Inhibition of Pseudothecial Development of *Venturia inaequalis* by the Basidiomycete *Athelia bombacina* in Apple Leaf Litter

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ABSTRACT

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An immunocytochemical stain was developed to detect the basidiomycete *Athelia bombacina* in apple leaf litter. The polyclonal antibodies for *A. bombacina* were sufficiently specific that only hyphae of this fungus were detected in immunocytochemically treated sections of dead leaves inoculated with *A. bombacina*. Apple leaves naturally infected with *Venturia inaequalis* were inoculated with *A. bombacina*, incubated outside from November 1986 to May 1987, and sampled monthly. Sections stained immunocytochemically showed that *A. bombacina* grew endophytically and epiphytically. The antagonist prevented neither growth of hyphae of *V. inaequalis* into the interior of leaves, nor initiation of pseudothecia. There was no particular spatial association between hyphae of the two fungi, nor any sign of direct parasitism of hyphae or pseudothecia of *V. inaequalis*. Pseudothecia in leaves with the antagonist did not mature further than the stage of producing pseudoparaphyses, reaching an average of 84 μm in length. Pseudothecia in leaves without *A. bombacina* developed asci normally and were 108 μm long by 1 May. These data were confirmed by results from an abbreviated sampling scheme of McIntosh apple leaves during 1987–1988 and by observations of pseudothecial inhibition in crab apple leaves following delayed application of the antagonist.

Additional keywords: apple scab, biological control.

The basidiomycete *Athelia bombacina* Pers. (Corticiaceae) has demonstrated potential as a biological control agent for apple scab caused by *Venturia inaequalis* (Cke.) Wint. When leaves naturally infected by *V. inaequalis* were inoculated in the fall with *A. bombacina* and allowed to overwinter in litter bags in the orchard, no ascospores were produced in spring (12). The stage of development of pseudothecia that was inhibited by *A. bombacina*, and the spatial distribution of *A. bombacina* within leaves relative to *V. inaequalis*, were not determined.

One approach to studying the interaction of *A. bombacina* and *V. inaequalis* in leaf litter is by direct observation. Immunocytochemical staining, based on specific antibodies produced against *A. bombacina*, could provide a means of visualizing hyphae of this basidiomycete among other fungi within dead leaves. In the present study, specificity refers to binding of antibodies only to the fungus against which they were produced and not to other species of fungi in the same habitat.

Production of a specific immunocytochemical stain involves preparation of a suitable antigen, appropriate methods for evaluating specificity of the antibodies, and development of the immunocytochemical staining procedure. Methods for preparing fungal antigens (e.g., 5,6,8,17,26) have been empirical, and the degree of specificity of the antibodies has varied. Nevertheless, fungal hyphae and spores have been visualized successfully in natural substrata with specific immunofluorescent (5) or nonfluorescent immunocytochemical stains (8) based on polyclonal antibodies.

The purpose of this study was to develop a specific immunocytochemical stain for *A. bombacina* for detection of hyphae of the fungus within apple leaf tissue, and to use the stain to examine the effect of the antagonist on pseudothecial development of *V. inaequalis*.

MATERIALS AND METHODS

Development of a specific immunocytochemical stain for *A. bombacina*. *Isolate of A. bombacina. A. bombacina*, isolated in March 1978 from apple leaf litter in an experimental orchard near Arlington, WI (2), was identified by Dr. H. H. Burdsall (Forest Products Laboratory, Madison, WI). One isolate (ATCC 20629) was maintained for further experiments. Stock cultures were stored on silica gel. For routine use, cultures of *A. bombacina* and all other fungi were maintained on potato-dextrose agar (PDA) under oil at 4 C (27).

Antigen preparation. A suspension of hyphal fragments of *A. bombacina* was prepared (12), and 1-ml samples were added to flasks containing 500 ml of Czapek solution (27); each flask was amended with 15 g of glucose and 0.5 g of yeast extract.

Flasks were incubated on a rotary shaker (50 rpm) for 10 days at room temperature. No basidiospores were produced in these culture conditions. Mycelium was collected on Büchner funnels and washed three times with distilled water. Approximately 20 g of hyphae was placed in a 250-ml side-arm flask, frozen by immersion in an alcohol-ice bath, and lyophilized. Hyphae were ground in liquid nitrogen with a mortar and pestle, then stored in a desiccator at −20 C. Soluble proteins were prepared from this mycelium by the method of Gerik et al (8), except that the hyphal suspension was sonicated at 75% maximum power (Biosonik II, Bronwill Scientific, Rochester, NY; maximum power 125 W) for 15 min in bursts of 30 sec. The final total protein concentration for *A. bombacina* (ATCC 20629) was 1,620 μg/ml, as determined by the Bio-Rad protein assay (3).

Production of antibodies. The protein solution was dialyzed for at least 6 hr at 4 C against three changes of chilled phosphate-buffered saline (PBS; 0.39 g of $NaH_2PO_4 \cdot H_2O$, 2.01 g of Na_2HPO_4, 8.18 g of NaCl, and 1 L of H_2O, pH 7.3). Approximately 500 μg of soluble protein was dialyzed for each immunization of each animal. Extracts were emulsified with equal volumes of Freund's complete adjuvant (Sigma Chemical Co., St. Louis, MO)

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for the first injection, and with incomplete adjuvant (Sigma) for all subsequent injections.

