J. Phytopathology, 116, 1—10 (1986)

ISSN 0031-9481

Department of Plant Genetics, The Weizmann Institute of Science, Rehovot, Israel

Phenols in Cotton Seedlings Resistant and Susceptible to *Alternaria macrospora*

YOAV BASHAN[1]

Author's address: Dr. Y. BASHAN, Department of Plant Genetics, The Weizmann Institute of Science, Rehovot (Israel).

With 5 figures

Received January 21, 1985; accepted October 26, 1985

Abstract

In healthy cotton seedlings, stems have a lower phenol content than leaves, but resistant plants have an altogether relatively higher phenol content than susceptible plants. Phenols extracted from infected plants can inhibit the growth of *A. macrospora in vitro*. In cotton plants infected with *A. macrospora*, phenols are oxidized by polyphenoloxidase rather than peroxidase and catalase. The main oxidative activity was around the developing necrotic area but activity was detected far from necrosis as well. Though pre-inoculation mechanical injuries operated the phenol oxidation mechanism in the plant, they neither prevented nor encouraged the increase in disease severity. Isozyme pattern showed that contribution of all participants in the pathological interaction to the oxidative mechanism occurred in the diseased plant. A negative linear correlation was found between polyphenoloxidase activity, phenol accumulation and resistance. This study suggests that the phenol oxidative mechanism participates in cotton plant resistance to *A. macrospora*.

Zusammenfassung

Phenole in Baumwollkeimpflanzen, resistent und anfällig gegenüber *Alternaria macrospora*

In gesunden Baumwollkeimpflanzen haben die Stiele einen geringeren Phenolgehalt als die Blätter, jedoch haben resistente Pflanzen im großen und ganzen einen relativ höheren Phenolgehalt als die anfälligen Pflanzen. Phenol, das man den infizierten Pflanzen entnimmt, kann den Wuchs von *A. macrospora in vitro* hemmen. In Baumwollpflanzen, die mit *A. macrospora* infiziert sind, werden Phenole eher durch Polyphenoloxidase als durch Peroxidase und Katalase oxidiert. Die hauptsächliche oxidierende Wirkung wurde um das sich entwickelnde nekrotische Gebiet gefunden, jedoch wurde die

[1] Recipient of a Sir Charles Clore fellowship.

Wirkung auch weiter entfernt von der Nekrose entdeckt. Trotz vor der Inokulation angebrachter mechanischer Verletzungen wirkte der Mechanismus der Phenoloxidierung in der Pflanze, weder verhinderten noch verstärkten sie die Schwere der Krankheit. Isozymmuster zeigten, daß Zusammenwirkung aller Beteiligten der pathologischen Wechselwirkung zu dem Oxidierungsmechanismus in der kranken Pflanze vorkamen. Eine negative lineare Korrelation wurde zwischen Polyphenoloxidase-Aktivität, Phenolanhäufung und Resistenz gefunden. Diese Untersuchung läßt vermuten, daß der phenoloxidierende Mechanismus in der Resistenz von Baumwollpflanzen gegenüber *A. macrospora* beteiligt ist.

Alternaria blight of cotton, caused by *Alternaria macrospora* Zimm. is one of the main cotton diseases in Israel (HADAS and JAKOBY 1981). The fungus has been detected in many countries, but relatively little information on the disease is available (VASUDEVA 1969, WATKINS 1981). The disease causes economic losses (BASHI *et al.* 1983) and is considered to be a seedling disease (EBBELS 1980). However, a recent study revealed that seedlings of both susceptible and "so-called" resistant cultivars (cv. 'Pima' and 'Acala', respectively) are equally infected (BASHAN 1984).

The purposes of this study were to find why resistant seedlings are also infected and if one of the resistance mechanisms operates through phenol oxidation.

Materials and Methods

Organisms, growth conditions, isolation methods, inoculation methods, pathogenicity tests and spore formation. These were as previously described (BASHAN 1984).

