Microbial Antagonism to the Imperfect Stage of the Apple Scab Pathogen, *Venturia inaequalis*

John H. Andrews, Flora M. Berbee, and Erik V. Nordheim

First and second authors, Department of Plant Pathology; third author, Departments of Forestry and Statistics, University of Wisconsin, Madison 53706.

Support by the College of Agricultural and Life Sciences, University of Wisconsin-Madison, and by NSF Grant DEB-7725783 is gratefully acknowledged, as is the technical assistance of S. Miller and J. Lane.

Accepted for publication 16 July 1982.

ABSTRACT

Andrews, J. H., Berbee, F. M., and Nordheim, E. V. 1983. Microbial antagonism to the imperfect stage of the apple scab pathogen, *Venturia inaequalis*. Phytopathology 73:228-234.

Fifty microorganisms from McIntosh apple leaves were screened for antagonism to two strains of the apple scab pathogen, *Venturia inaequalis*. The eight most antagonistic microorganisms (*Flavobacterium* sp., *Cryptococcus* sp., *Aureobasidium pullulans*, *Trichoderma viride*, *Chaetomium globosum*, *Microsphaeropsis olivacea*, and two unidentified actinomycetes) were ranked as to their efficacy. The ranking resulted from integration of data on inhibition of *V. inaequalis* from three in vitro and three in vivo assays: growth on nutrient agar, germination and germ tube lengths on agarose-coated slides, lesion size, overall symptom development, and conidial production on infected leaves. The best and most consistent antagonist was *C. globosum*. Average rank order of the antagonists was not influenced appreciably by the strain of *V. inaequalis* but, except for *C. globosum*, ranks differed markedly among assays. Impact of antagonists on germination and germ tube lengths of conidia of *V. inaequalis* on leaf surfaces was predicted by the agarose slide method 72 and 83% of the time, respectively. Ability of antagonists to reduce disease development was positively correlated with their suppression of conidial production by *V. inaequalis* ($\rho = 0.651$). The presumptive modes of antagonism determined in vitro are nutrient competition and antibiosis.

Additional key words: biological control of aerial or foliar plant pathogens, *Spilocaea pomi*, screening for microbial antagonists.

Current interest in leaf-surface microbiology (eg, 6,10) has been increased by the realization that an antagonistic epiflora reduces infection (20,22) and that leaf exudates contain substances that may either stimulate or inhibit microorganisms, including pathogens (5). Recently, in Europe, foliar biological control was undertaken by Fokkema and his colleagues (13,14) who used yeasts as antagonists, and Spurr (24) in the United States, who focused on antagonistic bacteria. The most familiar example of applied foliar biological control probably is the work of Bhatt and Vaughan (4). Rot of greenhouse strawberries caused by *Botrytis cinerea* was inhibited by 36 and 42% when senescent flowers were sprayed with propagules of *Aureobasidium pullulans* or *Cladosporium herbarum*, respectively.

Biological control of foliar diseases has not attracted as much attention as biological control of soilborne pathogens. However, we decided to investigate it as a strategy against the apple scab disease because of the relatively slow growth of *Venturia inaequalis* (Cke.) Wint. During the asexual cycle (*Spilocaea pomi* Fr.), its proximity as a subcuticular inhabitant to phylloplane antagonists suggested that biological control might be feasible. Furthermore, tolerance of *V. inaequalis* to certain key fungicides (17), accelerating cost of chemical controls (15), and increasing regulatory restrictions make development of biocontrol options attractive.

The purpose of the research reported here was to define the components of a comprehensive, integrated in vitro and in vivo procedure for evaluating microbial antagonism to *V. inaequalis*, and the relative efficacy of eight antagonists.

MATERIALS AND METHODS

Growth conditions required by apple seedlings, *V. inaequalis*, and microbial antagonists. Apple seeds (open pollinated) stratified in moist sand in aluminum flats at 3 C for 2 mo were germinated at 19 C for 1–2 wk. Seedlings were transplanted to 6-cm-diameter plastic pots containing a soil-sand-vermiculite (2:1:1, v/v) mixture and grown until inoculation under the following regime: temperature, days 26 C, nights 21 C; 12 hr photoperiod; illumination, approximately 270 $\mu E \cdot s^{-1} \cdot m^{-2}$ at pot level; periodic fertilization with modified Hoagland's solution (16).

V. inaequalis strains 3C5 per 5/1 (green) and 365-4 (olivaceous brown; wild-type), differing in color and in virulence to McIntosh apple, obtained from D. M. Boone, University of Wisconsin-Madison, were grown on cheesecloth wicks in 140-ml medicine bottles containing 25 ml of weak potato-dextrose broth at 16 C (18). Cultures were reinitiated about every 6 mo by a host plant passage and single-spore isolation to maintain conidial rather than mycelial production.

