Possible Role of Colonization and Cell Wall-Degrading Enzymes in the Differential Ability of Three *Ulocladium atrum* Strains to Control *Botrytis cinerea* on Necrotic Strawberry Leaves

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ABSTRACT

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Ulocladium atrum (strain 385) consistently reduced *Botrytis cinerea* sporulation on necrotic fragments of strawberry leaves. On these tissues, two strains of *U. atrum* (isolates 18558 and 18559) showed lower antagonistic activities than the reference strain 385. Colonization of strawberry leaflets by the three *U. atrum* strains appeared similar in the absence of *B. cinerea*, whether quantified by chitin or immunological assays. The second method (based on anti-*U. atrum* antibodies) revealed that strawberry leaflet colonization by *U. atrum* 385 was better than by the other *U. atrum* strains in the presence of *B. cinerea.* An immunoassay using anti-*B. cinerea* antibodies revealed that the colonization of *B. cinerea* in tissues was lower in the presence of *U. atrum* 385 than with the two other *U. atrum* strains. The enzymatic activities produced by *U. atrum* 385 during the colonization phases of necrotic tissues were compared to *B. cinerea* and *U. atrum* strains 18558 and 18559. *U. atrum* 385 had the highest lipase, pectate lyase, and cellobiase activities while *B. cinerea* had the highest endo-β-1,4-glucanase activity. The study of lytic activities hydrolyzing the fungal cell wall revealed higher β-1,3-glucanase activity with *U. atrum* 385, which was stimulated by *B. cinerea* on necrotic strawberry leaflets. These results suggest that plant and fungal cell wall-degrading enzymes produced by *U. atrum* 385 may play a complementary role in the competitive colonization of dead strawberry leaves against *B. cinerea*.

Additional keywords: gray mold, mode of action.

Botrytis cinerea Pers.:Fr., the causal agent of gray mold, is one of the most devastating pathogens in several crops worldwide. The abundant *B. cinerea* sporulation on dead and senescent plant tissues contributes to the development and the maintenance of an epidemic within a crop (31). The suppression of pathogen sporulation was proposed as a potential strategy of biological control (20, 32) to slow down epidemics of *B. cinerea*. Indeed, the application of the antagonistic strain 385 of the saprophytic fungus *Ulocladium atrum* G. Preuss on leaf onion tips (18,23) and cyclamen (21) consistently reduced both sporulation of *B. cinerea* and the development of symptoms on these crops.

Although experimental evidence is still missing, nutrient competition was suggested as a putative mechanism involved in the biocontrol activity of *U. atrum B. cinerea* sporulation (19). A reliable determination of biomass of fungi in vivo is needed to study interactions among biological control agent (BCA) and pathogens (9), as well as a method to evaluate nutrient competition itself. Two experimental strategies are reported to approach this problem. The first is based on the study of nutritive elements consumed by the antagonistic fungus during the biocontrol process, as previously performed by Filonow (12) on the model yeast-*B. cinerea*-apple. A second, indirect approach rests on the study of cell wall-degrading enzymes produced by the antagonistic fungus and presumably involved in the colonization (25). These enzymes contribute to release nutrients and are thus candidates as intermediary of competition between two organisms.

The present work compared the suppression of *B. cinerea* sporulation by different strains of *U. atrum* on necrotic strawberry leaves in relation to (i) tissue colonization and (ii) plant and fungal cell wall-degrading enzyme (PCWDE and FCWDE, respectively) activities.

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MATERIALS AND METHODS

Fungi. *B. cinerea* (strain 700 isolated from a gerbera flower) and strain 385 of *U. atrum* (isolated from a tip of onion leaf) were received from the Institute of Plant Disease of Wageningen (The Netherlands). Two other *U. atrum* strains (MUCL 18558 and 18559) were obtained from La Mycothèque Universitaire catholique de Louvain-la-neuve (MUCL, Louvain-la-neuve, Belgium). All were cultured in petri dishes on oat meal agar (20 g of oatmeal, 15 g of agar, 1,000 ml of tap water) at 20°C for 14 days. Conidial suspensions were obtained by flooding the colonies with sterile tap water containing 0.01% Tween 80, rubbing the removal conidia from fungal colonies, and filtering through a sterile nylon gauze with a mesh of 200 µm. The conidial suspensions were diluted to the required concentrations as determined with a Bürker cell.

Plant material. Strawberry plants (cv. Elsanta, provided by the Biotechnological Department of Gembloux) were grown in the greenhouse at 25°C with a photoperiod of 16 h.

Bioassays. Healthy green leaflets of strawberry were harvested in the greenhouse from 4-week-old plants, sterilized by gamma radiation of 40 krays (irradiator Gammacell 220 Co-60; Griffith-Mediris, Fleurus, Belgium), dried at room temperature under sterile atmosphere for 3 weeks, and stored in sealed plastic bags. Before using them for bioassay, leaflets were rehydrated with sterile tap water overnight and were washed thoroughly to remove soluble nutrients. Four washed leaflets were then placed in a petri dish (9 cm in diameter) on 0.75% water agar (wt/vol). The suspension of *B. cinerea* conidia (10^4 spores/ml) was sprayed to the leaf surface to deposit 5 µl per centimeter of area. After either 0 or 12 h at 20°C under a 16-h photoperiod, conidia of the antagonist

(2×10^6 spores per ml) were applied on the inoculated leaves as described for *B. cinerea*.

