Interaction of Four Antagonistic Fungi with *Botrytis aclada* in Dead Onion Leaves: A Comparative Microscopic and Ultrastructural Study

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Accepted for publication 5 March 1997.

ABSTRACT

Köhl, J., Bélanger, R. R., and Fokkema, N. J. 1997. Interaction of four antagonistic fungi with *Botrytis aclada* in dead onion leaves: A comparative microscopic and ultrastructural study. *Phytopathology* 87:634-642.

The colonization of dead onion leaves by *Botrytis aclada* and the fungal antagonists *Aureobasidium pullulans*, *Chaetomium globosum*, *Gliocladium catenulatum*, and *Ulocladium atrum* and the interactions between *B. aclada* and each of the four antagonists were studied at the microscopic and ultrastructural level. This approach was used in an attempt to understand the colonization pattern of these fungi and the nature of the biocontrol activity of the antagonists that have shown a potential to suppress spore production of *Botrytis* spp. on necrotic plant tissues. When applied alone, *B. aclada* and *U. atrum* were found throughout the leaf tissues in high densities after an incubation period of 6 days at 18°C in a moist chamber. *C. globosum* and *G. catenulatum* colonized only the outer portions of the leaf, whereas *A. pullulans* appeared to be concen-

trated in the leaf stomata. When pathogen and antagonists were applied together, ultrastructural observations revealed that cells of *B. aclada* were plasmolyzed in the presence of *G. catenulatum*, suggesting a reaction to antifungal molecules. Antibiosis also seemed to be involved, albeit to a lesser extent, in the antagonistic interactions between *B. aclada* and *A. pullulans* or *C. globosum*. No evidence of direct parasitism was recorded. On the other hand, *U. atrum* appeared to completely exclude *B. aclada* from dead onion tissues when both fungi competed for the substrate. Ultrastructural observations of the in vitro interaction between the two fungi did not reveal parasitism or antibiosis by either fungus. Based on previous records of its biocontrol potential and observations of its colonizing properties, it appears that *U. atrum* can compete for and utilize necrotic tissues rapidly and extensively, thus, excluding competitors without any other antagonistic action.

Additional keywords: biological control, cell damage.

Botrytis spp. cause severe damage on a wide range of crops. Biological control of necrotrophic pathogens such as *Botrytis* spp. can be achieved by the suppression of spore production by the pathogen on necrotic plant tissues, resulting in a lower density of initial or secondary inoculum of the pathogen within the crop (12,19,25). This may result in a delay of the start or progression of epidemics incited by the pathogen (17,19,26).

In a previous screening program, *Chaetomium globosum* Kunze:Fr., *Gliocladium catenulatum* Gilman & E. Abbott, and *Ulocladium atrum* G. Preuss efficiently suppressed sporulation of *B. aclada* Fresen. and *B. cinerea* Pers.:Fr. on dead onion leaves under laboratory conditions, whereas *Aureobasidium pullulans* (de Bary) G. Arnaud was moderately effective (21). Antagonism of *A. pullulans*, *C. globosum*, and *U. atrum* was not reduced when leaf wetness periods were interrupted to simulate field conditions, whereas *G. catenulatum* was sensitive to dry conditions. The four isolates were tested in a series of nine small-scale field experiments under a broad range of environmental conditions (20). *U. atrum* consistently reduced sporulation of *B. cinerea* by more than 90% in all experiments. Applications of *G. catenulatum* and *C. globosum* caused significant sporulation reduction in one and three experiments, respectively. *A. pullulans* did not reduce sporulation of *B. cinerea* under the experimental conditions.

Very little is known about the mechanisms involved in the antagonism by these four organisms. A better understanding of the mechanisms is essential for the further exploitation of the antago-

nists in necrotic tissues of various crops under different environmental conditions. Population densities of the antagonists in the necrotic substrates necessary to achieve sufficient control levels and the timing of antagonist applications may depend on the underlying mechanisms. Furthermore, necrotic tissues from different crops may have a differential effect on the expression of such mechanisms.

