Increased aggressiveness of *Alternaria macrospora*, a causal agent of leaf blight in cotton monoculture

GINA HOLGUIN AND YOAV BASHAN[1]

Department of Microbiology, Division of Experimental Biology, The Center of Biological Research, La Paz, P.O. Box 128, B.C.S. 23000, Mexico

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Consecutive, single-crop cultivation continues to be practiced worldwide as a matter of necessity. The monoculture of cotton now practiced in Israel results in annual outbreaks of leaf blight epidemics. Pathogenic isolates of the causal agent, *Alternaria macrospora*, were field collected for 6 years and inoculated onto cotton plants under controlled environmental conditions. We were able to demonstrate that the aggressiveness (virulence and fitness) of the pathogenic isolates increased over the years. The modified aggressiveness was manifested as follows: (*i*) an increase in the number of lesions per leaf; (*ii*) an increase in the size of the lesions; (*iii*) a decrease in the time required for symptom expression; (*iv*) an increase in the viability of the pathogen spores after exposure to a normally lethal temperature; and (*v*) a decrease in the wetting period required for symptom expression. We conclude that agricultural monoculture can accelerate evolution in the aggressiveness of this plant leaf pathogen.

Key words: leaf blight of cotton, *Alternaria*, aggressiveness, virulence, fitness.

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La pratique de la monoculture répétée continue de prévaloir partout au monde et apparait comme une nécessité. La monoculture du cotton telle que pratiquée en Israël, entraîne annuellement des épidémies de brûlure foliaire. À partir de ces cultures et au cours d'une période de 6 ans, les auteurs ont isolé des souches de l'agent responsable, l'*Alternaria macrospora*, et les ont inoculées sur des plants de cotton en conditions environnementales contrôlées. Ils ont pu démontrer que l'aggressivité (virulence et ajustement) des isolats pathogènes, augmente avec les années. La modification de l'aggressivité se manifeste par : (*i*) une augmentation du nombre de lésions par feuille; (*ii*) une augmentation de la dimension des lésions; (*iii*) une diminution du temps nécessaire pour l'expression des symptômes; (*iv*) une augmentation de la viabilité des spores du pathogène après exposition à une température normalement léthale; et (*v*) une diminution de la période humide pour l'expression des symptôme. Les auteurs concluent que la pratique agricole en monoculture répétée peut accélérer l'évolution de l'aggressivité de ce phytopathogène foliaire.

Mots clés : brûlure foliaire du cotton, *Alternaria*, aggressivité, virulence, ajustement.

[Traduit par la rédaction]

Introduction

Leaf blight is a destructive leaf disease of cotton plants. Management of this disease is a worldwide concern, except in the United States where it causes minimal damage (8, 17).

At present, *Alternaria macrospora* is considered the main causal agent of leaf blight in high quality Pima cotton (*Gossypium barbadense* L.) (8, 21) but not in Acala cotton (*Gossypium hirsutum* L.) (30). Cotyledons are the most susceptible plant organs (5). Although presumably dispersed primarily by wind, the seed-borne pathogen can be transferred locally by a variety of biotic vectors and abiotic vehicles (4, 6, 9). Recently, *Alternaria alternata* was proposed as an additional pathogenic agent of Alternaria leaf blight disease in cotton (11, 13). It has also been proposed that leaf blight of cotton is essentially a disease composite of two pathogens, *A. macrospora* and *A. alternata* (8, 12).

Variability in the aggressiveness of pathogenic agents is a fundamental concept in the plant pathology of either fungal or bacterial causal agents (1, 7). Increased aggressiveness of a fungal pathogen can be defined as the ability to induce disease symptoms that are more severe than the originally reported symptoms. These more aggressive strains replace the original strains and ultimately dominate the susceptible host. The term virulence and fitness is synonymous with aggressiveness. Aggressiveness can be determined by a number of factors such as host susceptibility (32), nutritional status of the pathogen prior to plant infection (2, 24), and environmental factors such as relative humidity (20) and temperature (18). It has been observed that aggressiveness of certain fungal species can increase when these strains are repeatedly isolated and inoculated onto new plants (19).

It has been speculated that monoculture of a susceptible crop increases disease occurrence over time (29), leading eventually to more severe crop damage. It has also been established that in soil-borne fungal pathogens, disease occurrence increases after several replantings of susceptible host plants in the same soil (26). This phenomenon is commonly associated with a buildup of pathogenic populations in the soil rather than with an increase in the aggressiveness of the pathogenic strains themselves (28).

The objective of this study was to evaluate quantitative data gathered over successive years to determine whether cotton monoculture selects for isolates of *A. macrospora* that are more aggressive to a standard cultivar and more fit to survive and rapidly establish infection under the environmental conditions of the region.

Materials and methods

Organisms, sampling, and growth conditions

Alternaria macrospora Zim (ATCC 62363; isolated by Y. Bashan in 1982 from fields in Bet-Shean Valley, northeastern Israel) served as the control strain for all the experiments. In addition, 83 local iso-

[1]Author to whom correspondence should be addressed.

