Ecology and Epidemiology

Recovery of *Athelia bombacina* from Apple Leaf Litter

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ABSTRACT

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A semiselective medium (PAB: potato agar amended with 50 μg a.i./ml of benomyl, 100 μg/ml of chlortetracycline HCl, and 200 μg/ml of streptomycin sulfate) was developed for detection in apple leaf litter of *Athelia bombacina*, an antagonist to *Venturia inaequalis*. The efficiency of PAB depended on inoculum level and varied between 14% (close to the detection threshold of 0.14×10^3 cfu/ml) and 81%, when expressed as recovery from inoculated, nonsterile apple leaves as related to recovery from inoculated, initially sterile leaves. Bacteria were eliminated and filamentous fungi and yeasts were reduced by 99.6 and 82.1%, respectively, on PAB as compared with potato-dextrose agar. The one other isolate of *A. bombacina* available did not grow on PAB. A mixture of inoculated and uninoculated apple leaves was incubated on the ground in mesh bags from November 1986 to May 1987 and November 1987 to mid-April 1988. Leaves were sampled every 4–8 wk and leaf pieces were incubated on PAB. During 1986–1987, recovery of *A. bombacina* from inoculated leaf pieces averaged 85% in the last four samples. During 1987–1988, recovery from inoculated leaf pieces averaged 96% in all samples. Recovery (averaged over all samples in both experiments) from uninoculated leaves was 21% less than that from inoculated leaves. The fungus grew 15 cm (1.2% recovery at this distance) across a mat of uninoculated leaves but did not cross a 1-cm gap of turf between adjacent mesh bags. Thus, extensive coverage by initial inoculum and a high survival rate of *A. bombacina* are probably the most important factors leading to extensive colonization of leaf litter.

Additional keywords: apple scab, biological control.

Apple scab, caused by *Venturia inaequalis* (Cooke) Wint., occurs worldwide in moist, temperate climates and is the most economically important disease of apples (15). Currently, fungicides are the mainstay of control; eight to 20 applications are needed each growing season in the eastern and midwestern United States to produce commercially acceptable fruit (12). Fungicides such as benomyl and dodine are declining in use because resistant strains of *V. inaequalis* have become widespread (5,11), and

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resistance to fungicides that inhibit ergosterol synthesis is a concern. Chemical control also affects nontarget organisms, with the impact continuing beyond leaf fall (1). Development of an alternative strategy, such as biological control, may be important to ensure successful management of apple scab in the future.

One approach to biological control of apple scab is to break the overwintering cycle of *V. inaequalis* by preventing the formation of pseudothecia, thus removing the source of ascospores, which are the primary inoculum in spring. Previous studies (3,4) showed that bacteria and fungi isolated from apple leaves can significantly reduce pseudothecial formation in leaves infected with *V. inaequalis*. Heye (7) tested 56 phylloplane fungi for their antagonism to the overwintering stage of *V. inaequalis*, and, of these, only the basidiomycete *Athelia bombacina* Pers. (*Corticiaceae*) completely inhibited formation of pseudothecia on initially sterile leaf disks incubated in vitro. When leaves naturally infected with *V. inaequalis* were inoculated with *A. bombacina* in the fall and allowed to overwinter in the orchard, no ascospores were produced in the spring (8).

In addition to antagonizing *V. inaequalis, A. bombacina* has features of commercial value, including the ability to grow rapidly in culture and to soften and degrade leaf litter (9). No evidence has been found that it is pathogenic to apple trees (16). However, ecological information on the behavior of *A. bombacina* in apple leaf litter is currently lacking and is needed to provide a logical basis for the development of the basidiomycete as a biological control agent.

The purpose of this study was to develop a selective isolation medium for *A. bombacina* and to use this medium to monitor the population density and growth of the antagonist in apple leaf litter. A preliminary report has been published (18).

MATERIALS AND METHODS

Isolate of *A. bombacina*. *A. bombacina* was isolated in March 1978 from apple leaf litter in an experimental orchard near Arlington, WI (1), and was identified by H. H. Burdsall (Forest Products Laboratory, Madison, WI). One isolate was maintained for experimental purposes. A subculture (ATCC 20629), deposited (8) at the American Type Culture Collection, Rockville, MD, was used in all experiments. Stock cultures were maintained on potato-dextrose agar (PDA) under mineral oil (14) at 4 C.

