Protective Applications of Conidia of Nonpathogenic Alternaria sp. Isolates for Control of Tobacco Brown Spot Disease

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Cooperative investigations of the Southern Region, Agricultural Research Service, U.S. Department of Agriculture, and the North Carolina State University Agricultural Experiment Station. Journal Series Paper No. 4995 of the North Carolina Agricultural Experiment Station, Raleigh.

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Appreciation and thanks are expressed to Eleanor T. Howard for technical assistance, Linda D. Daniel for statistical assistance, W. M. Williams for photographic assistance, and R. E. Welty for supplying isolates of *Alternaria*. Special appreciation is expressed to G. B. Lucas, Professor of Plant Pathology, North Carolina State University and E. G. Simmons, Professor of Botany, University of Massachusetts, Amhurst, for examining the taxonomic characteristics of *Alternaria* isolates F646 and A5.

Accepted for publication 15 June 1976.

ABSTRACT

SPURR, H. W., JR. 1977. Protective applications of conidia of nonpathogenic Alternaria sp. isolates for control of tobacco brown spot disease. Phytopathology 67:128-132.

Nonpathogenic isolates of *Alternaria*, applied to leaves of tobacco before inoculation with the pathogen, *Alternaria alternata*, reduced tobacco brown spot. Brown spot severity was reduced 60% in laboratory experiments and 65% in artificially induced field infections. Level of disease reduction was related to the concentration of conidia applied as a protectant. In the laboratory, protection was maximum when the protective conidia were applied to the leaves 2-3 days before inoculation. Nonpathogenic conidia germinated and grew on the leaf surface with no evidence of antagonism toward the pathogen. Colonization by the protective fungus on the leaf surface usually altered host penetration by the pathogen to one of epiphytic growth on the leaf surface.

Additional key words: biological control.

The epi- and endophytic mycoflora of green, growing flue-cured tobacco leaves in North Carolina have been identified and quantified (7, 8, 9). Nonpathogenic *Alternaria* spp. were the most common residents of tobacco leaf surfaces, and presumably were growing as saprophytes or as weak parasites unaccompanied by visible symptoms.

Brown spot, which is caused by the pathogenic *Alternaria alternata* (Fries) Keissler, is an economically important disease that develops on tobacco leaves during the middle to latter part of the growing season. Initial experiments showed that application of nonpathogenic *Alternaria* to tobacco leaves before inoculation with the pathogen could reduce severity of brown spot (5). Previous reports (3) also have indicated that interactions in the phyllosphere between various pathogenic and nonpathogenic microorganisms might reduce infection and disease development. It seemed probable, therefore, that an interaction on the leaf surface between these nonpathogenic and pathogenic *Alternaria* isolates could prevent or reduce brown spot disease. Thus, laboratory and field experiments were undertaken to define the interactions between these isolates of *Alternaria* and to develop practical biological methods for controlling foliar diseases of tobacco.

MATERIALS AND METHODS

In the laboratory, brown spot of tobacco was produced on leaf disks with the inoculation procedure previously described (6). This technique employs leaf disks 9 cm in diameter excised from the leaves of greenhouse-grown tobacco plants. Leaf disks were inverted on a tray over water in transparent plastic boxes (24 × 34 × 6 cm) which maintained a humid atmosphere. The leaf disks were inoculated by applying 12 0.01-ml drops of an aqueous suspension of pathogenic conidia (10,000 *Alternaria* A5 conidia per milliliter) on the lower, laminar surface between the veins. The boxes were left open for several hours to allow the water drops to dry, then closed and placed in an incubator at 21 C with an 8-hour photoperiod (2,099 lux).

Brown-spot development on the leaf disks usually was evaluated after eight days using a scale from 1 to 5 wherein 1 = no symptoms, 2 = light-yellow lesions, 3 =

dark-yellow lesions, 4 = dark-yellow lesions with brown necrosis, 5 = extensive brown necrosis. The 12 inoculation sites on each leaf disk were rated and the ratings from three leaf disks were averaged and used as one replicate; a minimum of three replicates constituted an experiment.