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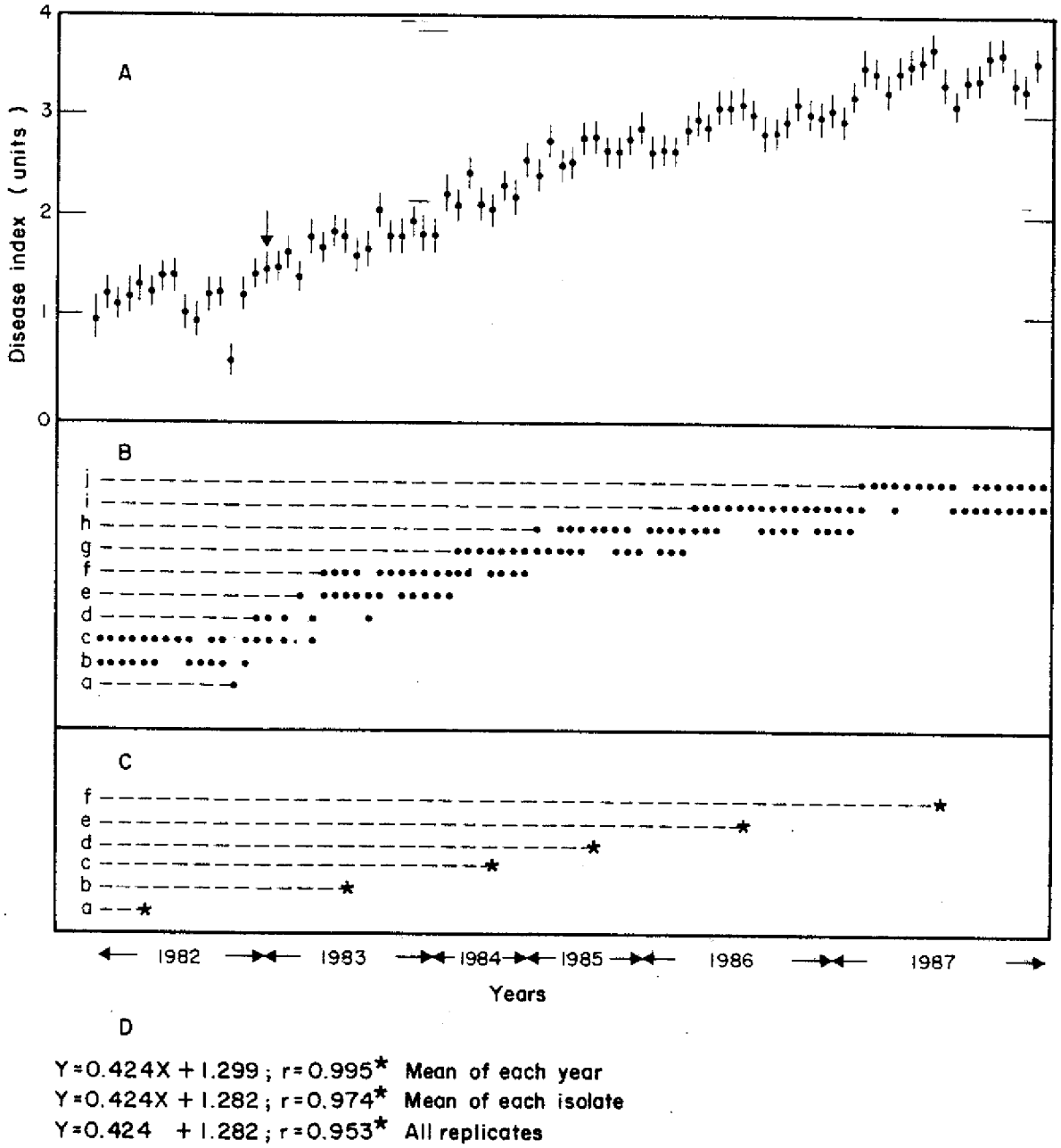

FIG. 1. Analysis of disease severity (expressed as disease index) of cotton leaf blight disease caused by 84 isolates of *A. macrospora* sampled from infected plants between 1982 and 1987 and inoculated simultaneously onto cotton plants in a controlled environment. (A) Mean disease index of each isolate. Bars represent standard error, and arrow locates the reference strain ATCC 62363. (B) One-way analysis of variance (ANOVA) of (A). Points preceded by a different letter differ significantly at $P \leq 0.05$. Broken lines were added for clarity. (C) One-way ANOVA of the mean of disease indices of all the isolates of a certain year. Stars preceded by a different letter differ significantly at $P \leq 0.05$. (D) Linear regression analysis of data representing all the replicates, the mean of each isolate, and the means of each year. Regression coefficient (r) followed by an asterisk is significant at $P \leq 0.01$.

lates of this fungus were isolated from leaf-blight infected plants during the years 1982–1987 and used as inoculum. Samples of diseased cotton leaves were collected from fields in the Bet-Shean Valley (4, 11, 12, 13), which had a summer monocultivation of cotton for over 30 years, primarily of *G. barbadense* cv. Pima S-5 during the years 1982–1987. The climatological features of this valley were described elsewhere (11).