The objective of our study was to elucidate the mechanisms involved in the antagonistic interactions between a *Botrytis* sp. and these four antagonists during the saprophytic colonization of necrotic plant tissues. Parasitism, antibiosis, and nutrient competition may be components of the antagonistic activity (27). Whereas in vitro tests can be useful to determine enzymatic or antibiotic activity of biocontrol agents, they cannot determine how and if these mechanisms take place when the organisms are interacting on their natural substrate. We used microscopic and ultrastructural studies to analyze and compare the behavior of the four antagonists as they developed on dead onion leaves. This is, to our knowledge, the first report of ultrastructural observations on the interference between a saprophytically growing pathogen and antagonists in dead plant tissues.

MATERIALS AND METHODS

Fungal material. *B. aclada* (synanamorph *B. allii* Munn), causing onion neck rot, was chosen as the model pathogen and was isolated from onion seed. The antagonists (with isolate number) *A. pullulans* (490), *C. globosum* (256), and *U. atrum* (385) were isolated from necrotic leaf tips of field-grown onions (21). *G. catenulatum* (162), originating from red clover root, was provided by P. Lüth, Prophya Biologischer Pflanzenschutz GmbH, Malchow, Germany.

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Publication no. P-1997-0421-01R
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Onion leaves. Symptomless green leaves were removed from 12-week-old field-grown plants of onion cv. Hyton, dried for several days at 60°C, and stored in paper bags at room temperature in the dark (21). Dried leaves were cut into 2-cm segments, sealed in plastic bags, and sterilized by gamma radiation of 4 Mrad.

Conidial suspensions. All fungi were cultured in petri dishes on oatmeal agar (20 g of oatmeal, 15 g of agar, and 1,000 ml of tap water), except *A. pullulans*, which was grown on basal yeast agar (10 g of bacteriological peptone, 20 g of sucrose, 1 g of yeast extract, 20 g of agar, and 1,000 ml of tap water). Cultures were incubated for 3 days for *A. pullulans*, 14 days for *C. globosum* and *G. catenulatum*, and 21 days for *U. atrum*. To obtain spore suspensions, cultures were flooded with sterile tap water containing 0.01% Tween 80. After gently rubbing with a rubber spatula to remove spores from fungal cultures, suspensions were filtered through a sterile nylon gauze with a mesh of 200 µm. Concentrations of spore suspensions were determined with a haemocytometer and adjusted with sterile tap water containing 0.01% Tween 80 to 1×10^6 spores/ml for antagonists and 1×10^5 conidia/ml for *B. aclada*.

Bioassay on dead leaves. Onion leaf segments for use in bioassays were washed thoroughly with tap water to remove soluble nutrients and subsequently blotted dry with sterile filter paper (21). Six leaf segments were placed in each of a series of moist chambers. Each chamber consisted of a sterile plastic petri dish (90 mm in diameter) containing two sterile filter papers (80 mm in diameter) and 1 ml of sterile tap water. The experiment was carried out in a completely randomized design with five replications (petri dish) with 10 treatments. The treatments were each of the four antagonists applied alone or in combination with *B. aclada* and *B. aclada* applied alone. In another control treatment, no fungi were applied to the leaf segments. Conidial suspensions of *B. aclada* or water plus surfactant were sprayed onto the leaf segments in the moist chambers, and the segments were incubated at 18°C for 24 h. Immediately after the incubation periods, spore suspensions of antagonists or water plus surfactant were sprayed onto the segments. Thereafter, leaves were further incubated at 18°C in the dark.

For light and electron microscopy observations, samples were taken 6 days after application of the antagonists, since preliminary work had established this time period to be optimal. Leaf pieces (2 × 2 mm) were cut from the central part of two randomly selected leaf segments of each replication of each treatment and were fixed and stored in glutaraldehyde (3%, vol/vol) in 0.1 M sodium cacodylate at pH 7.2. Samples from same treatments were bulked.

