6, 8, 10) at each hyphal penetration site. Sharp etching (Fig. 6) and cleavage (Fig. 7) of the intine layer were evident during the early stage of hyphal penetration. Further progress of hyphal penetration resulted in the complete breakdown of cell walls with wall remnants present at each penetration site (Figs. 6, 7), the presence of hyphae inside the pollen grain (Fig. 10), the separation of plasma membrane from the cell wall, and the disintegration of pollen cytoplasm resulting in formation of large vacuoles (Fig. 10).



Figs. 1-4. Scanning electron micrographs of cotton pollen. Healthy pollen grains with numerous spines (Sp) and an outer coating of mucilaginous substance (Figs. 1 and 2). *Verticillium dahliae*-colonized pollen grains with the formation of verticillate conidiophores (Fig. 3), and the penetration of the pollen cell wall (Fig. 4, arrow) by hypha of *V. dahliae* (Vd). Note the absence of the mucilaginous coating and the appearance of the pitted pollen cell wall (Fig. 4).



Figs. 5-10. Transmission electron micrographs of healthy (Fig. 5) and *Verticillium dahliae*-infected (Figs. 6-10) pollen grains of cotton. Fig 5. Cross-sectional view of a healthy pollen grain showing spines (Sp), the exine (Ex) and intine (In) layers of the cell wall, and the dense cytoplasm (Cyt) that contains numerous starch granules. Figs. 6-10. Hyphal penetration of pollen grains showing the breakdown of exine layer (Ex) and intine layer (In) with the cell wall remnants (Fig. 9, arrows) at the infection sites, as well as the disintegration of pollen cytoplasm associated with the formation of large vacuoles (Fig. 10). Note the etching (Fig. 6, arrow), swelling (Figs. 6,7) and breakdown (Fig. 7, asterisk) of the intine wall (In). Note also the presence of hyphal cells of *V. dahliae* (Vd) in the infected pollen grain (Fig. 10).

DISCUSSION

The stimulatory effects of cotton pollen diffusates on growth of germ tubes of *V. dahliae* indicates that substances leached from cotton pollen are important source of nutrients for growth and development of the fungal pathogen. Pollen grains are rich in protein, amino acids and sugars [26], but the amount of protein and other substances released varied with pollen species [23]. Olivier [20] observed that diffusates of pine pollen attracted the hyphal growth of *Retiarius superficiaris* sp. Nov. and *Retiarius bovicornutus* sp. Nov. The intense colonization and sporulation of *V. dahliae* on cotton pollen grains observed in present study (Fig. 3) suggests that cotton pollen diffusates may serve as important attractants for the pathogen. Colonization of pollen grains by *V. dahliae* resulted in the removal of the mucilaginous substance from the pollen grain (Fig. 4). Whether the mucilaginous substance affects the growth and development of *V. dahliae* remains unknown. The SEM and TEM studies reveals that cotton pollen is susceptible to infection by *V. dahliae*. Infection of pollen grains was achieved by direct hyphal tip penetration of the pollen cell walls without the formation of appressoria. This mode of hyphal penetration is similar to the infection of pollen grains of alfalfa (*Medicago sativa* L.) by *Verticillium albo-atrum* Reinke. & Berthold [9,11], *Sclerotinia sclerotiorum* (Lib.) de Bary [12], and *Botrytis cinerea* Pers. : Fr. [13], as well as infection of pollen grains of canola (*Brassica* spp) [14] and dry pea (*Pisum sativum* L.) [10] by *S. sclerotiorum*. Knox and Heslop-Harrison [15] studied pollen from 50 angiosperm and pine species and found that activities of enzymes such as acid phosphatase, ribonuclease, amylase and protease, were concentrated in the intine layer of the pollen grain, especially around germ pores. The intine enzymes are readily leachable and are believed to be involved in nutrition of the male gamete and also in digestion of the stigma as part of the fertilization process [15]. The evidence of etching and dissolving of exine and intine walls (Fig. 6) at each infection site suggests that enzymatic activity is also involved in the infection of cotton pollen by *V. dahliae*. The cleavage of the swollen intine layer (Fig.7) may be the result of strong enzyme activities of the pollen grains in response to infection by the pathogen.

The present study reveals that cotton pollen is a suitable habitat for *V. dahliae*, as it is readily colonized by this pathogen. Moreover, the infection of pollen grains by *V. dahliae* may be of epidemiological significance for Verticillium wilt of cotton. Previous reports indicated that pollen enhanced pathogenicity and severity of plant diseases caused by fungal pathogens, such as *B. cinerea* [2,5,19] and *S. sclerotiorum* [6,8]. Huang *et al*. [11] observed that alfalfa pollen grains infected by *V. albo-atrum* were carried by leafcutter bees (*Megachile rotundata*) foraging on diseased alfalfa plants in the field. Stelfox *et al*. [24] reported that pollen grains of rapeseed (*Brassica* spp.) contaminated with ascospores of *S. sclerotiorum* were transported by honey bees (*Apis mellifera*), causing head blight of rapeseed plants. Since *V. dahliae* has been reported to occur on the surface and inside the seeds of cotton [4], further investigations on the possibility of transmission of *V. dahliae*-infected pollen grains to cotton flowers by insects, pollinators in particular, are warranted.