All samples were field collected during the second annual outbreak of cotton leaf blight from July to September (11). Diseased leaves were randomly collected from the central part of the plants (20 cm below the upper canopy). The leaves were placed in small polyethylene sealed bags and immediately transferred to the laboratory. The leaves were lyophilized to dryness as this is known to preserve the virulence of pathogenic bacteria (10) as well as the viability and pathogenicity of pathogenic fungi (3, 14) for prolonged periods.

Cotton plants (*G. barbadense* cv. Pima S-5) were used as host plants. 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 (1:1:1, v/v/v) and fertilized with half-strength Hoagland's nutrient solution once a week.

Isolation, inoculation, and disease severity

The pathogen was recovered from all samples of dry leaves during 1 week (12, 16, 27), and spore formation in the developing colonies was induced (4). All isolates were cultured for approximately the

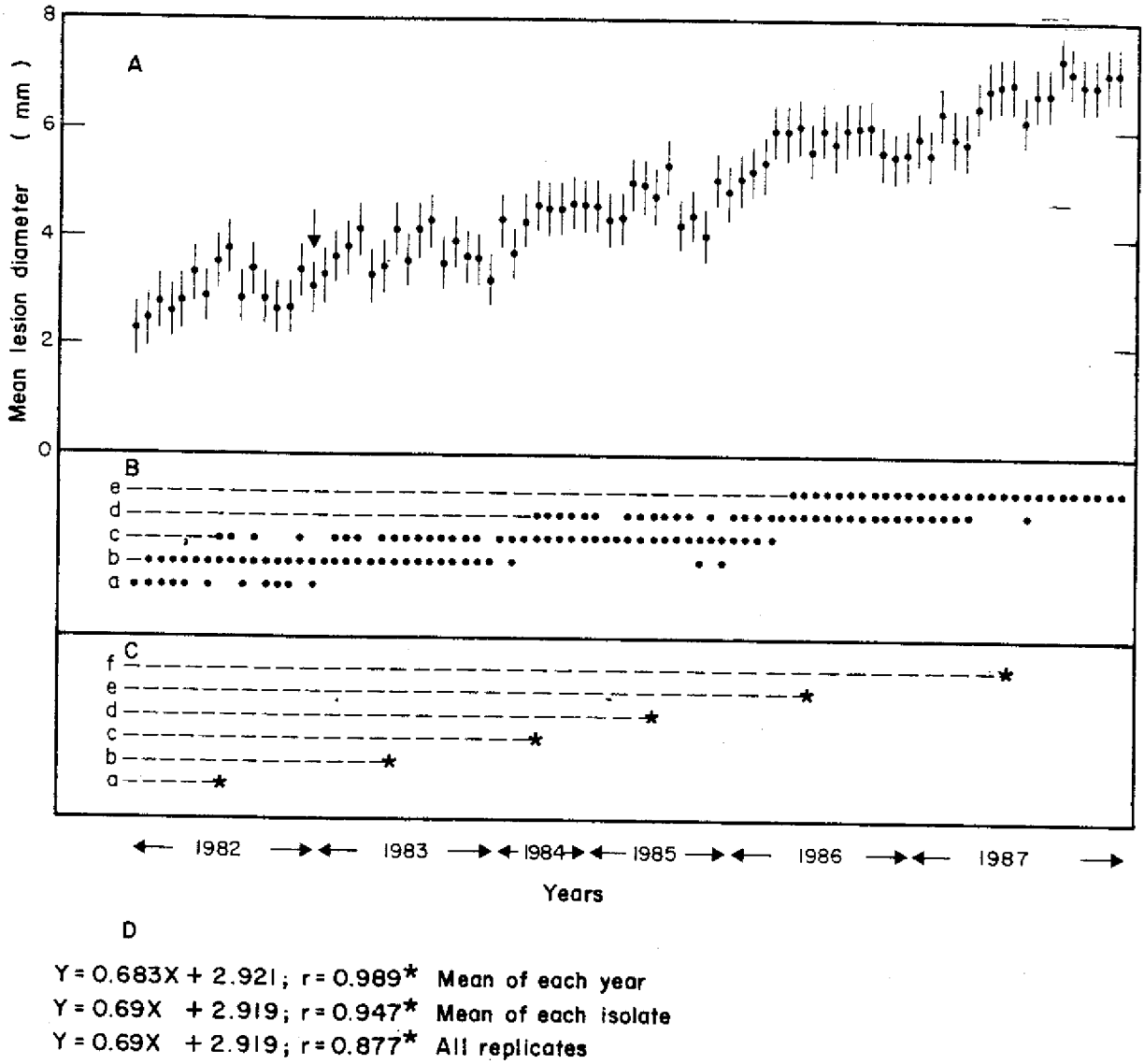

FIG. 2. Analysis of mean lesion diameter of cotton leaf blight disease caused by 84 isolates of *A. macrospora*, sampled from infected plants between 1982 and 1987 and inoculated simultaneously onto cotton plants in a controlled environment. (A) Presentation of the mean lesion diameter caused by each isolate. Bars represent standard error and arrow locates the reference strain ATCC 62363. (B) One-way analysis of variance (ANOVA) of (A). Points preceded by a different letter differ significantly at $P \leq 0.05$. Broken lines were added for clarity. (C) One-way ANOVA of the mean lesion diameter caused by all the isolates of a certain year. Stars preceded by a different letter differ significantly at $P \leq 0.05$. (D) Linear regression analysis of data representing all the replicates, the mean of each isolate, and the means of each year. Regression coefficient (r) followed by an asterisk is significant at $P \leq 0.01$.