Eight days after antagonists had been applied, the leaf area covered with conidiophores of *B. aclada* was estimated using classes from 0 to 5 that represented, respectively, 0%, 1 to 5%, >5 to 25%, >25 to 50%, >50 to 75%, and >75 to 100% of the leaf surface covered with conidiophores of the pathogen (21). From the number of leaf segments (n) of each class (0 to 5) a sporulation index (SI) ranging from 0 to 100 was calculated for each replication (petri dish) consisting of four leaf segments ($SI = (0 \times n_0 + 5 \times n_1 + 25 \times n_2 + 50 \times n_3 + 75 \times n_4 + 100 \times n_5)/4$).

Assay on agar. Petri dishes containing 15 ml of malt extract agar (10 g of malt extract, 15 g of agar, and 1,000 ml of tap water) were inoculated in four replicates with 5-mm agar discs with mycelium of *B. aclada* or *U. atrum* that had been obtained from the margins of young colonies of the fungi grown on the same medium. Petri dishes were inoculated with one agar disc, two discs of the same fungus, or two discs of different fungi. The distance between two discs was 50 mm. Plates were incubated at 18°C for 7 days. After 7 days, agar with mycelium was covered with a 1-mm layer of agarose (3%) (E. van Balen, personal communication). Small pieces of agarose-covered agar with mycelium were cut from the margins of colonies. From plates with two colonies, samples were taken at the margins of both colonies where they had first contact. Samples were fixed and stored in glutaraldehyde (3%, vol/vol) in 0.1 M sodium cacodylate at pH 7.2.

Light and electron microscopy. Both leaf and agar samples were postfixed in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer at 4°C for 1 h, before being dehydrated in an ethanol series and embedded in Epon 812. Sections (0.7 µm) of leaf tissues were cut and mounted on glass slides prior to being stained with toluidine blue (0.96 mg/ml of water) or methylene blue (10 mg/ml of water) and examined by light microscopy. Ultrathin sections (0.1 µm) of leaf and agar samples were stained for transmission electron microscopy (TEM) with uranyl acetate and lead citrate prior to being examined, using a JEOL 1200 EX electron microscope (J. B. Em. Services, Montreal, Quebec, Canada) at an accelerating voltage of 80 kV. For each treatment of bulked samples, a minimum of three samples were cut into serial sections and examined under both light microscopy and TEM. Unless otherwise stated, reported observations were representative of all samples.

RESULTS

Bioassay. Under the constant humid conditions, all four antagonists efficiently suppressed the sporulation of *B. aclada* as in earlier experiments (21). The sporulation index for *B. aclada* was 41.8 (with a standard deviation of 5.8) when the pathogen had been sprayed alone. On leaf segments treated with *B. aclada*, no sporulation of *B. aclada* was observed when *G. catenulatum* or *C. globosum* had been applied, and the sporulation index was 0.8 and 0.3 for leaf segments treated with *A. pullulans* or *U. atrum*, respectively. *B. aclada* was not observed on leaf segments that had been treated with antagonists without *B. aclada*. Growth of all antagonists, resulting in sporulation of *G. catenulatum* and *U. atrum* and blastospore formation of *A. pullulans*, could be observed on the leaf segments whether or not treated with the pathogen. No fungal development was observed on leaves that had not been inoculated with any of the fungal suspensions.

Agar plate assay. Colonies of *B. aclada* or *U. atrum* grown alone showed a regular radial growth. When two colonies of the same fungus had been inoculated on a petri dish, colonies were slightly flattened where growing towards each other. Similar colony shapes were observed when *U. atrum* and *B. aclada* were grown in combination on the same petri dishes. There were no distinct inhibition zones between colonies of the same or the two different fungi. However, the colonies did not seem to grow into each other during the 7 days of the experiment.