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LITERATURE CITED

1. Bell, A.A. 1992. Verticillium wilt. Pages 87-126 in: Cotton Disease. R. J. Hillocks, ed. CAB International, Oxon, United Kingdom.
2. Bekesi, P. 1982. New inoculation method for infecting sunflowers by *Botrytis cinerea* Pers. Acta Phytopathologica Academiae Scientiarum Hungaricae, 17:221-224.
3. Boodley, J.W., and Sheldrake, Jr., R. 1977. Cornell Peat-Lite Mixes for Commercial Plant Growing. N. Y. State Coll. Agric. Life Sci., Info. Bull. 43, 8pp.
4. Chen, J-L., Chen, S.S., Wang, J.Y., and Xu, C.X. 1980. Studies on interior seed borne Verticillium of cotton. Acta Phytophylacica Sinica. 7:159-164.
5. Chou, M.C., and Preece, T.F. 1968. The effect of pollen grains on infections caused by *Botrytis cinerea* Fr. Ann. Appl. Biol. 62:11-22.
6. Dillard, H.R., and Hunter, J.E. 1986. Association of common ragweed with Sclerotinia rot of cabbage in New York State. Plant Dis. 70:26-28.
7. Hartill, W.F.T. 1975. Germination of *Botrytis* and *Sclerotinia* spores in the presence of pollen on tobacco leaves. N. Z. J. Agr. 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. Agr. Res. 17:147-152.
9. Huang, H.C., and Kokko, E.G. 1985. Infection of alfalfa pollen by *Verticillium albo-atrum*. Phytopathology 75:859-865.
10. Huang, H.C., and Kokko, E.G. 1993. Infection of pea pollen by *Sclerotinia sclerotiorum*. Trends in Agric. Sci.-Plant Pathol. 1:13-17.
11. Huang, H.C., Richards, K.W., and Kokko, E.G. 1986. The role of the leafcutter bee in dissemination of *Verticillium albo-atrum* in alfalfa. Phytopathology 76:75-79.
12. Huang, H.C., Kokko, E.G., and Erickson, R.S. 1997. Infection of alfalfa pollen by *Sclerotinia sclerotiorum*. Phytoparasitica 25:17-24.
13. Huang, H.C., Kokko, E.G., and Erickson, R.S. 1999. Infection of alfalfa pollen by *Botrytis cinerea*. Bot. Bull. Acad. Sinica 40:101-106.

14. Huang, H.C., Kokko, E.G., Erickson, R.S., and Hynes, R. K. 1998. Infection of canola pollen by *Sclerotinia sclerotiorum*. Plant Pathol. Bull. 7:71-77.
15. Knox, R.B., and Heslop-Harrison, J. 1970. Pollen-wall proteins: localization and enzymic activity. J. Cell Sci. 6:1-15.
16. Kokko, E.G., B. Gowen, and C.R. Jahnke. 1990. An inexpensive water volume control device for ultramicrotomy. in: Electron Microscopy 1990; Proc. 12th Int. Congr. For Electron Microscopy. San Francisco Press, USA, pp. 732-733.
17. Ma, C., and Chen, Q-Y. 1992. Progress on studies of cotton resistant breeding. Fusarium and Verticillium wilts in China. Scientia Agricultura Sinica. 25:50-57.
18. Ma, P. and Li, S. Z. 1996. Occurrence and severity of Verticillium wilt of cotton in Hebei. Pages 16-19 in: Proceedings of Symposium of Fusarium root rot and Verticillium wilt of cotton in China. Phytopathol.Soc. of China. (In Chinese)
19. Ogawa, J.M., and English, H. 1960. Blossom blight and green fruit rot of almond, apricot and plum caused by *Botrytis cinerea*. Plant Dis. Rep. 44:265-268.
20. Olivier, D.L. 1978. *Retiarius* gen. Nov.: Phyllosphere fungi which capture wind-borne pollen grains. Trans. Br. Mycol. Soc. 71:193-201.
21. Shi, R. Y., and Jian, G. L. 1994. Factors and control measures for breakout of Verticillium wilt of cotton. Plant Prot. 20(1):34-35.
22. Spurr, A.R. 1969. A low-viscosity epoxy embedding medium for electron microscopy. J. Ultrastruct. Res. 26:31-43.
23. Stanley, R.G., and Search, R.W. 1971. Pollen protein diffusates. Pages 174-176 in: Pollen: Development and Physiology. J. Heslop-Harrison, ed. Appleton Century Crofts, Meredith Corporation, New York.
24. 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.
25. 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.
26. Yamakawa, T. 1984. The effect of pollen on the infection of fruit vegetables with conidia of *Botrytis cinerea*. Proc. Kansas Plant Prot. Soc. 26:1-8.

摘　要

馬平[1]、黃鴻章[2,4]、Eric G. Kokko[2]、唐文華[3]. 2000. 棉花黃萎病菌 (*Verticillium dahliae*) 侵染花粉. 植病會刊 9:93-98 ([1.] 河北農林科學院植物保護研究所，河北省保定；[2.] 加拿大農部，Lethbridge 研究中心；[3.] 中國農業大學植病系，北京；[4.] 聯絡作者：電子郵件 huangh@em.agr.ca，傳真 (403) 382-3156)

利用光學顯微鏡及電子顯微鏡觀察來研究棉花花粉與棉花黃萎病菌 (*Verticillium dahliae*) 的關係，結果顯示新鮮花粉的水溶性浸出液能促進黃萎病菌分生孢子的發芽及發芽管的生長。將新鮮花粉與分生孢子混合培養 4 天，以顯微鏡觀察，顯示棉花花粉易受黃萎病菌侵染。入侵過程是由菌絲頂端直接穿透花粉細胞的外壁 (Exine) 與內壁 (Intine)，進而使菌絲在細胞內大量增殖，終至造成花粉細胞質崩潰解體。在每一菌絲入侵部位均會造成細胞內壁澎大，以及內、外壁受病原菌切割及融解的現象。此種帶菌花粉在棉花黃萎病流行病學上的重要性也在本文加以討論。

關鍵詞：棉花、花粉侵染、*Gossypium hirsutum*、*Verticillium dahliae*、黃萎病