

Association between *Alternaria macrospora* and *Alternaria alternata*, causal agents of cotton leaf blight

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The association between *Alternaria macrospora* and *Alternaria alternata*, responsible for the development of alternaria blight disease in cotton, was evaluated in artificially inoculated greenhouse plants and in naturally infested field plants. When greenhouse plants were inoculated with suboptimal doses of both pathogens ($< 1.2 \times 10^4$ spores/mL) infection was greater than when separately inoculated by each pathogen at optimal dosage. In field-grown, naturally infected plants (*Gossypium barbadense*), both pathogens were found together in more than 40% of the plants. A second field-grown cotton species (*Gossypium hirsutum*) exhibited infection mainly by either *A. alternata* or both pathogens together. When both cotton species were naturally infected by both pathogens together, the number of *A. alternata* spores (either airborne or on the leaf surface) was greater than that of *A. macrospora*. We propose that *A. macrospora* together with *A. alternata* create a disease composite responsible for alternaria blight symptoms in cotton.

Key words: *Alternaria*, cotton diseases, *Gossypium barbadense*, *Gossypium hirsutum*.

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Sur des plantes artificiellement inoculées en serre et Sur des plantes naturellement infectées au champ, les auteurs ont évalué l'association de l'*Alternaria macrospora* et de l'*Alternaria alternata* responsables pour le développement de la brûlure alternarienne chez le coton. Lorsque les plantes en sen a sont inoculées avec des doses suboptimales des deux pathogènes ($< 1,2 \times 10^4$ spores/mL), l'infection est plus forte que lorsqu'on les inocule séparément avec chacun des pathogènes à son taux optimum. Chez les plantes cultivées au champ et naturellement infectées (*Gossypium barbadense*), les deux pathogènes se retrouvent ensemble chez plus de 40% des plants. Une deuxième espèce de coton cultivée au champs (*Gossypium hirsutum*) montre de l'infection surtout par l'*A. alternata* où encore par les deux pathogènes à la fois. Lorsque les deux espèces de coton sont naturellement infectées par les deux pathogènes à la fois, le nombre de spores de l'*A. alternata* (provenant par la voie des airs où Sur la surface foliaire) est plus élevé que celui de l'*A. macrospora*. Les auteurs proposent que l'*A. macrospora*, concurrentement avec l'*A. alternata*, provoque une maladie composite responsable des symptômes de la brûlure alternarienne chez le coton.

Mots clés : *Alternaria*, maladies du coton, *Gossypium barbadense*, *Gossypium hirsutum*.

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Introduction

Leaf blight is an important disease of cotton plants in Israel. Two pathogens are probably responsible for symptom production. *Alternaria macrospora* is considered the main causal agent for *Gossypium barbadense* cv. Pima plants (6, 15) but not for *Gossypium hirsutum* cv. Acala plants (14, 24). *Alternaria alternata* is capable of producing blight symptoms in both cotton species but is found mainly on *G. hirsutum* plants (9, 20, 27).

The epidemiological factors governing this disease are largely unknown. One pathogen, *A. macrospora*, is seedborne and can be transferred within the field by various biotic and abiotic factors (3, 5, 7). Cotton cotyledons appear to be the most susceptible host organ to this pathogen (4). The other pathogen, *A. alternata*, overwinters in wild beet plants (9) and can be transferred by the wind for short distances within the

field (10). Observations carried out in fields of both cotton species revealed a regular occurrence of heavy blight infections. However, the pathogen(s) directly responsible for this phenomenon remains to be determined (Y. Bashan, unpublished data).

The objectives of this study were (i) to define which of the fungal species is present in *Alternaria* blight lesions in two cotton species and (ii) to suggest a possible disease association between *A. macrospora* and *A. alternata* in blight-infected cotton leaves.

Materials and methods

Organisms

Alternaria macrospora Zimm. (ATCC 62363) and *A. alternata* (Fr.) Keissler (S-1) (9) were used as inoculum. Cotton plants (*G. barbadense* cv. Pima S5 and *G. hirsutum* cv. Acala SJ-2) were used as host plants.