The protective effect of *U. atrum* strains (385, 18558, and 18559) was quantified by counting *B. cinerea* conidia produced after 6 days of colonization. For this purpose, four leaflet disks (10 cm^2), cut with a cork-borer from one leaflet, were shaken in 20 ml of tap water containing 0.1% Tween 20. The number of *B. cinerea* conidia was determined with a Bürker cell (Marienfeld, Lauda-Königshofen, Germany) and expressed per square centimeter of leaflet surface. The detection limit (one single spore observed in the Bürker cell) corresponds theoretically to 15,625 conidia/cm^2 of leaf surface (calculated from the 10 cm^2 of leaf surface per replication). Data were means of four independent experiments over time with four replicates each, one replicate corresponding to one leaflet.

Measurement of fungal growth. The leaf treatment with *U. atrum* strains and *B. cinerea* was performed as described previously. The mycelial growth of *U. atrum* on dead strawberry leaflets was first determined in the absence of *B. cinerea* by the quantification of chitin content as described by Ride and Drysdale (27). The chitin content was expressed as glucosamine equivalents per square centimeter of leaflet area. This experiment was repeated independently three times, with two replicates each.

U. atrum colonization of dead strawberry leaflets was also quantified in absence and in presence of *B. cinerea* by plate trapped antibodies (PTA) enzyme-linked immunosorbent assay (ELISA) (17) using specific monoclonal anti-*U. atrum* antibodies Ua-PC3 received from M. Dewey (Oxford, UK). *U. atrum* strains and *B. cinerea* were applied simultaneously on leaf tissues. Standard curves have been performed for each *U. atrum* strain according to the incubation time (up to 6 days) in 1% (wt/vol) strawberry leaf extract medium (dead leaves ground in distilled sterile water). Standard curves have been performed up to 6 days of incubation using the strawberry leaf medium and were selected at 3 and 6 days for conversion of ELISA data from days 0 to 3 and days 4 to 6 of *U. atrum* colonization, respectively.

Similarly, *B. cinerea* colonization was assayed during 6 days of leaf colonization after a simultaneous inoculation with one of the *U. atrum* strains. Specific monoclonal anti-*B. cinerea* antibodies AD-Bc6 received from M. Dewey (Oxford, UK) were used for the immunoquantification. Lyophilized mycelium harvested from a 3-week-old liquid culture of 2% (wt/vol) oat meal agar, *B. cinerea*, or *U. atrum* (strain 385, 18558, or 18559) was used as reference standard in this immunoassay. The experiments based on PTA-ELISA were repeated independently two times with two replicates each.

Extract preparation. For the PCWDE and FCWDE activity assays, homogenates of leaflet disks (±40 cm^2 except for lipolytic assay, ±100 cm^2) colonized by one of the *U. atrum* strains or by *B. cinerea* were prepared according to the method of Somé and Tivoli (30). The extracts were frozen at –20°C before further enzymatic assays.

Enzyme assays. CWDE activities produced during the strawberry leaf colonization by *B. cinerea* or *U. atrum* were followed after growing incubation periods (from 0 to 6 days). All the results dealing with the enzymatic assays were means of two independent experiments with two replicates each.

Lipase (EC 3.1.1.3) activity was quantified by titrimetric assay using an emulsion of olive oil as substrate (5). The olive oil emulsion was first stirred to get an emulsion which remained stable for at least 1 h. Incubations were performed for 16 h at 20°C. The reaction was stopped by addition of 10 ml of an ethanol-acetone mixture (1:1, vol/vol) and 0.1 ml of phenolphthalein indicator solution (0.375% in methanol). One specific unit (SU) of lipase activity was defined as the amount of enzyme causing the release of 1 mmol of oleic acid per minute and per milligram of protein.

Pectate lyase (EC 4.2.2.2) hydrolyses pectate by transelimination, resulting in the release of double-bond substances. The pectate lyase (PL) activity was measured spectrophotometrically at 235 nm (34). One SU was defined as the amount of enzyme that caused an increase of 2.6 in absorbance at 235 nm, equivalent to the release of 1-µmol aldehyde groups per milligram of protein for specific activity (35), after 16 h of incubation.

The activity of cellobiase (EC 3.2.1.21) was determined using the glucose hexokinase method (Glucose/HK kit; Sigma, Bornem, Belgium). The spectrophotometric reading was carried out at 340 nm. The glucose concentration of the samples was determined using a concentration range of D-glucose (0 to 300 µg) as a standard. One SU of the cellobiase activity was defined as the amount of enzyme causing the release of 1 mmol of glucose per hour and per milligram of protein.

The activity of endo-β-1,4-glucanase (EC 3.2.1.4) was measured according to a colorimetric method utilizing xyloglucan (XG) as substrate (29). After circular stirring (125 rpm) at 40°C for 16 h, the absorbance of the samples was measured against blank (sample without XG) at 640 nm. The endo-β-1,4-glucanase activity was expressed in arbitrary units (AU) as follows: $AU/ml = [(Ac - As)/Ac] \times 100$, where *Ac* is the absorbance of the control per milliliter (containing XG with no enzyme) and *As* the absorbance of the leaf homogenate (per milliliter). The SU was expressed in AU/h and AU/mg of protein.

