Microbial Antagonists of Bipolaris maydis

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ABSTRACT

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Microorganisms antagonistic to *Bipolaris maydis* in culture were isolated from *B. maydis* conidia and lesions on maize leaves. When *B. maydis* conidia and cells of one bacterium, AN771, were mixed prior to inoculation, southern maize leaf blight was controlled in greenhouse tests. Disease control was 86-100% when $>10^8$ cells/ml of AN771 were used, but it was <50% with $<2.2 \times 10^7$ cells/ml. Bacterium AN771 inhibited germination of *B. maydis* conidia and germ tube growth in culture and on maize leaves. Most AN771 cells died when the leaf dried.

Additional key words: biological control, survival, antagonistic bacteria.

The possibility of controlling plant diseases with antagonistic microorganisms is appealing in an age of concern with pesticides (1). Microbial antagonists isolated from healthy or diseased leaf tissue, and antagonists derived from other sources have inhibited fungal leaf pathogens. Several reviews on biological control of the pathogens of aerial plant parts have appeared (1, 7, 8, 9).

Antagonists associated with spores of air-disseminated fungal pathogens deserve study, since antagonists useful for biological control might be carried on and disseminated by the spores. For example, cereal rust development was inhibited by antagonistic bacteria associated with the spores of the rust fungi (13, 15, 17). Similarly, an isolate of *Bacillus* sp. associated with *Sclerotinia fructicola* (Wint.) Rehm conidia reduced peach brown rot (5). Bacteria, fungi, and yeast have been isolated from spores of several basidiomycetous pathogens (2, 3, 4, 14, 18).

The purposes of this study were to determine: (i) if antagonistic microorganisms were associated with conidia of *Bipolaris maydis* (Nisik.) Shoemaker (=*Helminthosporium maydis* Nisikado & Miyake), which incites southern maize (*Zea mays* L.) leaf blight; (ii) if antagonists were associated with leaf lesions incited by *B. maydis* on maize or with healthy maize leaves; and (iii) the possibilities of using antagonists for biological control of southern maize leaf blight.

MATERIALS AND METHODS

Culture methods and microorganisms.—After initial isolation, potential antagonists were purified by streaking on agar medium and stock cultures were maintained on Difco nutrient agar (NA) slants at 4 C. Most lesions, isolation plates, and test plates for in vitro antagonism were incubated at 24 C.

Bipolaris maydis Race T isolate 8844 (originally obtained from E. S. Luttrell) was used in inoculations. Conidial suspensions of the pathogen were prepared from Difco potato-dextrose agar (PDA) cultures incubated at 28 C. Conidial concentration was estimated with a hemacytometer or a Sedgewick-Rafter counting chamber and adjusted to 882-2,907/ml. About 0.04 ml of wetting agent (Tween-20, J. T. Baker Chemical Co.) was added per 100 ml of inoculum to ensure uniform wetting. This chemical did not affect incidence of disease. *Bipolaris maydis* stock cultures were maintained on silica gel particles (19).

Antagonists associated with Bipolaris maydis conidia.—*Bipolaris maydis* lesions were collected from naturally-diseased Texas male-sterile (Tms) cytoplasm (susceptible) hybrid maize (Trojan 102, DeKalb XL 45, and other unknown hybrids) in Ohio fields. Diseased leaves were dried in the laboratory and stored at −20 C, except for leaves in one experiment, which were used immediately after collection. Lesions (necrotic tissue with some surrounding green tissue) were excised, affixed with double-stick tape to a sterile surface, and incubated in moist air for 3-5 days. The abundant *B. maydis* conidia formed were dislodged from the lesions onto an agar medium in a petri dish by a stream of filtered air or by sharp tapping. Conidia were collected from moist lesions or after they had dried 7-12 hours.

Selective media and favorable incubation temperatures for the growth of bacteria or yeasts were the same as those used by Leben (10), except the tetrazolium salt was not used in the bacterial medium.

It was assumed that bacterial or yeast colonies growing

in direct contact with *B. maydis* conidia arose from propagules carried by conidia when deposited on the medium. These organisms, as well as a number of common types not associated with conidia, were tested on maize seedlings for antagonism to *B. maydis*, as described below.