Preimmune blood samples were collected from two female white Dutch rabbits before the first injection. The rabbits were injected subcutaneously and intramuscularly on days 0, 9, 16, 45, and again at day 90. Rabbits were bled from ear veins before the first injection and then at weekly intervals starting on day 51. Serum was separated and stored in 10-ml aliquots of 1:1 serum/glycerol, with 0.01% NaN_3, at −20 C (8).

Antibody titer. Antibody titer was assessed by measuring the reactivity of antibodies with a hyphal homogenate of *A. bombacina* by the indirect ELISA methods from Mierendorf and Dimond (19), with the following modifications. For binding to plates, 500 mg of lyophilized, ground hyphae of *A. bombacina* was suspended per milliliter of carbonate coating buffer (CCB: 1.59 g of Na_2CO_3, 2.93 g of $NaHCO_3$, 1 L of H_2O, pH 9.6). The blocking solution used was skim milk (50 g of skim milk per liter of H_2O, adjusted to pH 7.4), and the incubation temperature for antibody steps was 37 C. The serum/glycerol (herein referred to as serum) was diluted 1:1,000 in PBS with 1% bovine serum albumin (BSA 99.9%, Sigma). Goat anti-rabbit alkaline phosphatase (GARAP; Sigma) was diluted 1:1,000 in PBS with 0.1% BSA. After washing, 50 μl of phosphatase substrate (*p*-nitrophenyl phosphate, Sigma, 1 mg/ml of H_2O) were added to each well. The reaction was stopped after 1 hr in the dark by addition of 50 μl of 2 N NaOH per well. Absorbances were read on an automatic ELISA reader (model EL310 EIA Autoreader, Biotek Instruments, Burlington, VA). The reactivity of the preimmune and weekly samples of serum was determined from the mean absorbance reading from four replicate wells for three dilutions of serum (1:10, 1:100, and 1:1,000).

Specificity of antibodies. Specificity of the antibodies for *A. bombacina* was determined by ELISA and by immunocytochemical staining with a range of fungi. For ELISA tests, antibodies were selected from one rabbit according to preliminary ELISA tests with serum from both rabbits. Antibodies from one sample date from one rabbit were chosen according to the ELISA specificity test results for the staining procedure. Fungi tested were selected to represent taxonomically similar and dissimilar basidiomycetes and other fungi commonly found in apple leaf litter (Table 1). Isolates of *A. bombacina*, other species of *Athelia*, and 10 additional basidiomycetes were obtained from Dr. H. H.

TABLE 1. Specificity of antibodies against *Athelia bombacina* 20629 as assessed by enzyme-linked immunosorbent assay (ELISA) and immunocytochemical staining

Fungal group	Name of fungus	Antibodies against *A. bombacina* 20629[a]	
		ELISA absorbance[b]	Stain intensity[c]
Isolates and species of *Athelia*	*A. bombacina* 20629	1.000 ± 0.173	5
	A. bombacina 5626	0.328 ± 0.003	5
	A. decipiens (V. Hoehnel & Litschauer) J. Eriksson 9376	0.182 ± 0.049	1
	A. decipiens 133365	0.284 ± 0.002	3
	A. epiphylla Persoon 8546	0.080 ± 0.021	4
	A. epiphylla 1333892	0.192 ± 0.003	2
	A. galzinii (Bourdot) Donk 5450	0.151 ± 0.022	4
	A. galzinii 4014	0.296 ± 0.002	3
Other Basidiomycetes	*Confertobasidium olivaceoalbum* (Bourdot & Galzin) Jülich	0.533 ± 0.008	3
	Fibulomyces septentrionalis (J. Eriksson) Jülich	0.150 ± 0.044	4
	Fomes fomentarius (L.: Fr.) Kickx	0.193 ± 0.024	4
	Heterobasidium annosum (Fr.) Brefeld	0.399 ± 0.087	3
	Phellinus everhartii (Ellis & Gall.) A. Ames	0.745 ± 0.002	5
	Postia placenta (Fr.) M. Larsen & F. Lombard	0.179 ± 0.017	5
	Stereum hisutum (Will.: Fr.) S. F. Gray	0.547 ± 0.002	3
	Typhula sp. 27 RW EN 6	0.363 ± 0.004	3
	Typhula sp. 27 RW EN 2	0.043 ± 0.009	1
	Typhula micans (Fr.) Berthier	0.085 ± 0.003	4
Apple leaf litter	*Alternaria* sp. A53	0.059 ± 0.000	0
	Alternaria sp. B151	0.003 ± 0.028	1
	Alternaria sp. D46	0.020 ± 0.003	3
	Cladosporium sp. B56	0.012 ± 0.014	2
	Epicoccum sp. A15	0.004 ± 0.022	1
	Epicoccum sp. A41	0.006 ± 0.020	0
	Microsphaeropsis sp. A59	0.003 ± 0.044	1
	Microsphaeropsis sp. B210	0.007 ± 0.007	1
Isolates of *Venturia*	*Venturia inaequalis* 3C5 5/1	0.042 ± 0.012	1
	V. inaequalis 365-4	0.017 ± 0.005	1
	V. inaequalis 222	0.019 ± 0.001	3
	V. inaequalis 282	0.029 ± 0.002	2
	V. inaequalis 1031-4	0.008 ± 0.012	3
	V. inaequalis 289	0.023 ± 0.023	3
	V. inaequalis G2-Br-1	0.028 ± 0.002	3

[a] Results shown are with postimmune, day 77 serum used in both ELISA and stain tests.
[b] Ratio of ELISA absorbance reading with fungus to absorbance with *A. bombacina* 20629. Each value represents the mean, with standard error, of four replicate wells.
[c] 0 = no stain, 5 = maximum stain.