All microbial antagonists were isolated from apple leaves (1,2), transferred by the standard pure culture methods (streaking, single-sporing, or hyphal-tipping), and were maintained either under mineral oil at 4 C on slants of potato-dextrose agar (PDA) (yeasts and filamentous fungi), nutrient agar (NA) (bacteria), or chitin agar (actinomycetes; see reference 1). The eight antagonists evaluated in this report were: *Flavobacterium* sp., *Cryptococcus* sp., *Aureobasidium pullulans* (de Bary) Arnaud, *Trichoderma viride* Pers. ex Fr., *Chaetomium globosum* Kunze ex Fr., *Microsphaeropsis olivacea* (Bonard.) Höhn., and two unidentified actinomycetes. Inoculum was increased by streaking antagonists on the appropriate medium in petri dishes and incubating the plates for 1 day at 28 C (yeasts and bacteria) or 7–30 days at 24 C (actinomycetes and filamentous fungi). Inoculum was harvested by flooding the plates with 0.01 M (pH 7.0) phosphate buffer solution, which contained 0.01% Tween-20, and agitating it with a sterile L-shaped glass rod. After filtration through three layers of cheesecloth, the cells of *V. inaequalis* and all antagonists were washed once by low-speed centrifugation. Concentrations of propagules in suspensions of *V. inaequalis* and fungi were standardized with the aid of a hemacytometer; actinomycete and bacterial numbers were estimated visually by comparison with McFarland's (21) barium sulfate standards. Conidial suspensions of *V. inaequalis* were used immediately after harvesting and those of the antagonists were stored in buffer overnight at 4 C before use.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. § 1734 solely to indicate this fact.

©1983 The American Phytopathological Society

Evaluating antagonistic activity. *In vitro assays.* Relative antagonism of the isolates to *V. inaequalis* was assessed macroscopically by growth inhibition on weak potato agar (WPA) (infusion from 40 g of peeled potatoes plus 20 g of agar per liter), and microscopically by reduction in germination and alteration in germ tube morphology on agarose-coated microscope slides. For the former, a narrow band ($\sim 3 \times 54$ mm) of *V. inaequalis* was imprinted on WPA in petri dishes by replica-plating (19) from a suspension of $\sim 10^6$ freshly prepared and washed conidia/ml. This band then was cross-streaked twice by drawing inoculum of an antagonist on the tip of a fine dissecting needle through the pathogen line. Observations on the relative growth of *V. inaequalis* were made following 12–14 days of incubation in the dark at 16 C.

Relative degree of antagonism was compared by rating test plates on a scale of 0 to 5, in which 0 = complete inhibition of *V. inaequalis* and 5 = growth equivalent to the control streak of *V. inaequalis* alone. After several preliminary trials without the full complement of antagonists, the entire experiment was conducted twice, both times consisting of at least five replicate plates per antagonist for each of the two strains of *V. inaequalis*. The Friedman procedure (9), described below under statistical analyses, was used to test the null hypothesis (H_0) that there were no differences among antagonist treatments.

Microscopic determinations were made on agarose-coated microscope slides (11) spread with 10 μl of a mixture of equal volumes of *V. inaequalis* at 10^6 conidia/ml and an antagonist at 10^7 cells per milliliter, distributed over an area of about 1,375 mm^2 with a sterile, L-shaped glass rod. The slides were incubated in petri-dish humidity chambers for 2 days at 16 C, then stained with lactophenol-cotton blue. Germination of conidia of *V. inaequalis* and germ tube lengths were assessed in at least two experiments, each of two to three replicate slides per antagonist per strain of *V. inaequalis*. For germination, five random microscope fields at $\times 160$ magnification, each containing at least 25 conidia, were examined from each slide. Total numbers and numbers of nongerminated conidia were tallied for each field, and the proportion of nongerminated conidia were calculated. Germ tube lengths were measured with an ocular micrometer for 90 spores from each slide.

For statistical analyses, arc sine square root transformations and log transformations were made of the germination proportion and the germ tube length data, respectively, to reduce variance heterogeneity (23). The Kruskal-Wallis test (9) was then applied to the transformed data, as described below under statistical analyses.

Effects assessed by the agarose slide method were compared with those on apple seedling leaves sprayed with *V. inaequalis*, with or without a selected antagonist from each microbial category. In two experiments, each of three replicates per treatment, leaves on plants in situ in an infection box (see following section), and excised leaves in moist chambers as for agarose slides, were incubated, and antagonism then was quantified as above from transparent adhesive Scotch® tape strippings of epidermal surfaces.

In vivo assays. Conditions required for infection.—A Plexiglas infection box installed in a growth chamber accommodated a small humidifier, hygrothermograph, and 100 potted seedlings (20 replicates of four antagonist treatments and 20 controls of *V. inaequalis* alone, per experiment). Plants representing the controls and all treatments were randomized within each of four blocks within the box. As exploratory analysis of the data showed no differences among blocks, subsequent analysis was conducted without formal inclusion of this blocking structure.

The unit was humidified for 2 hr before use and for 16 hr after inoculated seedlings were inserted to maintain relative humidity (RH) close to 100% and to insure free moisture on plant surfaces. Thereafter, the RH was maintained above 90%; in general, this necessitated 1.5 hr of humidification before illumination and darkness. The other environmental parameters for incubation were temperature, 19 C days, 16 C nights; photoperiod, 14 hr; illumination, 116–170 $\mu E \cdot s^{-1} \cdot m^{-2}$ at pot level.

Assay procedure.—Influence of antagonists on lesion development was determined on individual leaves inoculated with inoculum droplets; disease severity and conidial production were assessed on whole sprayed seedlings as described below.