Antagonists from ground leaf tissue.—Two adjacent field plots (approximately 9 m × 100 m), one of a susceptible (Tms cytoplasm) and the other a resistant (normal cytoplasm) cultivar of a single-cross hybrid (Oh51A × W64A), were established to produce diseased leaves for study. One plant per 100 m row was inoculated by spraying a conidial suspension of *B. maydis* into the leaf whorl when the plant was 50-60 cm high. The pathogen spread naturally throughout the plot from these inoculum sources. Healthy and diseased leaf tissue disks were collected for assay with a 1-cm diameter cork borer.

Two experiments comparing assay by washing or grinding disks (29 and 43 disks, respectively) indicated that many more bacteria and yeasts were isolated after grinding (mean:17,261/disk) than by washing (mean:4,924/disk); therefore, disks were ground to obtain the larger populations.

Leaf disks were ground individually in 1-2 ml of sterile water (SW) in a sterile mortar. One to three serial 10-fold dilutions were made, and 0.1 ml of the grindings and dilutions were spread on the surface of growth media in three petri dishes. Media were: nutrient glucose agar (NGA–nutrient agar, 23 g and glucose 5 g/liter), PDA, and corn extract agar (CEA). The CEA was prepared by boiling fresh maize leaf tissue (100 g/liter) for 15 minutes, filtering the preparation through cheesecloth, and adding glucose (5 g/liter) and agar (20 g/liter) to the filtrate before autoclaving.

Colonies appearing after 2-5 days were tested for inhibition of the pathogen by spraying the agar surface with a suspension of *B. maydis* conidia. Plates were examined for bacterial or yeast colonies surrounded by *B. maydis* inhibition zones after 2-6 days.

Selected antagonists were tested again in vitro. An isolate was streaked at one side of the agar medium in petri dishes and a suspension of *B. maydis* conidia was sprayed on the plate 2-4 days later. Isolates that yielded *B. maydis* inhibition zones >1-2 mm were tested for disease control. Some isolates produced zones >15 mm.

Testing antagonists for disease control.—A 12-cm segment of the third leaf of susceptible greenhouse-grown maize plants was sprayed, past runoff, as described previously (6), with a mixture of *B. maydis* conidia and potential antagonist cells, or with *B. maydis* conidia alone (control). Antagonist cells were obtained from 24- to 48-hour-old NA slant or Difco nutrient broth (NB) cultures (10 ml). Antagonist cells from slants were suspended in 10-15 ml of SW; turbid antagonist suspensions were combined directly with 10-15 ml of *B. maydis* conidial suspensions. Control plants were inoculated with a *B. maydis* conidial suspension diluted with an equal volume of SW. Following inoculation, plants were incubated in a mist chamber for 24-48 hours at 21-26 C before removal to the greenhouse. Lesions were counted 24-48 hours later, and isolates that reduced lesion counts >50% were tested further. Each test had three-to-eight replicate leaves per isolate.

Observations of the leaf surface.—The collodion epidermal impression technique (16) was used to study the effect of one antagonistic isolate (isolate AN771—see Results section) on germination of *B. maydis* conidia. Seedlings were inoculated with conidia or antagonist plus conidia. After removal of plants from the mist chamber, leaves were dried for 1-2 hours, and one to two coats of collodion were applied with a camel's hair brush. Collodion strips were peeled from the leaf, mounted in lactophenol containing 0.1% cotton blue, and observed at × 79-800 magnification.

Antagonist survival.—Turbid suspensions of one antagonist (isolate AN771) obtained from 24- to 48-hour-old NA slant cultures were sprayed on a 12-cm segment of the third leaf of maize seedlings. Treated plants were incubated in a mist chamber for 24-48 hours prior to drying. Leaf segments (six replicate leaves/treatment) were assayed for AN771 by grinding while still wet (control) and after drying for 1 hour. Bacterium AN771 formed characteristic, easily-recognized colonies on 'TTCC' bacterial medium (10). Progeny from representative colonies, applied with *B. maydis* to seedlings as described above, reduced disease, thus providing further verification of the identity of AN771.