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Plant growth conditions

Cotton plants were grown by one of the following methods. (i) In 50 and 85 ha commercial fields in the Bet-Shean valley, northeastern Israel (for climatic and geographical details regarding this region see ref. 10), 610 plants per metre per row were grown following standard procedures used in cotton production in Israel. Cotton plants of the two species were grown in separate fields. (ii) Cotton plants designated for artificial inoculation were grown from seeds in 5L pots containing peat - coarse vermiculite - volcanic dust (1:1:1, v/v/v), four plants per pot (two *G. barbadense* and two *G. hirsutum* plants) in a plastic-covered greenhouse lacking environmental control. Plants were fertilized (100 mUpot) with half-strength Hoagland's nutrient solution (18) every week after germination.

Pathogen growth conditions, plant inoculation, and pathogenicity tests

Pathogens were cultivated and inoculum was prepared as previously described (3). Cotton plants with three to five true leaves were inoculated with either *A. macrospora* or *A. alternata* at a rate of 1.2×10^4 conidia/mL, suspended in sterile deionized water. This concentration is the optimal spore concentration for initiating disease (3). Inoculation was accomplished by brushing the leaves with a small brush containing spore suspension at a rate of 1 mL/leaf (9). In dual inoculation experiments, the inoculum concentration of each pathogen was adjusted to different levels in sterile deionized water, combined, and inoculated onto the plants. Each pot was covered with a separate, loosely sealed, large, prewetted polyethylene bag and incubated in a controlled growth chamber in the dark for 16 h at $25 \pm 1^\circ\text{C}$. Later, the plants were returned to the greenhouse for an additional incubation of 5 days. The plastic bags were removed daily for a few minutes to improve aeration. No cross contamination of inoculated plants by the other fungal species was detected when plants were inoculated with only one pathogen. Control plants were identically treated with dead γ -irradiated spores (25 kGy) or with sterile water. Pathogenicity tests were performed as previously described (3). Disease incidence was calculated as the percentage of plants exhibiting symptoms (at least three leaves of each plant had more than one symptom per leaf).

Field sampling

During the sampling periods of 1986, most plants in each field exhibited *Alternaria* blight symptoms (10). Plants for detailed analyses were taken from the center of each field. Plants were randomly chosen by throwing a 1-m stick several times along a row to produce a total of 438 plants for study. Several randomly chosen leaves (three to five per plant) were sampled from the central part (20 cm below the upper canopy) of each plant, placed in small polyethylene sealed bags, and immediately transferred to the laboratory. The leaves were sorted into two categories, i.e., leaves with visible symptoms (>1 lesion per leaf) and leaves without symptoms. Only 2 out of the 438 plants sampled exhibited both infected and noninfected leaves. After examination, the leaves were lyophilized as previously described (8).

Detection of *A. macrospora* and *A. alternata* in dried leaves

Both pathogens were recovered from the dry leaves as follows: leaf samples (approximately 0.5 g each) were milled by a General Electric mill model 5XBGOOB (1725 rpm, 0.25 hp, 60 mesh) into a very fine powder under aseptic conditions. Portions (0.1 g) were suspended in 50 mL of liquid Czapek medium, supplemented with 250 mg/L chloramphenicol (CC) (12), in 125-mL Erlenmeyer flasks. The flasks were vigorously shaken for 4 h (250 strokes/min) at $28 \pm 2^\circ\text{C}$. The slurry was decimally diluted in the same medium, and 0.1 mL of aliquot was spread with a glass rod on the surface of solid CC medium supplemented with 1 mg/L of the surfactant Triton N-101 (alkylphenylpolyethyleneglycol) (CCT, 21) to prevent overgrowth of fungal colonies on the medium (Sigma Chemical Co., St. Louis, Mo.). None of the irradiated spores, used as controls, developed fungal colonies on the growth medium. Spore formation in the developing colonies was induced as previously described (3). The presence of each path-

ogen in each sample was determined according to spore morphology as described by Joly (19) and Simmons (25). Percentage of plants infected either by single pathogen or by both pathogens was calculated from this analysis.

