Antagonism of *Athelia bombacina* and *Chaetomium globosum* to the Apple Scab Pathogen, *Venturia inaequalis*

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ABSTRACT

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Athelia bombacina and *Chaetomium globosum*, applied to apple leaves, decreased production of ascospores by *Venturia inaequalis* and altered leaf decomposition. In autumn, inocula of the antagonistic fungi were sprayed on detached leaves or leaf disks naturally infected with *V. inaequalis*. Each antagonist was applied as a suspension of fungal propagules in buffer, as a suspension of propagules in carboxymethylcellulose:malt extract:yeast extract solution (36:46:18, w/w), or as a suspension of colonized bran particles in buffer. Leaf disks were incubated in the laboratory at 4 C and intact leaves were overwintered on the orchard floor. The fungi colonized the leaf disks and the intact leaves.

On leaf disks the antagonists reduced ascospore production by 40–100% on a logarithmic scale (~80–100% on an arithmetic scale). For the outside incubations, no ascospores were produced on the leaves treated with *A. bombacina* and production was decreased about 30% on a logarithmic scale (~ 90% on an arithmetic scale) on leaves treated with *C. globosum*. Those treated with *A. bombacina* were about 60% softer and weighed up to 50% less than control leaves. *C. globosum* affected leaf decomposition variably. The nutrient accompanying the antagonists altered the extent of leaf colonization, softening, and dry weight loss, but had no influence on the effect of either antagonist on ascospore production by *V. inaequalis*.

Additional key words: biological control, eradication, *Malus pumila*, *Spilocaea pomi*.

A practical means of controlling the apple scab pathogen, *Venturia inaequalis* (Cke.) Wint., involves breaking the overwintering cycle by eliminating the ascospores, which are the primary inoculum for infection in at least two major apple growing areas of the world (19,20). If successful, such measures would be likely to have a major impact wherever apples are grown.

Reports of microbial antagonism to *V. inaequalis* (9,10,26,28,32) have increased prospects for biological control. The effects of mixtures of apple phylloplane microorganisms and of individual organisms on the formation of pseudothecia by *V. inaequalis* in incubated leaf disks and in leaves placed in the orchard after inoculation have been investigated (6,10). The mixtures variously antagonized pseudothecial formation, but no attempts were made to identify antagonists within the mixtures. Among the individual microorganisms tested, *Fusarium sporotrichioides* and *Aerobacter aerogenes* reduced the number of pseudothecia. The effect of these antagonists on ascospore productivity was not assessed.

There are numerous reports on biological control of pathogens with *Chaetomium* spp. (eg, ref. 21), but with one exception (1) they all concern seed- or soilborne diseases.

Of 56 microorganisms isolated from apple leaves and apple leaf litter (2,3), six fungi inhibited the formation of pseudothecia on leaf disks sterilized prior to inoculation (17). Two of these, *Athelia bombacina* Pers. from litter (now in the American Type Culture Collection under ATCC 20629) and *Chaetomium globosum* Kze. from leaves and litter also colonized nonsterile leaf disks and prevented or inhibited pseudothecium formation (17).

This study was undertaken to investigate whether the microbial antagonism of *A. bombacina* and *C. globosum* to the apple scab pathogen, either with or without supplemental nutrients, can prevent or significantly inhibit ascospore production by *V. inaequalis*.

MATERIALS AND METHODS

Leaf material. Green leaves naturally infected with *V. inaequalis* were collected from an apple tree (*Malus pumila* Mill. 'McIntosh') in an orchard near Arlington, WI on 15 October 1980, approximately 3 wk prior to the expected end of natural leaf fall. Leaves were kept at 4 C for 2 wk in coarse nylon mesh bags until processed. Leaf disks, for the laboratory experiment, were cut with a No. 10 corkborer (2.54 cm^2; diameter, 1.8 cm), one disk from a lesion-bearing part per leaf. The leaves were equally divided between those with definitely margined and indefinitely margined lesions (34). The disks were kept humid at 4 C until application of the antagonists 1 wk later. Each treatment (see below) was applied to 50 leaf disks incubated individually in vials.

For the orchard assay, each treatment was applied to six groups, each of 100 whole leaves with petioles removed. The dry weight of each group was determined by multiplying the fresh weight and a conversion factor obtained by drying three subsamples of 10 g of leaf material to constant weight.