Burdsall. Leaf litter fungi were isolated on PDA and PAB (potato agar with 50 μg/ml a.i. of benomyl, 100 μg/ml of chlortetracycline HCl, and 200 μg/ml of streptomycin sulfate) (28) from apple leaves collected from the floor of an experimental orchard near Arlington, WI, in March 1985. The fungi were grown for 3 wk on PDA overlaid with dialysis membrane (Union Carbide Corp., Chicago, IL). Mycelia then were peeled off the membrane, lyophilized, ground, and wells of ELISA plates were coated as described above for *A. bombacina*. A 1:1,000 dilution of antibodies from each of four different sample times was tested with each fungus (preimmune, days 51, 77, and 112). For each serum date-fungus combination, four replicate wells were used. One well was used on each half of two replicate plates. Absorbance was read for wells coated with hyphal suspensions of *A. bombacina* on each plate in each experiment. Mean readings for each fungus and date of serum were expressed as a proportion of the corresponding readings for *A. bombacina*. The experiment was performed twice.

All fungi used in the ELISA specificity tests also were stained immunocytochemically. Each fungus was grown on PDA as described for the ELISA tests. Three pieces of mycelium, approximately 4–5 mm^2, were peeled off the dialysis membrane and incubated overnight in 100 μl of a 1:100 dilution of preimmune or day 77 serum in PBS with 1% BSA or in PBS-BSA only at 4 C in wells of a 96-well assay plate. Mycelia then were transferred to new wells and washed with three changes (30 min each) of PBS with 0.1% Tween 80 (Sigma), incubated in a 1:100 dilution of GARAP in PBS with 0.1% BSA for 2 hr at 37 C, and washed again as described above. Staining was carried out in the dark at room temperature in 200 μl of staining solution. (A stock substrate solution consisted of 0.15 g of naphthol-AS-phosphate [Sigma] dissolved in 2.5 ml of N-N dimethyl formamide [Sigma] and added to 17.0 g of Tris base [Sigma] in 500 ml of H_2O. The staining solution, prepared immediately before use, consisted of 1 ml of Fast Blue [Sigma] and 5 μl of 0.1 M $MgCl_2$ added per milliliter of stock solution) (8). Squash mounts of hyphae were examined with a light microscope after rinsing. Each fungus was assigned a random number. Intensity of hyphal staining was rated by two observers independently on an arbitrary scale of 0 (no stain) to 5 (maximum stain). Stain ratings were consistently reproducible between observers. To determine the variability of staining and conserve the antibody supply, the experiment was repeated once on a subset of 10 fungi.

Specificity of antibodies for *A. bombacina* also was tested by staining the fungus in sections of apple leaves containing other fungi, including *V. inaequalis*. Leaves infected with *V. inaequalis* were collected from litter in the orchard at Arlington in March 1986, and 10 leaves were inoculated with hyphal fragments of *A. bombacina*. After incubation at 24 C for 4 wk, leaves with and without *A. bombacina* were examined with a dissecting microscope for presence of mature pseudothecia of *V. inaequalis*. Pieces of leaves (approximately 3–4 mm^2) with *V. inaequalis* only (verified to be free of *A. bombacina* by incubating on PAB), with *A. bombacina* only (as confirmed by reisolation onto PAB), with both *V. inaequalis* and *A. bombacina*, or with neither fungus were fixed in FAA (70% ethanol, formaldehyde 37%, glacial acetic acid; 85:10:5, v/v/v), dehydrated in tertiary butyl alcohol, and embedded (15) in Paraplast (Monoject Scientific, St. Louis, MO). Sections 15 μm thick of each leaf type were cut with a rotary microtome, the paraffin removed with xylene (15), and the sections placed in moist chambers. To conserve antibody solutions, small Parafilm (American Can Co., Geenwhich, CT) strips were placed across the slides (1), and a clean slide was placed on top of the strips. Sections were incubated with PBS containing 1% BSA for 20 min at room temperature and then stained immunocytochemically by a procedure similar to the one used for the antibody specificity tests on pieces of mycelium. The serum and the GARAP were each diluted 1:100 and incubated with the sections for 2 hr at 37 C. Slides were rinsed in three, 3-min changes of PBS between each antibody step. The substrate-stain solution was filtered through Whatman No. 1 filter paper immediately before being applied to slides, and was incubated with the sections for no longer than 40 min in the dark before slides were rinsed in PBS and sections were mounted in glycerol jelly.

Growth of *A. bombacina* and effect on development of *V. inaequalis* in apple leaf litter. *Inoculum of A. bombacina.* Inoculum was prepared as described by Heye and Andrews (12), except that mycelial mats from 20 plates were homogenized in 50 ml of phosphate buffer, pH 7.4. In three separate inoculum preparations, the final concentration ranged from 4.08 to 7.94 × 10^5 colony-forming units (cfu)/ml, as assessed by dilution plating on PDA. This represented 31.4–66.6% of the direct counts, enumerated with a hemacytometer. Large clumps of hyphae, although highly visible, were not numerous relative to the number of individual hyphae. Basidiospores were not encountered in the microscope fields examined.