Counting of spores on the leaf surface

The inoculated, diseased leaves (2 to 4 g/sample, the third, fourth, and fifth leaves from plant top) obtained from both cotton species were cut into small pieces (0.5 cm² each), submerged in 50 mL 0.06 M potassium phosphate buffer, pH 7.0, supplemented with 0.15 M NaCl (PBS) in beakers, and subjected to a mild sonication (3 min at 10 W, 4°C; Sonifier B-12, Branson Sonic Power Co., CT). This sonication removed surface spores and had no effect on the viability of fungal spores of the two species when tested by treating spores from pure cultures. The leaf pieces were then filtered through a gauze. The supernatant containing spores of a pathogen was decimally diluted and plated on CCT medium. The resulting fungal species were determined as described above. All leaf pieces (after sonication and filtration) were examined under a stereoscopic microscope to confirm removal of nearly all surface spores. This method was found superior to macerating leaves by high-speed homogenizers that generally yielded a higher propagule number resulting from the development of colonies of mycelial fragments (7, 9).

Trapping of *A. macrospora* and *A. alternata* airborne spores in the field

Two gravity spore traps were operated in the "Eden" Regional Experimental Station in the Bet-Shean valley during the annual *Alternaria* blight epidemic period of August to September 1986. Traps were located downwind, one in a *G. barbadense* plot and the other adjacent to a *G. hirsutum* plot. Although not artificially inoculated, cotton plants that were grown in these plots exhibited *Alternaria* blight symptoms. Trap assemblage, sampling, and counting of airborne spores were described in detail elsewhere (10).

Experimental design and statistical analysis

Experiments were randomly designed in five replicates where two pots served as a replicate. Each experiment was repeated twice. Since usually both experiments share a similar trend, data from both experiments were combined and analyzed using Fisher's least significant difference (LSD) analysis or Student's *t*-test analysis at $P \leq 0.05$. Field samples were randomly taken as described above from regular commercial fields in five replicates. Data from all field samples, including data of airborne spores, were combined and subjected to LSD analysis.

Results

Occurrence of *A. macrospora* and *A. alternata* in visibly infected and in symptomless cotton plants in the field

An analysis of the presence of *A. macrospora* and *A. alternata* in leaves of two field-grown cotton species was performed. In *G. barbadense* plants showing symptoms, occurrence of each pathogen was similar ($28 \pm 3\%$ of all plants were infected by either of the pathogens). However, a significantly higher number of plants were infected by both pathogens (Fig. 1 A). In *G. hirsutum* plants exhibiting symptoms, most plants contained many *A. alternata* propagules but only a few *A. macrospora*. However, more than 40% of all plants contained both pathogens (Fig. 1 A).

In symptomless plants, similar trends were detected in both cotton species but at a lower magnitude (Fig. 1B). A significant number of symptomless plants were free of propagules of any pathogen. Thus, these plants were considered healthy (Fig. 1B).

Spore formation on the leaf surface of two cotton species after dual inoculation with *A. macrospora* and *A. alternata*

After dual inoculation (*A. macrospora*-*A. alternata*, $6 \times 10^3 : 6 \times 10^3$ spores/mL) spore counts on the cotton leaf sur-