Light microscopy. *Colonization of dead onion leaves.* Despite the fact that tissues and cells of irradiated dead onion leaves were quite disorganized, it was possible to observe a differential pattern of colonization among the five fungi 6 days after their inoculation. For instance, in all samples examined, *B. aclada* was found throughout the leaf section including the vascular tissue (Fig. 1A, arrowhead). In the same manner, *U. atrum* was found to be a very aggressive colonizer of onion leaves and was consistently observed in dense concentrations throughout the leaf tissues (Fig. 1B).

On the other hand, both *C. globosum* and, to an extent, *G. catenulatum* appeared to be confined primarily on the outer portion of the leaf surface (Fig. 2A). In all examined samples, *C. globosum* appeared less dense than *B. aclada* or *U. atrum*. *G. catenulatum* was found occasionally, although in low concentrations, in the parenchymal tissues and around the veins (data not shown). *A. pullulans* was characteristically concentrated in the leaf stomata (or between epidermal cells) (Fig. 2B and C), with a few fungal propagules being randomly dispersed underneath the epidermal cells (Fig. 2B and C, arrow).

Interactions between B. aclada and the fungal antagonists. Of the four interactions, it was nearly impossible to generate salient information under light microscopy, because of the difficulty of precisely differentiating the fungi. In the case of *Botrytis-Gliocladium*, differences in mycelium diameter between the two species made it somewhat easier to separate them. From all samples

studied, it was obvious that *B. aclada* was unable to colonize onion leaves to the extent observed in control treatments (Fig. 1A) when competing against *G. catenulatum* (Fig. 3A). In some instances, cells of *B. aclada* appeared dead, as evident by their lack of reaction with methylene blue (Fig. 3A, arrow).

For the *Botrytis-Ulocladium* interaction, abundant colonization and sporulation by the antagonist suggested that *U. atrum* had completely saturated the substrate (Fig. 3B). The antagonist was as abundant as in the controls (Fig. 1B), with the distinction that more spores were consistently observed on the leaf surface (Fig. 3B). In this situation, typical dictyospores of *U. atrum* covering the leaf surface (Fig. 3B, arrowheads) made it easy to identify the antagonist. In the numerous samples studied, it was impossible to confirm conclusively the presence of *B. aclada* under light microscopy.

Transmission electron microscopy. *Botrytis-Gliocladium*. Ultrastructural observations of dead onion leaves inoculated with *B. aclada* showed that fungal cells were of regular shape and contained a dense cytoplasm (Fig. 4A). Typical organelles such as nuclei and mitochondria were clearly visible, and the cells were surrounded by a thick fibrillar mucilage. By comparison, *G. catenulatum* cells were characteristically smaller than those of the pathogen (thus, confirming light microscopy observations) and were covered by a thin and irregular mucilage (Fig. 4B). Their cytoplasm was regular and contained a few large, electron-dense lipid bodies.

In the instances in which it was possible to observe both fungi in close vicinity, cells of *B. aclada* had lost all turgor pressure and were typically devoid of cytoplasmic content (Fig. 4C). They were reduced to empty shells shaped according to the presence of *G.*

Fig. 1. Light micrographs of cross sections of dead onion (*Allium cepa* cv. Hyton) leaves 6 days after being inoculated with **A**, *Botrytis aclada* (Ba) and **B**, *Ulocladium atrum* (Ua). Bar = 25 μ m. **A**, Cells of Ba can be found throughout the leaf tissues (arrows) from the epidermis (E) to the leaf vein (V). Note the presence of Ba within vein cells (arrowheads). **B**, Ua is present within the tissues in equal density from the epidermis (E) through the vein (V) cells (arrowhead).

catenulatum hyphae. The cell wall of the pathogen was thicker compared with controls (Fig. 4A), but was otherwise unaltered, and no samples revealed the presence of the antagonist within *B. aclada* cells (Fig. 4C).