same length of time. After recovery from the dry leaves, the pathogens were maintained on Czapek medium (16). After 1–2 months they were transferred to a fresh medium by cutting a small piece of culture from the edge of the colony using a cork borer 5 mm in diameter. None of the isolates were transferred more than three times during the experimental period. Leaves infected by an isolate later designated as ATCC 62363 were stored as described for the other isolates. Identification of colonies as *A. macrospora* was determined according to spore morphology (25, 31). The pathogen was cultivated and spores were harvested and prepared for inoculation (4). Cotton plants with three to five true leaves were inoculated with *A. macrospora* at a rate of 1.2×10^4 spores/mL deionized water until runoff (12). Control plants were treated identically with dead γ-irradiated spores (25 kGy) or with sterile water. Disease development after inoculation with each isolate was assessed 5 days after inoculation by a disease index employing a scale as follows: 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 (4, 13).

Lesion diameter

The diameter of each lesion on the second and third oldest leaf of each plant was measured 5–6 days after inoculation (17). Data from the two leaves were combined, and the mean lesion diameter of each plant was used in the statistical analysis.

Time required for appearance of symptoms

After inoculation, the plants were incubated in humid chambers (4, 12). After 16 h in the dark and a further 8 h under humid chamber conditions in an illuminated greenhouse, all the plants were transferred to normal greenhouse conditions (described above) and inspected daily for the appearance of symptoms. Infectivity was scored as positive when at least one of the five replicates of each isolate showed a disease severity of 2.0 (4).

Spore mortality

Spore mortality analysis *in vitro* was done by harvesting spores from 5-day-old cultures of *A. macrospora*. They were suspended in 0.06 M potassium phosphate buffer supplemented with 0.15 M NaCl (PBS) to a final concentration of 10^3 spores/mL (by hemacytometer)