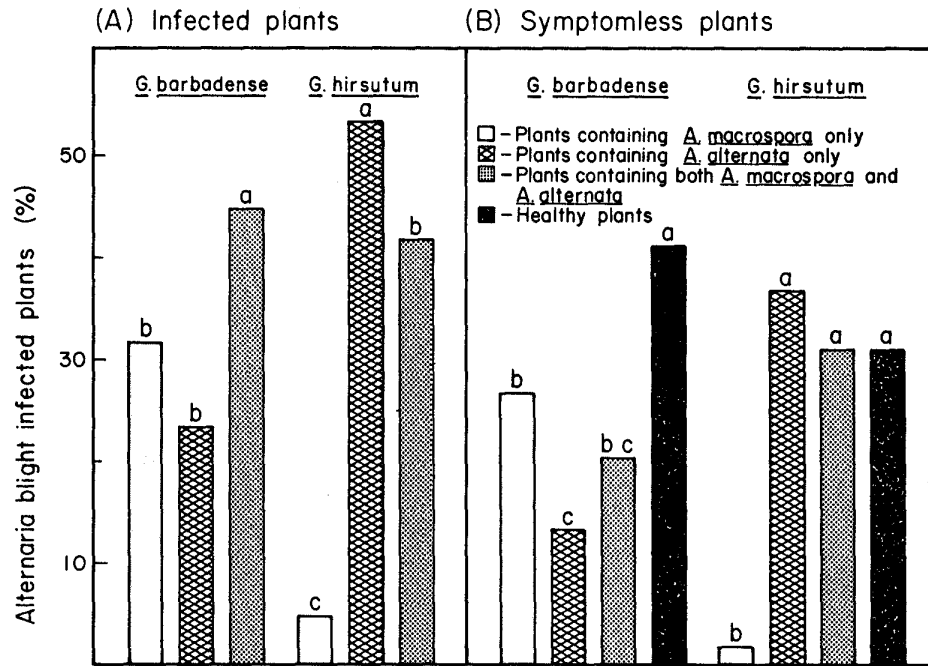


FIG. 1. Occurrence of *A. macrospora* and *A. alternata* in (A) visibly infected and (B) symptomless cotton plants in the field. For each cotton species within each subfigure, columns followed by different letters differ significantly at $P \leq 0.05$.

face revealed significantly more *A. alternata* spores on both cotton species (Fig. 2A). Spore production ratio on leaves between *A. macrospora* and *A. alternata* was 1:1.85 in *G. barbadense* plants and 1:4.3 in *G. hirsutum* plants.

Inoculation of the two species of cotton plants by a single pathogen showed that in *G. barbadense* plants, both pathogens produced a similar number of spores on the leaf surface. In *G. hirsutum* plants, *A. macrospora* produced a small number of spores. However, in this cotton species, *A. alternata* produced a higher amount of spores than its spore production in dual inoculation (Fig. 2B). Consequently, the spore production ratio (*A. macrospora*:*A. alternata*) increased from 1:1.1 in *G. barbadense* plants to 1:25.2 in *G. hirsutum* plants. Analysis of the host effect on spore production revealed a significant host effect in the two types of inoculations (Fig. 2).

Disease incidence in two cotton species after dual inoculation with *A. macrospora* and *A. alternata*

In *G. barbadense* plants, *A. macrospora* initiated symptoms in 61% of the inoculated plants and in only 3% of *G. hirsutum* plants (Fig. 3). The remaining inoculated plants of both species remained symptomless. Inoculation with *A. alternata* yielded visible symptoms in 85% of *G. barbadense* plants and in 56% of *G. hirsutum* plants. Dual inoculations with suboptimal concentrations of each pathogen increased disease incidence over inoculations with a sole pathogen (over 90% of all inoculated plants were visibly infected). In *G. hirsutum* plants the inoculum combination ratios (*A. macrospora*:*A. alternata*) responsible for causing the increase in disease incidence was 6×10^3 : 6×10^3 and 4×10^3 : 8×10^3 spores/mL. In *G. barbadense* plants the inoculum combination ratios were 8×10^3 : 4×10^3 , 6×10^3 : 6×10^3 , and 4×10^3 : 8×10^3 spores/mL (Fig. 3).