Lesion development was studied on adaxial surfaces of expanding apical leaves 6–10 mm long that were inoculated with 2–4 paired 5-μl droplets containing *V. inaequalis* plus an antagonist on one side of the midrib, and *V. inaequalis* alone on the opposite side. Inoculum was quantified and used at concentrations described above for the agarose slides. After inoculation, plants were placed in the infection box. Relative size of lesions and sporulation of *V. inaequalis* were recorded after 14 days. Antagonism was assessed by the relative ability of each microbe to restrict lesion development, rated on a scale of 0 to 5, in which 0 = absence of symptoms or signs and 5 = maximum development (eg, where *V. inaequalis* was present alone). Because of biological differences between the apical and basal portions of seedling leaves, droplet pairs in these two regions were analyzed as separate experiments. Two to four experiments were conducted per antagonist per strain of *V. inaequalis*, each consisting of 5–10 replicate pairs of droplets per antagonist. Data were analyzed by the Kruskal-Wallis test (9).

Whole-seedling trials were conducted on 3-wk-old potted plants sprayed until runoff with suspensions of *V. inaequalis* or *V. inaequalis* + antagonist, prepared and used at concentrations described for the agarose slides; a chromatography sprayer was used for inoculations. Inoculated seedlings were incubated for 14 days. Antagonism was judged by the ability of each antagonist to reduce disease severity, rated on a scale of 0 to 5, in which 0 = not diseased and 5 = the most severe expression. Each antagonist was tested in at least two experiments with 20 seedlings per strain of *V. inaequalis* per experiment. Data were analyzed by the Kruskal-Wallis test (9).

After determinations of disease severity, nonsusceptible basal leaves and those that emerged after inoculation were discarded. The remaining aerial portion from each plant was weighed to 1 mg, and washed in 0.01 M, pH 7.0, phosphate buffer containing 0.01% Tween-20 for 10 min on a reciprocating shaker operating at ~ 200 excursions per minute. Conidia of *V. inaequalis* in the suspension were concentrated by low-speed centrifugation and counted with a hemacytometer. Antagonism was quantified by measuring reduction in conidial production per gram fresh weight of leaf based on at least two experiments with 20 seedlings per strain of *V. inaequalis* per experiment. Frequently, counts of zero were recorded because antagonism reduced conidia of *V. inaequalis* below the sensitivity threshold of the hemacytometer. To enable calculations, these censored data were replaced with the lowest spore number detected in the corresponding experiment. The Kruskal-Wallis test (9) was applied to log transformations of the data.

Statistical analyses. In each assay, the eight microbial antagonists were ranked in descending order of effectiveness. Nonparametric methods (9) were used throughout because of their relatively simple nature and general applicability with limited distributional assumptions. Primarily two procedures were conducted: the Friedman test (see reference 9, page 299), which is a nonparametric analog to two-way analysis of variance, and the Kruskal-Wallis test (see reference 9, page 229), which is analogous to one-way analysis of variance. The Friedman procedure was restricted to assays for which all antagonists were present in each experiment; this situation pertained only to ratings of growth of *V. inaequalis* on WPA. Treatments were ranked within each experiment, treatment ranks were summed over experiments, and the test statistic was computed. The Kruskal-Wallis test was used for assays wherein all antagonists did not occur in each experiment, although the control was invariably present. To provide data appropriate for this analysis, means of each treatment were subtracted from the control mean. (As noted in the preceding subsections, the original data were transformed before computation of the means.) The treatment means, corrected for control, were arrayed in a one-way layout. Treatment means then were ranked across all experiments; rank sums were obtained for each treatment; and the test statistic was computed. The significance level taken for all our analyses was $\alpha = 0.05$. Multiple comparisons, used with both the Kruskal-Wallis and Friedman tests when the H_0 of no treatment differences was rejected, are the

nonparametric analog of the Fisher least-significant difference procedure (23), and were performed as described on pages 213 and 300 of Conover (9).

Mode of antagonism. Unless stated otherwise, each type of assay described below involved at least two experiments, with at least five replicates per antagonist per strain of *V. inaequalis*. In addition to a control without antagonists, four nonantagonistic isolates (a yeast, filamentous fungus, bacterium, and actinomycete) were included throughout. Relative inhibition (diameter of halos or growth of lawns of *V. inaequalis*) by each antagonist was rated as follows: (−) = no inhibition, effect equal to controls; (+) = slight inhibition; (++) = moderate inhibition; and (+++) = substantial inhibition.

Hyperparasitism. The occurrence of hyperparasitism was determined by examining the zones of interaction between the filamentous fungal antagonists and *V. inaequalis* on WPA. Observations were made with a dissecting microscope at 24, 48, and 72 hr and 7 days, and small slabs of agar were removed from the zones of interaction, stained in cotton blue-lactophenol, and viewed with a light microscope. Additional portions were examined by scanning electron microscopy following standard methods (1).

Volatile antibiosis. To test for gaseous inhibition, two of the four sections in a plastic compartmentalized petri dish were inoculated with conidia of *V. inaequalis* suspended in WPA at 40 C and the intervening two wells were inoculated similarly with an antagonist. Controls consisted of two wells with *V. inaequalis* and two with WPA alone; all four wells containing *V. inaequalis;* and nonantagonistic microbes from each microbial category substituted for the respective antagonist. Plates were incubated in darkness for 14 days at 16 C when the relative growth of *V. inaequalis* was assessed.