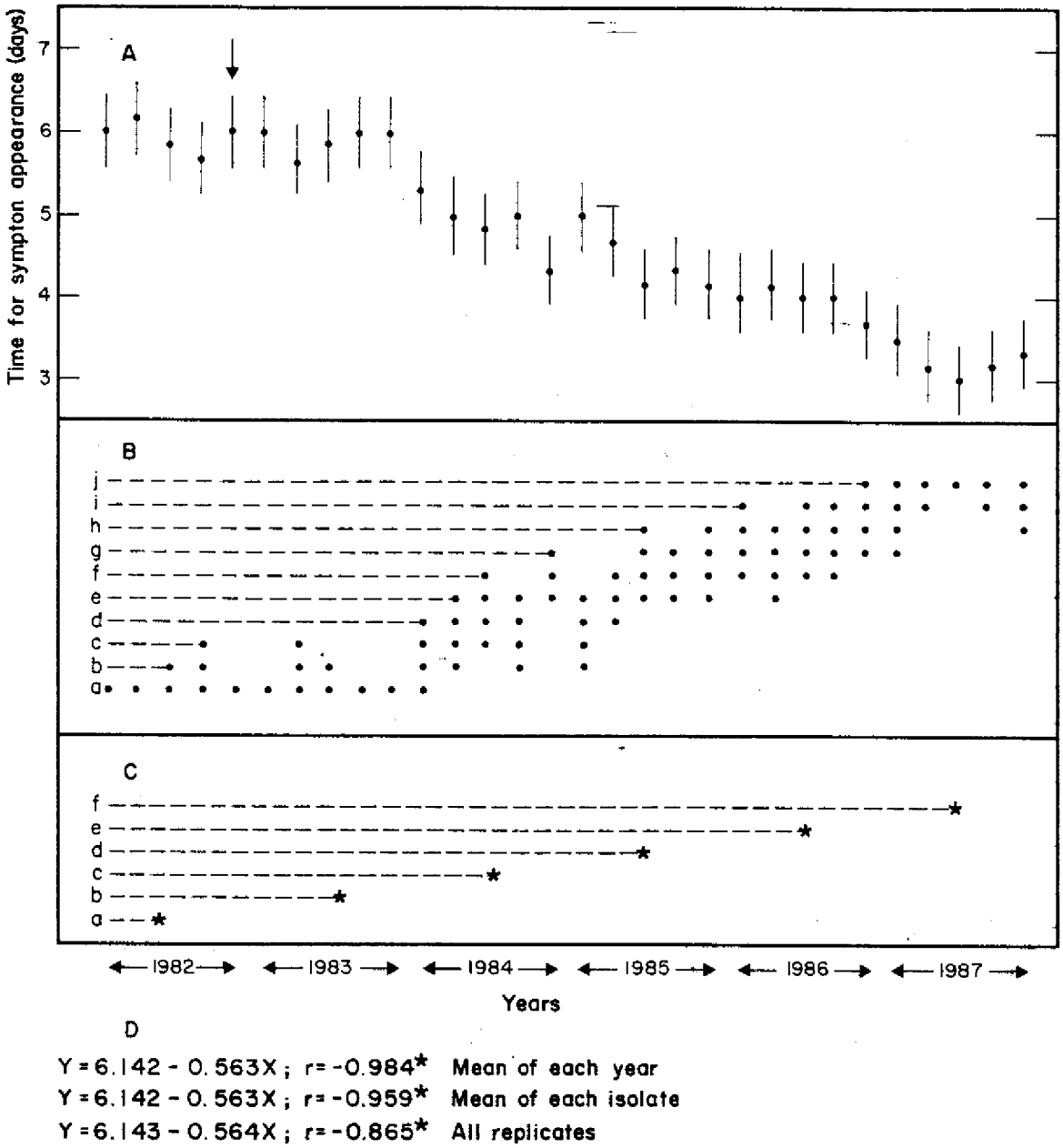

FIG. 3. Analysis of the time required for appearance of visible symptoms of cotton leaf blight disease caused by 30 isolates of *A. macrospora* sampled from infected plants between 1982 and 1987 and inoculated simultaneously onto cotton plants in a controlled environment. (A) Time required for symptom formation of each isolate. Bars represent standard error and arrow locates the reference strain ATCC 62363. (B) One-way analysis of variance (ANOVA) of (A). Points preceded by a different letter differ significantly at $P \leq 0.05$. Broken lines were added for clarity. (C) One-way ANOVA of the time required for symptom formation of all the isolates of a certain year. Stars preceded by a different letter differ significantly at $P \leq 0.05$. (D) Linear regression analysis of data representing all the replicates, the mean of each isolate, and the means of each year. Regression coefficient (r) followed by an asterisk is significant at $P \leq 0.01$.

in microtubes. The microtubes were sealed with aluminum foil and incubated at 45 ± 1°C for 6 h (18). Then the microtubes were gently stirred using a vortex stirrer, and 0.1-mL aliquots were spread with a glass rod on solid growth medium (16, 27) in 18-cm diameter Petri dishes. The dishes were incubated at 25 ± 2°C for 4 days, spore formation was induced (4), and the resulting *A. macrospora* colonies were counted. For controls, spores were incubated at 25 ± 2°C (optimal temperature for *A. macrospora* development) (17, 18). Viability of spores was calculated by dividing the number of developed colonies by the original spore concentration × 100.

Wetness periods required by A. macrospora *to induce visible symptoms of disease*

After inoculation, the plants in the humid chambers were incubated in the dark. At intervals, the humid chamber conditions were stopped

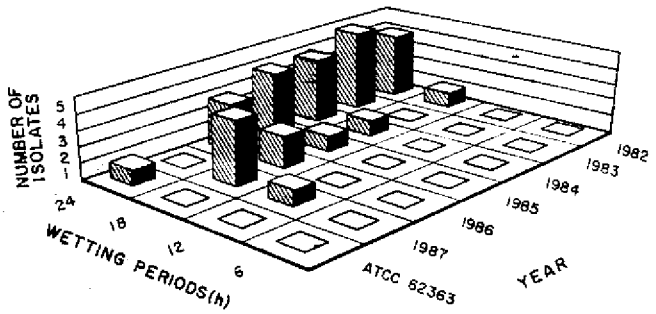

FIG. 4. Hours of wetting periods required for different isolates of *A. macrospora* to induce visible disease symptoms. ATCC 62363 is a reference strain.