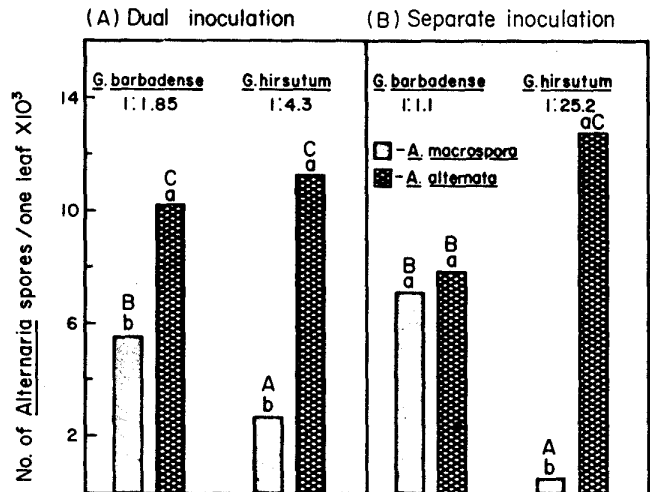


FIG. 2. Spore formation on leaf surfaces of two cotton species (A) after dual inoculation with *A. macrospora* and *A. alternata* and (B) after inoculation by each pathogen separately. Numbers represent the ratio between the numbers of *A. macrospora* and *A. alternata* spores produced on the leaf surface. For each cotton species within each subfigure, columns followed by a different lower case letter differ significantly at $P \leq 0.05$. Within each subfigure, columns followed by a different upper case letter differ significantly at $P \leq 0.05$.

Spore formation on the leaf surface of two cotton species exhibiting alternaria blight symptoms in the field

Alternaria macrospora and *A. alternata* spores were detected and enumerated in 47 leaf samples of field-grown plants exhibiting alternaria blight symptoms. In general, the number of *A. alternata* spores produced in all samples was

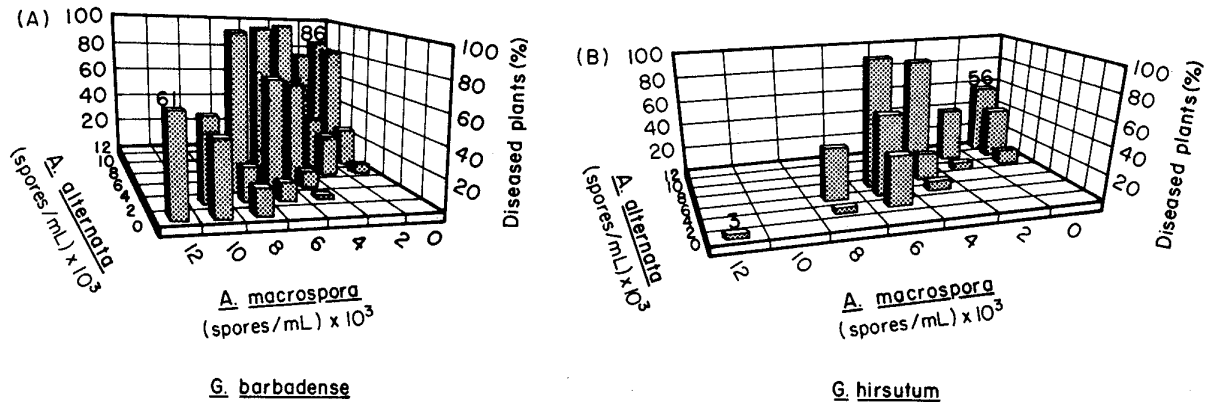


FIG. 3. Percentage of plants exhibiting symptoms on leaves of (A) *G. barbadense* and (B) *G. hirsutum* after dual inoculation with various concentrations of *A. macrospora* and *A. alternata*.

higher compared with the number of *A. macrospora* in both cotton species. On *G. barbadense* plants, *A. macrospora* produced an average of 4.4×10^3 spores per one leaf, while *A. alternata* produced statistically significantly higher number of spores (9×10^3 spores per one leaf). On *G. hirsutum* plants, *A. macrospora* produced an average of 2×10^3 spores per one leaf. *Alternaria alternata* also produced significantly more spores also on *G. hirsutum* plants (9.16×10^3 spores per one leaf). Spore production ratio (*A. macrospora*-*A. alternata*) varied from 1:1.14 to 1:21.4. The average spore production ratio between the pathogens in each cotton species revealed a 1:2 ratio in *G. barbadense* plants. The ratio increased to 1:4.6 in *G. hirsutum* plants.