Nonvolatile antibiosis. To test for a diffusible inhibitor, WPA plugs, presumed to contain diffusates from an antagonist, were placed on WPA that had been inoculated uniformly by replica-plating with conidia of *V. inaequalis*. Initially, plugs were taken adjacent to antagonist colonies grown for 10–14 days at 12 or 16 C; a preferable method, used subsequently, involved excising agar plugs from beneath cellulose dialysis membrane (M.W. cutoff 12,000–14,000; Scientific Products, McGraw Park, IL), which had been streaked with the antagonist 14 days previously and incubated at 16 C. Observations for halo formation in the *V. inaequalis* 'lawns' were made at 12 days following incubation in darkness at 16 C.

To determine whether inhibition was due to alteration in acidity, the pH of WPA in plates inoculated with a single streak of the antagonist was determined both within and outside zones corresponding to the area of inhibition visible on plates cross-inoculated with the antagonist and *V. inaequalis*. Growth of *V. inaequalis* alone between pH 4.4 and 7.7 also was tested. The pH was taken routinely by paper indicator strips placed in contact with the agar, and confirmed for representative samples with a pH meter.

Nutrient competition. Ability to restore growth of *V. inaequalis* by addition of nutrients, in the presence of a potentially inhibitory substance, was used to distinguish presumptively between nutrient competition and antibiosis. Assays were conducted in petri dishes with two layers of media. Underlays consisted of 10 ml of either water agar (WA) (which did not support the growth of *V. inaequalis*) or WPA. Overlays were soft WPA (1% agar) removed from beside or beneath colonies of the antagonists as described for nonvolatile antibiosis. The WPA removed was either heated gently to melting (90 C) in an Arnold Steamer (Wilmot Castle and Co., Rochester, NY) or autoclaved at 121 C for 15 min, and then poured as a 10-ml layer above the WA and WPA underlays. When the overlay WPA had solidified, it was inoculated uniformly with a conidial suspension of *V. inaequalis* by the replica-plating method. Relative growth of *V. inaequalis* was compared on the four media-treatment combinations (WA or WPA underlays; steamed or autoclaved WPA overlays) for each antagonist and the appropriate controls (WPA overlays removed from plates not inoculated with antagonists) following incubation of the plates in darkness at 16 C for 17 days.

RESULTS

Evaluating antagonists. *In vitro assays*. Plate assays.—The null hypothesis of no treatment differences was rejected. Multiple comparisons revealed significant differences among several pairs of treatments (Table 1). All eight antagonists appreciably reduced growth of *V. inaequalis* on WPA and, despite some variation in ranking of antagonism between the green vs the brown strain, relative performance was quite consistent.

Agarose slide assays.—Relative effects of antagonists on germination and germ tube lengths of *V. inaequalis* are ranked in Table 2. With respect to germination, the treatments were not significantly different. *C. globosum* was the only antagonist that consistently reduced germination of both the brown and green strains of *V. inaequalis* (by ~24 and 46%, respectively). *C. globosum* was also the most pronounced and consistent inhibitor of germ tube elongation of *V. inaequalis* (Table 2) resulting in germ tubes 53 and 83% shorter for brown and green *V. inaequalis*, respectively.

In general, organisms that inhibited germination also reduced germ tube elongation. *M. olivacea* was the only organism that increased above controls both germination of the brown and green strains of *V. inaequalis* (by 15 and 13%, respectively), and germ tube length (26 and 95%, respectively). Antagonists ranked quite comparably by both types of measurements in their activity against the green strain. Variability, reflected by the ranking inconsistencies, was higher with the brown strain. However, with

TABLE 1. Ranking of the eight microbial antagonists assessed by inhibition of growth of *Venturia inaequalis* on weak potato agar

	V. inaequalis strains					
	Green			Brown		
		Antagonist			Antagonist	
Antagonist	*V. inaequalis* growth[a]	Rank sums[b]	Rank[c]	*V. inaequalis* growth[a]	Rank sums[b]	Rank[c]
Chaetomium globosum	1.1	8.0	2	1.3	3.5	1
Trichoderma viride	1.4	10.5	4	1.8	5.5	3
Microsphaeropsis olivacea	1.7	12.5	5	2.8	12.0	6
Aureobasidium pullulans	2.0	18.5	6	2.5	10.0	5
Cryptococcus sp.	3.0	22.0	7.5	4.0	15.5	8
Actinomycete A-11	0.4	6.0	1	1.5	4.0	2
Actinomycete A-13	1.0	8.5	3	2.3	8.5	4
Flavobacterium sp.	3.1	22.0	7.5	3.3	13.0	7

[a] Scale: 0 = no growth of *V. inaequalis*; 5 = growth equal to that on control plates without antagonist. Means from two experiments each of five replicate plates per antagonist per *V. inaequalis* strain.

[b] Rank sums from the Friedman test. See text for details. Null hypothesis of no treatment differences rejected at $\alpha = 0.05$ ($P < 0.01$). Any two treatments whose rank sums are more than 2.5 units apart (for green *V. inaequalis*), or 1.9 units apart (for brown *V. inaequalis*), are unequal.