Inoculation, incubation, and sampling of apple leaves. McIntosh apple leaves infected with *V. inaequalis* and inoculated with *A. bombacina* (2.07 × 10^5 cfu/leaf) were incubated in mesh bags on an orchard floor from 1 November 1986 to 1 May 1987 (29). Every month for 6 mo, two inoculated leaves were chosen randomly and removed from each of four randomly selected litter bags, and two leaves were removed from each of four control bags, which contained only uninoculated leaves. The eight treated and eight control leaves were fixed in FAA. Areas infected with *V. inaequalis* from one leaf from each pair per bag were sectioned (see below; histology). In most cases, areas infected by *V. inaequalis* were examined from three leaves inoculated with *A. bombacina* and three uninoculated leaves per month from December to May.

To validate and extend the above results, two additional experiments were performed. First, McIntosh leaves collected on 11 October 1987 were inoculated with *A. bombacina* (5.61 × 10^5 cfu/leaf) and incubated from 2 November 1987 to 19 April 1988 as described for the 1986–1987 experiment. On 19 April 1988, three inoculated leaves from each of three bags, and three uninoculated leaves from each of three control bags were fixed in FAA. One leaf from each of the groups of three leaves (i.e., a total of three inoculated and three control leaves) was processed later for sectioning and staining (see below; histology).

Second, the ability of *A. bombacina* to inhibit maturation of pseudothecia was investigated in leaves where pseudothecia had begun to develop at the time of inoculation with the antagonist. On 14 December 1987, leaves infected by *V. inaequalis* were collected from beneath crab apple trees (*Malus* × *sublobata* (Dipp.) Rehd. 'Yellow Autumn Crab') on the University of Wisconsin-Madison campus. Hyphae of *V. inaequalis* already had grown into the mesophyll tissue. Pseudothecia were between stages 3 (formation of ascogonium) and 5 (pseudoparaphyses fill the lumen) (14). All leaves were stored dry at 4 C in coarse mesh bags until processed 15 days later. Disks 1.8 cm in diameter were cut from areas bearing lesions. Before inoculation with *A. bombacina*, each disk was incubated at 24 C for 24 hr in a 25- × 55-mm glass vial containing 10 ml of sterile vermiculite and 8 ml of sterile water (24). On 1 January 1988, half of the disks were inoculated with 0.1 ml of a hyphal suspension (3.1 × 10^6 cfu/ml) of *A. bombacina*, applied as a drop to the center of each disk. After inoculation, vials were capped, stored at 24 C for an additional 24 hr, then placed at 4 C. Other than for preliminary observations (see below), disks were sampled only at the end of the experiment on 12 May 1988. Three inoculated disks and three uninoculated disks were sampled, and sections from each disk were examined.

Initial growth of *A. bombacina* on crab apple leaves was observed on leaf disks with the aid of scanning electron microscopy (SEM). Two inoculated disks and two uninoculated disks were fixed in FAA immediately after inoculation, and after 24 hr at 24 C. Disks were prepared for SEM (22; amyl acetyl step omitted), and examined at 15 kV with an SEM (model S 5-70 with an LaB6 emission source; Hitachi, Tokyo, Japan). Whole mounts of cleared leaf disks, stained with trypan blue, also were examined with a light microscope after 1 mo of incubation at 4 C.

Histology. To observe distinctive subcuticular growth of *V. inaequalis* and pseudothecia, leaf disks first were placed in alcohol with dissolved chlorine gas (2 g of sodium chlorate was added

to 5 ml of concentrated hydrochloric acid and, immediately afterwards, 50 ml of 70% alcohol was added. Acid and alcohol were decanted off together and used immediately for bleaching.) in tightly capped vials for 1–4 hr until bleaching was complete. Leaf disks were washed in three, 30-min changes of water and cleared in hot chloral hydrate (23). Pieces approximately 4 × 8 mm were excised, embedded, and sectioned as described above. For immunocytochemical staining of sections, antibodies first were absorbed with McIntosh apple leaf material (9; leaves were ground to a powder in liquid nitrogen with a mortar and pestle and washed with acetone) to reduce background staining.

Immediately before staining, slides were cleared of paraffin, bleached in chlorine gas for 3 min, and placed in distilled water, with three, 3-min changes. Free aldehyde groups were blocked with 0.05% sodium borohydride (Sigma) (25) for 30 min. Slides were placed in three changes of water, then immunocytochemically stained as previously described. The controls used were: leaf sections with *A. bombacina* stained with one of the steps omitted from the procedure (serum, GARAP, or stain solution); leaf sections with *A. bombacina* stained with preimmune serum; leaf sections without *A. bombacina* stained with postimmune serum. Sections containing only *V. inaequalis* were immunocytochemically stained with postimmune antibodies in preliminary trials as an additional control. Thereafter, to conserve serum, one slide of these sections was treated with postimmune serum in each staining experiment, and the other slides were examined without staining.

Sections from at least two different areas of each leaf, and at least two slides from each area (not less than 100 sections), were examined. Sections first were examined qualitatively for the presence of hyphae of *A. bombacina* and *V. inaequalis*. Then, the position of the hyphae of each fungus on and within leaf tissue, particularly relative to each other, was noted. The size and stage of internal development of pseudothecia of *V. inaequalis* (14) in leaves with and without *A. bombacina* were determined from serial sections. Pseudothecia in sections from leaves inoculated with *A. bombacina* were rated only if hyphae of the antagonist were present in at least one part of each section. Identity of each pseudothecium was confirmed by the presence of hyphae connecting it with subcuticular stroma of *V. inaequalis* in one or more of the serial sections. The identity of hyphae of *V. inaequalis* was similarly confirmed by connections to the subcuticular stroma in at least one of the serial sections.

