Ethanol Treatment—A Valuable Technique for Foliar Biocontrol Studies of Plant Disease

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ABSTRACT

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Ethanol treatment of tobacco leaf surfaces increased infection and reduced variation in disease indexes from inoculations with *Alternaria alternata* conidia. The ethanol treatment decreased the normal (resident) bacterial and fungal leaf microflora of greenhouse-grown tobacco leaf tissue 91 and 100%, respectively, without changing relative response of cultivars differing in susceptibility. Protective biocontrol of tobacco Alternaria leafspot resulting from applications of nonpathogenic *A. alternata* conidia was effective on both ethanol-treated and untreated leaf tissue. This indicated that nonpathogenic conidia can control leafspot without interacting with other leaf surface microorganisms but does not mean that leaf surface interactions between the protective fungus and other microorganisms do not occur and thereby alter control efficacy. A computer analysis showed a positive linear regression for probit disease index vs. $\log_{10}$ inoculum dosage for all treatments. Comparisons of values calculated with the regressions indicated the ethanol and biocontrol treatments had separate but additive effects and may have altered disease indexes via the same mechanism. Ethanol treatment of leaf tissue should be valuable for studies of phyllosphere infection and interactions of microorganisms and for development of biocontrols.

Additional key words: epidemiology, tobacco brown spot.

The inoculation of greenhouse-grown plants by an established procedure for inducing disease sometimes gives unexpected results that may be attributed to "biological variation"—a familiar, catchall phrase biologists use. These variations are frustrating and may occur even after standardization of plant growth conditions, inoculation procedures, and incubation conditions. Such variations were observed occasionally in leafspot disease indexes on tobacco leaves inoculated with pathogenic *Alternaria alternata* (Fries) Keissler and may relate directly to fluctuating microfloral populations in the phyllosphere. This variation is particularly troublesome for biocontrol studies using the interactions of known microorganisms added to the leaf surface.

Microorganisms are abundant on leaf surfaces of greenhouse-grown and field-grown plants. Fluctuations in the resident microfloral population on tobacco leaves were described recently (8,11,12), and similar observations were made with other plant species (2,6,8). Some of these resident microflora were isolated and tested, and antagonistic bacterial and fungal isolates were selected for disease biocontrol studies (4,10). Inoculations of tobacco leaves with a nonpathogenic isolate of *A. alternata* or *Bacillus cereus* subsp. *mycoides* before inoculation with pathogenic *A. alternata* controlled disease. Thus, these and other examples (2,6,9) have shown that microflora on leaf surfaces interact and often may be the major determining factor in disease development.

The purpose of this investigation was to test the effect on variations in tobacco Alternaria leafspot after ethanol treatment of leaves, to explore the relationship of test variations to leaf microflora, and to determine if leaf microflora altered the efficacy of applications of nonpathogenic *A. alternata* for biocontrol.

MATERIALS AND METHODS

Disks 9 cm in diameter were excised from the leaves of greenhouse-grown tobacco (*Nicotiana tabacum* L.) plants (5). The Alternaria leafspot susceptible cultivar Coker 298 and the resistant Beinhart 1000-1 were used. The following treatments were applied to alter populations of microorganisms in the phyllosphere of leaf disks. Disks were (i) immersed in 70% ethanol for 30 sec and rinsed with deionized water, (ii) immersed in 1% sodium hypochlorite (NaClO) for 30 sec and rinsed three times with deionized water, (iii) immersed in deionized water, sonified for 15 sec, and rinsed with deionized water, (iv) immersed in deionized water containing 0.01% Tween 80, sonified for 15 sec, and rinsed with deionized water, or (v) rinsed in deionized water. Excess moisture was shaken off the disks after treatment, and the disks were dried for 1 hr in plastic boxes.

Leaf disks were inverted and placed on a tray 2 cm above water in plastic boxes to maintain a humid atmosphere. The leaf disks were inoculated by applying 12 0.01-ml drops of a pathogenic, conidial suspension of *A. alternata* on the lower laminar surface between the veins, as described previously (7). Two aqueous conidial concentrations of isolate A5 (5,000 and 10,000 conidia/ml) were used unless specified otherwise. A few hours after inoculation when the inoculum drops had dried, the boxes were closed and placed in an incubator at 21 C with an 8-hr photoperiod (2,099 lux).