[c] Rank of rank sums from the Friedman test.

few exceptions (Table 2), antagonists performed similarly against both *V. inaequalis* strains.

The noteworthy events on agarose slides compared with those on excised and attached leaves were as follows: Influence of the antagonists on the two strains of *V. inaequalis* in leaf assays was predicted by the agarose method 72 and 83% of the time for germination and germ tube lengths, respectively; there was no pattern of differences in behavior between the excised and intact leaf assays; leaves generally were the more variable system, ie, variance in germ tube lengths was about 30–50% higher than those from the agarose slides; regardless of the assay, the brown strain of *V. inaequalis* behaved more variably than the green strain.

In vivo assays. Lesion development.—As in previous assays, *C. globosum* was the most effective antagonist in inhibiting lesion development of both the brown and green *V. inaequalis* (Table 3). Other antagonists ranked inconsistently against the two strains. Antagonism reduced lesion development in all cases, except on leaves inoculated with the brown *V. inaequalis* in the presence of actinomycete A-13. However, for the green strain, the differences among effects of the various antagonists were insignificant ($0.10 < P < 0.25$), although against the brown strain the treatments differed significantly ($0.01 < P < 0.05$). In this latter case, multiple comparisons showed that *C. globosum* performed significantly better than antagonists rated third or lower. At the other extreme, whereas A-13 ranked eighth, its effect was not different statistically from *Cryptococcus* sp. or *A. pullulans*.

Disease severity.—For green *V. inaequalis*, differences among treatments were insignificant ($P > 0.25$), whereas for the brown strain, differences were significant ($0.01 < P < 0.05$) (Table 4). Nevertheless, *C. globosum* ranked first against both strains and, in the case of the brown strain, multiple comparisons showed that *C. globosum* performed significantly better than any other antagonist. All antagonists except the actinomycetes ranked similarly against both strains.

Conidial production.—Antagonists differed significantly ($0.001 < P < 0.005$ for both strains) in their ability to reduce conidial production by *V. inaequalis* (Table 5). The impact of all antagonists is underestimated due to substitution of the lowest detectable counts for zeros in the censored data. Clearly, however, *C. globosum* was again the best antagonist, and its rank was consistent against both strains.

One would expect antagonists that effectively inhibited lesion development to have also reduced conidial production. The correlation between disease index and numbers of conidia was tested in each experiment by the Spearman rank correlation coefficient (Spearman's ρ) (9). Based on 35 tests, the average value of ρ was 0.651 (range, 0.104–0.900) and, with three exceptions, correlations were significant at $\alpha = 0.05$. Clearly there was a

TABLE 2. Ranking of the eight microbial antagonists assessed by reduction in germination and germ tube length of *Venturia inaequalis* conidia on agarose-coated slides

	V. inaequalis strains											
	Green						Brown					
	Reduction in germination[a]	Antagonist Rank mean[b]	Antagonist Rank[c]	Reduction in germ tube length[d]	Antagonist Rank mean[b]	Antagonist Rank[e]	Reduction in germination[a]	Antagonist Rank mean[b]	Antagonist Rank[c]	Reduction in germ tube length[d]	Antagonist Rank mean[b]	Antagonist Rank[e]
Antagonist												
Chaetomium globosum	+0.541	2.8	1	0.843	2.9	1	+0.257	2.7	1	0.383	2.5	1
Trichoderma viride	+0.078	13.7	4	0.284	9.7	2	+0.083	12.3	6	−0.007	11.3	3.5
Microsphaeropsis olivacea	−0.058	18.0	8	−0.155	21.0	8	−0.090	18.5	8	−0.199	14.5	7
Aureobasidium pullulans	+0.059	12.5	3	0.206	11.3	3.5	+0.157	7.7	2	−0.033	7.7	2
Cryptococcus sp.	−0.038	14.5	6	0.100	18.5	7	+0.135	9.0	3	−0.235	16.3	8
Actinomycete A-11	−0.018	15.0	7	0.093	15.0	6	+0.068	12.0	4.5	−0.09	12.5	5.5
Actinomycete A-13	+0.039	14.3	5	0.193	12.7	5	+0.056	12.0	4.5	−0.086	11.3	3.5
Flavobacterium sp.	+0.043	10.5	2	0.227	11.3	3.5	+0.047	13.5	7	−0.091	12.5	5.5

[a] Calculated from arc sine square root-transformed germination proportion data, control–treatment. From two experiments each of two to three slides per antagonist per *V. inaequalis* strain. See text for details.
[b] Rank means from Kruskal-Wallis test. See text for details.
[c] Rank of rank means. Differences insignificant at $\alpha = 0.05$ ($0.10 < P < 0.25$ for both green and brown *V. inaequalis*).
[d] Calculated from log-transformed ocular micrometer units, control–treatment. See text for details.
[e] Rank of rank means. Differences significant for green *V. inaequalis* ($0.01 < P < 0.05$); for brown *V. inaequalis* insignificant ($0.10 < P < 0.25$).