Trapping of *A. macrospora* and *A. alternata* airborne spores in the field

Field sampling performed on four occasions during an *Alternaria* blight epidemic revealed that a larger amount of *A. alternata* spores compared with *A. macrospora* spores were trapped in fields of both cotton species. In a field of *G. barbadense*, the number of trapped *A. macrospora* airborne spores was an average of 56 spores per 300 cm² surface per 2 h, while the number of airborne spores of *A. alternata* was significantly higher (96 spores per 300 cm² surface per 2 h). In a field of *G. hirsutum*, the number of airborne spores of *A. macrospora* was 48 spores per 300 cm² surface per 2 h). Similar to the previous field, the number of airborne *A. alternata* was significantly higher (103 spores per 300 cm² surface per 2 h).

Discussion

This study presents evidence that the common, economically important *Alternaria* blight of cotton is caused by the association of at least two *Alternaria* species, *A. macrospora* and *A. alternata*. Dual colonization could be detected at the leaf level where the two pathogens were sharing the same cotton leaf. The relative humidity in the artificial inoculation experiments resembled in exposure time the natural dew hours in the Bet-Shean valley. On many nights, the cotton plants in the field are subjected to dew from a few hours after sunset until after sunrise (10).

Alternaria macrospora is solely a cotton pathogen. On the other hand, the pathogenicity of *A. alternata* strains varied. Strains belonging to the *A. alternata* complex of species (25) are pathogenic to numerous plant species (1, 13, 23) including cotton (2, 9, 27). However, this complex of species is better known as a common saprophyte (19). *Alternaria* propagules are abundantly detected everywhere. *Alternaria* sp. is being used as a common model organism for human allergy studies (17). Our study does not reveal which is the primary causal agent, or whether there is a primary and secondary disease agent. The higher amount of *A. alternata* spores found on infected leaves does not necessarily indicate that *A. alternata* is the main agent and that *A. macrospora* is the secondary one. Rather it may reflect a higher sporulation capability of *A. alternata*, without a direct relation to pathogenicity.

Another cotton leaf disease composed of two disease agents was previously reported for *Alternaria tenuis* and *Cercospora gossypina*. A single report by Calvert *et al.* (11) stated that this disease composite caused a severe and premature defoliation. Conversely, Miller (22) and Sinclair and Shatla (26) claimed that this is a minor disease and that the disease-causing organisms were non-aggressive pathogens that invaded only weakened leaves. This disease composite, exclusively detected in the United States, is not known in any other cotton-growing area of the world (6), and research in this direction has not been pursued any further.

Airborne spores of pathogenic fungi are an accurate indicator of their presence in the field (10,16). The present study shows that both pathogens produce viable airborne spores during the cotton crop season, although the number of *A. alternata* airborne spores is significantly greater than those of *A. macrospora*. Yet, as indicated earlier, this is not directly related to pathogenicity. It rather reflects higher numbers of *A. alternata* spores being produced on cotton leaves, an improved release mechanism of the spores to the air, a better capacity for dissemination because of smaller spores, or spore production by saprophytic *A. alternata* on debris under the crop. Nevertheless, our data indicate that since the spores of both pathogens are abundant in the air or upon cotton, the probability that a single plant can be infected by both pathogens is more common than rare.

In conclusion, this study shows that both *A. macrospora* and *A. alternata* are capable of simultaneously infecting cotton

leaves and are often associated with *Alternaria* blight symptoms on the same plant or even on the same leaf. Thus, we propose that *Alternaria* blight disease of cotton can be defined as a disease composite of two pathogens. The contribution of each disease agent to the overall disease requires further study.

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