Inoculum. Antagonists were grown on wheat bran and on potato-dextrose agar (PDA, medium 167 in [30] modified by the use of unpeeled potatoes and 20 g dextrose). Wheat bran for cultures was milled and sieved to a particle size ⩽300 μm. Ten grams of bran + 30 ml of H_2O were autoclaved in 500-ml Erlenmeyer flasks and inoculated with a mycelial macerate of *A. bombacina* or an ascospore suspension of *C. globosum*. Cultures, harvested after 30 days, were washed by repeatedly suspending the colonized bran particles in sterile 0.01 M phosphate buffer (pH 7.1) and centrifuging for 2 min at 1,465 *g* to obtain a clear supernatant. Final inoculum suspensions of bran were prepared in buffer at a ratio of 1:10 original dry weight of bran:buffer.

For PDA cultures of *A. bombacina*, a dialysis membrane (13) placed on the agar surface in a petri plate was inoculated with a plug of the fungus or with mycelial macerate. *A. bombacina* formed a thick hymenium with basidia and basidiospores on this membrane and was separated easily from agar and membrane. The mycelial layers of 40 plates per batch were peeled from the surface of 16-day-old cultures, placed aseptically in 140 ml of sterile buffer in

a sterilized 400-ml cup (model OM-1062) of a Sorvall Omni-Mixer (model OM-1150), and ground for 5 min at speed 5 (about 8,000 rpm). The macerate was washed in sterile buffer until a clear supernatant was obtained after centrifugation for 2 min at 1,465 g. The pellet was then suspended aseptically at about 1 g (wet weight) of macerate per 4 ml of either buffer or a solution of carboxymethylcellulose (C-8758, Sigma Chemical Corp., St. Louis, MO 63178) (0.9%, w/v), malt extract (1.125%, w/v), and yeast extract (0.45%, w/v) in buffer (CMY). All *A. bombacina* inocula had the consistency of a finely ground slurry.

Inocula of *C. globosum* in buffer and CMY were prepared similarly by using ascospore suspensions from 30-day-old PDA cultures.

Application. The inocula were applied in the laboratory by spraying both surfaces of leaf disks or of leaves in each group until they were uniformly wet (about 200 ml of inoculum for 600 leaves). The aspirator-type sprayer consisted of two 3-mm i.d. stainless steel tubes at right angles to each other. Air pressure (70×10^4 dynes per square centimeter) was applied to one of the tubes resulting in delivery of the antagonist suspension from the other tube as a spray at a rate of about 110 ml/min. The leaves were held between sterile galvanized wire mesh (1.5 cm² holes) during spraying. Rates of application to each surface of the leaves were: *A. bombacina* in buffer or CMY, 620 viable propagules per square centimeter of leaf (vp/cm²); *A. bombacina* as a suspension of colonized bran particles, 25 vp/cm²; *C. globosum* in buffer or CMY, 56×10^2 vp/cm²; *C. globosum* as colonized bran particles, 17×10^2 vp/cm².

Incubation of inoculated leaf material. Each treated disk was placed, abaxial surface uppermost, on a layer of 8 ml of vermiculite moistened with 10 ml of distilled water in a sterile 20-ml vial (27). The disks were incubated at 16 C for 8 days and then at 4 C for about 6 mo. Treated leaves were sealed in nylon mesh (15-μm-diameter holes) bags, 100 leaves per 30 × 30 cm bag. On a unit area basis, the number of leaves per bag approximated the cover of leaf litter in an orchard (25). The bags were incubated at 16 C for 1 wk to monitor colonization and then fastened on sod with wire pegs in a randomized complete block design in the orchard. The blocks represented different locations on the ground. The entire procedure was completed within 3 wk after collection. Leaf bags remained in the orchard until the green-tip stage of bud development (33) the following spring.

Colonization. For the laboratory trials, preliminary experiments showed that colonization could be rated visually. *A. bombacina* grew up the walls of the glass vials after growing across the leaf disks; this characteristic provided a rapid measure of colonization. *C. globosum* was considered to be present if perithecia characteristic of the fungus were observed with the aid of dissecting and light microscopes. Colonization in the orchard experiment was evaluated by reisolating the antagonists in the spring. One disk (0.2 cm²) was cut from each of 270 leaves per treatment. The disks were surface sterilized for 5 min in a 0.53% solution of sodium hypochlorite, rinsed in water, and plated on PDA containing chloramphenicol (250 ppm) and novobiocin (100 ppm) (15 disks per petri plate). Colonization was recorded as the average number of successful reisolations per plate and per treatment.