The comparative protective activity of nonpathogenic isolates of *Alternaria* from tobacco was determined as follows. An aqueous suspension of conidia of each isolate was prepared at a concentration of 10,000 conidia per milliliter unless stated otherwise. Both surfaces of 9-cm diameter leaf disks were sprayed lightly with a Hurricane hand sprayer (Afa Corp., Palatine, Ill.). The leaf disks then were placed in plastic boxes and incubated as described above for 3 days and then were inoculated as described above with a pathogenic isolate and incubated until evaluated for brown-spot disease.

The protective activity of isolate F646 also was tested in a field planting of flue-cured tobacco, cultivar Coker 298 at Oxford, N. C. Suspensions (50,000 conidia per milliter) were applied with Hurricane hand sprayers on 9 July to both surfaces of selected leaves in the middle- and upper-stalk positions. Nineteen days later F646-treated and control leaves were spray-inoculated on both surfaces with suspensions of the pathogen, isolate A5, at concentrations of 12,500, 25,000, or 50,000 conidia per milliliter. After two weeks, brown-spot disease was evaluated by counting the number of lesions per leaf.

TABLE 1. Decrease in tobacco brown spot severity on 9-cm leaf disks cut from greenhouse-grown cultivar Coker 298, resulting from inoculation of leaf surfaces with nonpathogenic *Alternaria* isolates three days prior to inoculation with pathogenic *Alternaria*

Treatment	Disease index[a]
None	1.1 A[b]
Protectant (F646)	1.5 A
Protectant (F646 + Pathogen (A5)	2.1 A
Pathogen (A5)	3.5 B

[a]Disease index rating scale: 1 = no disease, 5 = severe disease.
[b]Average of eight replicates; any two numbers followed by the same letter do not differ significantly ($P = 0.05$) according to Duncan's multiple range test.

TABLE 2. Protective efficacy of various concentrations of nonpathogenic *Alternaria* sp. (isolate F646) conidia when sprayed on tobacco leaf disks 3 days before inoculation with a pathogenic *Alternaria* sp. (isolate A5)

Protectant (conidia/ml × 10^3)	Disease index[a]
0	4.0 A[b]
0.5	3.6 AB
1.0	3.2 BC
2.5	3.4 ABC
5.0	3.0 BC
10.0	2.8 C
20.0	2.8 C

[a]Disease index rating scale: 1 = no disease, 5 = severe disease.
[b]Average of three replicates; any two numbers followed by the same letter do not differ significantly ($P = 0.05$) according to Duncan's multiple range test.

Each treatment was replicated three times and each replicate was three leaves.

To determine whether antagonistic metabolites were produced by the protectant, nonpathogenic *Alternaria* F646, spore germination tests were made in vitro; a drop of conidial suspension (10,000 A5 per ml) was mixed in a concavity slide with an equal volume of test solution and incubated for 24 hours at 21 C. Test solutions were aqueous, filtered extracts of conidia germinated in water, on cellulose-filter paper, or on tobacco-leaf disks. Twelve, 9-cm diameter leaf disks were shaken in 120 ml of deionized water 96 hours after the application of isolate F646. The washings were filtered through a 0.45-μm filter and concentrated 10-fold prior to use. The possibility of antagonsim also was examined by growing F646 and A5 together in the same petri dish on V-8 juice agar. All tests were replicated three times.

Histological examinations were made of leaf surfaces and of leaf cross-sections that were prepared as described previously (9). Observations of spore germination, host penetration, and fungal growth were facilitated by mounting tissues on slides in lactophenol containing 0.025% cotton blue. Attention was focused on developments after apray-applications of protective conidia (isolate F646) to leaf disks 3 days prior to inoculations with the brown-spot pathogen (A5). Numerous comparative observations were made on inoculated and protected vs. nonprotected tissues from more than three experiments conducted at different times.