Development of selective medium. Preliminary tests to develop a selective medium involved growing *A. bombacina* on seven basal media supplemented with one or more of 19 antifungal and antibacterial amendments at a range of concentrations. The media were: PDA (with 2% dextrose) (14), potato agar (PA; infusion from 200 g of unpeeled potatoes per liter and 2% agar) (14), V-8 juice agar (with 2% agar) (14), 2% water agar, carboxymethyl cellulose agar (13), apple leaf decoction agar (20 g of dry apple leaves were steamed in 500 ml of H_2O for 1 hr; in a separate container, 40 g of chopped, peeled potatoes were steamed in 500 ml of H_2O for 1 hr; the two solutions were strained through four layers of cheesecloth, combined, and 20 g of agar and H_2O to 1 L were added), and malt extract agar (14). Antifungal amendments were benomyl, dicloran, dodine, metiram, pentachloronitrobenzene, *o*-phenylphenol, alpha naphthol, iso-eugenol, cycloheximide, nystatin, amphotericin B, and 5-fluorocytosine. Antibacterial agents were chlortetracycline HCl, chloramphenicol, streptomycin sulfate, novobiocin, gentamycin, rose bengal, and polymyxin B sulfate.

A 5-mm plug of inoculum taken from the edge of an actively growing colony of *A. bombacina* was placed in the center of each of five plates of each medium or each amendment in PDA. Colony diameter was measured every 24 hr until the fungus covered the surface of the medium, or up to 12 days. Growth tests on each medium or amendment were performed twice.

Based on the results of these preliminary tests and those in which the amendments benomyl, chlortetracycline HCl, and streptomycin sulfate together suppressed more contaminating fungi, yeasts, and bacteria than other amendments, the final composition of the medium (designated PAB) was: PA amended with 50 μg/ml a.i. of benomyl, 100 μg/ml of chlortetracycline HCl (90–95%; Sigma Chemical Co., St. Louis, MO), and 200 μg/ml of streptomycin sulfate (Sigma) (18). Amendments were suspended individually in tubes of sterile water, mixed with a Vortexer unit (Scientific Industries Inc., Bohemia, NY), and added to the PA after it had cooled to 50 C. The medium was mixed before plates were poured.

Selectivity of medium and growth of *A. bombacina* on PAB. Apple leaf litter was collected from beneath apple trees (*Malus pumila* Miller 'McIntosh') in an abandoned orchard near Arlington, WI, in November 1984. Leaves were stored for 3 wk at 4 C in mesh bags until processed.

Three leaves, each approximately 20 cm^2, were selected arbitrarily. Each leaf was placed in the sterile cup of an Omnimixer (model OM-1061, Sorvall Inc., Norwalk, CT) with 50 ml of sterile 0.01 M phosphate buffer (pH 7.4) and homogenized for 5 min at speed five over ice. The resultant homogenate was diluted to 10^{-1}, and 0.1-ml samples were spread onto each of five PDA and five PAB plates. Plates were incubated inverted for 5 wk at 4 C, and then the number of colony-forming units (cfu) of filamentous fungi, yeasts, and bacteria were recorded for each plate. The 10^{-1} dilution was the lowest dilution of leaf homogenate that generally gave discrete, enumerable colonies of the organisms on PDA at 4 C. Means and standard deviations were calculated for each group of five plates from each leaf, and the numbers of colony-forming units on PAB were expressed as a percent of those on PDA.

The growth of two isolates of *A. bombacina*, ATCC 20629 and HHB 5626 (the latter obtained from H. H. Burdsall), was compared on PAB. Each isolate was transferred to five plates each of PDA and PAB, which were incubated and measured as described previously for preliminary tests to develop a selective medium. Growth rate on both media was calculated for each fungus. The above experiments were each performed twice.

Recovery efficiency of medium. Preliminary experiments to measure recovery efficiency of *A. bombacina* (ATCC 20629) on PAB involved detection by dilution plating of homogenates of initially sterile leaves inoculated with the fungus. However, the number of colony-forming units per leaf obtained after leaf homogenization was greater than the number of colony-forming units originally applied, because mycelia were further fragmented. Thus, measuring efficiency of recovery as a percentage of input was not feasible. In addition, the sensitivity of the dilution plating method was low (no *A. bombacina* was recovered on PAB if fewer than approximately 4.5×10^3 cfu were applied to a leaf). Therefore, recovery was quantified by placing leaf pieces on PAB (see below) and scoring presence or absence of *A. bombacina* that grew onto the medium. Recovery from initially sterile leaves was set at 100%, and recovery of *A. bombacina* from inoculated, nonsterile leaves was calculated as a percentage of recovery from inoculated, initially sterile leaves.