Botrytis-Chaetomium. In dead onion leaves inoculated with *C. globosum* alone, cross sections of fungal cells revealed a dense and uniform cytoplasm with a dark, lateral lipid body that sometimes covered most of the section (Fig. 5A). Leaf cell walls adjacent to fungal propagules were either degraded or characterized by a loose fibrillar appearance, suggestive of cellulolytic activity by the antagonist.

In the samples inoculated with both the pathogen and the antagonist, simultaneous presence of both fungi was rarely observed under TEM. In longitudinal sections, lipid bodies of *C. globosum* were quite numerous and distinctive, making it easy to identify the antagonist (Fig. 5B). Based on our observations, *C. globosum* was predominant over *B. aclada* within all treated leaf tissues. In a few instances, dead fungal cells completely devoid of their cytoplasm, appearing as empty hyphal shells, were observed in proximity to *C. globosum* (Fig. 5B and C). However, because of the advanced stage of degradation of these former cells, it was impossible to confirm whether they belonged to *B. aclada*.

Fig. 2. Light micrographs of cross sections of dead onion (*Allium cepa* cv. Hyton) leaves 6 days after being inoculated with **A**, *Chaetomium globosum* (Cg) and **B and C**, *Aureobasidium pullulans* (Ap). Bar = 25 μ m. **A**, Cg cells are typically concentrated on the leaf surface. **B and C**, Characteristic clustering of Ap cells in leaf stomata. A few cells can be found below the epidermis around the clusters (arrows).

Botrytis-Aureobasidium. In dead onion leaves inoculated with *A. pullulans*, cells of the antagonist were typically found in clusters (Fig. 6A) as observed under light microscopy (Fig. 2B). They were characterized by a very dense cytoplasm and the presence of dark lipid bodies.

Close contact between *B. aclada* and *A. pullulans* cells revealed that the pathogen was affected by the presence of the antagonist (Fig. 6B). In these observations, cells of *B. aclada* had lost their integrity, and the cytoplasmic content of damaged cells had retracted away radially from the cells of *A. pullulans*. At the point of direct contact between the pathogen and the antagonist cells, the

Fig. 3. Light micrographs of cross sections of dead onion (*Allium cepa* cv. Hyton) leaves inoculated with *Botrytis aclada* (Ba) 24 h prior to being inoculated with **A**, *Gliocladium catenulatum* (Gc) and **B**, *Ulocladium atrum* (Ua). **A**, Ba and Gc cells together in dead onion tissues 6 days after application of the antagonist. Cells of Ba are considerably less dense than in samples in which it is inoculated alone (Fig. 1). Note the presence of empty Ba cells (arrow). Bar = 25 μ m. **B**, Abundant Ua propagules under and above the epidermis. Note the formation and germination of typical conidia (dictyospores; arrowheads) of Ua on leaf surface 6 days after inoculation. No conclusive presence of Ba. Bar = 10 μ m. E = epidermis; V = vein.

cell wall of *B. aclada* had lost some of its definition and appeared distended and fibrillar (Fig. 6B, open arrow).

Botrytis-Ulocladium. In samples of dead onion leaves inoculated with *U. atrum* alone, the fungus was observed easily within

tissues. Its cells were characterized by a granular cytoplasm containing several large lipid bodies of variable electron density; its cell wall was covered by a regular hairy mucilage, making it easy to discriminate from the cells of *B. aclada* (Fig. 7A). The fungus

Fig. 4. Transmission electron micrographs of **A**, *Botrytis aclada* (Ba) cells; **B**, *Gliocladium catenulatum* (Gc) cells; and **C**, the interaction between Ba and Gc cells in dead onion (*Allium cepa* cv. Hyton) leaf tissues. **A**, Ba cells surrounded by a thick fibrillar mucilage (mu) and characterized by a dense cytoplasm filled with distinct organelles such as a nucleus (N) and mitochondria (M). Bar = 1 µm. **B**, Cells of Gc showing typical large lipid bodies (lb) and surrounded by a thin and irregular mucilage. Bar = 1 µm. **C**, A dead cell of Ba surrounded by three cells of Gc. External pressure by Gc induces wall deformation. Note thickness of the cell wall of Ba. Bar = 2 µm.

seemed to colonize all dead leaf tissues, and it was capable of degrading the plant cell walls (Fig. 7A, arrowhead). In the numerous samples observed, it was impossible to obtain conclusive evidence of the presence of *B. aclada* when both fungi were inoculated onto onion leaves (Fig. 7B). On the other hand, *U. atrum* was always conspicuously present and degraded the cell walls of onion leaves.