Ascospore production. To determine ascospore production on leaf disks, five disks were attached, abaxial leaf surface downward, with petroleum jelly to the inside cover of a 5-cm-diameter plastic petri plate. The disks were wetted after 24 hr with distilled water from an aspirator bottle and kept moist for 72 hr by rewetting. Ascospores were discharged into 3 ml of distilled water with 0.05% Tween-20 and 0.02% NaN₃ in the dish and were distributed more-or-less uniformly by agitating the water with a Pasteur pipette. After settling, spores were counted in a grid comprising six equally spaced bands, each of 29.75 mm² area, by using a compound microscope (×125). Counts were transformed to log₁₀ to stabilize the variance (29), and a mean and standard error (SE) were calculated per square centimeter of disk.

To determine ascospore production in vivo, the leaves were returned from the orchard in early April and stored at 4 C for 3 wk. The leaves from each of the six 100-leaf samples per treatment were divided into six subsamples, each of 200 cm² ± 2% leaf area as

determined with an electronic leaf area meter (model 3100; Lambda Inst. Corp., Lincoln, NE 68504). Ascospores were collected from both surfaces of these subsamples at each sampling time using a modified spore tower (14,18). Leaves were soaked in water for 10 min, blotted, and placed on a screen tray on top of a 19-cm i.d. Plexiglas cylinder, 32 cm away from an air jet (5-mm i.d.) in the bottom center of the cylinder. Vacuum, applied to the bottom section of the cylinder, pulled air containing ascospores through the jet (47 L/min). Spores impacted into a 9-mm i.d. cup placed under the air jet. Leaves became dry after 20–30 min of sampling in the tower, and were then kept at 16 C in petri plates until the next sampling. Leaf surfaces facing the air jet were alternated for each determination. Leaves were sampled until the ascospore supply was exhausted, generally four times. Ascospores were stored in the cups at laboratory temperatures until counting. For counting, spores were resuspended in 60 μl of distilled water containing 0.02% Tween-20 by stirring them with the disposable tip of a digital micropipette. Three counts per cup were made with a hemacytometer. Counts were transformed to log₁₀ to stabilize the variance (29) and means ± SE were calculated for each leaf bag replicate.

Leaf strength and dry weight loss. Leaf strength and dry weight loss were quantified, in the orchard experiment only, as an indication of the leaf-decomposing abilities of each antagonist. Leaf strength was measured on 20 disks cut randomly with a No. 10 cork borer (2.54 cm²) from each of the six leaf batches per treatment. The disks were clamped between two 6-mm-thick Plexiglas sheets (23 × 8 cm) containing 7-mm-diameter holes equally spaced on a 3 × 3-cm grid. The force necessary to punch through a leaf disk was determined with a penetrometer (model DPP, 500 g; John Chatillon and Sons, Inc., Kew Gardens, NY 11415) with a 6-mm-diameter notched head. Major veins were avoided. The force was recorded in dynes, and means ± SE were calculated for each of the six leaf batches per treatment.

The six groups of leaves of each treatment were weighed in the fall and in the spring. Their dry weight was calculated by multiplication with an oven-dry-weight conversion factor obtained by drying three subsamples, of five leaves each, per treatment to constant weight. Dry weight loss between fall and spring was expressed as percent of leaf dry weight in the fall.

Statistical analyses. Most variables were subjected to analyses of variance (ANOVA) followed in the laboratory experiment by a Duncan multiple range test (DMRT) or in the orchard test by orthogonal comparisons (16,29). Residual plots and tests for

TABLE 1. Analysis of variance with orthogonal comparisons for dry weight loss of apple leaves incubated in the orchard following application of *Athelia bombacina* or *Chaetomium globosum* as antagonists to *Venturia inaequalis*[a]

Source of variation	Degrees of freedom	Sum of squares	*F*-value	Prob. >*F*
Blocks	5	0.0157		
Treatments	6	0.1167	10.79	<0.01
Buffer vs. other treatments	1	0.0109	6.04	0.02
CMY[b] vs. bran	1	0.0212	11.77	<0.01
(*A. bombacina* + amendments) vs. (*C. globosum* + amendments) vs.	1	0.0549	30.47	<0.01
(Buffer + amendments) vs. (antagonists + amendments)	1	0.0171	9.50	< 0.01
Interactions:				
Amendments × antagonists	1	0.0078	4.31	<0.05
Amendments × background[c]	1	0.0048	2.65	0.11
Error	30	0.0541		
Total	41	0.1865		

[a] See Fig. 1.