Inoculum consisting primarily of hyphal fragments was prepared as described by Heye and Andrews (8), except mycelial mats were homogenized in 150 ml of sterile 0.01 M phosphate buffer (pH 7.4) twice for 5 min over ice. Inoculum concentration was 2.34×10^4 cfu/ml as assessed by dilution plating on PDA. From this concentration, 14 serial dilutions of 1:1 were prepared with the same buffer, i.e., 1:1 through 1:8,352.

Intact leaves (220), collected as described above, were placed individually in paper bags and assigned randomly to treatments. Half of the leaves were autoclaved for 20 min at 121 C. The 110 autoclaved bags were divided into 11 groups of 10 bags each. The leaf from each of the 10 bags in the first group was treated with 0.5 ml of buffer (control), whereas each leaf in the second group was inoculated with 0.5 ml of the 1:8,352 dilution of inoculum of *A. bombacina* (72 cfu). For each group, inoculum or buffer was placed on the abaxial surface of the first five leaves and on the adaxial surface of the other five leaves. Inoculum was spread evenly with a bent glass rod over each leaf as it was inoculated and allowed to dry completely (about 10 min in a laminar flow hood). Similarly, each leaf in the third through the 11th group received twofold increases in inoculum, viz., 0.14,

0.28, 0.57, 1.15, 2.29, 4.58, 9.16, 18.3, 36.7 × 10^3 cfu of *A. bombacina*, respectively. To increase the sensitivity of the assay for recovery of *A. bombacina*, each leaf was then cut into eight similarly sized pieces and incubated (four leaf pieces per plate of PAB) with the inoculated surface toward the medium. The 110 unautoclaved leaves were divided into 11 groups of 10 leaves, and the leaf groups were inoculated and incubated on PAB as described for the set of inoculated leaves.

Plates were incubated at 24 C for 3 days and then examined for growth of *A. bombacina*. Each leaf piece was rated as positive or negative for the presence of *A. bombacina*. Results were expressed as the mean for each leaf group of the percentage of pieces from each leaf from which *A. bombacina* was reisolated. Analysis of variance was used to test the significance of differences between recovery from sterile and nonsterile leaves, between recovery from the adaxial or abaxial surface of the leaves, and among recoveries at different inoculum levels. The experiment was performed twice with similar results; data from one trial are presented.

Recovery from inoculated leaf litter incubated in the orchard. Senescing leaves were collected from McIntosh apple trees in an abandoned orchard on 7 October 1986 and 11 October 1987. Leaves were kept at 4 C in coarse nylon-mesh bags until processed.

Inoculum of *A. bombacina* (ATCC 20629) was prepared as described for the experiment on recovery efficiency. Final inoculum concentrations were 2.9 and 8.5 × 10^5 cfu/ml for the 1986–1987 and 1987–1988 experiments, respectively. Inoculum was stored overnight at 4 C before use.

Leaves to be inoculated were held between galvanized wire mesh (12-mm mesh). Each leaf was marked by depositing small dots of latex paint at the tip and base of one surface. It was necessary to mark inoculated leaves in order to retrieve them from a mixture of inoculated and uninoculated leaves. Leaves were stored overnight at 4 C between the mesh before inoculation, and then a hyphal suspension of *A. bombacina* was sprayed onto both surfaces of the leaves until they were uniformly wet (about 700 μl per leaf). Dosages were approximately 2.07 and 5.61 × 10^5 cfu per leaf during 1986–1987 and 1987–1988, respectively, as estimated from volumes applied to each leaf. Buffer was sprayed on control leaves, which were marked with a different color of paint. All leaves were stored overnight at 4 C between the layers of wire mesh to allow the inoculum to dry.