The number of pseudothecia observed ranged from 2 to 157 (mean = 31, SE = 34) per leaf. In 28% of all leaf samples in the 1986–1987 experiment, particularly in the first 3 mo of sampling, infection was not extensive and fewer than 10 pseudothecia per leaf were available for rating. Unequal numbers of pseudothecia resulted in unbalanced data. Whereas unequal numbers of leaves can be dealt with easily in a statistical analysis, unequal numbers of leaves *and* pseudothecia result in a type of imbalance that is more difficult to analyze and interpret than a corresponding analysis without such imbalance (20). Although an analysis on the unbalanced pseudothecial data was conducted to yield approximate comparisons (and means, calculated from all observations), a more easily interpretable analysis on the stage of development of pseudothecia was performed with five observations, randomly sampled from each leaf. The mean stage of development of pseudothecia was calculated based on the five sampled observations, and these means were used in an analysis of variance with two factors: month of observation and presence or absence of *A. bombacina*. Differences between groups of inoculated and uninoculated leaves for each month were compared with Student *t* tests, with the mean square error from the analysis of variance on the balanced data set. Residual plots were examined to ensure that the assumptions for the statistical models were met. A similar (balanced) analysis was conducted on the observations on pseudothecial length. For both the 1988 McIntosh and crab apple experiments, at least 10 observations per leaf were available, and a (balanced) analysis was conducted on 10 observations randomly sampled from each leaf. All means and significance levels reported in the text and tables were from the analyses of data with five (1987) or 10 (1988) random observations per leaf.

Color photographs were taken with Kodak Ektachrome 100 Professional Plus film at ASA 100, with a combination of didymium and yellow filters and exposure times of 1/125 to 1/30 sec.

RESULTS

Development of a specific immunochemical stain for *A. bombacina*. In ELISA tests, a 1:1,000 dilution of antibodies from day 77 gave absorbance readings >0.9. These antibodies were used in all immunocytochemical staining tests. Preimmune antibodies at the same dilution gave absorbance readings of <0.02. According to ELISA tests, antibodies to *A. bombacina* (20629) were relatively specific; reactivity of antibodies with A. bombacina (20629) was at least three times higher than reactivity with other isolates of *A. bombacina* and most other fungi (Table 1). Several other basidiomycete species gave relatively high absorbance values, e.g., *Confertobasidium olivaceoalbum* (Bourdot & Galzin) Julich, *Phellinus everhartii* (Ellis & Gall.) A. Ames and *Stereum hirsutum* (Will.:Fr.) S. F. Gray. ELISA readings generally were low with isolates of *V. inaequalis* and other leaf litter fungi (Table 1).

The antibodies were less specific for *A. bombacina* in immunocytochemical tests than in ELISA tests (Table 1). Isolate 5626 of *A. bombacina*, and certain other basidiomycetes, e.g., *P. everhartii*, also had a rating of 5 (maximum stain). However, the majority of the *Athelia* spp. and other basidiomycetes were more lightly stained, with ratings of 1–4. Generally, these fungi have not been isolated from apple leaf litter (2), with the exception of an unidentified *Typhula* sp. Apple leaf litter fungi (mean rating 1.1) and isolates of *V. inaequalis* (mean rating 2.3) were lightly stained and easily distinguishable from stained hyphae of *A. bombacina*.

There was no significant correlation between ELISA absorbance readings and corresponding stain ratings for the fungi (R^2 = 19.6%, $P > 0.05$). Unlike the ELISA assay, the stain rating was subjective, and staining within each hyphal squash tended to be uneven. Thus, stain ratings varied more within groups of taxonomically similar fungi than did the ELISA results. Nevertheless, only hyphae of *A. bombacina* were stained (rating 5) in leaf sections containing *A. bombacina* and hyphae and pseudothecia of *V. inaequalis*, plus other microorganisms. Evidence for this included the presence of clamp connections on stained hyphae, hyphal dimensions consistent with *A. bombacina* (width 3–5 μm), and lack of staining in control sections from leaves without *A. bombacina*. In control tests, sections of leaves containing *A. bombacina* or *V. inaequalis*, both fungi, or neither fungus did not stain when preimmune antibodies were used, or when the serum or GARAP step was omitted. In representative slides of each staining experiment, sections of leaves containing only *V. inaequalis* did not stain when preimmune antibodies were used.

Growth of *A. bombacina* and effect in vivo on *V. inaequalis*. SEM of crab apple leaves showed that at application, inoculum consisted of large pieces of hymenial mats and numerous small hyphal fragments. After 24 hr at 24 C, a web of straight, relatively unbranched hyphae had grown across the surface of the leaf disk. There appeared to be no relationship between growth of the antagonist and lesions caused by *V. inaequalis*. After 4 wk at 4 C, the cuticle had separated from the epidermis, and hyphae of *A. bombacina* grew relatively straight and unbranched over the cuticle surface. Beneath the cuticle in stained leaves, the hyphae (blue, with many clamp connections) formed a dense, highly branched network over and between the subcuticular hyphae of *V. inaequalis* (distinctive through lack of stain). No specific spatial association was seen between hyphae of the two fungi. The same general relationship was noted in McIntosh leaves after 1 mo of incubation in the orchard.

In field-incubated McIntosh leaves, the web of exposed hyphae of *A. bombacina* usually thickened by spring, especially on the surfaces of the leaves facing the ground. This hymenium often formed basidia with basidiospores from mid-April onward.

Fruiting occurred on the crab apple disks within 2 mo.