The total activity of β-1,3-glucanase, including the endo- (EC 3.2.1.39) and the exo- (EC 3.2.1.58) form, was assayed spectrophotocally at 550 nm by the dinitrosalicylic procedure (33) modified as follows: the reaction mixture was prepared with 0.9 ml of 1% (wt/vol) laminarin in potassium acetate buffer (pH 5.2) containing 0.02% (wt/vol) sodium azide and 0.1 ml of leaf homogenate. The reaction mixture was incubated at 37°C with continuous stirring (125 rpm) for 16 h. One SU of β-1,3-glucanase activity was defined as the amount of enzyme causing the release of 1 mg of glucose per minute and per milligram of protein under the assay conditions. For this enzymatic assay, *U. atrum* strains (385, 18558, and 18559) were applied on leaflets untreated or precolonized for 24 h by *B. cinerea*. After the period of incubation of 24 h at 20°C, *B. cinerea* on necrotic tissues was killed by treatment with gamma rays (40 krays).

Protein determination. Protein concentration was determined according to Bradford (4) using the Bio-rad protein assay kit (Nazareth, Belgium). Bovin serum albumin was used as standard.

Statistical analyses. Data on the number of *B. cinerea* conidia, observed after 6 days of dead leaf tissue colonization in the presence of different strains of *U. atrum* (385, 18558, or 18559) were subjected to analysis of variance (ANOVA) after log transformation. The contrast analysis allowed comparison between the effect of each *U. atrum* strain in relation with the other ones when they were applied simultaneously or 12 h after *B. cinerea* inoculation. Values of *U. atrum* colonization (chitin or immunoassays) were also submitted to statistical analysis using univariate ANOVA to perform the analysis by comparison of treatments for each time of colonization (1, 2, 3, 4, and 6 days). Data corresponding to time 0 were null whatever the treatment and not taken into account. When a significant difference was established, the Snedecor-Newman-Keuls test allowed the multiple mean comparison.

Split-plot ANOVA was also performed considering parameters of "time" and "experiment" as blocks; treatment–experiment interaction was used as error a, whereas time–experiment interaction together with treatment–time–experiment interaction were considered as error b for the determination of *F* value and statistical significance (data not shown). All these statistical procedures were performed using the SYSTAT 5 program (SPSS Inc., Chicago).

RESULTS

Comparison of the antagonistic activity of *U. atrum* strains (385, 18558, and 18559). A reduction of *B. cinerea* sporulation was observed on necrotic fragments of strawberry leaves when *U.*

atrum 385, *U. atrum* 18558, and *U. atrum* 18559 were applied (Fig. 1). Whatever the incubation period between *B. cinerea* inoculation and *U. atrum* application (0 h and 12 h), *U. atrum* 18558 and *U. atrum* 18559 exhibited a significantly lower antagonistic activity (F was significant at $P = 0.0001$) than *U. atrum* strain 385. The comparison of F values also revealed that this difference was more significant when *U. atrum* strains and *B. cinerea* were applied simultaneously ($F = 126.67$), in comparison with $F = 29.35$ obtained when *U. atrum* strains were applied 12 h after *B. cinerea*.

Quantification of *U. atrum* and *B. cinerea* colonization. The chitin content in tissues colonized by *U. atrum* 385 alone increased slightly up to 1.98 µg glucosamine equivalents per square centimeter of leaflet area after 48 h of incubation (Fig. 2A). The values of chitin content reached 6.1 µg equivalents per square centimeter after 6 days of incubation. Chitin contents measured for the two other strains of *U. atrum* were slightly lower but not significantly different than with *U. atrum* 385 using univariate ANOVA ($F = 0.66$ at $P = 0.05$). In the absence of *B. cinerea*, similar colonization by the three *U. atrum* strains was confirmed statistically using the PTA-ELISA method ($F = 0.63$ at $P = 0.05$). These results were confirmed by split-plot ANOVA (data not shown).

In the presence of *B. cinerea*, the *U. atrum* 385 content (4.98 mg/ml) was higher (2.5-fold) than the amount of *U. atrum* strain 18559 and *U. atrum* strain 18558 after 6 days of colonization (Fig. 3A). Univariate ANOVA indicated the difference was significant ($F = 20.04$, $P = 0.047$) after 6 days of colonization. The comparison by Snedecor-Newman-Keuls test between leaf treatments with different *U. atrum* strains confirmed that the colonization by *U. atrum* 385 was significantly higher than the two other *U. atrum* strains ($P = 0.05$).

On the other hand, the measurement of *B. cinerea* colonization by PTA-ELISA on necrotic strawberry leaflets revealed similar colonization profiles when *B. cinerea* was inoculated alone or in the presence of the strains 18558 and 18559 (Fig. 3B). In contrast, the amount of *B. cinerea* mycelium was significantly lower (approximately five times) in the presence of the reference strain 385 after 6 days of incubation. This significance was established by univariate ANOVA ($F = 11.97$ at $P = 0.035$), followed by Snedecor-Newman-Keuls test ($P = 0.05$). Split-plot ANOVA confirmed the significant difference ($F = 15.372$ at $P = 0.05$).

Activities of PCWDEs produced by *B. cinerea* and the three *U. atrum* strains during the different stages of strawberry leaf colonization. Nine classes of enzymatic activities involved in the degradation of plant cell walls were estimated in homogenates extracted from strawberry necrotic tissues colonized by *U. atrum* alone (strains 385, 18558, or 18559). Similar activities of nonspecific esterase, cutinase, lipase, pectin lyase (PnL), polygalacturonase (PG), and pectin methyl esterase (PME) were detected in extracts of tissue treated with *U. atrum* whatever the strain (data not shown). Only lipase, PL, cellobiase, and β-1,4-glucanase exhibited higher activities in extracts of tissue colonized by the most efficient antagonistic strain 385 of *U. atrum* in comparison with other strains. These four enzymatic activities were compared to those produced by *B. cinerea* alone on necrotic strawberry leaflets (Fig. 4).