During the 1986–1987 field experiment, four groups of 20 inoculated leaves each and four groups of 20 uninoculated leaves each were randomly selected and stored overnight at 4 C in paper bags. Remaining leaves were placed in nylon mesh bags (1.4- × 1.6-mm mesh), 30 × 15 cm. In each of 24 bags, designated "a," 25 marked, inoculated leaves were mixed with 25 unmarked, uninoculated leaves and distributed evenly. Twenty-five marked, buffer-sprayed leaves were mixed with 25 unmarked, uninoculated leaves and distributed inside each of another 12 "a" bags. Each bag contained 50 leaves and was stapled closed. In each of 36 additional bags, designated "b", 50 unmarked, uninoculated leaves were distributed. The bags were assigned into pairs of "a" with "b" bags and stored overnight at 4 C. On 1 November 1986, 36 pairs of bags were fastened to the ground in a completely randomized design in the orchard from which the leaves were initially collected. In each pair of bags the longer sides were adjacent and separated by a 1-cm gap.

The first sample of the experiment, on 1 November 1986, consisted of the 80 inoculated and 80 uninoculated leaves that were selected at random and not taken to the orchard. Thereafter, four pairs of bags with inoculated leaves and two pairs of bags with buffer-sprayed leaves were chosen randomly on the first day of each month from December 1986 to May 1987. From each "a" bag, 20 inoculated leaves and 20 uninoculated leaves were removed; from each "b" bag, 20 uninoculated leaves were chosen. Each leaf was cut aseptically into eight similarly sized pieces and incubated (four leaf pieces per plate of PAB) with adaxial or abaxial surface randomly toward the medium. Half of the leaves were incubated at 24 C and examined after 3–4 days, whereas the other leaves were incubated at 4 C and examined after 5–6 wk. Colonization for each month was recorded as the percentage of leaf pieces from which *A. bombacina* was reisolated.

During 1987–1988, the field experiment was repeated to confirm the results of the 1986–1987 study and to extend these results by examining spread of the fungus across a mat of uninoculated leaf litter. Immediately before placing leaves in mesh bags on 1 November 1987, five groups of 10 inoculated leaves each and five groups of 10 uninoculated leaves each were selected randomly and stored at 4 C overnight in paper bags. One hundred uninoculated, unmarked leaves were spread evenly throughout a 30- × 30-cm end portion of a 45- × 30-cm mesh (1.4 × 1.6 mm) bag (Fig. 1). In the remaining 15- × 30-cm area, 25 marked, inoculated leaves were mixed with 25 unmarked, uninoculated leaves, contiguous with the first lot of 100. Bags were stored overnight at 4 C. On 2 November 1987, 24 bags were fastened securely on the ground of the orchard in a completely randomized design; each bag was placed beneath a different tree. Another group of 100 uninoculated, unmarked leaves was spread under the coarse nylon netting (14-mm mesh) of each bag, such that the leaves were held directly in contact with the ground and were also adjoining the inoculated leaves at the open end of the fine-mesh enclosure. Coarse mesh was used to allow removal of leaves by earthworms and, thus, provide a comparison of survival of *A. bombacina* in confined leaves (i.e., between two layers of mesh) with survival in relatively unconfined leaves possibly exposed to a different microenvironment. Twenty bags contained marked, inoculated leaves, and four control bags contained marked leaves sprayed with buffer.

Because of obvious changes in the temperature of the bags over time, noted in the 1986–1987 experiment (the bags varied from being in direct sunlight, under complete snow cover, or encased in ice), temperatures were monitored hourly within two of the bags during 1987–1988 with a CR-21 data recorder (Campbell Scientific, Logan, UT). Two temperature sensors were placed in each bag.

The first sample of the experiment, on 2 November 1987, consisted of the inoculated and uninoculated leaves that were not taken to the orchard. The bags were sampled on 10 December, 10 February, 24 March, and 19 April. Five bags with inoculated leaves and one control bag with uninoculated leaves were chosen randomly on each sample date. Leaves under the netting portion were removed in the orchard, placed in paper bags, and returned to the laboratory. The rest of the litter bag was removed intact, and leaves were sampled in the laboratory. Ten inoculated leaves and 10 uninoculated leaves were randomly sampled from the central portion of each mesh bag. Additionally, at each distance

