

Cytochemical Study of the Antagonistic Activity of *Sporothrix flocculosa* on Rose Powdery Mildew, *Sphaerotheca pannosa* var. *rosae*

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ABSTRACT

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By using a wheat germ agglutinin-ovomucoid gold complex as a specific probe for localizing chitin distribution in cells of the rose powdery mildew fungus, *Sphaerotheca pannosa* var. *rosae*, and a reported antagonist fungus, *Sporothrix flocculosa*, we revealed a difference in cell wall composition of the two fungi as judged by the intensity of the labeling and the cytoplasm compactness. The same probe was used for indirectly determining whether or not chitinolytic activity was involved in the antagonistic action of *S. flocculosa* against *S. p. rosae*. Within 12 h after inoculation, the fungal host started to suffer some damage, characterized by a local retraction of the plasmalemma and an increase in cytoplasm compactness. Twenty-four hours after inoculation, close contact between

the two fungi was sometimes associated with the penetration of the host cells by the antagonist that caused considerable changes in the cytoplasm but no discernible alteration in the chitin labeling distribution over the cell walls. At this advanced stage of antagonism, *S. flocculosa* caused complete plasmolysis of host hyphae, which were reduced to only cell walls. Close contact between the interacting fungi did not appear necessary before the antagonistic effect was induced. Our results, based on ultrastructural observations and cytochemical localization of *N*-acetylglucosamine, suggest that antibiosis rather than chitinolytic activity is involved in the antagonistic process of *S. flocculosa* against *S. p. rosae*.

A number of fungi have been reported to be natural mycoparasites of powdery mildew fungi, and their potential value as biocontrol agents has often been suggested (2,3,27). The antagonistic relationship between the epiphytic yeastlike fungi, *Stephanoascus flocculosus* Traquair, Shaw & Jarvis (anamorph: *Sporothrix flocculosa* Traquair, Shaw & Jarvis) or *S. rugulosus*, and the powdery mildew pathogens of rose and cucumber has been described (10,13). Under controlled conditions, application of 1×10^6 spores per square milliliter of *S. flocculosa* to leaves of rose infected with *Sphaerotheca pannosa* (Wallr.:Fr.) Lév. var. *rosae* Woronichin reduced powdery mildew colonies by more than 80% within 48 h (10). Although it was reported that *S. flocculosa* could severely alter mycelial growth and spore production by the pathogen, the mechanisms underlying this antagonistic interaction are still unclear.

It is now well established that successful antagonism of plant pathogens by saprophytic microorganisms operates by nutrient competition, hyperparasitism, and/or antibiosis (2,8,24,29). However, in the case of obligate parasites such as powdery mildews, nutrient competitors are unlikely to be effective (26). In the case of *Sporothrix*, direct antagonism is one of the main mechanisms that has been suggested to explain activity against powdery mildew fungi (13).

In mycoparasitism, cell surface components of both partners play an important role in cell-to-cell interactions and cell recognition (16-19). Chitin, a β -1,4-linked homopolymer of *N*-acetyl-D-glucosamine, is a ubiquitous wall component of many plant pathogenic fungi (30). Previous studies have shown that fungal chitin is a potential substrate for plant and mycoparasite chitinases. These enzymes have been shown to inhibit hyphal growth and cause lysis of spores and hyphal tips when applied to fungi in vitro (5,9,22). Because the antagonistic activity against powdery mildew fungi can only be visualized in the host plant, localization

of carbohydrate-containing molecules at the fungal cell surface may be a valuable approach for indirectly determining whether or not hydrolytic enzymes are involved in the process.

The wheat germ agglutinin has multiple binding sites that are complementary to a sequence of three β -1,4-linked *N*-acetyl-D-glucosamine residues and therefore bind strongly to chitin and chitin oligomers (1,7). Wheat germ agglutinin, in conjunction with ovomucoid-conjugated to colloidal gold, has been used widely as a cytochemical probe to detect and localize chitin in cell walls of a large variety of fungi (1). In the present study, the wheat germ agglutinin-ovomucoid gold-complex was applied to rose leaves infected with *S. p. rosae* and treated with *S. flocculosa*. Our main objectives were to investigate the localization of a potential substrate for chitinase in healthy as well as in damaged hyphae of *S. p. rosae* and to gain a better insight into the antagonistic activity of *S. flocculosa* against the rose powdery mildew pathogen.