The lipolytic specific activity showed a sharp peak in the strawberry leaflets after 12 h of colonization by *U. atrum* 385, whereas the activity was not detected in extracts of leaflets colonized by the two other strains (18558 or 18559) and by *B. cinerea* (Fig. 4A).

After 2 days of colonization, the extracts of strawberry leaflets colonized by *U. atrum* 385 exhibited the highest PL specific activity (9.16 SU). Then, the activity rapidly decreased to reach 3 SU after 4 days. In contrast, activity profiles in extracts of leaflets colonized by the other strains (*U. atrum* 18558 and *U.*

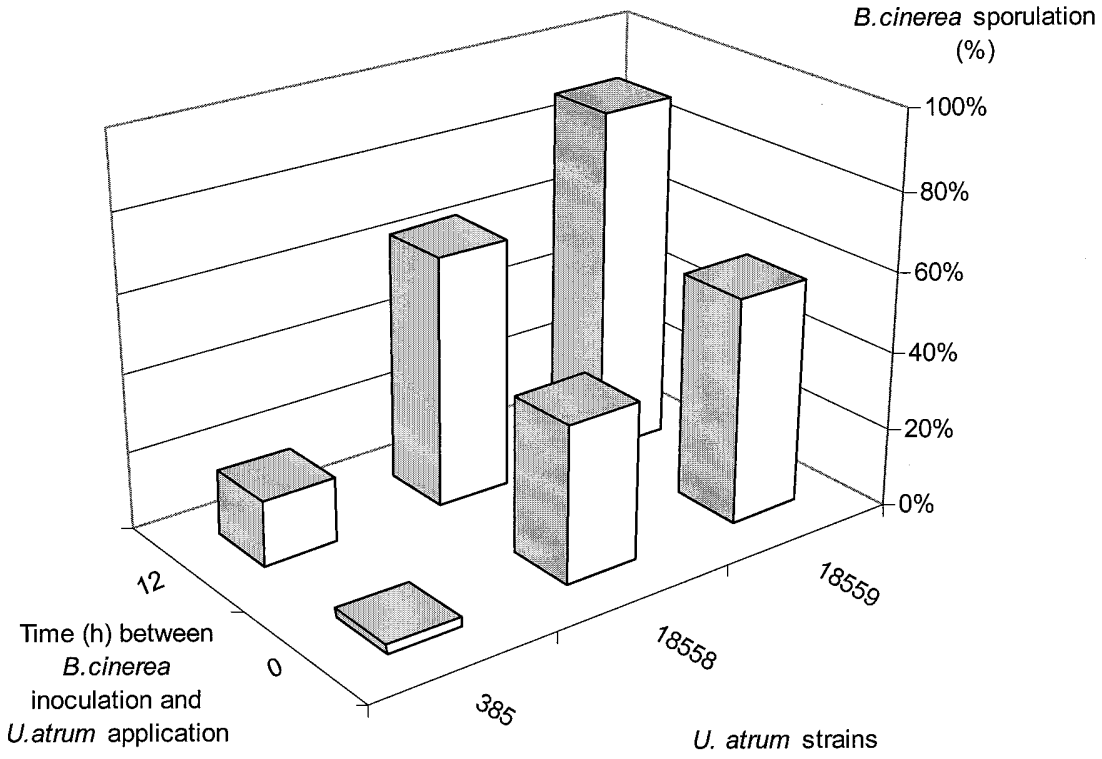

Fig. 1. Effect of different *Ulocladium atrum* strains (isolates 385, 18558, and 18559) on number of *Botrytis cinerea* spores produced on necrotic fragments of strawberry leaves. Conidial concentrations of *B. cinerea* inoculation and *U. atrum* (strains 385, 18558, and 18559) application were adjusted to 10^4 spores/ml and 2×10^6 spores/ml, respectively, at a sprayed volume of ≈5 µl/cm^2 of leaf area. *B. cinerea* sporulation was determined with a Bürker cell from washings of four leaflet disks (10 cm^2) cut after 6 days of colonization. Mean percentage of *B. cinerea* sporulation is expressed in percent of the control and is the result of four independent experiments with four replicates each.

atrum 18559) revealed a slighter increase after 2 days of incubation (≈2 SU) and then slowly decreased for the whole period of the experiment (Fig. 4B). There was no difference between the extracts prepared from leaflets colonized by the different strains (385, 18558, or 18559) after 4 days. During the same period, low PL activity was detected in *B. cinerea* extracts (≈0.5 SU) in comparison with the activity measured the second day with *U. atrum* 385 or with both of the other *U. atrum* strains.

Cellobiase specific activity went up steadily from 48 h to 6 days, when it reached ≈5 SU in extracts of strawberry leaflets colonized by *U. atrum* 385. After 4 days of colonization, the increase was three times higher in leaf extracts colonized by *U. atrum* 385 than by the other *U. atrum* strains (Fig. 4C). Low cellobiase activity (≈0.5 SU) was measured in dead strawberry leaflets colonized by *B. cinerea* whatever the incubation time. After 6 days, a 10-fold higher activity was observed in *U. atrum* 385 extracts than in *B. cinerea* extracts.