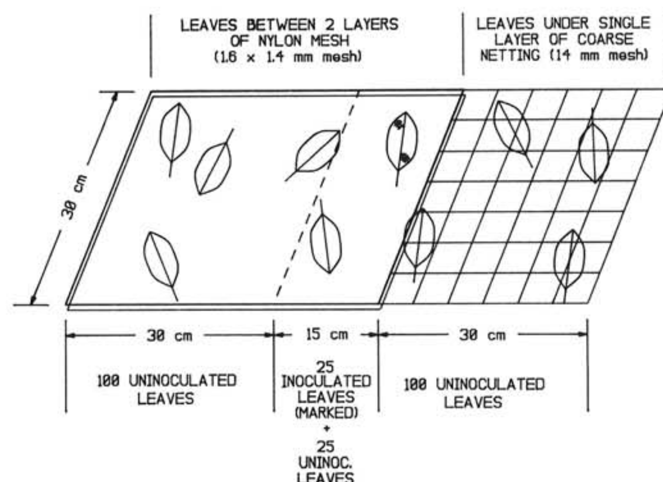

Fig. 1. Modified litter bag used in the 1987–1988 field experiment to monitor growth of *Athelia bombacina* from leaves inoculated with hyphal fragments to uninoculated leaves in a mixture of these leaves, and for investigating ability of *A. bombacina* to spread across a mat of uninoculated leaves held between nylon mesh or in contact with the ground under coarse netting.

of 5, 15, and 25 cm from this section in both directions (i.e., from within the bag and from under the netting), 10 leaves were sampled randomly. Leaves were processed and plated as described before, except that all incubations were made at 24 C.

Arcsin-square root transformations were used for all leaf recovery data to stabilize variance. All significance levels for differences in recovery between the groups of leaves were calculated from t tests on the transformed data. Residual plots were used to ensure that the assumptions for the statistical models were met. All means reported in the text and figures were calculated from untransformed data.

RESULTS

Selectivity of medium and growth of *A. bombacina* on PAB. The percentage of colony-forming units of filamentous fungi and yeasts from leaf litter that grew on PAB was 0.4 (SD = 0.3) and 17.9% (SD = 4.3), respectively, of those that grew on unamended PDA. No bacteria grew on PAB. Incubation of PAB plates at 4 C was preferable to incubation at 24 C, because, at 4 C, the size of yeast colonies and growth of filamentous fungi were reduced. Results of the two experiments were similar.

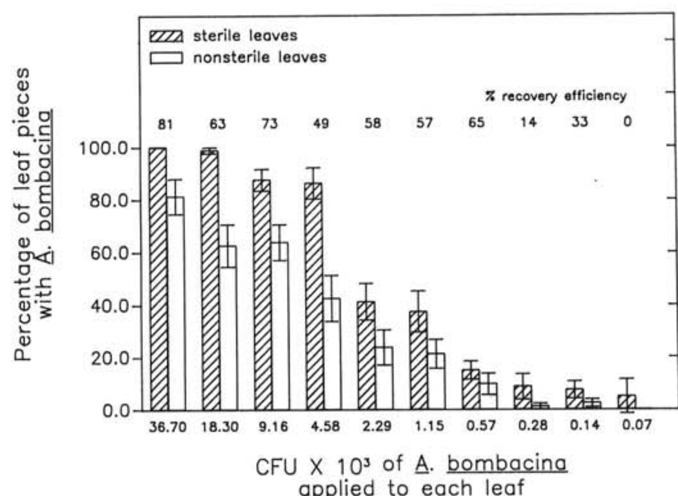

Fig. 2. Recovery of *Athelia bombacina* from initially sterile and nonsterile apple leaves inoculated with different quantities of hyphal fragments. Each leaf was cut into eight pieces and incubated. Each bar represents the mean percent recovery from 10 leaves, with standard error. Recovery efficiency is defined as recovery from nonsterile leaf pieces as a percentage of recovery from initially sterile leaf pieces.

The growth rate of *A. bombacina* (ATCC 20629) was 10.4 (SD = 0.1) mm/day on PDA at 24 C, compared with 1.2 (SD = 0.3) mm/day for isolate HHB 5626. On PAB, the growth rate of *A. bombacina* (ATCC 20629) was 6.6 (SD = 0.2) mm/day. HHB 5626 did not grow on PAB.

Recovery efficiency of medium. Colonies of *A. bombacina* were whiter and more distinct and contaminating organisms grew more slowly on the potato agar basal medium (i.e., without amendments) than on other media screened. Benomyl, commonly used in selective media for basidiomycetes (6,10), was the best amendment for inhibiting extraneous filamentous fungi and yeasts. *A. bombacina* was not sensitive to at least 50 μg/ml of benomyl.