[b] CMY = carboxymethylcellulose (0.9% w/v) + malt extract (1.125% w/v) + yeast extract (0.45% w/v) in water, and milled bran (≤300 μm) suspended in water were the amendments. Each antagonist was applied in combination with either amendment.

[c] Background = buffer or antagonist.

normality (16) were used to ensure that the underlying assumptions for the statistical models were not violated. Data from treatments with zero ascospores or zero colonization were excluded from the analyses, and transformed data were used where indicated. The ANOVA for dry weight loss data is shown in Table 1, together with the orthogonal comparisons to illustrate the general format used. The ANOVA for leaf strength followed the same format; those for colonization and ascospore production of field test data differed in the degrees of freedom because treatments with zero values had to be excluded (cf, Fig. 1).

RESULTS

Leaf disks incubated in the laboratory. No ascospores of *V. inaequalis* were produced on disks that were extensively colonized by *A. bombacina* applied with buffer or CMY (Table 2). Ascospore production on disks inoculated with *A. bombacina*/bran was significantly lower than on controls ($P < 0.05$, DMRT), even though these disks were less colonized than those that received the other two treatments with *A. bombacina. C. globosum* applied in buffer did not colonize leaf disks well. Ascospore production by *V. inaequalis* was, however, prevented or significantly decreased on

leaf disks treated with *C. globosum* together with either amendment (Table 2). Ascospores were detected from about 70% of all pseudothecia; spore discharge was only triggered once. The type of scab lesion did not affect ascospore production. The experiment was performed twice with similar results.

Intact leaves incubated in the orchard. Both antagonists colonized the leaves (Fig. 1A) and were reisolated the following spring. The extent of colonization depended on the nutrient amendment accompanying the antagonist, which indicated an interaction between the antagonists and amendments (Table 3). This interaction was significant ($P < 0.01$). CMY was the better amendment for *A. bombacina*, whereas bran was better for colonization by *C. globosum* (Fig. 1A).

A. bombacina completely prevented ascospore production by *V. inaequalis* (Fig. 1B), regardless of the amendment. Additional field trials in 1981/1982 with *A. bombacina*, using the same experimental design, confirmed this finding. *C. globosum* was less effective, but ascospore production by *V. inaequalis* was still significantly lower than on the controls ($P < 0.01$). The two *C. globosum* treatments were not significantly different ($P = 0.67$) (Table 3). *C. globosum* reduced ascospore production by about 30% on a $\log_{10}$ scale, or about 93% for untransformed ascospore numbers.

Leaves treated with *A. bombacina* were generally softer than control leaves, ie, strength of treated leaves was decreased by about 40% (Fig. 1C). There was a large difference between leaves treated with *C. globosum* in CMY and those treated with bran colonized with *C. globosum*. The latter were much softer than controls, whereas the former were not. The average of the two treatments with *A. bombacina* differed significantly from the average of the two treatments with *C. globosum* ($P < 0.01$) (Table 3). Both the amendment $\times$ antagonist and the amendment $\times$ background interactions were significant ($P < 0.01$) (Table 3). Thus, the difference between *Athelia* and *Chaetomium* depended on the amendment and the amendment acted differently on the two backgrounds (ie, buffer or antagonist).