Lesions produced on the leaf disks were rated after eight days by a disease index (DI) in which 1 = no symptoms, 2 = light-yellow

lesions, 3 = dark-yellow lesions, 4 = dark-yellow lesions with brown necrosis, and 5 = extensive brown necrosis. The 12 inoculation sites on each leaf disk were rated, and the ratings from six disks were averaged as one replicate.

The quantitative effect of the ethanol treatment on leaf surface bacteria was determined as follows: 20 disks (10 mm diam) were cut with a sterile cork borer from six ethanol-treated disks (9 cm diam). Untreated leaf disks were sampled in the same manner. The 20 disks were homogenized in 1 ml of sterile water using a sterile glass grinder. The homogenates were diluted 1–10 serially six times, and 0.1-ml samples of each dilution were plated on nutrient agar and incubated. Bacterial colonies that grew on the plates were counted and recorded as colony-forming units (CFU) per square centimeter of leaf surface. Endophytic fungi were quantified by immersing 10-mm diameter disks in 1% NaClO for 60 sec, then rinsing them three times in sterile water. The disks were plated on agar (100 per replicate) and incubated as described previously (11).

Ethanol-spray treatment consisted of lightly spraying both surfaces of leaf disks (9 cm diam) with 70% ethanol until thorough coverage was obtained without runoff. After a 30-min drying period, spraying and drying were repeated. The tissue was then inoculated.

Protective activity of the nonpathogenic *A. alternata* isolate F646 was determined as in the procedure described previously (10). An aqueous suspension of F646 conidia was prepared at 10,000 conidia per milliliter and sprayed on both surfaces of 9-cm diameter leaf disks. The disks were placed in plastic boxes and incubated for 3 days, inoculated with pathogenic *A. alternata,* and incubated until rated. The results were analyzed with the Statistical Analysis System · 76 Research Triangle Institute computer. Inoculum dosages were transformed to $\log_{10}$ inoculum dosage and DI to probit DI (DI 3 = 50% percent disease = probit DI 5). The probit procedure of SAS · 76 (1) based on the probit analysis described by Finney (3) was followed. This procedure calculates the linear regressions, including intercept, slope, and natural response rate, of the biological assay along with the mean and the standard deviation (sigma) of the stimulus tolerance. The inoculum dosage giving 50% disease (ED_{50}) was calculated for each treatment and tested for significance.

Visual observations with a light microscope were made of cross sections cut from leaf tissues with a Hooker Plant Microtome and stained with lactophenol containing 0.025% cotton blue, as described previously (12). Leaf surfaces were viewed with a scanning electron microscope (SEM).

RESULTS

The two sonifier treatments were ineffective and were discontinued. The other treatments were repeated in four additional tests. The ethanol treatment consistently resulted in larger DIs at both inoculum dosages when compared with other treatments (Table 1). The NaClO and the water treatments did not alter the DI. The differences among inoculum dosages, treatments, and their interactions were highly significant by analysis of variance (Table 2). The ethanol treatment was less variable across tests than the other treatments at both inoculum dosages; this was proved by a test for homogeneity of variance. Fewer test failures resulted with ethanol-treated leaf tissues than with untreated controls.

Inoculum efficiency on ethanol-treated leaf tissue was shown with five inoculum dosages. The lowest dosage was 7 pathogenic conidia per inoculation site and the highest was 100 (Table 3).

The average DI on resistant Beinhart 1000-1 was 2.0 on ethanol-treated tissue and 2.2 on untreated tissue after inoculation with 50,000 conidia per milliliter. This difference was not significant and indicated the ethanol treatment did not alter genetically inherited resistance.