Disease index

Disease severity was estimated using the following scale: 0 = no lesions; 1 = 1—5 lesions; 2 = 6—10 lesions; 3 = 10—20 lesions; 4 = over 20 lesions; 5 = leaf shedding resulting from heavy infection. The index was determined on the third, fourth and fifth upper leaves of each plant. Number of lesions per leaf were counted separately and the mean for the three leaves was considered to be the disease index for the plant.

Plant injuries and sampling

Leaves were injured by brushing them with a very fine carborundum powder (300 grid) using a soft brush. When injuries and inoculation treatments were combined, leaves were injured 24 h before inoculation. Leaf samples, for enzymatic activity, were taken with an 8 mm cork borer (25—30 discs per replicate) and extracted as later described.

In one experiment, plants (susceptible and resitant) were separated into nine groups. Each group showed the same disease index (D.I.) (approximately 20 plants per D.I. unit; at 0.5 D.I. intervals) ten days after inoculation. Resistant plants, cv. 'Acala', showed D.I. of 0 to 0.5, whereas susceptible plants, cv. 'Pima', showed D.I. of 2 and above. Data from the two types of plants were combined and analyzed (Fig. 5).

Enzyme extraction and assays

Leaves and stems were homogenized in an ice bath first with a high speed shaft homogenizer (Ystral, FRG), then in an Omni-mixer (Sorvall), again with the Ystral homogenizer and finally with a fine glass pestle (Kontes, U.S.A.).

The homogenate obtained was centrifuged at 12000 xg for 10 min and the supernatant was subjected to two further centrifugations at 30000 xg for 30 min at 4 °C. Supernatant proteins were partially purified by gel filtration, first on a G-25 and then on a G-200 Sephadex column (60 × 2 cm, elution rate 0.2 ml/min, 0.06 M potassium phosphate buffer, pH 7.0). Fractions showing enzymatic activities were lyophilized and redissolved in the phosphate buffer before further testing. Enzymes extracted from the fungal culture by filtration of the fungal mycelium through Whatman No. 42 filter paper and the supernatant were partially purified as were those obtained from diseased cotton plants.

Polyphenoloxidase (PPO) was measured according to LEONARD (1971). The reaction mixture, containing 0.5 ml 0.03 M phosphate buffer, pH 6.5; 0.4 ml of 5 mM 3,4-dihydroxyphenylalanine (DOPA) dissolved in the same buffer and 0.1 ml enzyme preparation, was incubated for two min at 30 °C in the spectrophotometer and recorded at 475 nm. Mixtures without enzyme or substrate or with a boiled or commercial enzyme preparation (Sigma), were used as controls. Enzyme units were defined as increase in 0.1 absorbance units/min/mg protein. Peroxidase (PO) activity was measured according to LEE (1973). The reaction mixture, containing 0.5 ml of 0.06 M phosphate buffer, pH 5.8; 0.4 ml of a solution (1:1, V/V) made of 1 % guaiacol (dissolved in absolute ethanol) and 15 mM H_2O_2 (dissolved in distilled water); and 0.1 ml enzyme preparation, was incubated in a spectrophotometer for 2 min at 30 °C and recorded at 470 nm. Controls were as described for PPO measurements, including commercial peroxidase. Enzyme units were defined as increase in 0.1 absorbance unit/min/mg protein.

Catalase activity was measured according to BEERS and SIZER (1952). The reaction mixture, containing 3 ml of 0.03 M phosphate buffer, pH 6.8, in which 3.6 µl 30 % H_2O_2 were dissolved and 0.02 ml freshly prepared enzyme preparation dissolved in the same buffer, was prepared inside the spectrophotometer and immediately recorded at 240 nm. Controls were as for the above mentioned enzymes. Enzyme units were defined as increase in 0.01 absorbance unit/sec/mg protein.

Electrophoresis and isozyme staining

Electrophoresis was carried out using slab gel electrophoresis on 7 % polyacrylamide gels with slight modifications of the DAVIS method (1964). After a pre-run (1 mA per sample site), 20 µl samples, each containing 10 µg protein, were run in 200 V, 40 mA for 4 h. Polyphenoloxidase isozyme staining was carried out using the DOPA staining method (STAFFORD and GALSTON 1970). The stained gels were then scanned on a spectrophotometer.

