Symptom expression and ethylene production in leaf blight of cotton caused by *Alternaria macrospora* and *Alternaria alternata* alone and in combination

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The interaction between the cotton leaf pathogens *Alternaria macrospora* and *Alternaria alternata* was studied using dual inoculation at dosages ($\approx 10^3$ spores/(mL · pathogen)) that did not produce symptoms with either pathogen alone. This dual inoculation produced the typical disease symptoms (spots and shedding) and disease severity similar to inoculation with 10^4 spores/mL of *A. macrospora* alone. Neither pathogen produced ethylene in culture; however, they induced production of ethylene concentrations by diseased tissue that were correlated to both disease severity and leaf shedding. Plants infected by both pathogens produced the highest concentration of ethylene. Leaf discs either from leaves exhibiting symptoms or from symptomless infected leaves produced similarly high concentrations of ethylene. Inoculation of any site of the leaf with *A. macrospora* alone or with both pathogens resulted in shedding of the leaf. Pretreating inoculated plants with several ethylene inhibitors or an auxin decreased ethylene production, disease severity, and leaf shedding. *Alternaria alternata* apparently triggers symptom expression by *A. macrospora* in leaf blight disease of Pima cotton, and disease is manifested by the production of ethylene that leads to the typical leaf shedding symptom.

Key words: *Alternaria macrospora*, *Alternaria alternata*, cotton leaf blight, defoliation, ethylene, fungus–fungus interaction, leaf spot of cotton, symptomless infections, virulence.

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Les auteurs ont étudié l'interaction entre les champignons pathogènes du coton, l'*Alternaria macrospora* et l'*Alternaria alternata* en utilisant une double inoculation à des densités de spores (environ 10^3 spores/(mL · pathogène)) qui ne produisent pas de symptôme, lorsqu'un seul des pathogènes est inoculé. Cette double inoculation produit les symptômes typiques de la maladie (tache et chute des feuilles) et une sévérité semblables à ceux obtenus avec l'inoculation de 10^4 spores/mL de l'*A. macrospora* seul. Aucun des champignons pathogènes ne produit d'éthylène en culture; cependant, ils induisent la production par les tissus malades, de concentrations d'éthylène qui sont corrélées à la fois avec la sévérité de la maladie et la chute des feuilles. Les plantes infectées par les deux champignons pathogènes produisent les plus fortes concentrations d'éthylène. Des disques foliaires venant de feuilles montrant les symptômes ou de feuilles infectées sans symptômes, produisent tous de fortes concentrations d'éthylène. L'inoculation à n'importe lequel point de la feuille avec l'*A. macrospora* seul, ou avec les deux champignons, entraîne également la chute des feuilles. Un pré-traitement des plants inoculés avec plusieurs inhibiteurs de l'éthylène ou avec une auxine diminue la production de l'éthylène, la sévérité de la maladie et la chute des feuilles. L'*A. alternata* déclenche apparemment l'expression des symptômes par l'*A. macrospora* chez la brûlure foliaire du cotton Pima, où la maladie se manifeste par la production d'éthylène, conduisant au symptôme typique de la chute des feuilles.

Mots clés : *Alternaria macrospora*, *Alternaria alternata*, brûlure de la feuille du cotton, défoliation, éthylène, interaction entre champignons, tache foliaire du coton, infections sans symptômes, virulence.

[Traduit par la rédaction]

Introduction

Alternaria leaf blight of cotton is a disease of secondary economical importance. Epidemics occur on an intermittent basis with mild losses of up to 15% (29). The causes of this intermittent occurrence are largely unknown. Cotton monoculture in Israel has been practiced for decades, but no significant climatic changes have been recorded during the summer cotton season (The Meteorological Service of Israel data base), nor have new, major agrotechnical practices been introduced. The aggressiveness of *Alternaria macrospora* is seen in increases in leaf spotting and blight over years in cotton monoculture (21). Symptomless infections also occur and are apparently related to plant stress conditions (4).

The identity of the pathogen(s) responsible for the disease is not completely clear. *Alternaria macrospora* is a causal agent of leaf blight in Pima cotton (*Gossypium barbadense*), and *Alternaria alternata* may cause a similar disease in Acala cotton (*Gossypium hirsutum*) (5). However, alternaria leaf blight of cotton also may be caused by a mixture of the two pathogens (8).