A protective application of F646 conidia lowered the DI on both ethanol-treated and untreated tissues at the four pathogen dosages tested (Fig. 1A). Computer analysis of these results showed the regressions were linear and their slopes did not differ significantly. The plot of regressions (probit DI vs. $\log_{10}$ inoculum dosage) was made using the means of the intercept and slope values for each treatment (Fig. 1B). Since the slopes did not differ significantly, the lines were mathematically parallel; they are not parallel in Fig. 1B because the means were plotted. On ethanol-treated tissue, the ED_{50} was 7.1 for that protected with F646 and 5.3 for that not protected (A5 conidia/inoculation site). The ED_{50} was 29.2 on untreated, unprotected tissue and 95.2 on untreated protected tissue. All ED_{50} values were significantly different.

Bacterial microflora of untreated, greenhouse-grown leaf tissue

TABLE 1. Effect of several leaf surface treatments on tobacco Alternaria leafspot indexes on 9-cm leaf disks cut from greenhouse-grown cultivar Coker 298 and inoculated with two dosages of pathogenic *A. alternata* isolate A5

	Treatment			
Test (no.)	Ethanol	NaClO	Water	None
100 conidia per inoculation site				
1	4.7[a]	3.2	2.8	3.4
2	4.6	3.4	4.4	3.8
3	4.7	3.1	4.0	3.3
4	4.9	4.4	3.6	3.5
Average	4.7	3.5	3.7	3.5
LSD ($P = 0.01$) = 0.4				
50 conidia per inoculation site				
1	4.5	2.7	3.2	3.0
2	4.8	2.0	3.0	3.6
3	4.4	4.1	3.5	2.8
4	4.7	3.5	2.5	2.5
Average	4.6	3.1	3.1	3.0
LSD ($P = 0.01$) = 0.4				

[a] Disease index rating scale (average of three replicates): 1 = no disease, 5 = severe disease.

TABLE 2. Summary of analysis of variance of several leaf surface treatments on tobacco Alternaria leafspot indexes at two inoculum dosages

Source of variation	Degrees of freedom	Mean square[a]
Tests	3	0.90*
Inoculum dosages	1	9.32**
Treatments	3	21.78**
Inoculum dosages × tests	3	1.52**
Treatments × tests	9	2.12**
Inoculum dosages × treatments	9	1.24**
Error	140	0.27

[a] * = Significance at $P = 0.05$, ** = significance at $P = 0.01$.

TABLE 3. Tobacco Alternaria leafspot disease indexes after inoculation of ethanol-treated and untreated leaf disks with five dosages of pathogenic *A. alternata* A5

Conidia/inoculation site	Disease index [y]
Ethanol treated	
100	4.7 a[z]
50	4.2 b
25	3.6 c
13	3.2 d
7	2.9 e
Untreated	
100	2.9 e
50	2.6 e
25	2.1 f
13	1.8 f
7	1.8 f

[y] Disease index rating scale (average of two replicates): 1 = no disease, 5 = severe disease.

[z] Numbers followed by the same letter do not differ significantly ($P = 0.05$) according to Duncan's multiple range test.

averaged 393 CFU per square centimeter of leaf surface. The CFU value was decreased 91% to 35 after ethanol immersion treatment and 78% to 86 after ethanol-spray treatment. Endophytic fungi were less prevalent in greenhouse-grown leaf tissue (11 colonies/100 disks) than in field-grown tissues (200 colonies/100 disks). Ethanol immersion decreased endophytic fungi 100% in greenhouse-grown tissue and 59% in field-grown tissue. Ethanol spray decreased endophytic fungi 70% in greenhouse-grown tissue and 26% in field-grown tissue.

Cross sections from tissue exposed to 70% ethanol for 30 sec and then stained showed no differences in the cuticle cell compression or dye absorption when compared with untreated tissues. When tissue was exposed to 95% ethanol for 60 sec, some dehydration of cells and leaf hairs was observed. Tissue exposed to 70% ethanol for 30 sec or 95% ethanol for 2 min and viewed with the SEM did not change in appearance. However, when tissue was exposed for 2 min to acetone, extensive disruption of the epidermis and leaf hairs was noted.