MATERIALS AND METHODS

Fungal cultures and inoculations. *S. flocculosa* was kindly provided by W. R. Jarvis and J. A. Traquair, Agriculture Canada, Harrow Research Station, Ontario. Cultures of *S. flocculosa* were maintained at room temperature on 2% malt extract agar. Conidial suspensions of the antagonist were obtained from 5-day-old cultures by washing the colonies with sterile water. Concentration of the suspension was adjusted to 1×10^6 conidia per square milliliter according to Jarvis et al (13). Rose plants (cv. Ruiredro) naturally infected with *S. p. rosae* were maintained in a glasshouse at 21 C. Individual leaflets were selected for study when approximately half of their surface area was covered with mildew colonies. They were excised and placed on moist filter paper in petri dishes and were sprayed to wetness with a spore suspension of *S. flocculosa*. For controls, infected leaflets collected from the same plants were sprayed with sterile distilled water. The dishes were left open for 30 min and then closed and incubated at 26 C under

mixed fluorescent and incandescent light at $30 \mu\text{E m}^{-2} \text{s}^{-1}$ for a 12-h photoperiod.

Tissue processing for electron microscopy investigations. Twelve and 24 h after inoculation with *S. flocculosa*, leaflet samples were cut into 1-mm² pieces and immersed in glutaraldehyde (3%, v/v) in 0.1 M sodium cacodylate at pH 7.2 for 2 h at 4 C, then rinsed with the same buffer, and postfixed with osmium tetroxide (1%, w/v) for 1 h at 4 C in sodium cacodylate buffer. Leaflets were dehydrated in a series of ethanol solutions graded in 10% steps and embedded in Epon 812. Ultrathin sections were collected on Formvar-coated nickel grids and processed for cytochemical labeling.

Preparation of the gold-complexed probe and cytochemical labeling. The colloidal gold suspension was prepared as described by Benhamou (1). Because of its low molecular weight, the wheat germ agglutinin, a lectin with *N*-acetylglucosamine-binding specificity, could not be directly complexed to gold and was used

in a two-step procedure for studying the distribution of chitin, a linear polysaccharide composed of β -1,4-linked *N*-acetylglucosamine units. The ovomucoid, a high molecular weight protein from egg white, was complexed to colloidal gold at pH 4.8 and was used as a second-step reagent.

Sections were first incubated on a drop of phosphate-buffered saline (PBS) at pH 7.2 for 5 min and then were transferred on a drop of wheat germ agglutinin (10 $\mu\text{g/ml}$) in PBS at pH 7.2 for 30 min at room temperature. They were rinsed with PBS and incubated on the gold-complexed ovomucoid for 30 min. Sections were subsequently washed with PBS and distilled water, and then contrasted with uranyl acetate and lead citrate. Specimens were examined with a JEOL 1200 transmission electron microscope with an accelerating voltage of 80 kV.

Cytochemical controls. Sections were incubated on the lectin previously adsorbed with *N*-*N'*-*N''*-triacetylchitotriose and then treated with the gold-complexed protein.