Ethylene is produced during pathogenic interactions in intact plants infected with fungi (11, 13, 15, 20, 22, 31, 35) and bacteria (10, 18, 32). Ethylene production is associated with leaf abscission and can be manipulated by ethylene inhibitors and precursors (10, 12, 14, 15, 31).

The aim of this study was to further investigate the possible interaction between the two *Alternaria* species in triggering symptom expression (spots and defoliation) and ethylene production during development of leaf blight of cotton.

Materials and methods

Organisms

Alternaria macrospora Zim (ATCC 62363) and *A. alternata* (Fr) Keissler (S-1) were used in all experiments. Cotton plants (*G. barbadense* cv. Pima S-5) were used as host plants.

Plant growth conditions

Plants were grown in the greenhouse in a controlled environment (25±2°C, 60% relative humidity, and natural illumination) in 5-L pots containing a mixture of peat – vermiculite – volcanic dust

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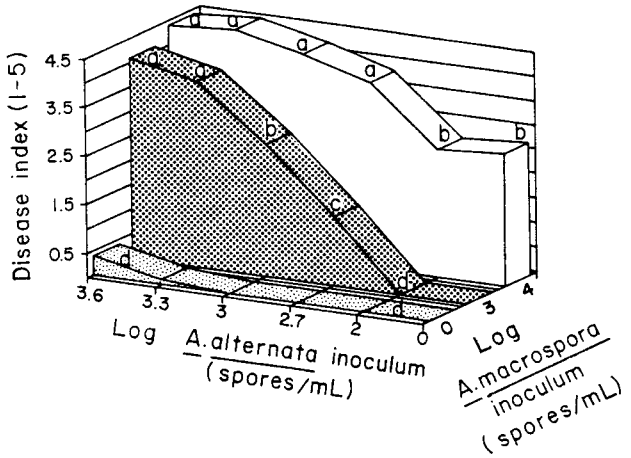

FIG. 1. Disease severity of cotton plants caused by dual inoculation with various inoculum levels of *A. macrospora* and *A. alternata*. Points denoted by a different letter differ significantly at $P \leq 0.05$ in ANOVA.

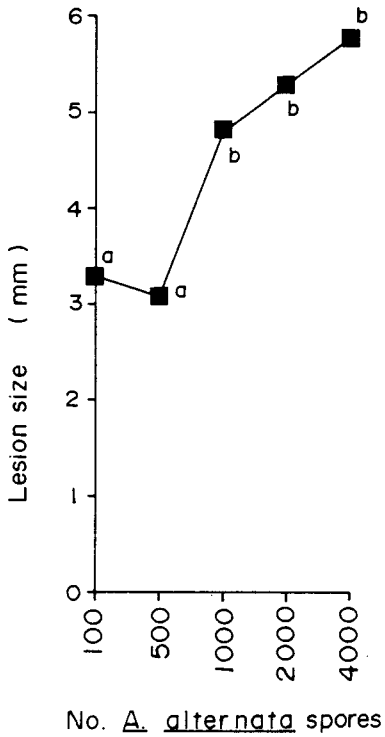

FIG. 2. Lesion size on cotton leaf caused by dual inoculation of constant inoculum of 1000 spores/mL of *A. macrospora* and various inoculum levels of *A. alternata*. Points denoted by a different letter differ significantly at $P \leq 0.05$ in ANOVA.

(1:1:1, v/v/v) and fertilized with half-strength Hoagland's nutrient solution once a week (21).

Fungal culturing and inoculum production

The pathogens were maintained on Czapek medium (1). Every 2–3 months they were transferred to fresh medium by cutting a small piece of culture from the edge of the colony using a cork borer 5 mm in diameter. Identification of the colonies was according to their spore morphology (30, 36). The pathogens were cultivated, and spores were harvested and prepared for inoculation at various concentrations.