Leaves treated with *A. bombacina* lost 26–80% more dry weight between fall and spring than did control leaves (Fig. 1D). This weight loss was significantly greater than in leaves treated with *C. globosum* ($P < 0.01$) (Table 3). Dry weight loss was significantly greater for leaves treated with the antagonists in CMY than when
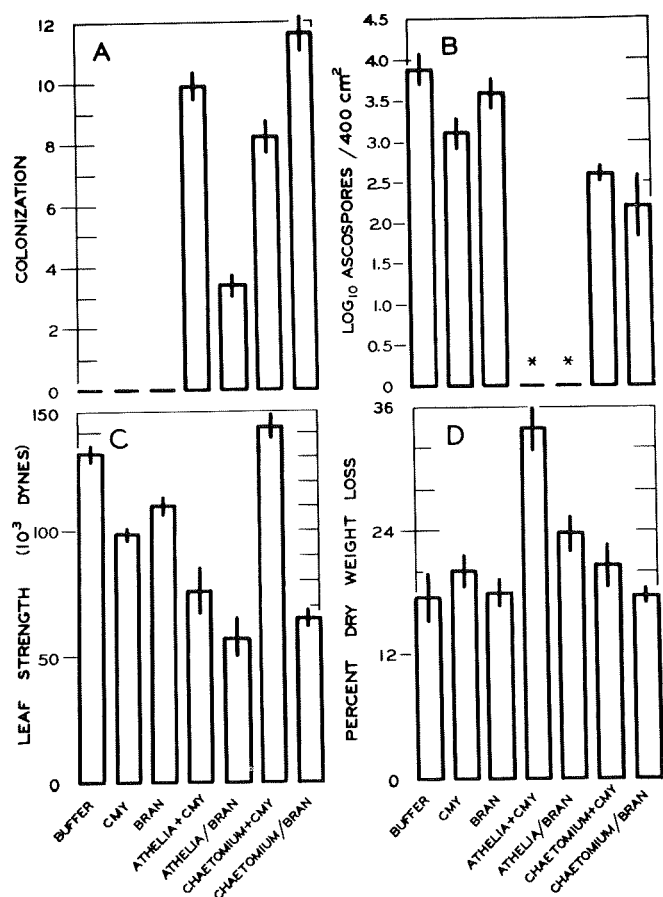

Fig. 1. Colonization by *Athelia bombacina* and *Chaetomium globosum* of **A,** whole McIntosh apple leaves naturally infected by *Venturia inaequalis*; **B,** impact of these antagonists on ascospore production by *V. inaequalis*; and **C** and **D,** on leaf decomposition. Antagonists were applied in a buffer solution of CMY = carboxymethylcellulose (0.9% w/v) + malt extract (1.125% w/v) + yeast extract (0.45% w/v), or as a suspension in buffer of bran particles colonized by either antagonist. Ascospore yields are from measurements on 36 leaf subsamples per treatment, each of ~200 cm² area. Ascospores were collected from both surfaces of leaves in each subsample. * = no ascospores detected. Colonization is the mean number of successful reisolations of antagonist from 15 leaf disks per petri dish (270 leaves evaluated for each bar on the graph). For leaf strength, each bar represents 120 measurements with a penetrometer. For percent dry weight loss, each bar corresponds to six measurements. Vertical lines represent standard error.

TABLE 2. Colonization of apple leaf disks by *Athelia bombacina* and *Chaetomium globosum* and the effect on ascospore production from disks of leaves naturally infected by *Venturia inaequalis*, inoculated with the antagonists with or without amendments, and incubated under controlled conditions

Treatment	Antagonist colonization[a]	Ascospores[b] Mean	SE
Buffer	0	1.894 A	0.131
CMY[c]	0	2.256 A	0.170
Bran[d]	0	1.871 A	0.277
A. bombacina in[e]			
buffer	98	0.0	0.0
CMY	100	0.0	0.0
bran	20	1.191 B	0.205
C. globosum in[f]			
buffer	18	0.613 C	0.169
CMY	76	0.0	0.0
bran	36	0.320 C	0.216

[a] Disks (%) visually colonized, based on 50 disks total.
[b] $\log_{10}$ ascospores + 1/cm² of disk, the mean of ten five-disk samples. Means followed by the same letter are not significantly different according to Duncan's multiple range test at $\alpha = 0.05$.
[c] Carboxymethylcellulose (0.9% w/v) + malt extract (1.125% w/v) + yeast extract (0.45% w/v) in buffer.
[d] Wheat bran milled to a particle size $\leqslant$300 μm.
[e] Antagonist inoculum: 620 (buffer and CMY) and 25 (colonized bran) viable propagules of isolate CO57 per square centimeter of disk.
[f] Antagonist inoculum: 56×10^2 (buffer and CMY) and 17×10^2 (colonized bran) viable propagules of isolate CO22 per square centimeter of disk.

TABLE 3. Significance levels of orthogonal comparisons[a] used to partition the treatment sum of squares in analyses of variance for four variables evaluated in the orchard test of *Athelia bombacina* and *Chaetomium globosum* as antagonists to *Venturia inaequalis*

	Variable			
Source of variation	Colonization by antagonists	Ascospore production	Leaf strength	Dry weight loss of leaves
1. Buffer vs. other treatments	†	<0.01	<0.01	0.02
2. (CMY[b] cont. treatments) vs. (bran cont. treatments)	†	†	<0.01	<0.01
3. (*A. bombacina* + amendments[b]) vs. (*C. globosum* + amendments)	<0.01	†	<0.01	<0.01
4. (Buffer + amendments) vs. (antagonists + amendments)	†	†	<0.01	<0.01
5. Amendments × antagonists	<0.01	0.06	<0.01	<0.05
6. Amendments × background[c]	†	†	<0.01	0.11
(CMY cont. antagonists) vs. (bran cont. antagonists)	<0.01	0.67	...	...
(Buffer + amendments) vs. (*C. globosum* + amendments)	...	<0.01	...	...