RESULTS

Antagonists associated with B. maydis conidia.—In

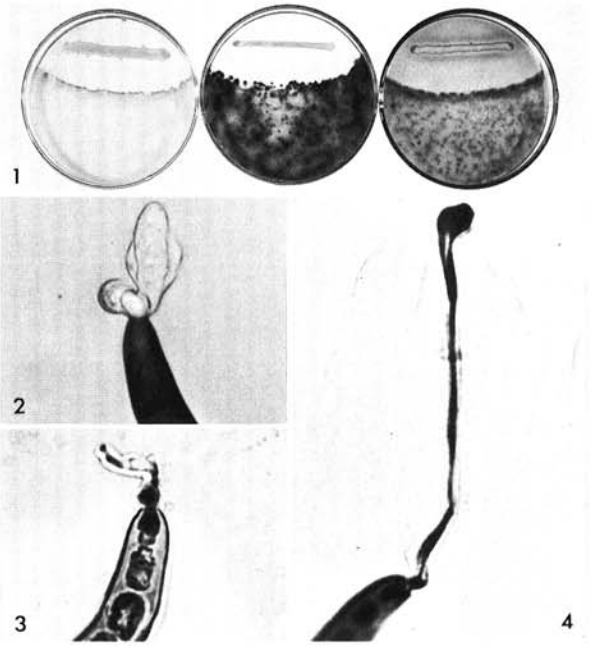

Fig. 1-4. 1) Inhibition of growth of *Bipolaris maydis* by the bacterial antagonist AN771 (horizontal streak) on three media. Left, nutrient glucose agar; center, potato-dextrose agar; and right, corn extract agar (see text). 2) Portion of *B. maydis* conidium midway in the inhibition zone of culture plate (Fig. 1), showing inhibited and malformed germ tube. (3-4) Photomicrographs of collodion impressions of maize leaf surface. 3) Portion of *B. maydis* conidium on leaf treated with bacterial antagonist AN771 showing inhibited and malformed germ tube. 4) Portion of conidium on leaf not treated with AN771 showing normal germ tube and appressorium.

eight experiments with conidia from 200 leaf lesions, 28 bacterial- and one yeast isolate were associated with conidia, as determined by the growth of colonies in contact with conidia. Less than 1% of the conidia observed were associated with bacteria or yeast colonies, but many other bacteria and yeast colonies were present (called "independent colonies"). These 29 isolates along with 209 independent colonies, were tested for disease inhibition, without first making in vitro tests for antagonism. Three of the 29 isolates and 23 of the independent colonies reduced lesion numbers by 50% or more. Five of the most effective isolates had been obtained from leaf lesions assayed immediately after collection from the field, and two were associated with conidia. One of the bacterial isolates from conidia, designated AN771, was selected for further study.

TABLE 1. Reduction of *Bipolaris maydis* conidia germination and appressoria formation on the leaf by the bacterial antagonist AN771

Response of *B. maydis*	Percentage of conidia[a]	
	Antagonist-treated	Non-treated
Not germinated	21	6
Germinated, but no appressorium formed	78	19
Germinated, appressorium formed	1	75

[a]Average of three tests, 318 antagonist-treated and 331 untreated conidia as determined by the impression technique.

Conidia were collected from field lesions between 1100 and 1500 hours by gently tapping diseased leaves to dislodge conidia onto the surface of NGA (with 50 mg/liter of the fungicide cycloheximide added). In three experiments, six bacterial isolates were associated with conidia on 43 isolation plates, but none was antagonistic in culture. They were not tested for disease control.

Antagonists from ground leaf tissue.—In these tests, inhibition of *B. maydis* in vitro was a prerequisite for selection of isolates to be tested for disease control. In 31 experiments, 408 lesioned and 25 healthy disks from the susceptible cultivar and 104 lesioned and 15 healthy disks from the resistant cultivar were assayed. Forty-five bacterial isolates were antagonistic to *B. maydis* in culture tests, but none controlled disease when sprayed on maize leaves with *B. maydis*. Thus, there was no correlation between inhibition in vitro and disease control. Pink yeasts commonly were isolated from lesions but were not inhibitory in vitro to the pathogen.