Plant inoculation procedure, inoculation levels, pathogenicity tests, and estimation of disease severity

Cotton plants with three to five true leaves were inoculated with inoculum suspended in deionized water until runoff. Control plants were treated identically with dead τ-irradiated spores (25 kGy) or with sterile water. A mixture of 10^3 spores/mL of *A. macrospora* and 2×10^3 spores/mL of *A. alternata* was used for most dual inoculations. At these concentrations, neither pathogen alone caused visible symptoms on Pima cotton under conditions conducive to leaf blight (9). Inoculation with 1.2×10^4 spores/mL of *A. macrospora* was used as a control (a level that produced visible symptoms).

Development of typical disease symptoms (described later) was recorded 5 days after inoculation. Disease severity was assessed by a disease index using the following scale: 0, no symptoms; 1, 1–3 lesions/leaf; 2, 4–10 lesions/leaf; 3, 11–20 lesions/leaf; 4, 21–30 lesions/leaf; and 5, more than 30 lesions/leaf, indicating heavy infection (21).

Procedures for inoculating plants at precise locations

Four agar discs (two of each pathogen, 5 mm in diameter) covered with mycelium and spores were cut from 4-day-old cultures grown on solid Czapek medium and placed face down on the leaf blade, the leaf petiole, or the junction between the leaf petiole and the stem. After inoculation, the plants were incubated for 16 h in a dark, humid chamber at 30 ± 2°C, then transferred to a growth chamber at the same temperature and at 60 ± 8% relative humidity, 120 μmol · m^{-2} · s^{-1} for an additional 5–8 days. Symptoms (spots and shedding leaves) were recorded daily. Ethylene production was measured from leaf discs, 1-cm lengths of petiole cut from the sites where the inoculum discs were applied, and the tissue lacking visible symptoms around these sites.

Ethylene determination

The three fully expanded upper leaves beneath the growing tip of the plant were usually sampled. The leaves were placed in 150 mL rubber-stopped bottles and incubated at 30 ± 1°C for 24 h. Ethylene production over a 24-h period was measured using a gas chromatograph fitted with a flame ionization detector. The rate of ethylene production was measured as picomoles per gram dry weight per hour using purified ethylene as a standard. Controls were noninoculated leaves or leaf parts, leaves inoculated with dead pathogens and subjected to the same cutting procedures, or empty stopped bottles.

Leaves for dry weight determinations were dried in a forced draught oven for 48 h at 80°C and weighed after cooling.

Ethylene production by the pathogens in culture

The pathogens were grown for 3 days at 30 ± 1°C (separately or together) in 60-mL serum bottles sealed with cotton stoppers in 20 mL Czapek medium or Czapek medium supplemented with 10 mM methionine. The bottles then were sealed with rubber stoppers, and ethylene production was measured 24–96 h later as described for leaves.

Ethylene cells

Three cotton plants with five to seven fully expanded leaves were placed in hermetically sealed 11-L glass flasks. One millilitre of purified ethylene was injected through a rubber bridge into each flask. The flasks were incubated in the temperature-controlled growth chamber described earlier. Flasks without added ethylene served as controls (10). Leaf shedding was recorded 8 days later.

Lesion measurements

These were done by an image analyzer scanning the entire leaf for typical alternaria lesions. The symptoms are characterized as small, necrotic spots, with a purplish halo. They may expand to about 1 cm in diameter. The center of the spot is gray and cracked. The spots can be better defined on the upper surface of the leaf.

Application of IAA, ethylene inhibitors, and an ethylene precursor

Cotton plants were treated with the following substances: indole-3-acetic acid (IAA; 2×10^{-3} M); the inhibitors silver thiosulfate (ST;