DISCUSSION

Ethanol treatment decreased biological variation in leafspot DIs in the laboratory tests and decreased test failures, probably because of the increased efficiency of pathogenic *A. alternata* conidia on ethanol-treated tissue. This could result from a decrease in the resident bacterial and fungal microflora, from removal or alteration of leaf surface substances, or from a physiological change in the leaf resulting from ethanol treatment. Since ethanol-spray treatment gave similar results without removing any substance(s) from the leaves and since examinations of tissue cross sections and SEMs of the leaf surfaces did not reveal changes, it seemed unlikely that the increased DIs resulted from physico-chemical alterations of the leaves by ethanol treatment. The decrease in DI by the addition of selected antagonists to the leaf surface and the failure of ethanol treatment to alter the DI of a resistant host also supported the idea that the primary mechanism or effect of ethanol treatment was on leaf microflora. However, the reduction in endophytic microflora of the leaf by ethanol treatment indicates the tissue was penetrated by the ethanol, and a resulting physiological change in the tissue of the susceptible cultivar Coker 298 cannot be completely ruled out.

Since ethanol treatment was effective, why were NaClO and water treatments ineffective? NaClO is toxic to microorganisms and some of it is adsorbed to leaf tissue, where it is not removed easily by water. Therefore, NaClO eliminated some leaf microflora and residual NaClO reduced the infectivity of pathogenic *A. alternata*. Water treatment was probably ineffective because it did not alter or remove a high percentage of the microflora from the leaf surface. Certain phyllosphere microorganisms adhere tenaciously to the leaf surface (5). Ethanol treatment provided a means to increase inoculation efficiency with pathogenic *A. alternata* because of ethanol's toxic activity to leaf microflora, its nonresidual nature, and its apparent nondamaging effect to the leaf tissue.

An interaction between leaf microflora and *A. alternata* F646 applied as a protectant seemed likely for two reasons. First, large numbers of bacteria were associated with the dissolution of the protective hyphae 3–8 days after application of the conidia (10). Second, bacteria capable of inhibiting infection by pathogenic *A. alternata*, ie, *B. cereus* subsp. *mycoides*, were isolated originally from tobacco leaf surfaces and also inhibited protective nonpathogenic *A. alternata* (4). Beneficial interactions between