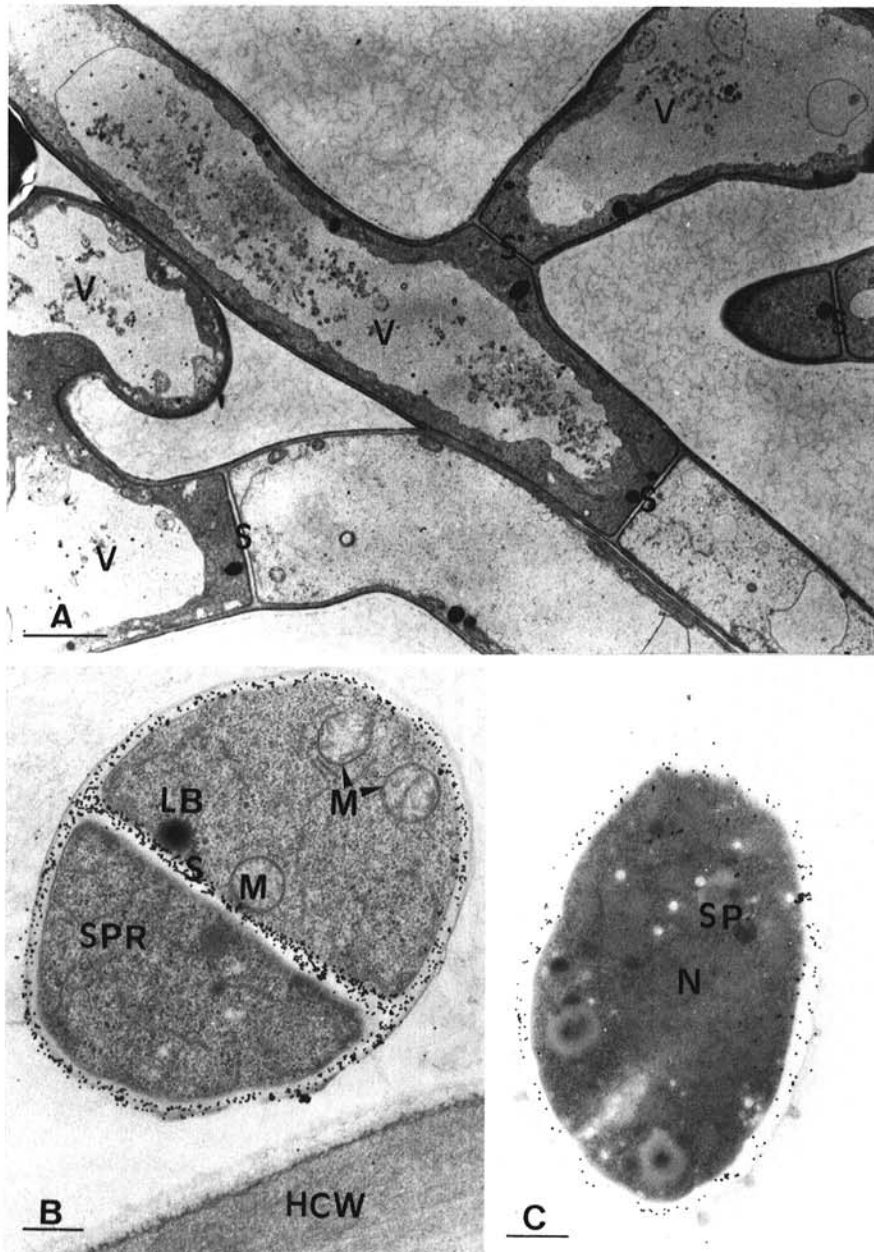


Fig. 1. Transmission electron micrographs of cells of *Sphaerotheca pannosa* var. *rosae* (SPR) and *Sporothrix flocculosa* (SP) treated with wheat germ agglutinin-ovomucoid gold complex. **A**, Control test. Labeling is absent over the vegetative structure of *S. p. rosae* after incubation with wheat germ agglutinin-ovomucoid gold complex, which was previously adsorbed with *N*-*N'*-*N''*-triacetylchitotriose. VA = vacuole; S = septum. **B**, An intense deposition of gold particles over the wall and septum (S) of *S. p. rosae*. Cytoplasm, lipid bodies (LB), mitochondria (M), and the plant cell wall (HCW) are unlabeled. **C**, Numerous gold particles distributed over the electron-lucent wall of *S. flocculosa* are observed. Cytoplasm and nucleus (N) are devoid of labeling. Bar = 0.25 μm .

RESULTS

Ultrastructural features and chitin distribution in *S. p. rosae* and *S. flocculosa*. Examination of the vegetative structures of the pathogen, *S. p. rosae*, showed that the mycelium was composed of regularly septate hyphae surrounded by a thin cell wall (Fig. 1A). In most cases, hyphal cells were branched at diverging angles with septum delimiting each element. The main ultrastructural feature of these cells was the presence of a large, centrally located vacuole in which numerous vesicular structures accumulated. The cytoplasm was usually appressed against the cell wall and contained several mitochondria and other typical organelles such as endoplasmic reticulum. Incubation with the wheat germ agglutinin-ovomuroid gold complex resulted in a specific and regular deposition of gold particles over cell walls and septa (Fig. 1B). In contrast, the cytoplasm, the organelles, and the plant host cell wall were unlabeled.

Observation of sections of *S. flocculosa* revealed the occurrence of hyphal cells that differed from those of *S. p. rosae* by their more compact cytoplasm and by the absence of large vacuoles (Fig. 1C). A number of organelles, often hardly discernible, included structures resembling lipid bodies and appeared embedded in the dense cytoplasm. After incubation with the wheat germ agglutinin-ovomuroid gold complex, the electron-lucent cell wall was the only structure to be labeled (Fig. 1C). Gold particles were scattered and apparently less numerous than over *S. p. rosae* cell walls.