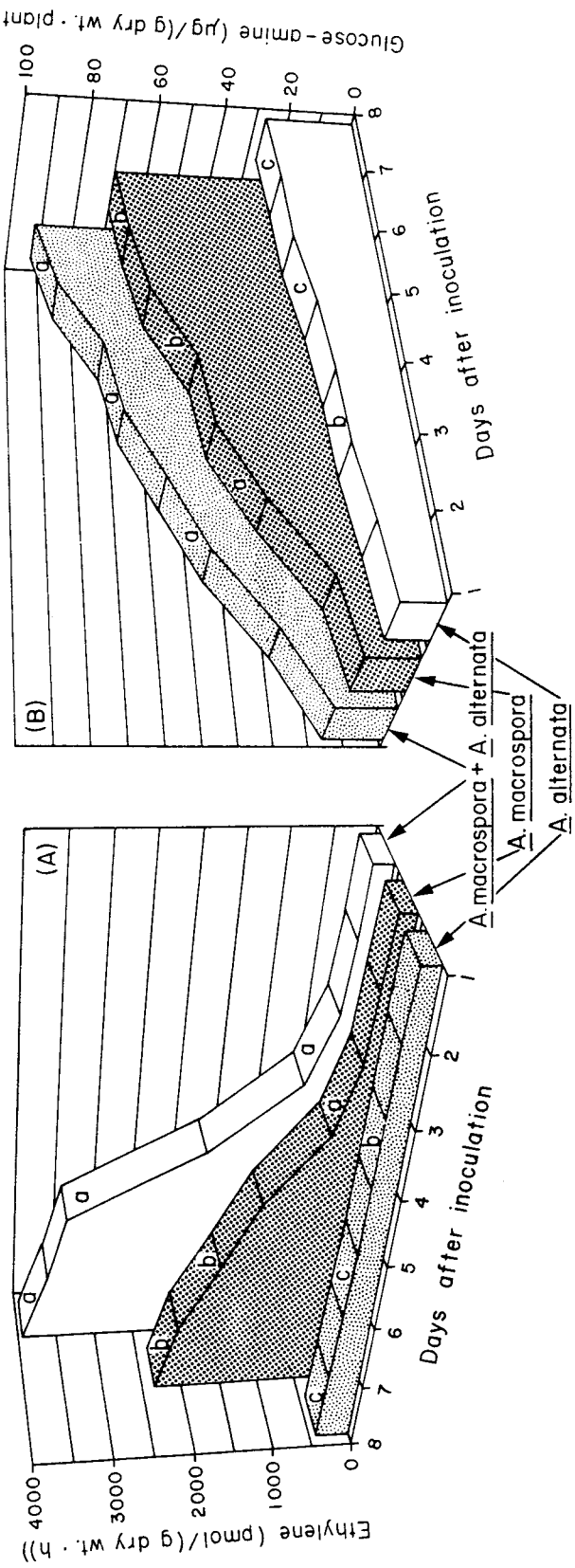

FIG. 3. Ethylene production (A) and the amount of fungal material (B) within the tissue of diseased cotton plants inoculated with 12 000 spores/mL of *A. macrospora*, 2000 spores/mL of *A. alternata*, or dual inoculation with 1000 and 2000 spores/mL of *A. macrospora* and *A. alternata*, respectively. Points denoted by a different letter differ significantly at $P \leq 0.05$ in ANOVA.

TABLE 1. Leaf shedding caused by dual inoculation with *A. macrospora* and *A. alternata*

Inoculation type (spores/mL)	No. of leaves shed
10^3 Am + 2 × 10^3 Aa	15.4*a*
10^3 Am	0.22*b*
2 × 10^3 Aa	0.12*b*
1.2 × 10^4 Am	14.8*a*
1.2 × 10^4 Am + 4 × 10^3 Aa	16.2*a*
Noninoculated	0.18*b*

NOTE: Numbers followed by different letters differ significantly at $P \leq 0.05$ in ANOVA. Am, *A. macrospora*; Aa, *A. alternata*.

10^{-5} M), aminooxy acetic acid (AOA; 10^{-4} M), and aminoethoxyvinylglycine (AVG; 10^{-4} M); and the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC; 10^{-4} M). All substances were dissolved in distilled water containing 0.01% Tween-20, and sprayed onto leaves until runoff. Leaves were inoculated immediately after the solutions on the leaf surface had dried.

Measurement of fungal material within the leaf

The amount of chitin in the leaves was measured by analyzing glucoseamine according to the method of Elson and Morgan (6, 16).

Experimental design and statistical analysis

Experiments were repeated two or three times. The replicates, arranged in a randomized design, consisted of 10 plants, 10 plant parts, 10 slants, or four ethylene cells. Results are the mean of all experiments analyzed at $P \leq 0.05$ by one-way analysis of variance (ANOVA).