A. bombacina was never recovered from initially sterile or nonsterile leaves treated with buffer but was recovered on PAB from at least some initially sterile leaf pieces that were inoculated with each concentration of inoculum of *A. bombacina* (Fig. 2). Overall, recovery on PAB from initially sterile and nonsterile leaves decreased with lower concentrations of inoculum. Recovery from nonsterile leaves was significantly less ($P < 0.01$) than recovery from sterile leaves for some inoculum levels. The difference in recovery was not uniform across inoculum levels (i.e., a significant interaction was found [$P < 0.01$] between inoculum level and sterile and nonsterile treatments). The recovery efficiencies ranged from 81 to 14% (Fig. 2) for inoculum applications ranging from 36.7×10^3 to 0.14×10^3 cfu per leaf, respectively. Thus, the detection threshold (lowest number of colony-forming units applied to a nonsterile leaf that could be recovered on PAB) was 0.14×10^3 cfu per leaf. Although mean recovery efficiency appeared low for low inoculum applications of 0.28×10^3 cfu per leaf or less, the percent recovery values used to calculate these efficiencies were highly variable. Therefore, it was not possible to conclude that recovery efficiency decreased significantly with decreasing inoculum. No significant difference was found ($P < 0.01$) between recovery from leaves inoculated on the adaxial or abaxial surface.

Recovery of *A. bombacina* from field-incubated leaves. During 1986–1987, no significant difference was found in recovery of *A. bombacina* between leaf pieces incubated at 4 C (plates examined after 5 wk) or 24 C (plates examined after 4 days). Therefore, data were pooled for subsequent analysis, and all plates in the 1987–1988 experiment were incubated at 24 C.

A. bombacina was recovered throughout the experiment from inoculated leaves in both field tests. During 1986–1987, recovery was low (approximately 38%) on 1 December and 1 January but increased to approximately 85% during the remainder of the experiment (Fig. 3). The highest recovery from inoculated leaves was during 1987–1988, when *A. bombacina* was reisolated from

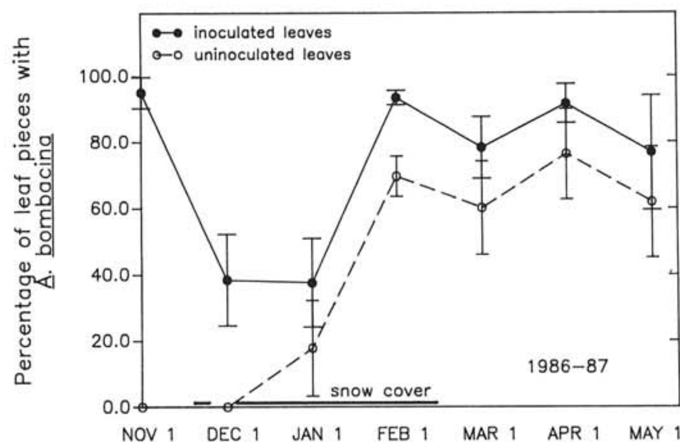

Fig. 3. Recovery of *Athelia bombacina* from apple leaves incubated in mesh bags in the orchard from 1 November 1986 to 1 May 1987. Each 15- × 30-cm bag contained 25 leaves inoculated with *A. bombacina* mixed with 25 uninoculated leaves. Twenty leaves were sampled from each bag, and each leaf was cut into eight pieces and incubated. Each point represents the mean recovery, with standard error, from four bags.

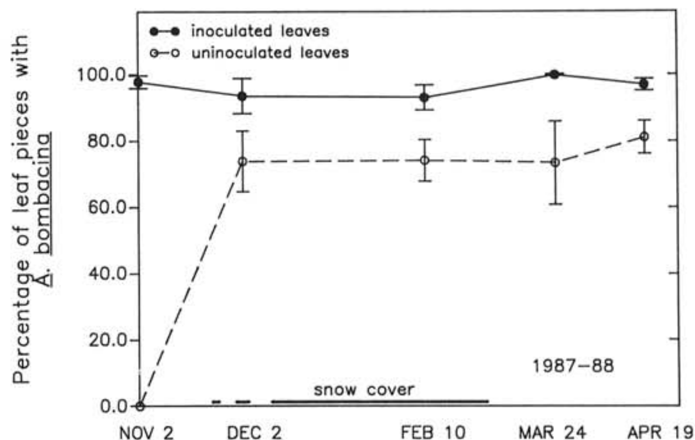

Fig. 4. Recovery of *Athelia bombacina* from apple leaves incubated in mesh bags in the orchard from 2 November 1987 to 19 April 1988. Twenty-five leaves were inoculated with hyphal fragments of *A. bombacina*, mixed with 25 uninoculated leaves and assigned to the central 15- × 30-cm part of a 75- × 30-cm bag. Ten leaves were sampled from this part of each bag, and each leaf was cut into eight pieces and incubated. Each point represents the mean recovery, with standard error, from five bags.

an average of 96% of inoculated leaf pieces in all samples (Fig. 4).