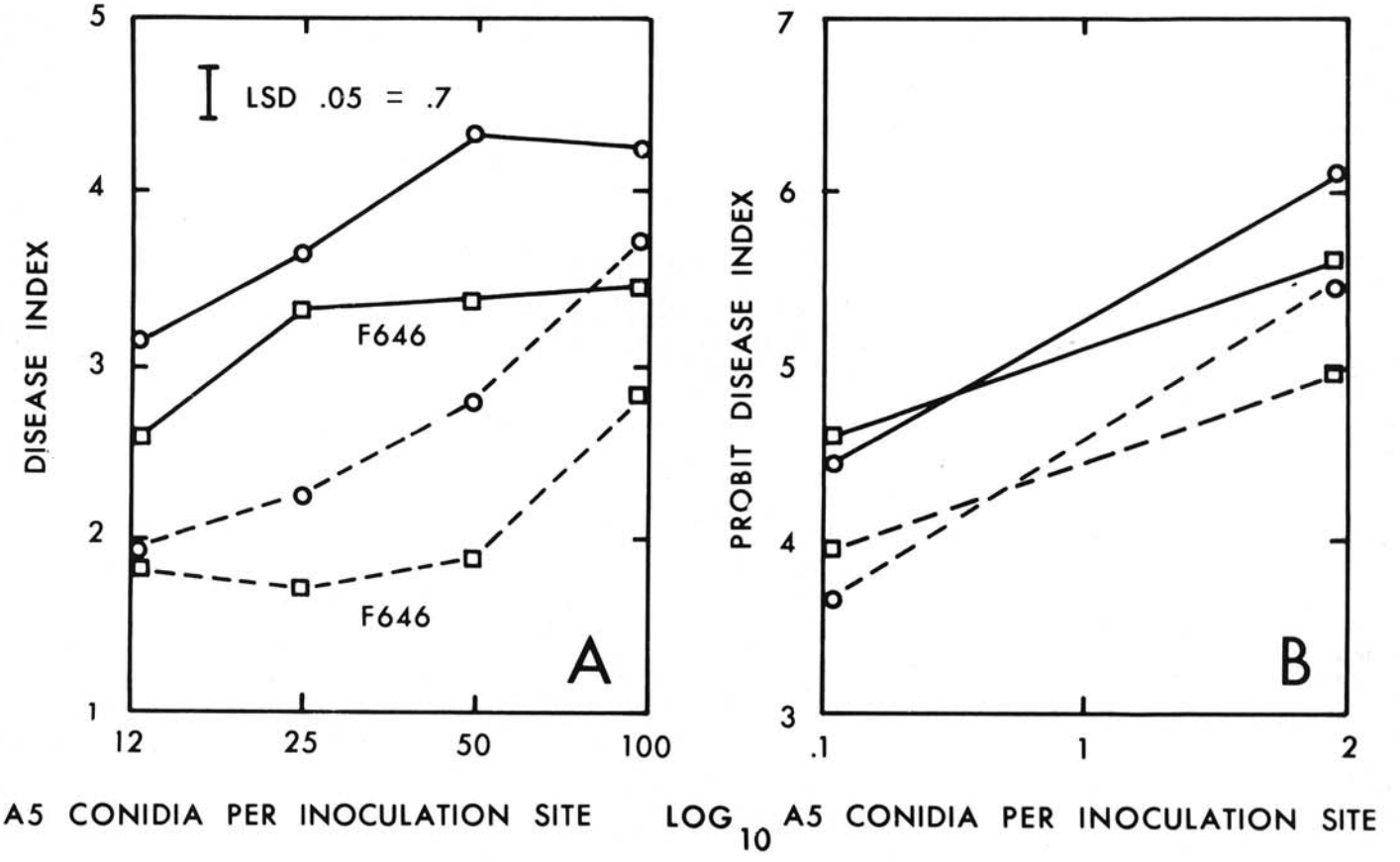

Fig. 1. Tobacco Alternaria leafspot from four inoculum dosages on ethanol-treated leaf surfaces (solid lines) and on untreated leaf surfaces (dotted lines). Leaf surfaces labeled F646 received a protective spray application of nonpathogenic *A. alternata* isolate F646 (10,000 conidia per milliliter) 3 days before inoculation with pathogenic *A. alternata*. **A,** Linear plot of the results. **B,** Plot of the linear regressions made from the mean intercept and slope values. These were determined by computer analysis of the data transformed to probits of disease index and $\log_{10}$ conidia per inoculation site. The symbols identify the treatments and do not represent specific data values.

protective *A. alternata* and leaf microflora or between pathogenic *A. alternata* and leaf microflora are also possible but have not been identified. Nevertheless, the protective activity of nonpathogenic *A. alternata* was effective on ethanol-treated leaf tissue, which indicated the protective mechanism was independent of other leaf microflora. However, other leaf microflora may enhance the effectiveness of the protective application. Since the slopes of the regressions of the ethanol and the F646 protective treatments did not differ but the ED_{50} values did, the treatments have separate effects. They probably work through the same mechanism, however, indicating that the protective activity of F646 was independent of any interactions with resident microflora.

Interpretations or extrapolations of control effectiveness from applications of protective conidia based on the DI as defined here should be made with caution. The DI is a measure of lesion development. A decreased DI value on the lower portion of the scale corresponding to relatively undeveloped lesions may be more significant epidemiologically, ie, secondary spread of disease, than a quantitatively similar decrease at a more advanced stage (higher on the scale) in lesion development. Therefore, effective biocontrol in the field by use of this approach may depend as much on the timing of a protective application as on the absolute quantity or efficacy of control.

Ethanol treatment as described is an effective method for eliminating most leaf microflora, increasing inoculum effectiveness, or decreasing experimental variation in disease studies. It may be of considerable value in studying phyllosphere interactions and in developing biocontrol in the phyllosphere. The technique also may be useful in studying resistance, physiology of disease, etc., by removing an unwanted variable.

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