The specificity of labeling observed with the wheat germ agglutinin-ovomuroid gold complex in this study was assessed through the negative results obtained after preadsorption of the lectin with *N-N'-N''*-triacetylchitotriose (Fig. 1A).

Ultrastructural features and chitin distribution in a *S. p. rosae*-*S. flocculosa* interaction. Close contact between both fungi was established by 12 h after inoculation (Fig. 2A). A layer of fine

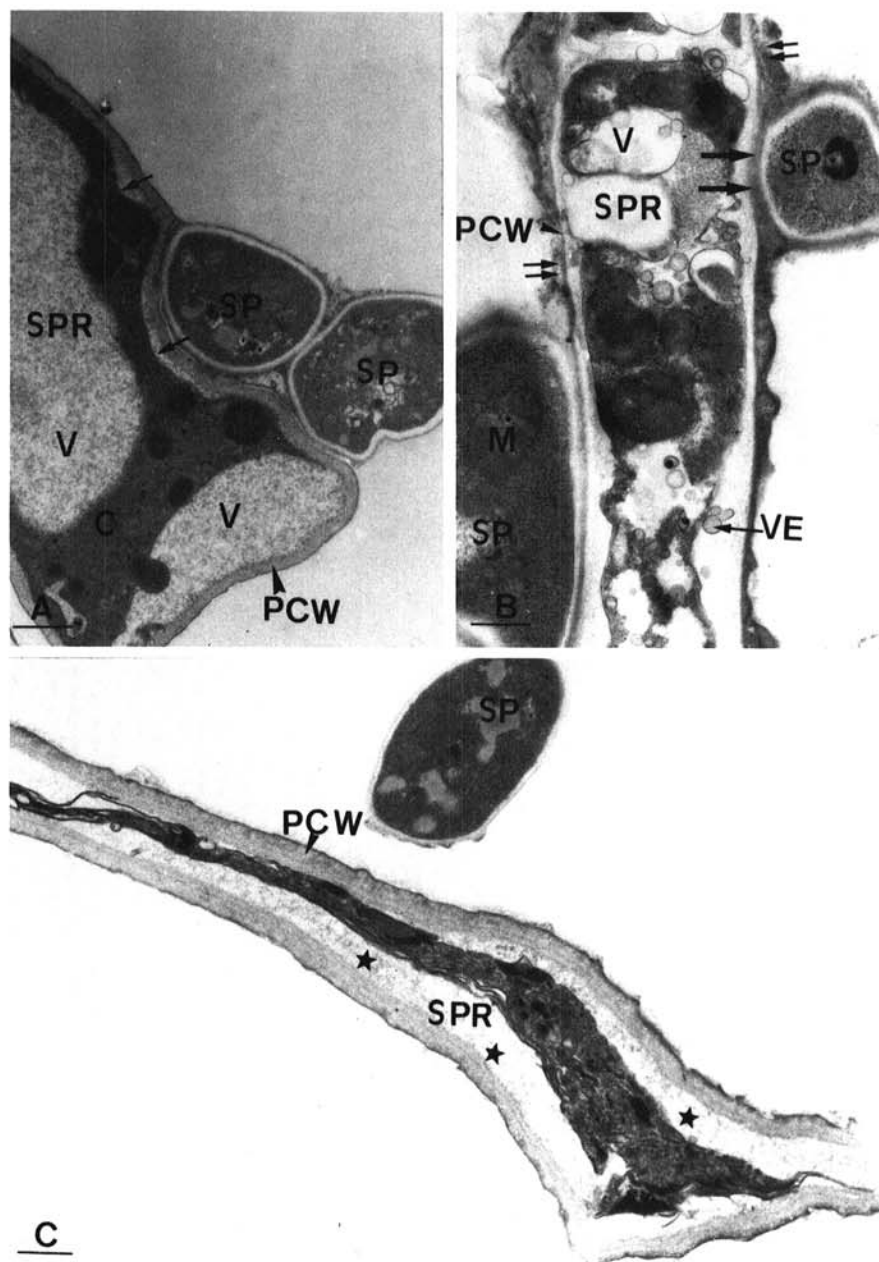


Fig. 2. Transmission electron micrographs of *Sphaerotheca pannosa* var. *rosae* (SPR) inoculated with *Sporothrix flocculosa* (SP). **A**, At 12 h after inoculation, close contact between the two fungi, plasmalemma retraction (arrows), and increase of cytoplasm (C) compactness of the fungal host are observed. The vacuoles (V) appear granular. **B**, At 24 h after inoculation, alteration of host cytoplasm occurs. Fragmentation of the large vacuole (V) into small polymorphic ones and the presence of vesicles (VE) are observed. PCW = pathogen cell wall; M = mitochondria. **C**, No contact between *S. flocculosa* and *S. p. rosae* results in host plasmalemma retraction, cytoplasm aggregation, and accumulation of granular material (stars) between the pathogen cell wall (PCW) and the plasmalemma. Bar = 0.25 μ m.

fibrillar material surrounded *S. flocculosa*, separating the antagonist wall from the pathogen cell wall. At this time, there was a local retraction of the plasmalemma (Fig. 2A, arrows) and an increase in cytoplasm compactness in *S. p. rosae*. Most organelles were hardly discernible and appeared as electron-opaque structures of various sizes and shapes. In contrast, the wall was apparently unaltered, as judged by its well-preserved architecture.