TABLE 3. Ranking of the eight microbial antagonists based on reduction in size of foliar scab lesions on apple seedlings inoculated with paired micropipetted droplets of *Venturia inaequalis* and of *V. inaequalis* plus antagonist

	V. inaequalis strains					
	Green			Brown		
	Reduction in lesion size[a]	Antagonist Rank mean[a]	Antagonist Rank[c]	Reduction in lesion size[a]	Antagonist Rank mean[b]	Antagonist Rank[c]
Antagonist						
Chaetomium globosum	2.6	5.6	1	2.4	2.5	1
Trichoderma viride	0.3	23.3	8	0.7	13.9	4
Microsphaeropsis olivacea	1.1	13.7	3	1.2	7.2	2
Aureobasidium pullulans	0.6	19.1	7	0.5	18.4	6
Cryptococcus sp.	0.9	15.5	4	0.4	19.8	7
Actinomycete A-11	2.0	12.8	2	0.7	14.5	5
Actinomycete A-13	0.5	18.3	6	−0.2	25.1	8
Flavobacterium sp.	1.6	15.4	5	0.8	13.8	3

[a] Calculated as lesion index control–treatment. Indices: 0 = healthy to 5 = maximum lesion size. From two to four experiments, each of five to 10 replicate pairs of droplets per antagonist per strain of *V. inaequalis*.
[b] Rank means from the Kruskal-Wallis test. See text for details.
[c] Rank of rank means. Differences in ranking insignificant at $\alpha = 0.05$ ($0.10 < P < 0.25$) for green *V. inaequalis*; for brown *V. inaequalis* differences significant at $\alpha = 0.05$ ($0.01 < P < 0.05$).

tendency for larger values for disease indices and conidial numbers to be paired.

Ranking of antagonists in all the in vitro and in vivo assays is summarized in Table 6. The most striking observation is that, except for one test, *C. globosum* rated first, and its performance was the most consistent with respect both to the type of assay and strain of *V. inaequalis*. The other organisms fluctuated in rank, although relative activity against brown and green *V. inaequalis* was quite constant. Except for *C. globosum* there is not close agreement in ranking by the in vitro vs the in vivo battery of assays.

Mode of antagonism. In the cases for which it was tested, hyperparasitism appeared either to be nonoperative, or the results were inconclusive. Although hyphae of *C. globosum* and *T. viride* coiled about conidia and germ tubes of *V. inaequalis*, there was no indication from plate trials, agarose slides, or epidermal strippings that the pathogen was perforated. There was no inhibition of either strain of *V. inaequalis* in the plate trials of all antagonists for a volatile inhibitor.

Most antagonists (and also the nonantagonist controls) caused the pH of the WPA medium to rise slightly (range, 4.5–6.0); however, the agar zones of elevated pH did not correspond to the inhibition patterns. *V. inaequalis* grew throughout the pH range tested (4.4–7.7).

Varied degrees of inhibition occurred when plugs of WPA, removed near colonies of *C. globosum*, *T. viride*, *Flavobacterium* sp., and the actinomycetes, were transferred to lawns of germinating conidia of *V. inaequalis*. Except for the compound(s) produced by *Flavobacterium* sp., the inhibitory principles were destroyed by autoclaving but not by heating to ~90 C.

Addition of nutrients as an underlay of WPA permitted normal growth of *V. inaequalis* when it was replica-plated onto an overlay of WPA removed from the zones of inhibition induced by *M. olivacea*, *A. pullulans*, and *Cryptococcus* sp. Growth of *V. inaequalis* was promoted, but not fully restored, by nutrients when the overlays were removed from inhibitory zones induced by actinomycete A-11 and *Flavobacterium* sp. Supplementary nutrients as underlays of WPA did not enhance growth of *V. inaequalis*, relative to that on control plates with WA underlays, when the pathogen was replica-plated onto overlays of WPA from zones adjacent to *C. globosum*, *T. viride*, and A-13.

DISCUSSION

To screen numerous isolates of phylloplane microflora for antagonism to *V. inaequalis*, we sought to develop a battery of assays that would permit detection of antagonism, ranking of antagonists, and determination of presumptive modes of antagonism. Although further removed from natural conditions than the seedling tests, the in vitro trials were included because they were considerably faster and easier to conduct, and they provided a visual clue as to the effect of the antagonist on the pathogen. Of the 50 microorganisms studied over the past 3 yr, the most promising eight antagonists are the subject of this paper. Thus, even *Cryptococcus* sp., which ranked eighth overall, exhibits some activity against the pathogen.