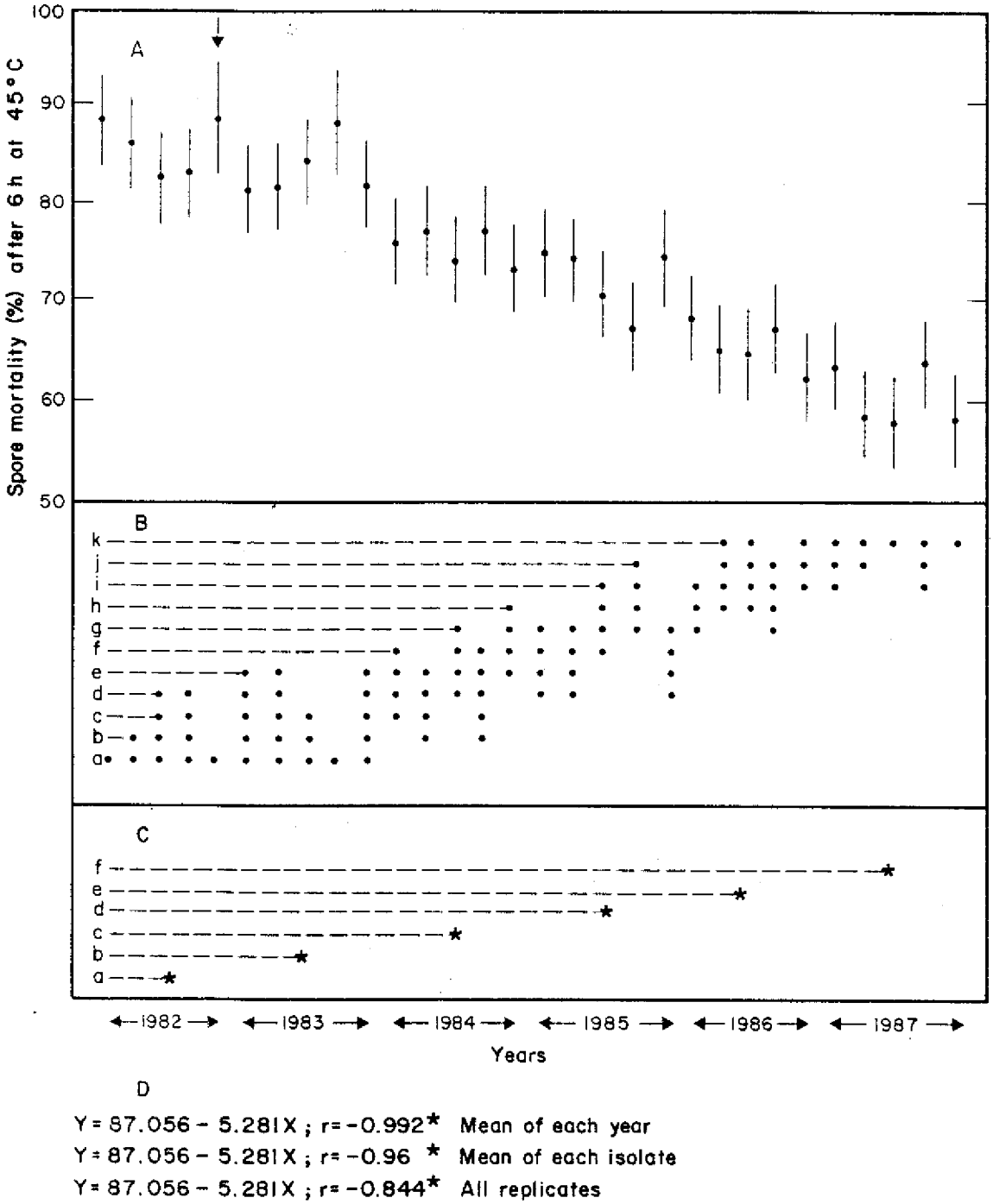

FIG. 5. Analysis of *A. macrospora* spore mortality after 6 h exposure to 45°C in 30 isolates of *A. macrospora* sampled from infected plants between 1982 and 1987. (A) Mean spore mortality of each isolate. Bars represent standard error and arrow locates the reference strain ATCC 62363. (B) One-way analysis of variance (ANOVA) of (A). Points preceded by a different letter differ significantly at $P \leq 0.05$. Broken lines were added for clarity. (C) ANOVA one-way of spore mortality of all the isolates of a certain year. Stars preceded by a different letter differ significantly at $P \leq 0.05$. (D) Linear regression analysis of data representing all the replicates, the mean of each isolate, and the means of each year. Regression coefficient (r) followed by an asterisk is significant at $P \leq 0.01$.

for a portion of the plants by drying the leaf surface with a hair drier operated at minimal heat and low air speed. Scoring of the symptoms was as described above.

Experimental design and statistical analysis

Experiments were carried out in a completely randomized design with three replicates per treatment and two pots per replicate. Each experiment was conducted twice. Data from the two experiments were combined (six replicates) and analyzed together using one-way ANOVA ($P \leq 0.05$) or linear regression analysis ($P \leq 0.01$).

Results and discussion

In Israel where the monoculture of cotton incurs large investments for pest management, we observed an increase in the disease severity (expressed as the disease index of the plants plus the size of the developing lesions) of 84 isolates of *A. macrospora* sampled between 1982 and 1987. Although disease severity varied greatly between the isolates, the trend of increased disease severity was statistically significant when the disease index was assessed (Fig. 1). A similar trend was

observed when the lesion diameter caused by these isolates was analyzed (Fig. 2). Disease severity induced by a control strain, *A. macrospora* ATCC 62363, was similar to the average of the 1982 isolates.