The endo-β-1,4-glucanase profiles of all *U. atrum* strains remained stable at ≈60 SU for the first 4 days. Then, the specific activity of extracts from *U. atrum* 385-colonized leaflets continued to increase and reached 116.9 SU after 6 days, while the activity remained unchanged for the other strains (isolates 18558 and 18559). In contrast, the endo-β-1,4-glucanase activity in tissues colonized by *B. cinerea* was much higher throughout the 6 days of incubation (Fig. 4D). The highest value was recorded after 3 days (5,573 SU).

Assays of fungal cell wall-degrading enzymes. Residual activities were detected in control extracts (leaflets precolonized only by *B. cinerea*) and were subtracted from the activities linked to *U. atrum* colonization on *B. cinerea* precolonized leaflets (Fig. 5). Regardless of the time of precolonization of *B. cinerea* (0 or 24 h), the β-1,3-glucanase activity measured after 4 and 6 days of incubation in extracts of leaflets colonized by *U. atrum* 385 was significantly higher than in extracts of leaflets colonized by the other *U. atrum* strains (Fig. 5A). The β-1,3-glucanase activity linked to *U. atrum* 385 colonization was increased twofold (7.95 SU) in the presence of *B. cinerea* biomass on necrotic tissues of strawberry (Fig. 5B) as compared with activity in the absence of *B. cinerea* (3.30 SU) (Fig. 5A). Similar results were obtained on leaflets with 12 h of *B. cinerea* precolonization (data not shown). No stimulating effect was observed with the strains 18558 and 18559 of *U. atrum*.

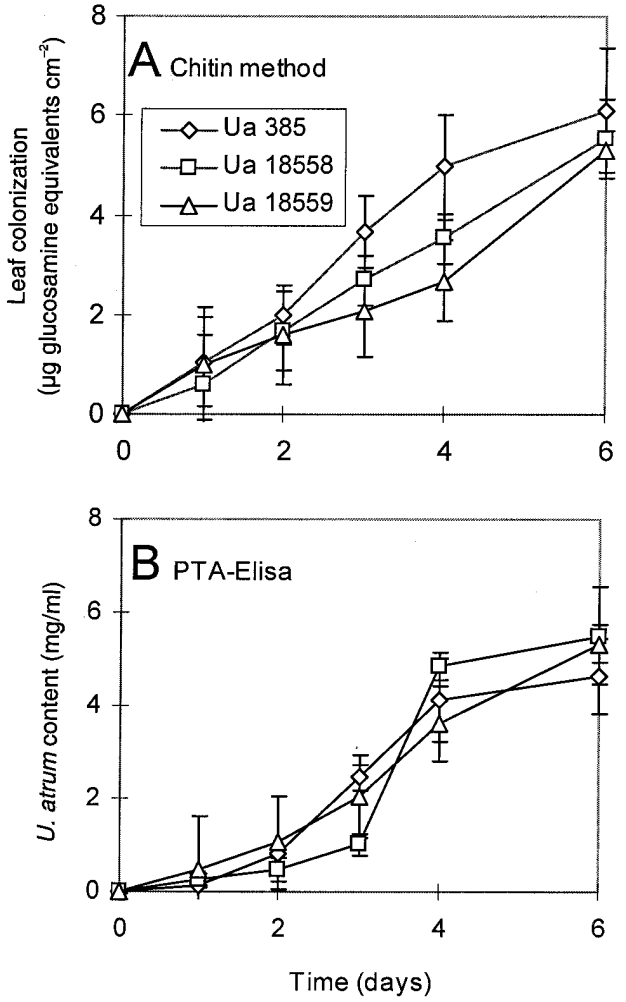

Fig. 2. Comparison of necrotic strawberry leaflet tissues colonization by three *Ulocladium atrum* strains (385, 18558, and 18559) in the absence of *Botrytis cinerea*. *U. atrum* (strains 385, 18558, and 18559) was applied at a concentration of 2×10^6 spores/ml on dead leaf tissues by spraying 5 µl/cm^2 of leaf area. **A,** Quantification of colonization by chitin content method. Data are means of three independent experiments with two replicates each. **B,** Immunoquantification of leaf colonization by plate trapped antibodies enzyme-linked immunosorbent assay using specific anti-*U. atrum* antibodies. Data are means of two independent experiments with two replicates each. Vertical bars represent standard error of the mean.