By 24 h after inoculation, more obvious damage to the cells of *S. p. rosae* was noticeable (Fig. 2B,C). The plasmalemma was highly retracted, and the cytoplasm was reduced to strands of fibrillo-granular material in which electron-opaque bodies and vesicular structures were present. Fragmentation of the large, centrally located vacuole into small polymorphic vacuoles also was observed. The fibrillar matrix surrounding the antagonist cell wall extended along the pathogen cell wall that, in some places, was slightly altered (Fig. 2B, double arrow). Severe pathogen cell alteration was observed even without close contact between both fungi (Fig. 2C). Indeed, plasmalemma retraction, severe cytoplasm aggregation, and deposition of an anamorphous

material between the cell wall and the plasmalemma occurred in cells of the pathogen even when the pathogen was relatively far away from the antagonist.

Incubation of sections with the wheat germ agglutinin-ovomucoid gold complex revealed an apparent preservation of cell wall structures as judged by the regular and intense labeling observed over the pathogen cell wall (Fig. 3A-C). In contrast, disintegration and necrosis of the cytoplasm of *S. p. rosae* were consistently observed (Fig. 3A,B). Sometimes, close contact between both partners was associated with the inclusion of *S. flocculosa* in *S. p. rosae* hyphae, thus causing considerable changes in the host cell cytoplasm (Fig. 3C). Interestingly, the occurrence of *S. flocculosa* inside hyphae of *S. p. rosae* did not apparently result in an alteration of the labeling distribution over the cell walls.

At a later stage after inoculation (24 h), *S. flocculosa* caused complete plasmolysis of *S. p. rosae* cells (Fig. 4A-C). All addressed host cells or nearby cells of *S. flocculosa* were completely depleted of their cytoplasm, and in certain cases only some remnants of