In both experiments, *A. bombacina* grew from inoculated leaves to adjoining uninoculated leaves. Colonization of these leaves was 21% (SE = 2.5) less (averaged over samples taken from December onward in both years, i.e., omitting leaves sampled at the beginning of November) than colonization of inoculated leaves in the same bag. The difference in colonization was relatively constant between sampling dates and between the two experiments. During 1986–1987, the difference in recovery between inoculated and uninoculated leaves in the "a" bags was significant ($P < 0.005$) for the first four samples (November—February), but was not significant for the samples taken through 1 March, 1 April, and 1 May. During 1987–1988, the difference was significant for all of the samples ($P < 0.005$) (Fig. 4).

In the 1986–1987 experiment, *A. bombacina* did not grow across the 1-cm gaps from bags containing inoculated leaves to those containing uninoculated leaves. The fungus was never recovered from control (buffer sprayed) leaves in either experiment.

The farthest spread of *A. bombacina* across a continuous mat of uninoculated leaves was 15 cm in the 1987–1988 experiment, which was recorded in the 10 February and 24 March samples. The highest recovery for leaves under netting at 15 cm was only 1.2% (recorded in the 24 March sample) (Fig. 5). The spread of *A. bombacina* across leaves confined within mesh was not significantly different ($P > 0.01$) from growth across leaves under netting in all samples up to and including 24 March. By 19 April, most of the leaves had been removed from under the netting by soil fauna.

Temperatures in the bags under snow cover were 0 to −1 C. Before snow fall or after snow melt, temperatures in the bags followed the pattern of air temperature, with large diel fluctuations. Temperatures ranged from −11 to 17 C before snow cover and from −9 to 41 C after snow melt. Temperatures were similar inside the mesh bag and under the netting. The duration of snow cover was similar in both years, except that snow fall and snow melt were earlier in 1986–1987 than in 1987–1988 (continuous snow cover from 2 December to 8 February, and 15 December to 25 February, respectively), and the overall snow depth was less during 1986–1987.

A. bombacina was visible macroscopically on leaves in the last sample of both field experiments (1 May 1987, 19 April 1988). The fungus formed a thin, white, pellicular layer on many of the inoculated leaves and adjacent uninoculated leaves, particularly on the undersurfaces. Some parts of this hymenium were producing basidia with basidiospores. *A. bombacina* could be recovered from a leaf even when a hymenium was not visible.

Leaves from which *A. bombacina* was reisolated, especially in the last 2 mo of each experiment, were decomposed to a greater extent than control leaves (assessed qualitatively). The cuticles on these leaves were separated from the leaf tissue; many were either peeling away or completely absent.

DISCUSSION

PAB performed adequately as a semiselective recovery medium for *A. bombacina* (ATCC 20629). The distinguishing morphological characteristics of *A. bombacina* developed, viz., clamp connections at each septum, basidia, and basidiospores, although the latter did not form on all plates where the fungus was recovered. Thus, if necessary, identity could be confirmed by examination at 100× magnification with a light microscope. *A. bombacina* (ATCC 20629) was resistant to at least 50 μg/ml of benomyl, whereas many basidiomycetes tested are not tolerant of more than 25 μg/ml (2,6). We have only one isolate of *A. bombacina* from orchard sampling and cannot determine whether benomyl tolerance is unique to isolate ATCC 20629 or common in *A. bombacina* as a species. More isolates need to be recovered and tested to determine the general utility of this medium for *A. bombacina*.

Incubation of leaf pieces placed flat on the medium increased the likelihood of recovering *A. bombacina* compared with dilution plating of leaf homogenate. The disadvantage of the former approach is the lack of quantification of the amount of *A. bombacina* on an individual leaf, but this method facilitated a 10-fold increase in the number of leaves sampled. The detection threshold of PAB for nonsterile leaves was such that the quantity of inoculum initially applied to leaves would be likely to yield high recovery from leaf pieces plated on PAB. Where recovery was low (e.g., 5% or less), the efficiency of PAB was also low (33% or less). Thus, caution would be needed in interpreting low recovery results.