Daily monitoring of the first symptoms to appear after artificial inoculation with 30 aggressive isolates (5 collected from each year) revealed that less time was required for symptom appearance by isolates from 1987 than by those from 1982 (a decrease from 6 days to 3–4 days on average). The variation between the isolates was large, but the reduction in the time required for symptom formation was statistically significant (Fig. 3). The time required for symptom formation by *A. macrospora* ATCC 62363 was similar to the average of 1982 isolates (6 days). The wetting period required for symptom induction also decreased. Isolates from 1987 required 12–18 h of wetting to induce symptoms while 1982 isolates (as well as the ATCC 62363) required about 24 h (Fig. 4).

For *A. macrospora* isolates, spore viability after 6 h exposure to a lethal temperature of 45°C (18) increased over the years. Isolates from 1987 were significantly more resistant to the heat treatment than those from 1982 (Fig. 5). Spores kept at an optimal growth temperature (25 ± 2°C) maintained high viability (90–100% of all spores that produced colonies), regardless of the year of origin.

Cotton monoculture of *G. barbadense* in Bet-Shean Valley has been susceptible to *A. macrospora* infection for many years. This pathogen was first isolated in Israel in 1958 (15), though related infection was considered minor until the late 1970s when severe epidemics focused attention. The isolates evaluated in this study were obtained from consecutive seasons having severe epidemics that were inefficiently controlled by chemical means (Y. Zacks and S. Biton, unpublished data). This disease appears sporadically, i.e., some years produce heavy epidemics while other years do not, as in the case of 1991 when its appearance was negligible (R. Or, unpublished data). This variability is probably dependent on environmental conditions that have yet to be identified. This pathogen is frequently isolated from hot-temperature regions worldwide (22).

While it is well established that leaf diseases are greatly affected by environmental conditions (23), the evidence in these experiments was gathered by measuring common plant pathological parameters under identically controlled environments. Thus, the possibility of a contribution by environmental factors to the increased aggressiveness of the strains was eliminated. By isolating the infection process from the environmental factors, we demonstrated that more aggressive strains of the pathogen evolved with time and that these new strains dominated the pathogen field populations in later years, as evidenced by random isolation of more aggressive strains every year. The abundance and permanence of monocultured cotton host plants over the years probably favors an accelerated evolution of the pathogen that would not occur under crop rotation conditions.

This study cannot overrule the possibility that during the long storage of lyophilized leaves, surviving fungal propagules may not be representative of the original population and that our results may have been influenced by the different lengths of time that the isolates were in storage. However, lyophilization of other pathogenic fungi does not appear to affect pathogenicity, even after extended periods of 25–34 years after lyophilization (7, 14). Furthermore, lyophilization is one of the most favored methods of culture collection used by scientific institutions.

The genetics of host–pathogen interaction for cotton leaf blight is unknown (8). However, from the variety of pathogenic factors that have been assessed and shown to have changed, it is assumed that selection in the field population of the pathogen is in more than one gene.

The phenomenon of increased aggressiveness of pathogenic agents over time under monoculture conditions was detected in one of the least studied plant diseases (8). It is essential to further explore whether this is a general phenomenon in phytopathology or a special case of leaf blight in cotton.

In summary, a common agricultural practice, the monoculture of cotton, increased the aggressiveness of one of its most common leaf diseases and should be regarded as indirect man-made interference in the evolution of this plant pathogen.

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1. Alcock, S., and Wheeler, B. E. J. 1983. Variability of *Cryptostroma corticale*, a causal fungus of sooty bark disease of sycamore. Plant Pathol. **32**: 173–176.
2. Arora, D. K., Filonow, A. B., and Lockwood, J. L. 1985. Decreased aggressiveness of *Bipolaris sorokiniana* conidia in response to nutrient stress. Physiol. Plant Pathol. **26**: 135–142.
3. Ashcar, H., Rodrigues-Paula, C., and Petrocini, V. R. 1988. Maintenance of fungi by lyophilization (observations after 34 years). [In Portuguese.] Rev. Microbiol. **19**: 422–424.
4. Bashan, Y. 1984. Transmission of *Alternaria macrospora* in cotton seeds. Phytopathol. Z. **110**: 110–118.
5. Bashan, Y. 1986*a*. Phenols in cotton seedlings resistant and susceptible to *Alternaria macrospora*. J. Phytopathol. **116**: 1–10.
6. Bashan, Y. 1986*b*. Field dispersal of *Pseudomonas syringae* pv. *tomato*, *Xanthomonas campestris* pv. *vesicatoria*, and *Alternaria macrospora* by animals, people, birds, insects, mites, agricultural tools, aircraft, soil particles, and water sources. Can. J. Bot. **64**: 276–281.
7. Bashan, Y. 1988. Are bacteria–plant cell interactions specific? *In* Experimental and conceptual plant pathology. *Edited by* R. S. Singh, U. S. Singh, W. M. Hess, and D. J. Weber. Oxford and IBH Publishers Pvt. Ltd., New Delhi, India. pp. 183–220.
8. Bashan, Y., and Hernandez-Saavedra, N. Y. 1992. Alternaria blight of cotton: epidemiology and transmission. *In Alternaria*—metabolites, biology and plant diseases. *Edited by* J. Chelowski. Elsevier Scientific Publishers, Amsterdam. pp. 233–266.
9. Bashan, Y., and Levanony, H. 1987. Transfer of *Alternaria macrospora* from cotton seeds to seedling: light and scanning electron microscopy of colonization. J. Phytopathol. **120**: 60–68.
10. Bashan, Y., and Okon, Y. 1986. Diseased leaf lyophilization: a method for long term prevention of loss of virulence in phytopathogenic bacteria. J. Appl. Bacteriol. **61**: 163–168.
11. Bashan, Y., Levanony, H., and Or, R. 1991*a*. Wind dispersal of *Alternaria alternata*, a cause of leaf blight of cotton. J. Phytopathol. **133**: 225–238.
12. Bashan, Y., Levanony, H., and Or, R. 1991*b*. Association between *Alternaria macrospora* and *Alternaria alternata*, causal agents of cotton leaf blight. Can. J. Bot. **69**: 2603–2607.
13. Bashan, Y., Levanony, H., and Or, R. 1991*c*. Wild beets as an important inoculum source of *Alternaria alternata*, a cause of leaf blight of cotton in Israel. Can. J. Bot. **69**: 2608–2615.