The most antagonistic microorganism tested was *C. globosum*. This is perhaps not surprising in view of its effectiveness as a biological control agent elsewhere (eg, 8,25). What is striking is its

TABLE 4. Ranking of the eight microbial antagonists based on reduction in severity of scab on apple seedlings sprayed with *Venturia inaequalis* or with *V. inaequalis* plus antagonist

	V. inaequalis strains					
	Green			Brown		
		Antagonist			Antagonist	
Antagonist	Disease reduction[a]	Rank mean[b]	Rank[c]	Disease reduction[a]	Rank mean[b]	Rank[c]
Chaetomium globosum	1.3	5.8	1	1.5	2.0	1
Trichoderma viride	0.2	14.7	7	0.6	11.5	6
Microsphaeropsis olivacea	0.6	10.8	5	0.6	10.5	3
Aureobasidium pullulans	1.0	6.7	2	0.7	9.0	2
Cryptococcus sp.	0.6	10.5	4	0.6	10.8	4
Actinomycete A-11	0.7	8.3	3	0.4	13.5	7
Actinomycete A-13	0.1	15.0	8	0.5	11.3	5
Flavobacterium sp.	0.5	12.3	6	0.1	19.5	8

[a]Calculated as disease index control–treatment. Indices: 0 = healthy to 5 = maximum severity. From two experiments, each of 20 seedlings per antagonist per strain of *V. inaequalis*.
[b]Rank means from the Kruskal-Wallis test. See text for details.
[c]Rank of rank means. For green *V. inaequalis* ranking differences insignificant ($P>0.25$); for brown *V. inaequalis* differences significant ($0.01<P<0.05$).

TABLE 5. Ranking of the eight microbial antagonists based on reduction in conidial production by *Venturia inaequalis* on apple leaves

	V. inaequalis strains					
	Green			Brown		
		Antagonist			Antagonist	
Antagonist	Reduction in conidia[a]	Rank mean[b]	Rank[c]	Reduction in conidia[a]	Rank mean[b]	Rank[c]
Chaetomium globosum	0.6283	2	1	0.5050	1.5	1
Trichoderma viride	0.0794	10.5	5	−0.0165	12.5	7
Microsphaeropsis olivacea	0.3559	7	3	−0.1817	5	2
Aureobasidium pullulans	0.5643	3	2	−0.0222	10.5	5.5
Cryptococcus sp.	0.1055	12	7	0.0623	10.5	5.5
Actinomycete A-11	0.2092	9.5	4	0.0915	9	4
Actinomycete A-13	0.0257	13	8	−0.0987	13	8
Flavobacterium sp.	0.1682	11	6	0.1727	6	3

[a]Calculated as log (conidia per fresh weight), control–log treatment. From two experiments, each of 20 seedlings per antagonist per strain of *V. inaequalis*.
[b]Rank means from the Kruskal-Wallis test. See text for details.
[c]Rank of rank means. Differences in ranking are significant ($0.001<P<0.005$) for both strains of *V. inaequalis*.

TABLE 6. Synopsis of ranking of the eight microbial antagonists of *Venturia inaequalis* in all assays

V. inaequalis strain	Assay	Antagonist							
		C. globosum	*T. viride*	*M. olivacea*	*A. pullulans*	*Cryptococcus* sp.	Actinomycete A-11	Actinomycete A-13	*Flavobacterium* sp.
Green	In vitro								
	Growth	2	4	5	6	7.5	1	3	7.5
	Germination	1	4	8	3	6	7	5	2
	Germ tube	1	2	8	3.5	7	6	5	3.5
	In vivo								
	Lesion size	1	8	3	7	4	2	6	5
	Disease severity	1	7	5	2	4	3	8	6
	Conidial production	1	5	3	2	7	4	8	6
	Rank total	7	30	32	23.5	35.5	23	35	30
	Rank[a]	1	4.5	6	3	8	2	7	4.5
Brown	In vitro								
	Growth	1	3	6	5	8	2	4	7
	Germination	1	6	8	2	3	4.5	4.5	7
	Germ tube	1	3.5	7	2	8	5.5	3.5	5.5
	In vivo								
	Lesion size	1	4	2	6	7	5	8	3
	Disease severity	1	6	3	2	4	7	5	8
	Conidial production	1	7	2	5.5	5.5	4	8	3
	Rank total	6	29.5	28	22.5	35.5	28	33	33.5
	Rank[a]	1	5	3.5	2	8	3.5	6	7

[a] Rank of rank total.

consistency in performance regardless of the strain of *V. inaequalis* or the type of assay. Whether this reflects some unique characteristics of the organism, or merely the fact that any outstanding antagonist would have constant ranking, requires further investigation. The other seven antagonists ranked variably among the assays. In particular, ranking in the in vitro trials gave no good measure of rank among the in vivo trials. Therefore, although a prospective phylloplane antagonist might not be eliminated from further consideration based on performance in vitro, such assays if used alone could be quite misleading. This conclusion, drawn from the rank synopses, is reinforced by comparisons of the agarose slide data on germination and germ tube lengths with actual events on leaf surfaces observed from epidermal strippings.

The results of our work show no evidence of target-strain specificity among the antagonists tested. Despite considerable variability among individual assays, the antagonists ranked essentially the same overall against the moderately virulent, brown, wild-type strain, and the more virulent, green, mutant strain.

The presumptive modes of antagonism for the eight microbes tested are antibiosis and/or nutrient competition, with the possible involvement of hyperparasitism for *C. globosum* and *T. viride*. More specifically, the results suggest nutrient competition is operative for *M. olivacea*, *A. pullulans*, and *Cryptococcus* sp.; nutrient competition plus antibiosis for actinomycete A-11 and *Flavobacterium* sp.; and antibiosis for *C. globosum*, *T. viride*, and actinomycete A-13. Our evidence is largely circumstantial. More importantly, it is restricted mainly to assays in vitro that do not necessarily reflect the relative importance of events in vivo, as is well known (3,12). However, information from plate trials is consistent with that obtained by microscopy of tape strippings from leaves, and the data provide a starting point for testing hypotheses as to how antagonistic organisms may function on the apple phylloplane. Further studies, eg, involving isotopic tracers to demonstrate competition for ^{14}C-labelled nutrients on leaves (7), are needed before unequivocal conclusions can be drawn.