Because of the inability to observe interactions in planta between *B. aclada* and *U. atrum*, in vitro confrontations were stud-

ied in an attempt to understand the basis of the antagonistic activity of *U. atrum*. In control samples, as observed in the bioassay, neither *B. aclada* nor *U. atrum* were markedly altered by a self-confrontation. Confrontations between the pathogen and the antagonist failed to show typical reactions of parasitism or antibiosis in the bioassay. On the other hand, TEM observations at the zone of contact revealed that fungal cells from both fungi showed varying degrees of alteration at the immediate zone of contact.

Fig. 5. Transmission electron micrographs of **A**, *Chaetomium globosum* (Cg) cells in dead onion (*Allium cepa* cv. Hyton) leaf tissues and **B and C**, Cg cells and dead fungal cells 6 days after inoculation of the antagonist on dead onion leaf tissues that had been inoculated with *Botrytis aclada*. Bar = 1 μ m. **A**, Cg is characterized by a dense cytoplasm and the presence of large and electron dense lipid bodies (lb). The plant cell wall (cw) is highly degraded and near complete disruption in certain areas (open arrow). **B and C**, Presence of empty hyphal shells (ehs) in proximity of Cg cells. Note typical lb of the antagonist.

This ranged from detached plasmalemma to slight disorganization of the cytoplasm (Fig. 7C and D). These alterations subsided gradually away from the contact zone, and neither fungus appeared to overcome the other.

DISCUSSION

All four antagonists tested in this study suppressed sporulation of *B. aclada* almost completely in the bioassay. This agrees with earlier results obtained by Köhl et al. (21) that had shown the relative biocontrol potential of *A. pullulans*, *C. globosum*, *G. catenulatum*, and *U. atrum*. However, microscopic observations of the interactions have highlighted some important differences among the four fungi in terms of their antagonistic activity against *B. aclada*. This information is of key importance in establishing the respective behavior of the antagonists and how this behavior can influence the eventual success of biocontrol of *Botrytis* spp.

Based on our light and electron microscopy studies, *A. pullulans* grew in a yeastlike manner. Clusters of cells, most probably blastospores, were formed on the leaf surface or inside stomata, whereas hyphae were rarely found inside the tissue. This can be explained by the limited formation of hyphae in solid media by this dimorphic fungus (1). Considering this colonization pattern, it

is unlikely that nutrient competition within the substrate would play a major role in antagonism, since the substrate would only be depleted locally at the site where colonies are formed. On the other hand, *A. pullulans* could be an efficient antagonist for the protection of leaf surfaces and man-made wounds, as previously reported (11,13). The in vitro production of antibiotic compounds by *A. pullulans* has been described (4,23). In the present work, in the rare instances in which close contact between *A. pullulans* and *B. aclada* was found, our observations appeared to support a chemical antibiosis by *A. pullulans* (Fig. 6B).