Results

Symptom expression in cotton leaves caused by dual inoculation of A. macrospora *and* A. alternata

Dual inoculation of cotton plants with 1000 spores/mL of *A. macrospora* (which is normally unable to induce spot symptoms) coupled with 2000 spores/mL of *A. alternata* induced disease severity greater than that from inoculation with an optimal concentration (12 000 spores/mL) of *A. macrospora* alone (Fig. 1). *Alternaria alternata* at concentrations as low as 500 spores/mL, inoculated together with 1000 spores/mL of *A. macrospora* also initiated symptom appearance on cotton leaves. No significant enhancement was found when the optimal inoculum level of *A. macrospora* was mixed with concentrations of *A. alternata* greater than 500 spores/mL. The strain of *A. alternata* used here produced a few spots at the highest tested concentration (Fig. 1).

Lesion size caused by dual inoculation

Different inoculum concentrations of *A. alternata* were mixed with 1000 spores/mL of *A. macrospora* and dually inoculated on cotton plants. *Alternaria macrospora* alone did not induce any visible lesions. However, regular size lesions (~3 mm in diameter) were induced when the level of *A. alternata* in the inoculum mixture was 100–500 spores/mL. The lesion size increased significantly when *A. alternata* concentrations were higher than 1000 spores/mL (Fig. 2).

Leaf shedding caused by dual inoculation

Dual inoculation with 1000 and 2000 spores/mL of *A. macrospora* and *A. alternata*, respectively, caused leaf shedding in cotton plants similar to 12 000 spores/mL of *A. macrospora* alone (Table 1). When inoculated separately, both pathogens produced negligible shedding equal to noninoculated plants. The addition of *A. alternata* to the optimal concentration of *A. macrospora* did not further increase shedding (Table 1).

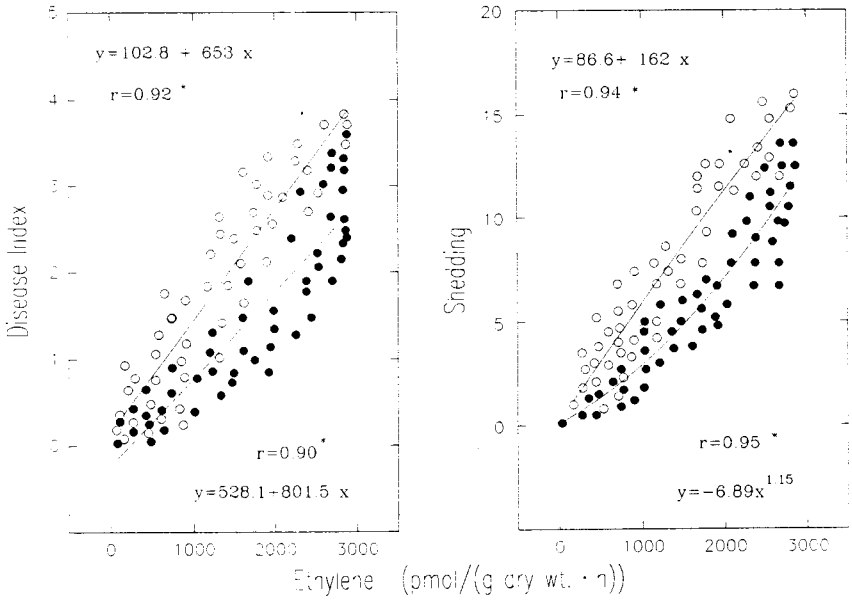

FIG. 4. Regression analyses of ethylene production in relation to disease severity and leaf shedding caused by *A. macrospora* inoculation and by dual inoculation with *A. macrospora* and *A. alternata*. Regression coefficient (r) followed by an asterisk is significant at $P \leq 0.01$. ●, *A. macrospora* inoculation; ○, dual inoculation.