Spore-forming (heat-resistant) bacterial antagonists might be better suited for survival under adverse environmental conditions in the field. To obtain spore-

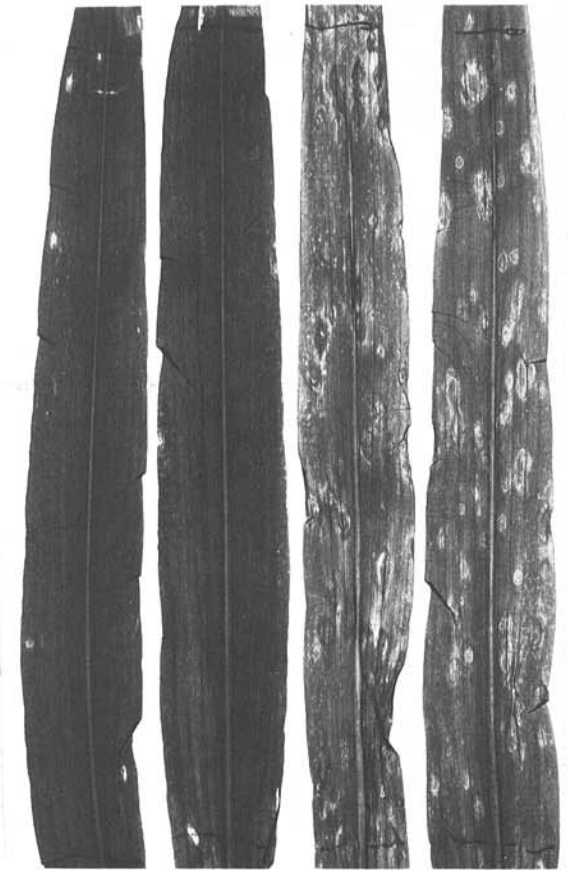

Fig. 5. Disease control of *Bipolaris maydis* provided by the bacterial antagonist AN771, two leaves on the left. Control, two leaves on the right.

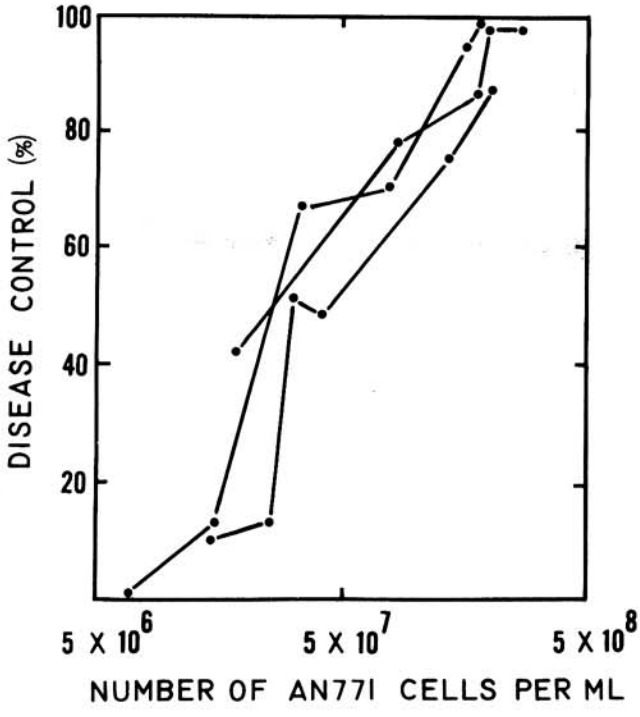

Fig. 6. Effect of different cell concentrations of the bacterial antagonist AN771 on reduction of *Bipolaris maydis* leaf lesions. Each line represents one experiment (six replicate leaves for each cell concentration).

formers, 68 lesioned leaf tissue disks (three tests) were ground, treated at 80 C for 12-15 minutes, and the grindings cultured on NGA. Few bacteria survived this treatment and none of the survivors controlled disease.