In McIntosh leaf sections stained immunocytochemically, hyphae of *A. bombacina* were detected growing along the epidermis of both surfaces, vertically between palisade cells, and horizontally across intercellular spaces in the mesophyll. There always appeared to be more fungal biomass within than on the surface of McIntosh and crab apple leaves. Hyphae of the antagonist frequently were observed clustered in intercellular spaces of the senescent leaves and in empty pycnidia of other leaf litter fungi. Pycnidia were distinguishable from pseudothecia by their thicker, more irregular walls, less differentiated contents, and tendency to develop at either surface of the leaf rather than internally. Hyphae of *A. bombacina* were seen in areas of leaf sections with and without hyphae of *V. inaequalis*, and grew across and adjacent to hyphae and pseudothecia of *V. inaequalis*. However, there was no evidence of direct parasitism of *V. inaequalis* by *A. bombacina*, e.g., no intertwining of hyphae and no growth into pseudothecia by *A. bombacina*. Although no stain was used for *V. inaequalis*, its hyphae were detectable when sections were bleached immediately before staining immunocytochemically for *A. bombacina*.

Analysis of variance of the observations on pseudothecial stage that were sampled randomly from each leaf, followed by *t* tests, gave results similar to those from the analysis on the data set that had unequal subsample (pseudothecial) numbers. Both analyses indicated a highly significant interaction between month of observation and presence or absence of the antagonist ($P = 0.0025$, from the analysis based on the randomly sampled pseudothecia). From 1 December 1986 through 1 March 1987, there was no significant difference ($P < 0.5$) in development of pseudothecia in McIntosh leaves with or without *A. bombacina* (Table 2). However, in leaves treated with the antagonist, pseudothecia did not progress, on average, beyond stage 3 or 4 (Table 2; Fig. 1a, c, e, g). In leaves without *A. bombacina*, pseudothecial development progressed steadily from February onward from stage 3 to reach a mean of stage 8 by April (Table 2; Fig. 1b, d, f, h). From 1 April until 1 May, pseudothecial development in control leaves was significantly different ($P < 0.001$) from that in leaves inoculated with *A. bombacina*.

Pseudothecia in the 1987–1988 McIntosh leaves treated with the antagonist developed, on average, to stage 5.0 ± SE 0.5 (range 3–7; 62 pseudothecia observed in total). Pseudothecia in leaves without *A. bombacina* reached a mean of stage 8.5 ± SE 0.2 (range 8–10; 116 pseudothecia observed), which was significantly different ($P < 0.001$) from development in leaves with the antagonist. Pseudothecia were arrested in the antagonist-inoculated crab apple leaf disks at stage 3.6 ± SE 0.03 (range 3–4; 54 pseudothecia observed) despite the more advanced development of *V. inaequalis* at the time of application of the antagonist. In the absence of *A. bombacina*, pseudothecia matured in crab apple leaf disks to the point of producing olivaceous ascospores (stage 12.3 ± SE 0.3; range 9–14; 170 pseudothecia observed), which was significantly different ($P < 0.001$) from development in the presence of the antagonist.

Length of pseudothecia in the 1986–1987 experiment increased from 52.4 ± SE 1.73 μm on 1 December 1986 to a mean of 112.6 ± SE 6.2 μm on 1 May 1987, in leaves without *A. bombacina* (Fig. 2), whereas they increased from 53.7 ± SE 8.1 μm to 81.2 ± SE 17.9 μm in leaves treated with the antagonist. Similar results were obtained in the 1988 McIntosh leaves. In crab apple disks inoculated with *A. bombacina,* pseudothecia were 51 ± SE 1.3 μm long after incubation for 5.5 mo compared with 125 ± SE 2.4 μm in control leaves.

DISCUSSION

ELISA and the immunocytochemical staining procedure gave differing results on specificity of the antibodies to *A. bombacina*. Here, as in previous work with polyclonal antibodies against fungi (e.g., 8,16), antibodies used with ELISA were specific for the isolate used as antigen, with intermediate reactivity occurring only in the case of taxonomically similar fungi. In the staining test with the same range of fungi there was less specificity of the antibodies; cross reactions occurred with taxonomically dissimilar fungi and with taxonomically similar fungi. These results are comparable to those from previous work with an indirect fluorescent antibody staining technique (4,7,18). Where both an ELISA and a staining assay have been conducted with the same set of antibodies, specificity was lower with the staining test (8).

The difference in specificity may be attributed partly to the more subjective rating of staining results, to uneven staining within a hyphal squash mount, and to binding of antibodies to different antigens in the two tests. Soluble antigens are bound in ELISA, whereas antibodies bind to insoluble surface antigens in staining tests. Surface antigens may include cell wall components common to different fungi (8). Nevertheless, antibodies against *A. bombacina* were 'specific' in that no stain was seen in field-incubated leaf material in the absence of the antagonist.

Specific staining of hyphae of *A. bombacina* within bleached leaf sections, where hyphae of *V. inaequalis* also were distinguishable, allowed the spatial arrangement of the two fungi to be observed. There was no evidence for parasitism of hyphae or pseudothecia of the scab fungus, and other modes of antagonism could not be determined. Apparently, the antagonist produces pectinases (R. Spear, *personal communication*). This may explain the removal of cuticles on treated apple leaves, since pectinases have been used to remove intact cuticles (21). Pectinase, together with cellulase production (13), evidently accounts for accelerated decomposition of leaves (10,11). A likely mode of antagonism, now under investigation, is nutrient competition. It also is possible that pseudothecial development may be inhibited by disruption of the leaf structure by *A. bombacina*.