RESULTS

Leaf disks sprayed with nonpathogenic *Alternaria* F646 conidia consistently were protected from brown

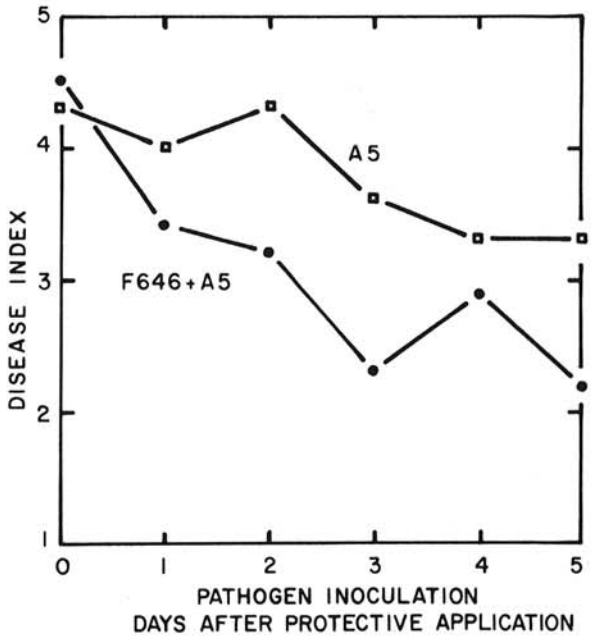

Fig. 1. Effect of time interval between applications to tobacco leaf disks of protective and pathogenic *Alternaria* conidia on disease index.

spot resulting from inoculations with pathogenic *Alternaria* A5 conidia. This control was evidenced by a significant reduction in disease index (Table 1).

A dosage-response was observed between the concentration of F646 conidia and brown-spot severity (Table 2). As the number of protective conidia per milliliter was increased, brown-spot severity decreased. The maximum protective efficacy, 60%, was reached at 10,000 F646 conidia per milliliter in laboratory tests.

As the time between application of the protective fungus and inoculation with the pathogen was increased from 0 to 3 days, brown-spot severity generally was decreased (Fig. 1). Maximum disease reduction (60%) resulted when the pathogen followed the protectant by 2-3 days. Beginning 2 days after the disks were excised, there also was a gradual decrease in disease development in unprotected and inoculated leaf disks (Fig. 1).

TABLE 3. Protective efficacy of nonpathogenic isolates of *Alternaria* when sprayed on tobacco leaf disks three days before inoculation with pathogenic *Alternaria* sp. (isolate A5)

Isolate[a]	Disease index[b]
F646	2.1
V5	2.9
V6	3.0
V13	3.1
V19	3.2
5 AB	2.5
41 AB	2.8
45 AB	2.5
63 B	2.9
187	3.1
216	2.5
None	3.5

[a]All isolates were from healthy tobacco leaf tissue and were applied at a concentration of 10,000 conidia/ml.
[b]Disease index rating scale: 1 = no disease, 5 = severe disease.

Eleven nonpathogenic isolates of *Alternaria* from tobacco varied in protective efficacy (Table 3), but none of the isolates was as effective as F646 when each was compared at 10,000 conidia per milliliter.

In a field test, isolate F646 sprayed on leaves prior to spray-inoculations with A5 resulted in a significant reduction (65%) in the number of brown-spot lesions (Table 4). Results were similar with leaves in both the middle- and upper-stalk positions. The application of F646 conidia at concentrations of 50,000 per milliliter did not decrease the number of naturally-occurring leaf lesions. Increasing the concentration of A5 inoculum from 12,500 conidia per milliliter to 50,000 per milliliter significantly increased the number of brown-spot lesions per leaf.