14. Bazzigher, G., and Kanzler, E. 1985. Long-term conservation of living fungal pathogens. Eur. J. For. Pathol. **15**: 58–60.
15. Chorin, M., and Rotem, J. 1958. Leaf disease of cotton. [In Hebrew.] Hassadeh, **38**: 648–649.
16. Commonwealth Mycological Institute. 1968. Plant pathologist's pocketbook. Commonwealth Mycological Institute, Kew, England.
17. Cotty, P. J. 1987*a*. Evaluation of cotton cultivar susceptibility to Alternaria leaf spot. Plant Dis. **71**: 1082–1084.
18. Cotty, P. J. 1987*b*. Temperature-induced suppression of Alternaria leaf spot of cotton in Arizona. Plant Dis. **71**: 1138–1140.
19. Cunfer, B. M. 1984. Change in virulence of *Septoria nodorum* during passage through barley and wheat. Ann. Appl. Biol. **104**: 61–68.
20. Diab, S., Bashan, Y., Okon, Y., and Henis, Y. 1982. Effect of relative humidity on bacterial scab caused by *Xanthomonas campestris* pv. *vesicatoria* on pepper. Phytopathology, **72**: 1257–1260.
21. Ebbels, D. L. 1980. Cotton diseases. Outlook Agric. **10**: 176–183.
22. Ellis, M. P., and Holliday, P. 1970. *Alternaria macrospora*. *In* Descriptions of pathogenic fungi and bacteria. No. 246. Commonwealth Mycological Institute, Kew, England.
23. Henis, Y., and Bashan, Y. 1986. Epiphytic survival of bacterial leaf pathogens. *In* Microbiology of the phyllosphere. *Edited by* N. J. Fokkema and J. van den Heuvel. Cambridge University Press, Cambridge. pp. 252–268.
24. Johnson, L. F., Hsieh, C., and Sutherland, E. D. 1981. Effects of exogenous nutrients and inoculum quantity on the virulence of *Pythium ultimum* to cotton hypocotyls. Phytopathology, **71**: 629–632.
25. Joly, P. 1964. Le genre *Alternaria*. Lechevalier Publications, Paris.
26. Lifshitz, R., Sneh, B., and Baker, R. 1984. Soil suppressiveness to a plant pathogenic *Pythium* species. Phytopathology, **74**: 1054–1061.
27. Madelin, T. M. 1987. The effect of a surfactant in media for the enumeration, growth and identification of airborne fungi. J. Appl. Bacteriol. **63**: 47–52.
28. Paulitz, T. C., Park, C. S., and Baker, R. 1987. Biological control of Fusarium wilt of cucumber with nonpathogenic isolates of *Fusarium oxysporum*. Can. J. Microbiol. **33**: 349–353.
29. Rhoades, R. E., and Johnson, L. 1991. The world's food supply at risk. Natl. Geogr. Mag. **179**: 74–105.
30. Sciumbato, G. L., and Pinckard, J. A. 1974. *Alternaria macrospora* leaf spot of cotton in Louisiana in 1972. Plant Dis. Rep. **58**: 201–202.
31. Simmons, E. G. 1981. *Alternaria* themes and variations. Mycotaxon, **13**: 16–34.
32. Yunis, H., Bashan, Y., Okon, Y., and Henis, Y. 1980. Weather dependence, yield losses and control of bacterial speck of tomato caused by *Pseudomonas tomato*. Plant Dis. **64**: 937–939.