The colonization of dead onion tissues by *C. globosum* was poor, and its hyphae were mostly restricted to the leaf surface. However, the ultrastructural studies indicated that this antagonist, which is known for its strong cellulolytic activity (9), has the potential to degrade plant cell walls. *C. globosum* was previously found to be antagonistic against several plant pathogens such as *Pythium ultimum* (7) and *Venturia inaequalis* (17) and is known to produce two antifungal substances (7). The production of antibiotics by *C. globosum* may also explain its antagonistic activity against *B. cinerea* under certain field conditions, regardless of the presence of an actively growing population of the antagonist on the substrate (20). In our ultrastructural studies, highly disorganized fungal cells were associated with cells of *C. globosum*, whereas such dead cells were not found when the antagonist was grown alone. This would support the manifestation of in situ antibiotic activity by *C. globosum*. However, it was not possible to confirm whether these dead cells belonged to *B. aclada*, but it can be assumed, supported by the information given in literature and our observations, that the antagonist did induce cell death of *B. aclada*. Because of its limited colonization potential, it would appear that antibiosis, if not exclusively, is at least an important component of the biocontrol ability of *C. globosum*.

In previous experiments, *G. catenulatum* displayed strong antagonism against *Botrytis* spp. under continuously moist conditions favorable for fungal growth (21). Our studies revealed that *G. catenulatum* could colonize all parts of necrotic tissues, but that this colonization was not very extensive. Nevertheless, colonization of *B. aclada* was considerably reduced in the presence of the antagonist. Antibiosis was clearly one of the modes of action of *G. catenulatum*, based on cell reactions of *B. aclada*, which were typical of toxic activity such as cytoplasm degradation and loss of turgor pressure (16,28). No clear signs of hyperparasitism such as cell wall degradation at sites of contact between hyphae of the two organisms were found. While no information is given in the literature about the mode of action of *G. catenulatum*, such information abounds about other species of *Gliocladium* and the closely related *Trichoderma* spp. (6,24). For instance, *G. virens* has been described as an antibiotic producer, and its activity against *Pythium ultimum* and *Rhizoctonia solani* is strongly related to the antibiotics it produces (18). Di Pietro et al. (8) suggested that enzymes such as beta-glucanases provided a synergistic effect with the toxins, but evidence of hydrolytic activity of *G. virens* in situ has never been reported. In addition, while some *Trichoderma* spp. have been shown to degrade their host cells enzymatically, it is well established that they produce a number of antibiotic substances (14). In the chronological events associated with the antagonism of *Trichoderma harzianum* against *B. cinerea*, Bélanger et al. (2) showed that antibiosis preceded penetration and suggested that the enzymatic cell wall degradation was more a saprophytic action than the cause of death in *B. cinerea*.

In the case of *U. atrum*, there are no previous reports on microscopic studies of the colonization of necrotic plant tissue or possible mechanisms of antagonism by this fungus. Among the four antagonists in our study, *U. atrum* expressed the best colonization potential on dead onion leaves and also the strongest antagonism on leaves that had been exposed to field conditions in a previous study (20). This antagonist also showed a high ecological competence for the habitat of above-ground necrotic plant tissues and

Fig. 6. Transmission electron micrographs of **A**, *Aureobasidium pullulans* (Ap) cells in dead onion (*Allium cepa* cv. Hyton) leaf tissues and **B**, Ap and *Botrytis aclada* (Ba) cells in dead onion leaf tissues inoculated with both the pathogen and the antagonist. Samples were taken 6 days after inoculation with Ap. Bar = 1 μ m. **A**, Cells of Ap are frequently found in clusters and characterized by a very dense cytoplasm. **B**, Interaction between Ba and Ap. Note Ba cytoplasm retracted radially from Ap (arrows). Note change in Ba cell wall definition and density at point of direct contact with Ap (open arrow).

was able to germinate and colonize the substrate under various environmental conditions in the presence of *Botrytis* spp. and other naturally occurring saprophytic fungi (20). This ability to colonize necrotic tissues at least under high humidity, a prerequisite for strong competitors, was confirmed in this work. Indeed, *U. atrum* mycelium was found to be present and distributed throughout necrotic leaf tissues, and *B. aclada* seemed to be completely excluded from that niche. Ultrastructural studies showed that *U. atrum* degraded plant cell walls actively so that it could grow through the dead plant cells walls. Because *B. aclada* could not be detected in necrotic leaf tissues in the presence of *U. atrum*, it was not possible to determine if antibiosis or parasitism complemented its competition for substrate on onion leaves. However, when the interaction between both fungi was studied on agar, there were no