Conidia of the pathogen A5 consistently germinated (nearly 100%) in 24 hours in drops of water in concavity slides. Mixing A5 conidia with extracts from F646 conidia germinated in water or on cellulose filter paper or tobacco leaf disks did not decrease the percentage of germination. Tests were examined for 4 days after germination. When A5 and F646 were plated 5 cm apart on V-8 juice agar and incubated at 21 C, they grew over each other within a few days with no indication of antagonism or other visible effects.

Histological examinations confirmed that A5 conidia germinate in a few hours and begin to penetrate susceptible leaf tissue within 24 hours after inoculation (Fig. 2-A). Yellow, chlorotic spots were visible after 3 days and necrotic lesions with characteristic halos by 7 days after inoculation.

Nonpathogenic F646 conidia germinated within 24 hours after spray-application to tobacco leaf disks. When disks were sprayed with concentrations of 10,000 conidia per milliliter, 42 conidia per square centimeter were observed on the tissue surface (average of 10 counts). An abundance of mycelial growth was observed on the leaf surface from 2 to 8 days after inoculation (Fig. 2-B). No observations were made after 8 days. Occasionally penetration of the tissue was observed, but no symptoms resulted from these penetrations. The formation of

TABLE 4. Decrease in tobacco brown-spot lesions per leaf on field-grown Coker 298 after spray-application of protectant nonpathogenic *Alternaria* sp. (isolate F646) 19 days before inoculation of the indicated pathogenic *Alternaria* sp. isolates

Treatment	Inoculum (conidia per ml × 10^3)	Brown-spot lesions per leaf	
		Middle leaves	Upper leaves
Noninoculated		16.6 A[b]	9.0 A
Protectant (isolate F646)	12.5	19.3 A	24.6 AB
	25.0	18.2 A	28.9 ABC
	50.0	13.4 A	18.2 AB
Pathogen (isolate A5) + Protectant (isolate F646)[a]	12.5	38.4 AB	41.2 ABCD
	25.0	44.0 AB	25.1 AB
	50.0	72.0 B	51.6 BCD
Pathogen (isolate A5)	12.5	118.8 C	62.8 CD
	25.0	124.3 C	71.2 DE
	50.0	180.1 D	100.7 E

[a]The concentration of protectant (isolate F646) was 50,000 conidia/ml.
[b]Average of three replicates; any two numbers followed by the same letter do not differ significantly ($P = 0.05$) according to Duncan's multiple range test.

conidia was not observed. The F646 mycelium on the leaf surface collapsed or disintegrated after 3 to 8 days. This degeneration was associated with increased numbers of bacteria which were abundant along the strands of superficial mycelium (Fig. 2-C).

When A5 conidia were applied to leaves 3 days after the spray-application of F646 conidia, the A5 conidia germinated as usual within a few hours, but appressoria were not formed. Mycelial growth on the surface was similar to that observed with isolate F646 alone and mycelium of A5 adjacent to F646 on the leaf showed no signs of inhibition. Few penetrations of the leaf surface by A5 were observed compared to when it was the only isolate on the surface. These few penetrations probably accounted for the limited development of symptoms.