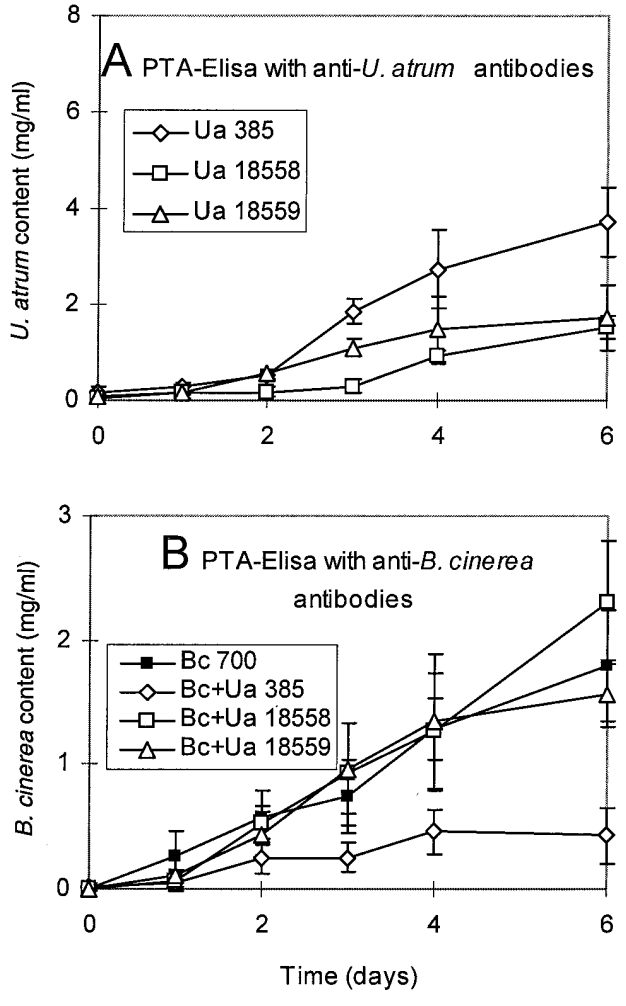

Fig. 3. Comparison of leaflet tissues colonized by three *Ulocladium atrum* strains (385, 18558, and 18559) or by *Botrytis cinerea* in antagonistic condition. Conidial concentrations of *B. cinerea* inoculation and *U. atrum* (strains 385, 18558, and 18559) application were adjusted to 10^4 spores/ml and 2×10^6 spores/ml, respectively, at a sprayed volume of 5 µl/cm^2 of leaf area. *B. cinerea* and *U. atrum* were sprayed simultaneously on dead strawberry leaflets. **A,** Immunoquantification of strain 385, 18558, or 18559 of *U. atrum* in the presence of *B. cinerea*. **B,** Immunoquantification of *B. cinerea* in the presence or absence of *U. atrum.* Data are means of two independent experiments with two replicates each. Vertical bars represent standard error of the mean.

DISCUSSION

U. atrum 385 was first isolated by Köhl et al. (22,23) for its ability to suppress *B. cinerea* sporulation on necrotic onion tips and lily leaves. This antagonistic effect of *U. atrum* was confirmed with stem wounds of tomato in greenhouse crops (13) and with grapevine in field conditions (28). The efficacy of *U. atrum* against *B. cinerea* has not been tested on strawberry leaves yet, although *U. atrum* showed a potential to reduce fruit rot on strawberry (20). Our results confirmed the antagonistic effect of *U. atrum* on this model by reducing the *B. cinerea* sporulation on necrotic leaves. Such reduction in production of *Botrytis* inoculum by application of *U. atrum* 385 can contribute to slow down the gray mold epidemy spread when the population of *B. cinerea* is developing independently of exogenous inoculum (18). On the other hand, we revealed that two other *U. atrum* strains (18558 and 18559) were consistently less effective.

Classical techniques are limited in their ability to quantify mycelial development on plant substrates by different fungi (2,8). Although chitin is strictly associated with the fungal cell wall (27), its assessment cannot distinguish between the pathogen and the antagonist present on the same biological niche. On the other hand, immunoquantification methods developed by Karpovitch-Tate et al. (18) allowed specific monitoring of the growth of *U. atrum* or *B. cinerea* in strawberry necrotic tissues in the presence of the other fungal competitor in biocontrol conditions.

The respective mycelial development of the three *U. atrum* strains on dead strawberry tissues was first evaluated without the pathogen and appeared similar in term of chitin content. This similar colonization of leaf tissues by the *U. atrum* strains observed with chitin assays in the absence of *B. cinerea* was confirmed by immunoassays. In contrast, when *U. atrum* and *B. cinerea* colonized the leaves together, the better colonization of *U. atrum* 385 in comparison with the other *U. atrum* strains established the best biocontrol efficacy of the reference strain 385. Moreover, the *B. cinerea* leaf colonization was reduced in the presence of *U. atrum* 385 compared to that observed alone or in presence of the less efficient strains of *U. atrum* (18558 and 18559), suggesting a close relationship between biocontrol efficacy and level of colonization both by the antagonist and the pathogen.

Filonow (12) used a radioactive labelling technique to show that nutrient competition was involved in biocontrol, with antagonistic yeasts against *B. cinerea* assimilating ^{14}C-sucrose more rapidly than the pathogen. The study of CWDE associated with the colonization of necrotic tissues represents an alternative used in this article to tentatively connect competitive colonization with competition for nutritive elements.

Three PCWDEs (lipase, PL, and cellobiase) were proposed as candidates which could be involved in the competition, due to their higher enzymatic activities in leaf extracts colonized by *U. atrum* 385 than by *U. atrum* strains or *B. cinerea*. Enzymatic activities of nonspecific esterase, cutinase, PME, PG, and pectin lyase produced by *U. atrum* 385 were also compared to those of the other *U. atrum* strains, but the levels were low and they did not appear to be involved in the increased efficiency of the colonization of strawberry leaf tissue by *U. atrum* 385.