The reduction in recovery of *A. bombacina* in the first and second field samples of the 1986–1987 experiment may have been caused by heavy rain shortly after the bags were first placed in the orchard, which probably removed much of the initial inoculum. This was followed by an unusually cold period (temperatures of −25 C) in December before snow cover, which would have slowed growth of the remaining inoculum. The initial reduction of inoculum may explain why recovery from inoculated leaves at subsequent samplings was not as high as those in the following year.

The level of recovery from uninoculated leaves mixed with inoculated leaves appeared to depend on the successful establishment of *A. bombacina* on inoculated leaves. Moreover, the difference in recovery between the two categories of leaves was not large. Adequate inoculum coverage and a high survival rate on inoculated leaves are probably the most important factors leading to extensive growth and colonization on adjacent uninoculated leaves.

Growth of *A. bombacina* across a mat of uninoculated leaves was slower than expected, based on the rate of colonization of mixed uninoculated and inoculated leaves. Spread across leaves may have been hindered by their progressive loss from February onward due to faunal activity. Further, *A. bombacina* did not grow across 1-cm gaps between adjacent bags. Thus, to achieve good colonization, inoculum would have to be applied extensively, either to leaves before defoliation, or to litter.

A. bombacina survived and grew at temperatures below 0 C before snow cover and under snow. This is important for biological control, because leaves must be colonized during the winter before maturation of pseudothecia of *V. inaequalis*. It is not known how late during pseudothecial development of *V. inaequalis* that

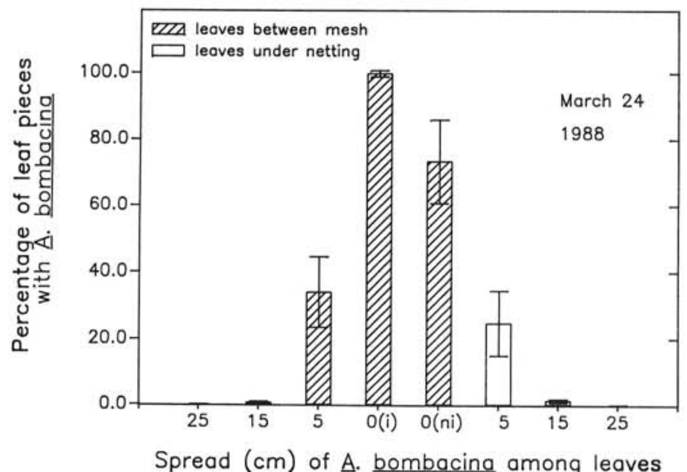

Fig. 5. Recovery of *Athelia bombacina* on 24 March 1988 from uninoculated apple leaves at 0, 5, 15, and 25 cm from inoculated leaves incubated in an orchard beginning 2 November 1987. Twenty five leaves (i, distance 0 cm) were inoculated with *A. bombacina*, mixed with 25 uninoculated leaves (ni, distance 0 cm), and placed in the central 15- × 30-cm portion of a mesh bag. On either side, and contiguous with these leaves, were placed 100 uninoculated leaves either within a 30- × 30-cm area of 1.4- × 1.6-mm mesh or beneath a 30- × 30-cm area of 14-mm coarse netting. Ten leaves from each bag were sampled at each distance. Each leaf was cut into eight pieces and incubated. Each bar represents the mean recovery, with standard error, from the same position in each of five bags.

A. bombacina can be applied and still cause inhibition. During 1986–1987, leaves in the inoculated bags were mostly colonized during January, whereas equivalent leaves during 1987–1988 were colonized in November, but ascospore production in spring was inhibited in leaves from both experiments (17).

A. bombacina did not sporulate until the end of each experiment, which means that spread within bags was by mycelial growth. No naturally occurring *A. bombacina* was detected in these experiments, and no inoculum survived between the two field experiments, as shown by the lack of recovery from uninoculated control leaves. If inoculum were to survive during the summer, it would have to utilize a substrate other than apple leaves, because these leaves are removed by litter fauna from the orchard by the end of spring or early summer. Thus, the antagonist probably would have to be reapplied each fall for effective biological control.

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