[a] Orthogonal comparisons identified in Table 1. Some comparisons were not possible, and are designated by †. For colonization and ascospore production, the orthogonal comparisons below the blank line were performed instead of comparisons two and four, respectively, because of zeros for the controls and *A. bombacina*, respectively (see Fig. 1). Each column provides the same information for each respective variable as the column headed by Prob. >F does for dry weight loss in Table 1.

[b] CMY = carboxymethylcellulose (0.9% w/v) + malt extract (1.125% w/v) + yeast extract (0.45% w/v) in 0.01 M phosphate buffer and milled wheat bran (≤300 μm particle size) suspended in buffer were the amendments. Each antagonist was applied in combination with either amendment.

[c] Background = buffer or antagonists.

applied as colonized bran particles ($P < 0.01$) (Fig. 1D). The interaction between amendments and antagonists was significant ($P < 0.05$); however, the amendment × background interaction was not significant ($P = 0.11$) (Table 1).

DISCUSSION

A. bombacina and *C. globosum*, when applied with or without amendments to apple leaves, were effective antagonists to *V. inaequalis*. *A. bombacina* prevented, and *C. globosum* significantly reduced, production of ascospores on naturally infected leaves, provided leaf colonization had been successful. Although the isolate of *C. globosum* evaluated here was identical to that ranked best among eight antagonists to the conidial state of *V. inaequalis* (1), clearly it was inferior to *A. bombacina* against the sexual state. Results from these studies with nonsterile leaf material extend data from preliminary screening trials wherein apple leaf disks sterilized by gamma irradiation were used (17).

Antagonism was demonstrated both on leaves incubated in the laboratory and on those incubated over the winter in nylon mesh bags in an orchard. Agreement between the laboratory and orchard results may be attributed to the fact that a natural substrate, ie, leaf material, rather than agar media, was used for the experiments (cf, 24).

Adding nutrients or amendments to the antagonist inoculum does not necessarily improve colonization or activity of antagonists. Whereas Tveit and Wood (31) found certain substrates stimulated colonization of unsterile soil by *Chaetomium* spp., Kommedahl et al (22) stated that adding nutrients reduced the effectiveness of biological seed treatments. One of the few reports on the phyllosphere (12) showed that yeast populations applied to leaves increased 100-fold within 1 wk when applied together with sucrose and yeast extract. *C. globosum* applied with amendments more frequently colonized disks than when applied with buffer alone. However, extent of colonization was not necessarily associated with a commensurate reduction in ascospore numbers.

In contrast to the hyperparasitic activity common among other basidiomyceteous antagonists (eg, 7,11), hyperparasitism was not evident from preliminary scanning electron microscope studies. *A. bombacina* apparently acts mainly through competition for nutrients and by antibiosis (C. C. Heye, *unpublished*). The antagonist is strongly cellulolytic; this was demonstrated by its ability to degrade the cellulose dialysis membrane in PDA plates. This explains, in part, the leaf-softening and decomposing action of

A. bombacina, and makes nutrient competition a plausible mode of antagonism. Also, leaf softening caused by *A. bombacina* may indirectly control pseudothecium production by promoting leaf removal by earthworms, which are known to prefer softer leaves (15).

The effect of treatments with *A. bombacina* on disease incidence remains to be established. Additional aspects awaiting study include methods of application, mode of antagonism, nontarget effects (4,8), and experimental design as it relates to results with confined versus unconfined litter (23).

Provided successful control of *V. inaequalis* can be demonstrated consistently, *A. bombacina* has characteristics that make it attractive for commercial development. We have therefore filed a patent application (35). Unlike many basidiomycetes, this fungus grows rapidly on nutrient media over a large temperature range. It produces basidiospores on agar, and can also be grown in liquid culture. Air-dried preparations of cultures on PDA have survived at 4 C for 18 months (C. C. Heye, *unpublished*). *A. bombacina* tolerates 5% urea (17), which has been used as an eradicant spray in the fall (5). Thus, this antagonist could constitute one component of an integrated program for apple scab control.

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