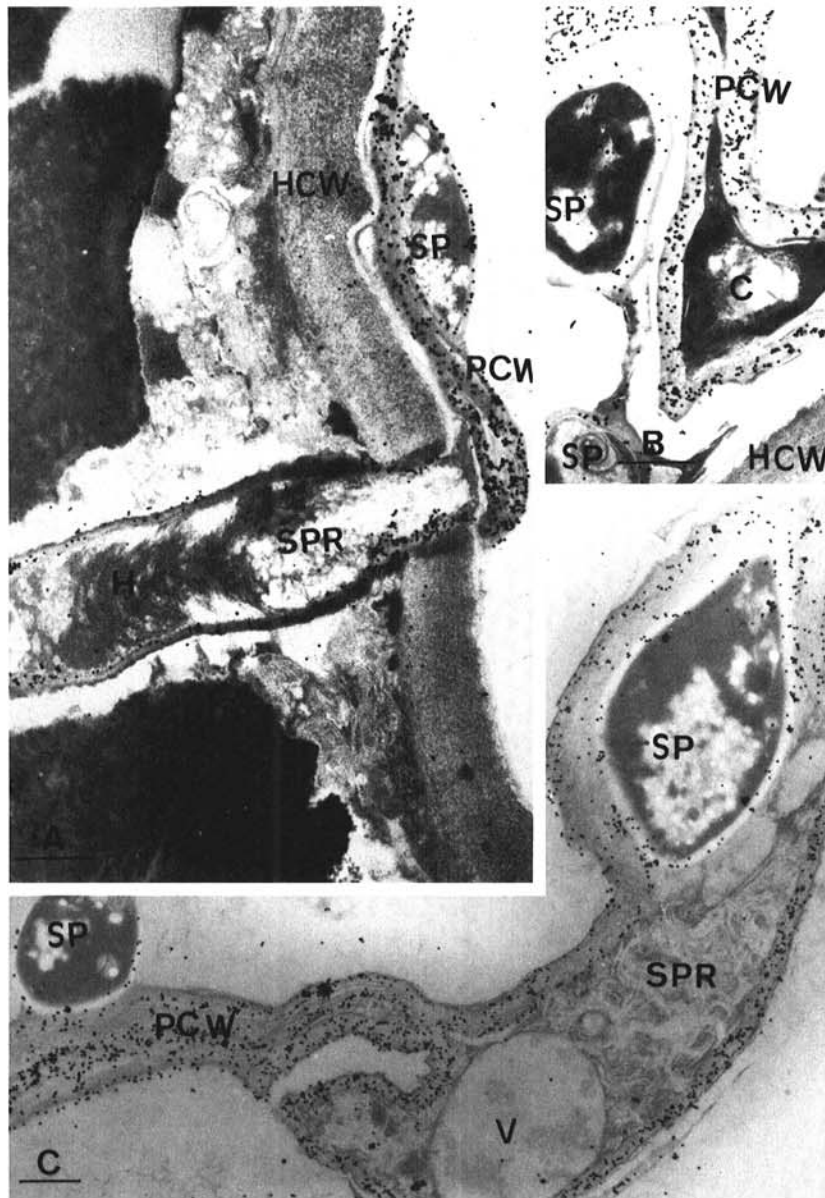


Fig. 3. Transmission electron micrographs of *Sphaerotheca pannosa* var. *rosae* (SPR) 24 h after inoculation with *Sporothrix flocculosa* (SP). Labeling is with a wheat germ agglutinin-ovomucoid gold complex. **A**, Penetration of plant epidermal cell by *S. p. rosae* and development of haustorium (H). Plasmolysis of external hyphae under attack by *S. flocculosa* is observed. HCW = host cell wall; PCW = pathogen cell wall. **B**, Preservation of *S. p. rosae* wall structures and necrosis of the cytoplasm (C) occurs. **C**, Penetration of *S. flocculosa* inside the hyphae of *S. p. rosae* resulted in a cytoplasm alteration. PCW = pathogen cell wall; V = vacuole. Bar = 0.25 μ m.

the plasmalemma were still present (Fig. 4A,B). Application of the wheat germ agglutinin-ovomuroid gold complex resulted in a strong deposition of gold particles over the convoluted pathogen cell walls (Fig. 4A,C). The labeling pattern was similar to that obtained at earlier stages of infection, although *S. p. rosae* was reduced to only branched cell walls.

DISCUSSION

For most mycoparasites, including *Trichoderma* spp. and *Gliocladium* spp., the mechanisms of enzyme and antibiotic production as well as hyphal interference involved in the antagonistic process have been extensively studied (12,25,28). In the case of *S. flocculosa*, however, little is known about its mode of action at the cellular level. The only available reports, which describe the cell surface events related to parasitic activity of this fungus against some powdery mildew pathogens, were derived from observations of scanning electron micrographs (10,13). According to these results, *S. flocculosa* causes rapid plasmolysis and collapse of

the conidia and hyphae of their fungal host. This led Jarvis et al (13) to postulate that excreted enzymes or antibiotics were likely involved in this mycoparasitic interaction. Conclusive evidence as to the exact role of such metabolites, however, could not be reached in the absence of in situ investigation. In this study, the use of the wheat germ agglutinin-ovomuroid gold complex as a specific probe for localizing chitin made it possible to gain new information regarding the presence of this polymer in the walls of the two interacting fungi. Differences in cell wall composition of the two tested fungi were reflected clearly by the intensity of labeling and the electron opacity. Based on a qualitative evaluation of the labeling intensity over the walls of *S. p. rosae*, it is likely that larger amounts of chitin are present in the walls of *S. p. rosae* than in those of *S. flocculosa*. Although wheat germ agglutinin has a high affinity for chitin oligomers, the lectin also can bind to glycoconjugates containing accessible *N*-acetyl-D-glucosamine residues (20). In this study, the binding of wheat germ agglutinin to fungal walls appeared to be highly specific, because it was completely inhibited by a preadsorption