Growth and ethylene production in the culture of the two pathogens

When grown together on solid agar, no visible inhibition or growth enhancement of either pathogen was observed when compared with their separate growth (data not shown). After several days of mutual growth on solid medium in Petri dishes the mycelia of both pathogens intermixed. Light microscopic preparations taken from the cultivation on the agar surface after induction of spore production by light exposure (1, 3) showed that both types of spores were present in the same preparations (data not shown). Ethylene production in cultures of each fungus growing alone or in a mixture, with or without methionine, was undetectable above the level of the background noise of the gas chromatograph.

Ethylene production in the diseased plant

Inoculation with *A. macrospora* alone at 12 000 spores/mL produced high amounts of ethylene, while inoculation with 2000 spores/mL of *A. alternata* alone produced almost nondetectable amounts. Dual inoculation with 1000 and 2000 spores/mL of *A. macrospora* and *A. alternata*, respectively, produced the highest amounts of ethylene (Fig. 3A). The amount of fungal material in cotton leaves was higher with the dual inoculation than with the *A. macrospora* alone. Inoculation with *A. alternata* alone produced the smallest amount of fungal material in plant tissue (Fig. 3B).

Ethylene production in relation to disease severity and leaf shedding

Regression analyses of ethylene produced by the dual inoculation correlated linearly and significantly with different levels of disease severity and shedding of leaves (Fig. 4). The disease severity produced by the *A. macrospora* alone was also linearly correlated with ethylene production and exponentially correlated with leaf shedding in infected cotton leaves.

Ethylene production and leaf shedding in relation to the inoculation and sampling sites

Plants infected by both pathogens produced the highest amount of ethylene. Leaf discs either from leaves exhibiting symptoms or from symptomless inoculated leaves produced similarly high amounts of ethylene, whereas plants inoculated with the *A. alternata* alone produced small amounts of ethylene, similar to noninoculated plants (Table 2). Leaf shedding was not directly related to the site of inoculation. Inoculation with *A. macrospora* or with both pathogens at any site of the plant foliage resulted in shedding of the nearest leaf (Table 2). Plants exposed to ethylene atmosphere also shed their leaves.

TABLE 2. Ethylene production and leaf shedding in relation to inoculation and sampling sites

Site	Ethylene (pmol/(g dry wt. · h))	No. of leaves shed
Entire diseased leaf		
Am + Aa	3834*a*	12.6*a*
Am	3001*ab*	11.4*a*
Aa	297*d*	0.4*b*
Leaf discs with symptoms		
Am + Aa	2948*b*	—
Am	2228*b*	—
Aa	217*d*	—
Leaf discs without symptoms		
Am + Aa	2745*b*	—
Am	1649*bc*	—
Aa	188*d*	—
Petiole (Am + Aa)	945*c*	13.8*a*
Connection between petiole and stem (Am + Aa)	0	11.7*a*
Noninoculated leaf	108*d*	0.3*b*
Noninoculated petiole	0	0.2*b*
Plants in ethylene atmosphere	—	10.4*a*
Plants in cells with normal atmosphere	—	0.6*b*

NOTE: Numbers followed by different letters in each column differ significantly at $P \leq 0.05$ in ANOVA. Am, *A. macrospora*; Aa, *A. alternata*.

TABLE 3. The effect of preapplication of auxin, ethylene inhibitors, and an ethylene precursor to cotton plants on ethylene production, disease severity, and shedding in cotton leaves inoculated by dual inoculation

Treatment	Ethylene (pmol/(g dry wt. · h))	Disease severity (1–5)	No. of leaves shed
Inoculated untreated plant	4223*a*	3.8*a*	12.4*a*
Healthy plants	386*c*	0	0.6*c*
IAA + inoculation	2641*b*	1.6*b*	8.1*b*
IAA + healthy plants	148*c*	0	0.2*c*
AOA + inoculation	2246*b*	1.9*b*	6.9*b*
AOA + healthy plants	86*d*	0	0.8*c*
AVG + inoculation	1896*b*	1.7*b*	8.3*b*
AVG + healthy plants	181*c*	0	0.4*c*
ST + inoculation	2003*b*	1.6*b*	7.8*b*
ST + healthy plants	214*c*	0	0.6*c*
ACC + inoculation	3848*a*	3.6*a*	12.7*a*
ACC + healthy plants	678*c*	0	0.8*c*

NOTE: Numbers followed by a different letter in each column differ significantly at $P \leq 0.05$ in ANOVA. IAA, indole-3-acetic acid; ST, silver thiosulfate; AOA, aminooxy acetic acid; AVG, aminoethoxyvinylglycine; ACC, 1-amino-cyclopropane-1-carboxylic acid.