obvious signs of hyperparasitism or antibiosis, as the fungi seemed unaltered. This would indicate that *U. atrum* relies exclusively on competition for gaining access to its ecological niche over *B. aclada*. The ability to colonize and utilize necrotic tissues rapidly may enable *U. atrum* to exclude competitors from the substrate without any other antagonistic interaction. These findings may have practical implications for the proper timing of application of the antagonist and for predicting its limitations. Most efficient control of substrate colonization and subsequent sporulation of the pathogen may be achieved on freshly senesced tissues or in freshly pathogen-induced lesions in which mycelium of the pathogen is not established in high density to allow profuse sporulation. Since the antagonist seems to lack the ability to kill fungal cells by toxins or hyperparasitism, a curative application of *U. atrum* would

Fig. 7. Transmission electron micrographs of **A**, *Ulocladium atrum* (Ua) cells in dead onion (*Allium cepa* cv. Hyton) leaf tissues; **B**, Ua cells in dead onion leaf tissues inoculated with both *Botrytis aclada* (Ba) and Ua; and **C and D**, Ba and Ua cells taken at the meeting point of two colonies growing on agar plates. Leaf samples were taken 6 days after inoculation with Ua. Bar = 1 μ m. **A**, Plant cell walls (cw) of onion leaves are highly degraded in the presence of Ua. Note the passage of an Ua cell through the plant cell wall (arrowhead). **B**, In the interaction *B. aclada*-*U. atrum*, only cells of the antagonist are observed within the plant tissues. Note the degradation of the plant cw. **C and D**, Cells of both fungi show varying degrees of alteration when grown in confrontation on agar medium.

be unlikely to reduce the pathogen population already established in high densities. However, nutrient competition resulting in reduced spore yields of the pathogen may also play a role when *U. atrum* colonizes substrates where *Botrytis* mycelium is already established. Further research is needed to study how the initial density of mycelium of *Botrytis* spp. and *U. atrum* affect subsequent substrate colonization by both fungi.

The exploitation of biological control agents with mechanisms that are mainly based on competition for nutrients and space can provide distinct advantages against necrotrophic plant pathogens that rely on their saprophytic phase for establishing a critical base population. Firstly, the antagonistic interaction is not specific for certain pathogen-antagonist relationships. For example, *U. atrum* is known to compete with and suppress sporulation of a range of *Botrytis* spp. and other saprophytic fungi such as *Cladosporium* spp. and *Alternaria* spp. (20). Secondly, there is no strict necessity for the simultaneous presence of antagonist and pathogen. The antagonist can utilize the limited amounts of nutrients in necrotic tissues long before the pathogen arrives and, thus, restricts access to the substrate. Organisms acting strictly by hyperparasitism depend on the presence of the pathogen for activity. In addition, the process can be relatively slow and often times only activated when the pathogen itself is under stress (2). In the case of antibiosis, while efficient against biotrophs such as powdery mildews (3), it is also subject to a precise timing of release against necrotrophs, because antibiotics produced early before arrival of the pathogen may be degraded or may be adsorbed to cell surfaces (5,10). Thirdly, it is unlikely that resistance against competition will be developed by *Botrytis* populations. This point is of particular significance considering the ability of *B. cinerea* to develop resistance against fungicides (15). This phenomenon was also reported against two antibiotics produced by *Bacillus subtilis* after nine subsequent crops of *Astilbe* had been sprayed with the antagonist (22).

In conclusion, our study is the first to report ultrastructural observations of a fungal pathogen and potential antagonists in situ as they colonize and interact on their natural substrate, dead onion leaves. These ultrastructural observations have provided insight into the mode of action of these antagonists and have highlighted the importance of understanding such phenomena in the selection and development of biocontrol agents.

ACKNOWLEDGMENTS

We thank C. Labbé and W. M. L. Molhoek for technical assistance.

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