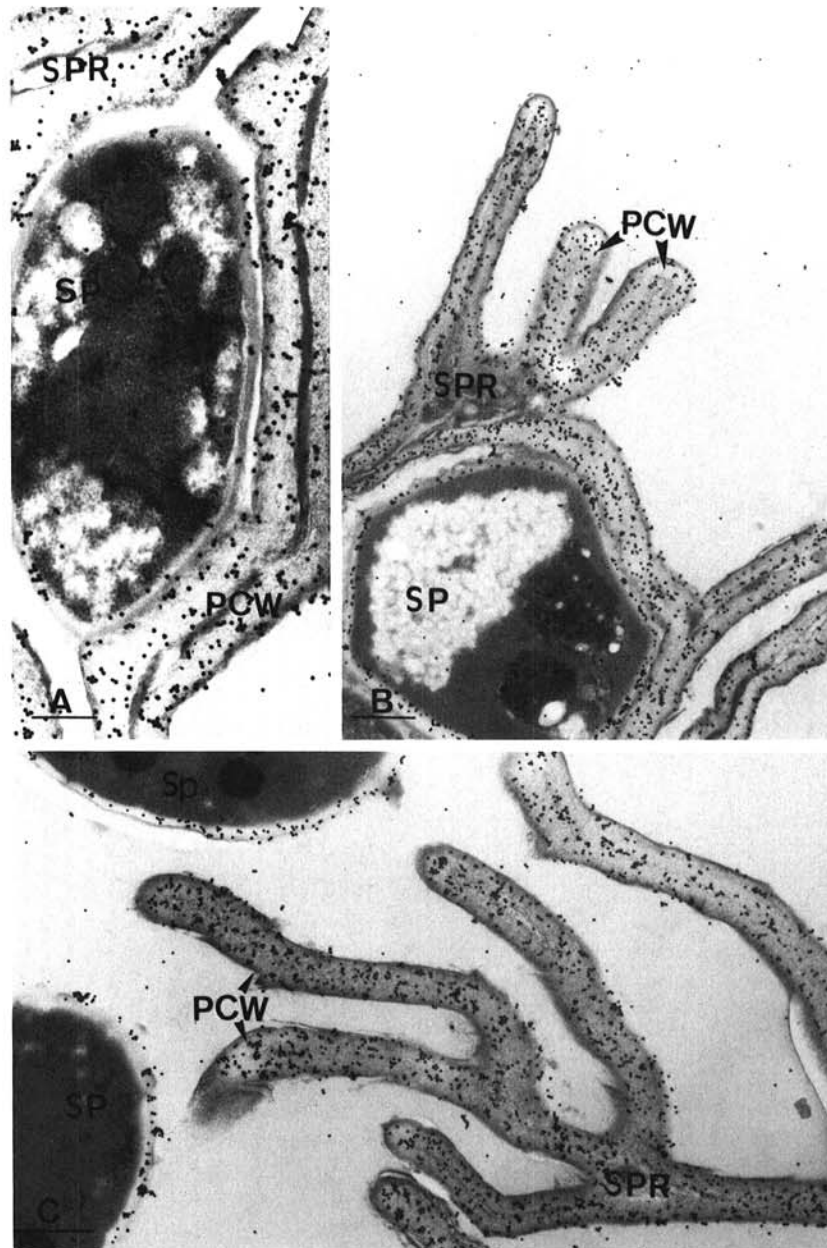


Fig. 4. Transmission electron micrographs of *Sphaerotheca pannosa* var. *rosae* (SPR) 24 h after inoculation with *Sporothrix flocculosa* (SP). Labeling is with a wheat germ agglutinin-ovomuroid gold complex. **A**, Close contact between the interacting fungi resulted in hyphal plasmolysis. Remnants of the plasmalemma are observed. Bar = 0.25 μ m. PCW = pathogen cell wall. **B-C**, Collapsed hyphae of *S. p. rosae* were completely depleted of their cytoplasm. The strong deposition of gold particles over the convoluted pathogen cell walls (PCW) is observed. Bar = 0.5 μ m.

with *N-N''*-triacetylchitotriose.