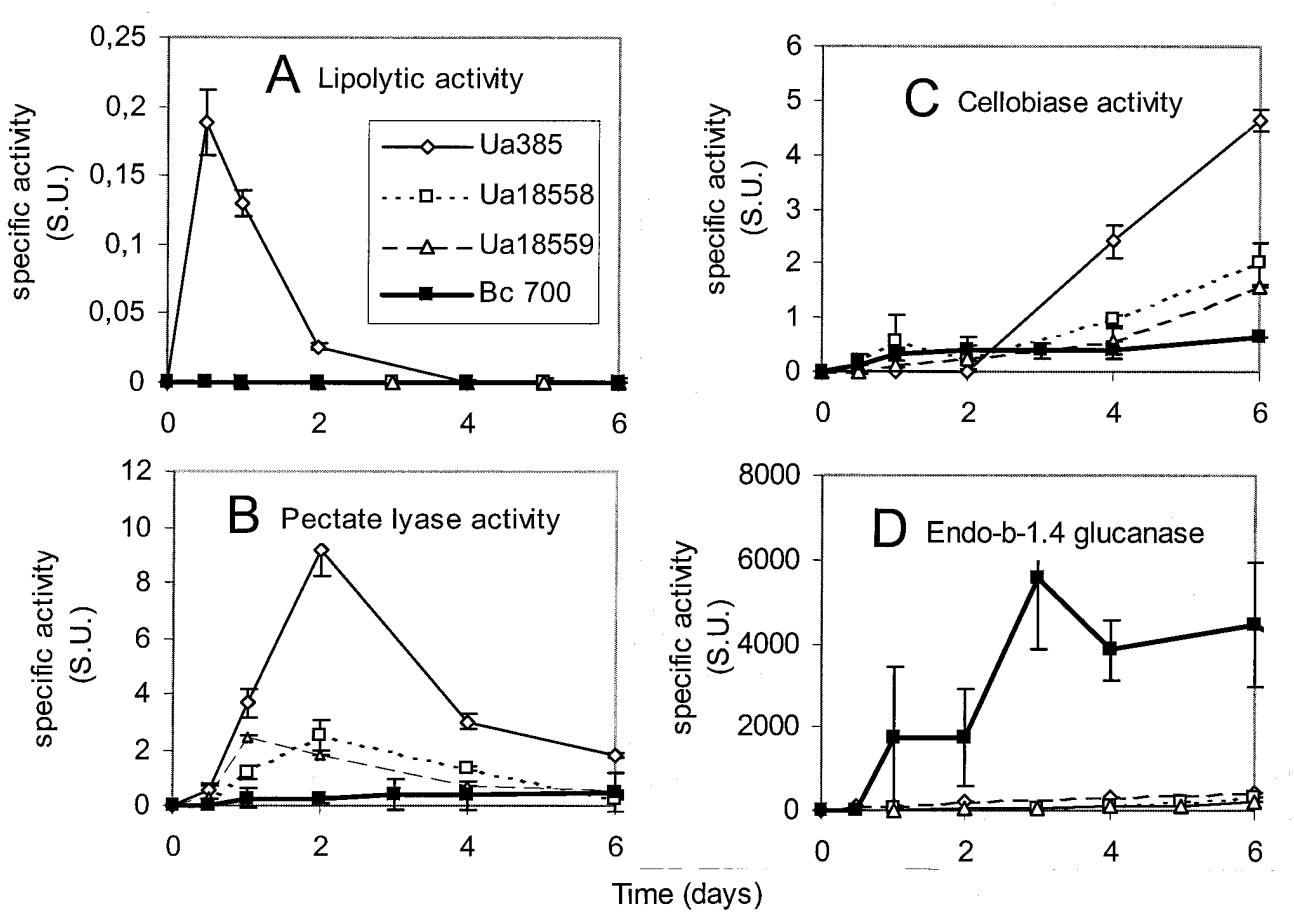

Fig. 4. Specific activities of plant cell wall–degrading enzymes in extracts of strawberry necrotic leaflets after growing periods of colonization by one of the three different *Ulocladium atrum* strains (385, 18558, or 18559) or by *Botrytis cinerea*. *B. cinerea* inoculation (10^4 spores/ml) and *U. atrum* application (strains 385, 18558, and 18559 at 2×10^6 spores/ml) were simultaneously sprayed at 5 µl/cm^2 of leaf area on dead strawberry leaflets. **A,** Lipolytic activity, **B,** pectate lyase activity, **C,** cellobiase activity, and **D,** endo-β-1,4-glucanase activity. Respective specific units (SU) are detailed in Materials and Methods section. Data on *U. atrum* 385 extracts are means of three experiments with three replicates each. Data on *B. cinerea* and *U. atrum* 18558 and 18559 extracts are means of two experiments with two replicates each. Vertical bars represent standard error of the mean.

A lipolytic activity was reported to be involved in the first steps of plant infection of *B. cinerea* (5), and Garcia-Lepe et al. (14) proposed that lipase activity could be also involved in fungal autolysis. In the framework of our study, lipase could be associated with the biocontrol effectiveness of *U. atrum*. Its exclusive detection during the first 24 h of *U. atrum* colonization suggested a relation with esterified polymers degradation of plant epicuticular waxes or cutin (1). This phenomenon does not support the hypothesis of a synergistic action of lipase with other FCWDE (3) by exolysis on *B. cinerea* cell membrane degradation.

PL is considered as a major enzyme for hydrolyzing pectin polymers (15), although the pectin degradation appeared more successful when PL was acting synergistically with pectin lyase and pectin methyl esterase (16). The last selected PCWDE was cellobiase, able to degrade cellobiose, a dimer of glucose, into a glucose unit. Cellobiose is released with other oligomers after the enzymatic hydrolysis of cellulose layers of plant cell walls by β-1,4-glucanases (7). In this study, endo-β-1,4-glucanase and cellobiase activities were only detected after 4 days of colonization, when all the pectinolytic enzymes have been detected (6). Endo-β-1,4-glucanase was dismissed from our selection of candidate enzymes involved in antagonistic properties of *U. atrum* according to the important levels of activity produced by *B. cinerea* during strawberry leaf colonization, compared with the endo-β-1,4-glucanase activities produced by *U. atrum* strains. However, cellobiose produced by hydrolysis of cellulose caused by *B. cinerea* β-1,4-glucanases could be preferentially used by *U. atrum* 385, which revealed higher cellobiase activity than the other *U. atrum* strains. This observation is in agreement with the similar strawberry leaflet colonization by the three *U. atrum* strains in the absence of *B. cinerea*, but does not exclude a possible synergistic effect between endo-β-1,4-glucanase and cellobiase activities produced by *U. atrum* 385.