The advantages of using a system of field-incubated leaves naturally infected with *V. inaequalis* include good establishment of the pathogen before application of the antagonist, normal growth of hyphae and pseudothecia of *V. inaequalis* within leaves, and confirmation of the antagonist's ability to inhibit pseudothecial development under natural conditions. Disadvantages of this system include asynchronous initiation of pseudothecia, difficulty in locating pseudothecia, and dependence of pseudothecial maturation on the weather. In an attempt to overcome these disadvantages, detached, senescent, gamma-irradiated apple leaves from the field were inoculated with two compatible strains

TABLE 2. Mean stage of pseudothecial development of *Venturia inaequalis* in McIntosh apple leaves[a], with and without inoculum of *Athelia bombacina*

Sample date	Stage of development[b]			
	With *A. bombacina*[c]		Without *A. bombacina*	
	Stage	Range	Stage	Range
1 December 1986	2.9 ± 0.4	2–3	2.5 ± 0.5	2–3
1 January 1987	3.1 ± 0.3	3–4	3.7 ± 0.4	3–4
1 February 1987	3.4 ± 0.6	3–4	3.2[d]	3–4
1 March 1987	3.9 ± 0.2	3–4	5.1 ± 0.1	5–6
1 April 1987	3.8 ± 0.2	3–4	8.0 ± 1.1[e]	5–9
1 May 1987	3.7 ± 0.7	3–4	7.8 ± 0.6[e]	7–9

[a] McIntosh apple leaves overwintered from 1 November 1986 to 1 May 1987 in 1.4- × 1.6-mm mesh nylon bags on the orchard floor.

[b] Mean stage of development, ± standard error, based on pseudothecia sampled randomly from each leaf. Stage of development ranged from 2 (pseudothecial initial showing coiling of hyphae) to 12 (ascospores pigmented and mature) (14).

[c] Inoculum of *A. bombacina* applied on 30 October 1986.

[d] No estimate of SE available; only one leaf available.

[e] Significantly different ($P < 0.001$) from development in leaves inoculated with *A. bombacina* for the same sample date, as indicated by *t* tests on balanced data obtained by randomly choosing five observations from each leaf.