Inhibition of B. maydis by bacterium AN771.—Bacterium AN771 produced inhibition zones 16-22 mm wide in culture tests (Fig. 1). Conidia near the AN771 did not germinate, as determined by microscopic inspection of the agar surface. Those farther away germinated, but germ tube growth was inhibited (Fig. 2).

Observations of conidial germination with the impression technique indicated a similar inhibition on the leaf surface (Table 1 and Fig. 3). Germ tubes of AN771-treated conidia were malformed and much shorter than germ tubes of untreated conidia (Fig. 4). Germ tube inhibition by AN771 apparently prevented appressorial formation, since only 1% of the AN771-treated conidia formed appressoria on the leaf surface compared with 75% of the untreated conidia.

Control of disease by bacterium AN771.—In four trials in which more than 10^8 colony-forming-units per milliliter (hereafter designated cells/ml) of AN771 were applied to leaves with *B. maydis* conidia, disease control was 86-100%. In one of these tests, the mean number of lesions per 12-cm leaf segment (six replicate leaves per treatment) was reduced from 55 for the control to three for AN771-treated leaves (Fig. 5). Comparable control (87-91%) was obtained in two additional tests with AN771 cells which had been washed once in water. Further tests demonstrated that a large number of AN771 cells was requisite for control (Fig. 6); disease control with concentrations below 10^8 cells/ml was greatly reduced.

Survival of the AN771 bacterium on maize leaves.—Most AN771 bacterial cells (96-99%) died on the leaf surface when the leaf dried. In two trials, cell numbers (=colony-forming-units) were reduced from a mean of 339,127 cells per 12-cm leaf segment for the wet treatment to 3,939 cells per 12-cm leaf segment for the 1-hour dry treatment.

Characteristics of bacterium AN771.—Bacterium AN771 was a non-sporing, Gram-negative, rod-shaped bacterium, which was motile by one-to-several polar flagella (viewed with the electron microscope). Also it was nonfluorescent, oxidase-positive, aerobic, and did not hydrolyze starch nor did it oxidize 10% ethanol to acetic acid (for method citations, see reference 20).

DISCUSSION

Inhibition zones produced by microorganisms on agar medium have been reported to be caused by acids, antibiotics, or nutrient deprivation. The zones produced by AN771 apparently were not due to acids, since the pH of 2-day-old NB cultures of AN771 was approximately 8.5 (NB without the bacteria was pH 6.5). Additionally, nutrient deprivation did not seem to be involved, since dried suspensions of dead AN771 cells applied to maize leaves inhibited lesion development by *B. maydis*.

Disease control provided by AN771 appeared to be produced by a diffusible compound, such as an antibiotic, which inhibited germination and germ tube growth of *B. maydis* conidia both in culture and on the maize leaf surface. As washed-cell tests indicated, the inhibitory substance may have been present in the cells when they were put on the leaf or it may have been produced on the leaf. If produced on the leaf and metabolites were required, nutrients could have been supplied by leaf and/or conidial exudates. In any event, bacterium AN771 apparently differed from other bacteria that were antagonistic in vitro, because it was the only isolate that also significantly reduced numbers of lesions.

Our results indicate control of disease in the field with AN771 is unlikely, since so many AN771 cells were required for disease control and since nearly all of the AN771 cells died when the leaf dried. Another factor detrimental to survival may be ultraviolet light, but this was not tested on AN771. Failure to survive on the leaf was believed to be the reason why a bacterial antagonist failed to control fungal diseases in the field (12). Survival of antagonists might be increased by materials that protect against drying and ultraviolet radiation damage. With respect to bacteria, studies for inducing hypobiosis (reduced metabolism) promise to be helpful (11).

Isolations through two growing seasons failed to demonstrate an association of an antagonistic bacterium or yeast with *B. maydis* conidia, as reported for cereal rust fungal spores (13, 15, 17). We conclude, therefore, that *B. maydis* conidia only rarely carry antagonistic microorganisms.

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