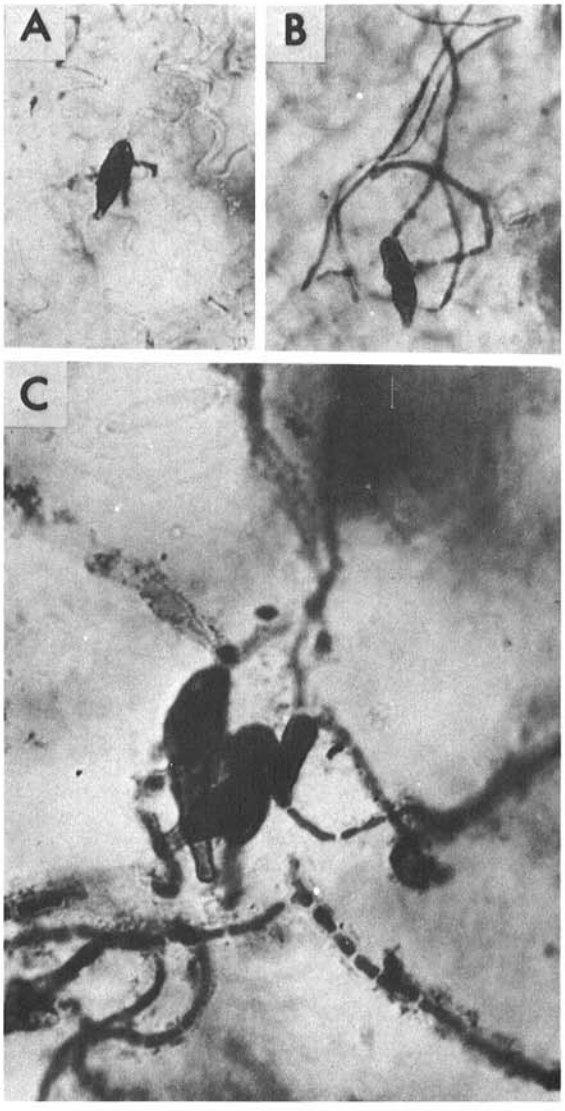

Fig. 2-(A to C). **A)** Pathogenic *Alternaria* conidium germinating and beginning to penetrate tobacco leaf tissue within 24 hours after inoculation. **B)** Protective, *Alternaria* conidium, germinating and growing on the surface of leaf tissue within 48 hours after application. **C)** Disintegration of protective *Alternaria* mycelium associated with large numbers of bacteria on the leaf surface 3 to 8 days after application of conidia.

DISCUSSION

The protectant isolate F646 and the pathogenic isolate A5, both were isolated from tobacco grown near Oxford, N. C. These isolates were examined by G. B. Lucas who considered both to be *Alternaria alternata*. Emory Simmons, however, considered that F646 was one of a group of species similar to, but not identical with *A. alternata* and that A5 was *A. longipes*. Lucas (4) discussed the taxonomic criteria for *A. alternata,* but resolution of the problem is beyond the scope of this paper.

The interaction on the leaf surface between nonpathogenic and pathogenic *Alternaria* significantly reduced the incidence and severity of brown-spot disease both under laboratory and field conditions. An attempt was made to increase disease reduction by using the most protective isolate of *Alternaria* available, by adjusting the number of conidia in the protective application, and by varying the time of inoculation with the pathogen. With these adjustments, disease reduction ranged from 25 to 60% in the laboratory and 65% in the field. Microscopic observations indicate that this pathogen is influenced by the presence of nonpathogenic fungi and resident bacteria on the leaf surface. Thus, additional factors, such as the influence of the host and the microflora of the host, especially bacterial residents, need to be examined for their influence on this method of disease control.

The application of nonpathogenic or saprophytic fungi to leaves of other hosts reduced infection by pathogenic *Alternaria* (1, 2). Heuval reported an antagonistic reaction between saprophytic *A. tenuissima* and pathogenic *A. zinniae* on bean leaves (2). His evidence suggested that a substance produced by *A. tenuissima* inhibited germination of *A. zinniae* spores. With his model system, lesion formation was reduced 80%. Fokkema and Lorbeer (1) reduced infection by *A. porri* on onion leaves with several saprophytic fungi other than *Alternaria* sp. *Aureobasidium pullulans,* which was the most effective, reduced infection by 55%. Spore germination was not reduced, but significant reduction in the length of germ tubes per spore might have accounted for the reduced infection. Measurement of the carbohydrates, amino acids, and pH of the phyllosphere did not explain the disease reduction.

In the several examples of reduced infection by pathogenic *Alternaria,* only a partial reduction, 25 to 80%, was observed and the mechanism(s) of reduction was not definitely determined. Also, in these studies, the natural microflora present in the phyllosphere could have influenced the results. A complete understanding of these interactions may be requisite for the development of methods for biological control of foliar diseases in the field.

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