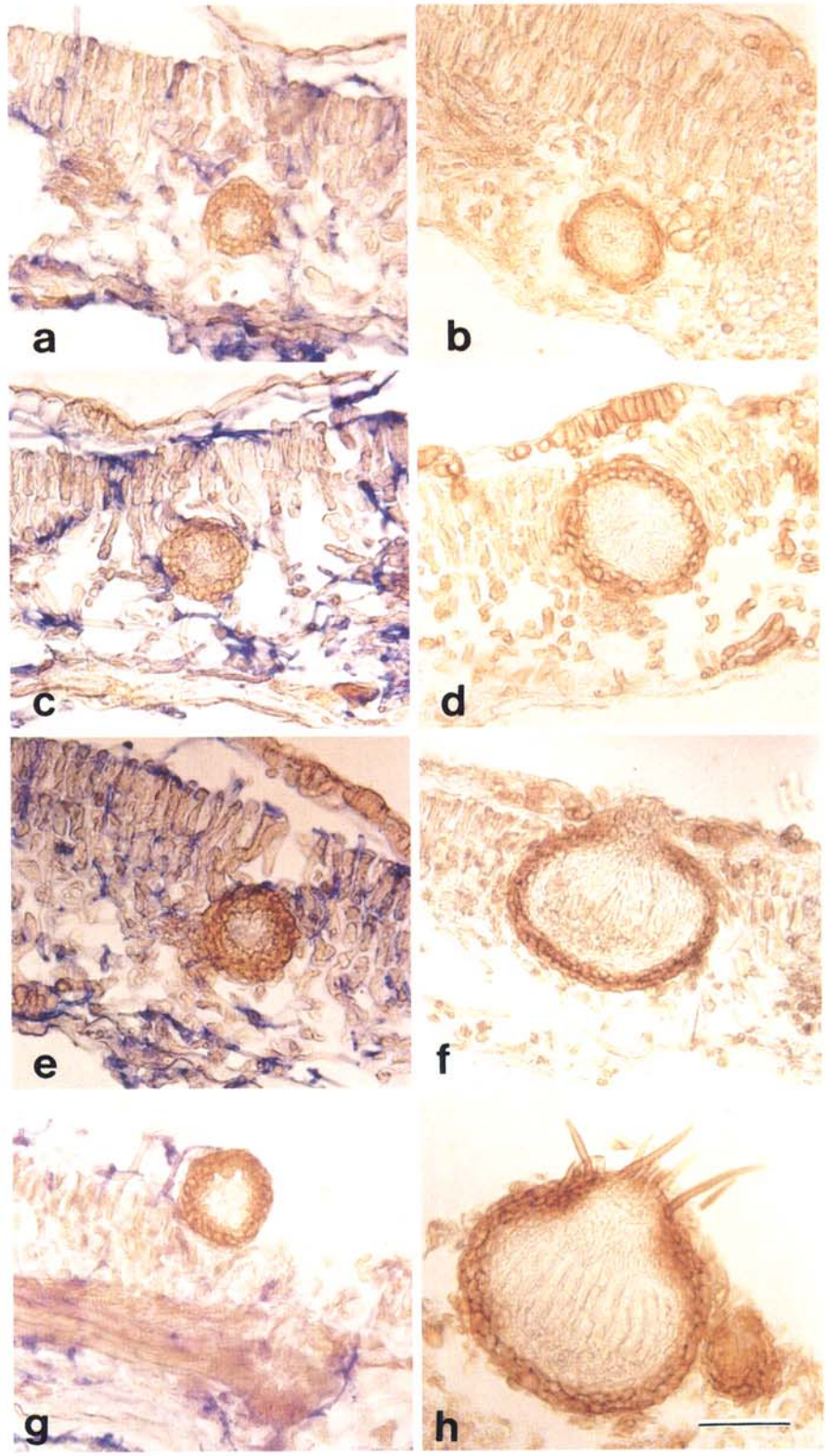

Fig. 1. Pseudothecial development of *Venturia inaequalis* in naturally infected McIntosh apple leaves, with (a, c, e, g; blue hyphae of *Athelia bombacina* within leaf sections) and without (b, d, f, h) colonization by *Athelia bombacina*. Leaf sections in a, c, e, and g were stained immunocytochemically for *A. bombacina*. Leaf sections in b, d, f, and h were not immunocytochemically stained; representative sections in each experiment were stained for *A. bombacina*; no staining of hyphae was observed in those sections where *A. bombacina* was absent. Leaves were inoculated on 29 October 1986 and incubated on the orchard floor from 1 November 1986 to 1 May 1987. See James and Sutton (14) for descriptions of stages of pseudothecial development (237 ×; bar = 55 μm). **a and b,** 1 February 1987. Stage 3 (formation of ascogonium from initial). **c and d,** 1 March 1987. c, stage 3 and d, stage 5 (pseudoparaphyses fill lumen of pseudothecium, which has increased in diameter). **e and f,** 1 April 1987. e, stage 4 (pseudoparaphyses appear in lumen), and, f, stage 8 (asci formed but their contents not differentiated). **g and h,** 1 May 1987. g, stage 4, and h, stage 8 (pseudothecium almost full size, but ascospores not differentiated).

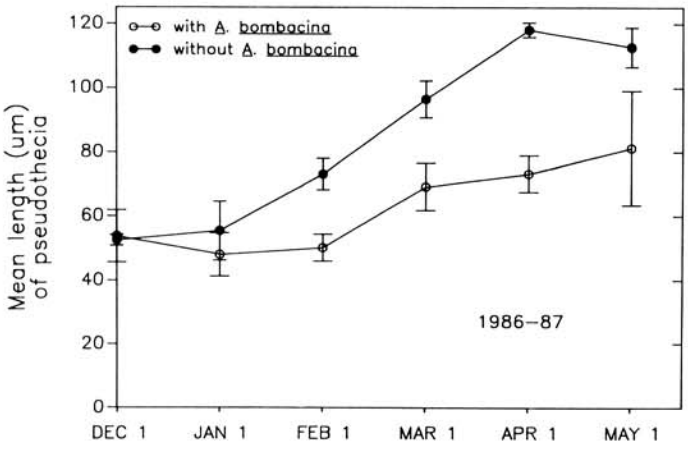

Fig. 2. Increase in size of pseudothecia of *Venturia inaequalis* in apple leaves, with and without the antagonist, *Athelia bombacina*. Each point represents the mean of all observations from, typically, three leaves; bars = standard error.

of *V. inaequalis* and incubated under controlled, humid conditions (24). Additionally, green leaves of potted seedlings inoculated with compatible strains were removed following lesion development and incubated similarly. However, in both of these systems, most pseudothecia were formed on or away from the surface of leaves, amongst an extensive surface growth of hyphae of *V. inaequalis*. This is unlike saprophytic growth in nature, which is mostly within leaf tissue, and an examination of the spatial distribution of the antagonist and apple scab fungus would not have been relevant to the field situation. Incubation of naturally infected leaves in vitro resulted in a small amount of surface growth of *V. inaequalis*, but pseudothecia developed normally within leaves.

The presence of pseudothecia in infected field-incubated leaves that were inoculated with *A. bombacina* may be attributed to insufficient colonization by the antagonist in the first 2–3 wk of the experiment. This was suggested by Heye and Andrews (13) who showed that, when colonization of leaf disks was not extensive, incipient, but not mature, pseudothecia developed. In contrast, few or no incipient pseudothecia reportedly were formed if colonization by the basidiomycete was extensive. Those observations were made with a dissecting microscope on cleared leaves, and pseudothecial initials may have been present but not mature enough to be visible. In the present study, results were based on a more sensitive histological method.

No conclusions can be made whether treated leaves had reduced numbers of pseudothecia compared with control leaves, because portions of leaves to be sectioned were selected nonrandomly for infected areas, and an effort was made to examine a given number of pseudothecia from each of these areas. However, even if the number of pseudothecia was not reduced, these pseudothecia did not mature in treated leaves. We confirmed this with the crab apple system, because young pseudothecia (stage 3–4) present at the time of inoculation with *A. bombacina* did not mature.

In overview, *A. bombacina* applied to McIntosh leaves when hyphae of *V. inaequalis* were still subcuticular neither prevented growth of hyphae of *V. inaequalis* down into the mesophyll, nor initiation of pseudothecia. However, at the conclusion of the experiment, these pseudothecia had not matured or produced ascospores. Even delayed applications of *A. bombacina* to crab apple leaves, when pseudothecial initials of *V. inaequalis* had already formed, prevented pseudothecia from maturing in vitro. Further work is needed to determine how early or late inoculum could be applied and still result in inhibition of both pseudothecial development and ascospore production. There may be a period of several months during which an application of *A. bombacina* would prevent ascospore production in the spring.

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