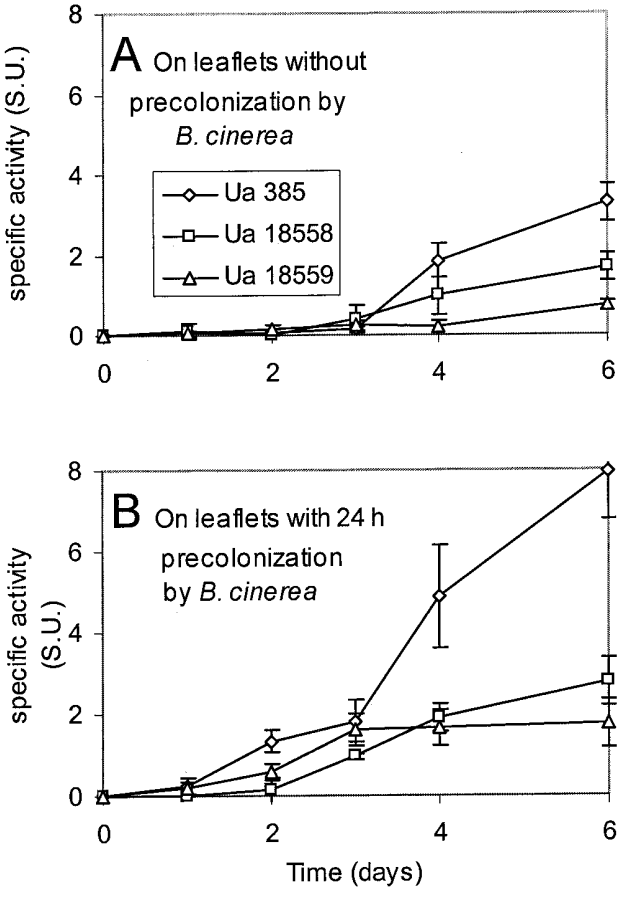

Fig. 5. β-1,3-Glucanase activity produced by different strains of *Ulocladium atrum* (385, 18558, and 18559) on strawberry necrotic leaflets precolonized by *B. cinerea*. After increasing period of incubation (**A,** 0 h and **B,** 24 h) at 20°C, *Botrytis cinerea* on necrotic tissues was killed by treatment with gamma rays (40 krays). *U. atrum* strains (385, 18558, and 18559) were then applied on these precolonized leaflets. One specific unit of β-1,3-glucanase activity was defined as 1 mg of glucose released per hour and per milligram of protein. Data are means of two experiments with two replicates. Vertical bars represent standard error of the mean.

The involvement of two or more mechanisms has been demonstrated in several antagonistic systems (24). FCWDEs are associated with degradation of hyphae of many pathogens (11), but few results dealing with this phenomenon in relation to *B. cinerea* are reported. In this context, the specific stimulation of higher β-1,3-glucanase activity produced by *U. atrum* 385 (compared with *U. atrum* strains 18558 and 18559) on leaf extracts precolonized by *B. cinerea* could play an additional role in the antagonistic strategy of *U. atrum* 385 against *B. cinerea*. Nevertheless, the function of the β-1,3-glucanase activity enhancement remains unclear. This enhancement could rest on the direct interaction between *U. atrum* 385 and *B. cinerea*, but could also result in a metabolic process (26), leading to a better dead cell wall degradation of either *B. cinerea* or *U. atrum* itself. The endochitinase activity was also measured in the same extracts prepared for the β-1,3-glucanase assays. No significant difference was observed between the different strains (data not shown). Therefore endochitinase activity should not be involved in the antagonism of *U. atrum*. The production of enzymes that degrade *B. cinerea* cell wall have already been observed (10,17). However, the importance of mycoparasitism in biocontrol of *B. cinerea* in vivo is open to doubt because it may be too slow to be effective alone against a pathogen that germinates and colonizes host tissues quickly (11).

The finding that *U. atrum* 385 colonized the strawberry necrotic tissues more extensively than two other strains with lower antagonistic activities only when the pathogen is present highlights the contribution of competitive colonization in biological control. However, our data underlined the complexity of mechanisms involved in the biological suppression of *B. cinerea* sporulation. Competitive colonization, regarded as an important mechanism in biocontrol strategy of *U. atrum*, does not exclude a combination with another mechanism, involving FCWDE activity. Moreover, toxins or other secondary metabolites produced by *B. cinerea* could interact differently on the physiology of the different *U. atrum* strains during the strawberry leaf colonization and reveal a variation in sensitivity of *U. atrum* strains.

Our results on differential colonization and CWDE activities of *U. atrum* strains associated with strawberry necrotic tissues suggested that nutritive competition could play a role in the mechanism of mode of action, as previously proposed by Köhl et al. (19). To confirm the importance of CWDE, additional studies are needed on the impact of stimulation of CWDE activities on the protection of *U. atrum* 385 against *B. cinerea*. The use of mutants disrupted in production of specific enzymes could complete these studies, but this second approach will require the genetic characterization of *U. atrum* 385 and the development of molecular tools.

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