The ultimate test of these antagonists as biocontrol agents is their performance against apple scab in an orchard. Field experiments are in progress.

LITERATURE CITED

1. Andrews, J. H., and Kenerley, C. M. 1978. The effects of a pesticide program on non-target epiphytic microbial populations of apple leaves. Can. J. Microbiol. 24:1058-1072.
2. Andrews, J. H., Kenerley, C. M., and Nordheim, E. V. 1980. Positional variation in phylloplane microbial populations within an apple tree canopy. Microbiol. Ecol. 6:71-84.
3. Baker, K. F., and Cook, R. J. 1974. Biological Control of Plant Pathogens. W. H. Freeman & Co., San Francisco. 433 pp.
4. Bhatt, D. D., and Vaughan, E. K. 1962. Preliminary investigations on biological control of gray mold (*Botrytis cinerea*) of strawberries. Plant Dis. Rep. 46:342-345.
5. Blakeman, J. P. 1973. The chemical environment of the leaf surface with special reference to spore germination of pathogenic fungi. Pestic. Sci. 4:575-588.
6. Blakeman, J. P. (ed.). 1981. Microbial Ecology of the Phylloplane. Academic Press, New York. 502 pp.
7. Blakeman, J. P., and Brodie, I. D. S. 1977. Competition for nutrients between epiphytic microorganisms and germination of spores of plant pathogens on beetroot leaves. Physiol. Plant Pathol. 10:29-42.
8. Chang, I.-P., and Kommedahl, T. 1968. Biological control of seedling blight of corn by coating kernels with antagonistic microorganisms. Phytopathology 58:1395-1401.
9. Conover, W. J. 1980. Practical Nonparametric Statistics, 2nd ed. John Wiley & Sons, New York. 493 pp.
10. Dickinson, C. H., and Preece, T. F. (eds.). 1976. Microbiology of Aerial Plant Surfaces. Academic Press, New York. 669 pp.
11. Fokkema, N. J. 1971. The effect of pollen in the phyllosphere of rye on colonization by saprophytic fungi and on infection by *Helminthosporium sativum* and other leaf pathogens. Neth. J. Plant Pathol. 77, Suppl. No. 1. 60 pp.
12. Fokkema, N. J. 1976. Antagonism between fungal saprophytes and pathogens on aerial plant surfaces. Pages 487–506 in: Microbiology of Aerial Plant Surfaces. C. H. Dickinson and T. F. Preece, eds. Academic Press, New York. 669 pp.
13. Fokkema, N. J., den Houter, J. G., Kosterman, Y. J. C., and Nelis, A. L. 1979. Manipulation of yeasts on field-grown wheat leaves and their antagonistic effect on *Cochliobolus sativus* and *Septoria nodorum*. Trans. Br. Mycol. Soc. 72:19-29.
14. Fokkema, N. J., and van der Meulen, F. 1976. Antagonism of yeastlike phyllosphere fungi against *Septoria nodorum* on wheat leaves. Neth. J. Plant Pathol. 82:13-16.
15. Green, M. B., Hartley, G. S., and West, T. F. 1977. Chemicals for Crop Protection and Pest Control. Pergamon Press, New York. 291 pp.
16. Hoagland, D. R., and Arnon, D. I. 1950. The water culture method for growing plants without soil. Calif. Agric. Exp. Stn. Circ. 347. 31 pp.
17. Jones, A. L., and Walker, R. J. 1976. Tolerance of *Venturia inaequalis* to dodine and benzimidazole fungicides in Michigan. Plant Dis. Rep. 60:40-44.
18. Keitt, G. W., and Palmiter, D. H. 1938. Heterothallism and variability

in *Venturia inaequalis*. Am. J. Bot. 25:338-345.
19. Lederberg, J., and Lederberg, E. M. 1952. Replica plating and indirect selection of bacterial mutants. J. Bacteriol. 63:399-406.
20. Leben, C. 1965. Epiphytic microorganisms in relation to plant disease. Annu. Rev. Phytopathol. 2:209-230.
21. Lennette, E. H. (editor-in-chief). 1980. Manual of Clinical Microbiology. 3rd ed. Am. Soc. Microbiol., Washington, DC. 1044 pp.
22. Sinha, S. 1965. Microbiological complex of the phyllosphere and disease control. Indian Phytopathol. 18:1-20.
23. Snedecor, G. W., and Cochran, W. G. 1980. Statistical Methods, 7th ed. Iowa State Univ. Press, Ames. 507 pp.
24. Spurr, H. W., Jr. 1981. Experiments on disease control using bacterial antagonists. Pages 369–395 in: Microbial Ecology of the Phylloplane. J. P. Blakeman, ed. Academic Press, New York. 502 pp.
25. Tveit, M., and Moore, M. B. 1954. Isolates of *Chaetomium* that protect oats from *Helminthosporium victoriae*. Phytopathology 44:686-689.