Since the initial report that suggested the possible involvement of chitinase in the lytic action against fungal as well as bacterial pathogens (4), the role of this enzyme in mycoparasitism has been emphasized in several reports (21,23,25). Our results, based on ultrastructural observations and cytochemical localization of *N*-acetylglucosamine residues, do not support the implication of a chitinolytic activity during the interaction between *S. flocculosa* and the powdery mildew fungus under study. Although the key role of β -1,3-glucanases, cellulases, and proteinases in fungal wall lysis and degradation has been reported in other fungal interactions, in this study, the preservation of both the cell wall structure and the wheat germ agglutinin binding sites does not support the hypothesis of an enzymatic process. Although several authors conclusively demonstrated the widespread capacity to produce cell wall degrading enzymes among saprophytic fungi in vitro, there are few references for the lysis of living material, except in cases in which enzymatic preparations were used to generate protoplasts from fungal spores (6). If one considers that living hyphae would normally react to a heterolytic effect during mycoparasitic attack (14), it seems reasonable to assume that under natural conditions hyphal breakdown may occur only in situations in which hyphae are dead or dying. This may explain, at least partially, the absence of fungal wall breakdown after treatment with the antagonist. Lifshitz (15), making a time-course analysis of the interaction between species of *Trichoderma* and *Pythium*, attributed to antibiosis a significant role in halting the pathogen before invasion and lysis. In the case of the *S. p. rosae*-*S. flocculosa* interaction, however, the absence of wall lysis in collapsed hyphae raises again the question as to the exact modes of attack and defense. A logical explanation regarding the absence of wall degradation in this mycoparasite interaction could be the inability of *S. flocculosa* to produce extracellular enzymes capable of digesting *S. p. rosae* cell walls, and/or to host wall resistance. In this latter case, it was reported that in some phytopathogenic fungi such as *Fusarium oxysporum*, high protein content in cell walls makes them more resistant to attack by *T. harzianum* (25) and *Pythium nunn* (5). Unfortunately, the absence of data concerning the cell wall composition of *S. p. rosae* does not allow speculation on the role of proteins or other wall substances in the resistance to an enzymatic lysis induced by the antagonist.

Research on mycoparasitism as a mechanism of biocontrol raises two important questions: Is physical contact between both partners necessary for host disintegration, and how important are enzymes and other extracellular compounds? Our time-course investigation revealed that *S. flocculosa* did not need to establish intimate contact with *S. p. rosae* to cause severe internal disorganization, cytoplasm aggregation, and plasmalemma retraction, which lead to host cell death. In addition, the rapidity with which *S. flocculosa* attacks *S. p. rosae* suggests the action of extracellular metabolites. In some cases, close contact between the interacting fungi resulted in the penetration of *S. p. rosae* hyphae by *S. flocculosa*. This phenomenon of hyperparasitism, observed for the first time with *Sporothrix*, has been previously reported for *Ampelomyces quisqualis* on cucumber powdery mildew (11). Subsequent to penetration, *S. flocculosa* grows within the dead or dying hyphae, the content of which is probably utilized by the antagonist as nutrients. That penetration occurs through a mechanical rather than an enzymatic process is suggested by the absence of host wall dissolution.

Involvement of enzymes in biocontrol blurs the distinction between parasitism and antibiosis (29). In this study, the absence of wall dissolution associated with a rapid collapse of host hyphae leads to speculation on the importance of antibiosis in the antagonistic activity of *S. flocculosa* on *S. p. rosae*. Antibiosis may provide advantages over other types of antagonism for biological control. Because antibiotic substances may diffuse rapidly, direct contact between the two partners is not necessary. Our ultrastructural observations reveal that the need for contact or presence in close proximity to the antagonist is not essential before the harmful effect is induced. A similar mode of action has been previously reported as a general feature of necrotrophic parasitism

in which lytic enzymes and toxic metabolites are implicated with antibiosis, but a more important role is ascribed to antibiosis (12,21).

In conclusion, results obtained from this study indicate that the enzymatic activity of *S. flocculosa* is not a primary determinant of an adverse effect on the pathogen survival and consequently is probably not important in the antagonistic process against *S. p. rosae*. Rather, it seems that antibiosis acting directly on cell contents could play a key role in the activity of *S. flocculosa*. The possible implication of antibiosis in this process is currently being investigated.

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