Effect of preapplication of auxin, ethylene inhibitors, and an ethylene precursor to cotton plants on ethylene production, disease severity, and leaf shedding after dual inoculation

Pretreating inoculated plants with IAA or with the ethylene inhibitors AOA, ST, and AVG reduced ethylene production by the diseased plants (Table 3). Concomitantly, disease severity and leaf shedding were also reduced. IAA and the ethylene inhibitors AVG, ST, and AOA had only a marginal effect on ethylene reduction in healthy plants (Table 3). The ethylene precursor ACC had no significant effect on ethylene production, disease severity, and leaf shedding (Table 3).

Discussion

Alternaria disease often refers to (*i*) more than one specific disease, (*ii*) a disease that can be caused by more than one *Alternaria* species, or (*iii*) a disease complex caused by two different *Alternaria* species. Such is the case of alternaria dark leaf spot of the Brassicas that can be caused by either *A. brassicicola* or *A. brassicae* (23). Similarly, alternaria seed blight of linseed can be caused by *A. alternata*, *A. infectoria*, or *A. linicola* (17), leaf blight of carrot by *A. radicina* or *A. dauci* (33), alternaria early blight of potato by *A. solani* or *A. alternata* (24), and leaf blight of cotton by *A. macrospora* and (or) *A. alternata* (8). The interactions between the different symptom-producing *Alternaria* species are largely unknown. The universal fungus *A. alternata* is generally thought to be associated with numerous plant pathogens but as a secondary opportunistic parasite or a saprophyte of necrotic tissues (25).

Both *A. macrospora* and *A. alternata* are common pathogenic flora in cultivated cotton. *Alternaria alternata* is constantly airborne in a cotton growing region (7) and persists in wild beet plants in the absence of cotton (9). *Alternaria macrospora* is seed-borne (1), and possibly airborne dispersed by many biotic and abiotic vectors (3), and can overwinter in plant debris (1, 5). Both species can cause visible disease symptoms in cotton plants (5). Infections by *Alternaria* species start as early as the cotyledon stage and occur twice a season (2, 6, 28). Each *Alternaria* species is reported to infect a different species of cotton (5). However, recent evidence shows that both pathogens can exist in a single plant, and it is unclear which is the primary pathogen, or whether there is a primary pathogen (8). This study demonstrates that the two pathogens are interacting and together create what is commonly known as alternaria leaf blight of cotton.

Relatively low inoculation levels of the combined species induced disease severity, lesion size, and leaf shedding similar to high levels of *A. macrospora*. Either *Alternaria* species alone at the low levels, which exist in the air spora of cotton fields in Israel (7), may create symptomless plants (4). This study together with the previous one (8) show synergism between pathogens in leaf blight of cotton.

Since shedding of infected leaves is a major symptom of alternaria leaf blight (29), ethylene might be involved in disease development following dual inoculation. Production of ethylene levels sufficient to cause abscission of flower buds of cotton plants results from infestation with cotton fleahopper (12, 19, 26, 27), and ethylene also has been implicated as a cause of defoliation in verticillium wilt of cotton (34). Alternaria leaf blight of cotton also is an ethylene-associated disease. Substances that inhibit ethylene biosynthesis also decrease disease development, whether expressed in spot symptoms or in leaf shedding. The *Alternaria* species did not produce ethylene in culture; however, very few pathogens that induce ethylene synthesis in diseased plant tissue also produced ethylene in culture (35). The pattern of ethylene production during alternaria leaf blight development in cotton is similar to other fungal diseases in which symptoms are manifested via ethylene production and leaf abscission (10, 14, 18, 20).

In conclusion, I propose that *A. alternata* triggers symptom expression by *A. macrospora* in leaf blight disease of Pima cotton, which is manifested through the production of ethylene and leading to leaf shedding.

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