Phenol extraction, absorption and phenol supplementation to fungal culture

The following procedures were carried out separately and their products were added to the fungal cultures as described in Table 2. Phenol samples were extracted using the ethanol: hexane: water method (WALTER and PURCELL 1979). Phenol absorbtion to polyvinylpyrrolidone (PVP) was as follows: Phenols were mixed with a methanol: water (1 : 4) mixture and the pH was set at 3.5 with 1 N HCL. Ten ml of the mixture were combined with 0.25 g PVP and vigorously shaken for 1 h. After phenol absorption by PVP and removal of the pellet by centrifugation at 30000 xg for 10 min, the methanol was evaporated under reduced pressure at 35 °C. The final solution was neutralized with 1 N NaOH before testing.

Fifteen mg/l of the reducing compound dithioerythritol (DTT) and 10^{-3} M of ethylenediaminetetra acetic acid (EDTA) (which neutralizes PPO) were added to other phenol extractions.

Analytical methods: Total protein content of the extracts was analyzed by the Coomassie brilliant blue method (SEDMAK and GROSSBERG 1977) and phenols by the $FeCl_3$ — $K_3F_e(CN)_6$ method (KRITZMAN and CHET 1980).

Statistical analysis

All experiments were repeated twice with five to ten replicates, using 20 plants or 10 samples per replicate. Results given are from a representative experiment. Significance is given by $P \leq 0.05$ in Duncan's Multiple Range Test.

Results

Phenol content of stems and leaves of healthy susceptible and resistant cotton seedlings

First true leaf seedlings showed a significant difference in phenol content between resistant and susceptible cultivars and also between stems and leaves of the same cultivar. The phenol content of cv. 'Acala' leaves was higher than that of cv. 'Pima', but the difference was not statistically significant. However, the phenol content of cv. 'Acala' stems was significantly higher than that of cv. 'Pima' stems (Fig. 1).

Changes in PO, PPO activity and phenol content during disease development in seedlings

No catalase specific activity could be detected during disease development. Specific activity of PO was low during the first six days of disease development, slightly increasing in leaves but not in stems, 4—6 days after inoculation (Fig. 2a).

PPO specific activity in leaves increased in inoculated plants of both cultivars but was significantly higher in the resistant ones, reaching its highest level 100 h after inoculation (Fig. 2b). Phenol content, though significantly higher, throughout the experiment, in the resistant plants, increased during disease development in both cultivars (Fig. 2c). No difference was found between PPO activity in inoculated resistant and susceptible stems. However, phenol accumulation, higher in resistant stems than in the susceptible ones, was still lower than in leaves.

PPO specific activity was very high in areas very close to the necrotic spot, but even 2 cm from necrosis high PPO activity was detected. Similarly, the highest phenol content was also found close to the necrotic spot and far from necrosis it was still high (Table 1).

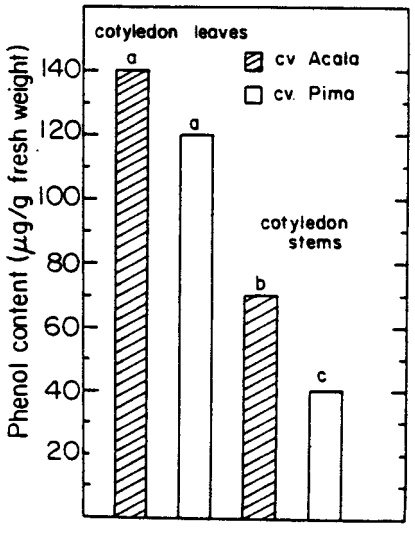

Fig. 1. Phenol content of stems and leaves of healthy susceptible and resistant cotton seedlings. Histogram values followed by different letters differ significantly at $P \leq 0.05$

Effects of mechanical injuries in leaves with or without inoculation, on PO, PPO activity and phenol content

Though PO specific activity increased 24 h after mechanical injury, its level was low and no further increase was detected during disease development. In

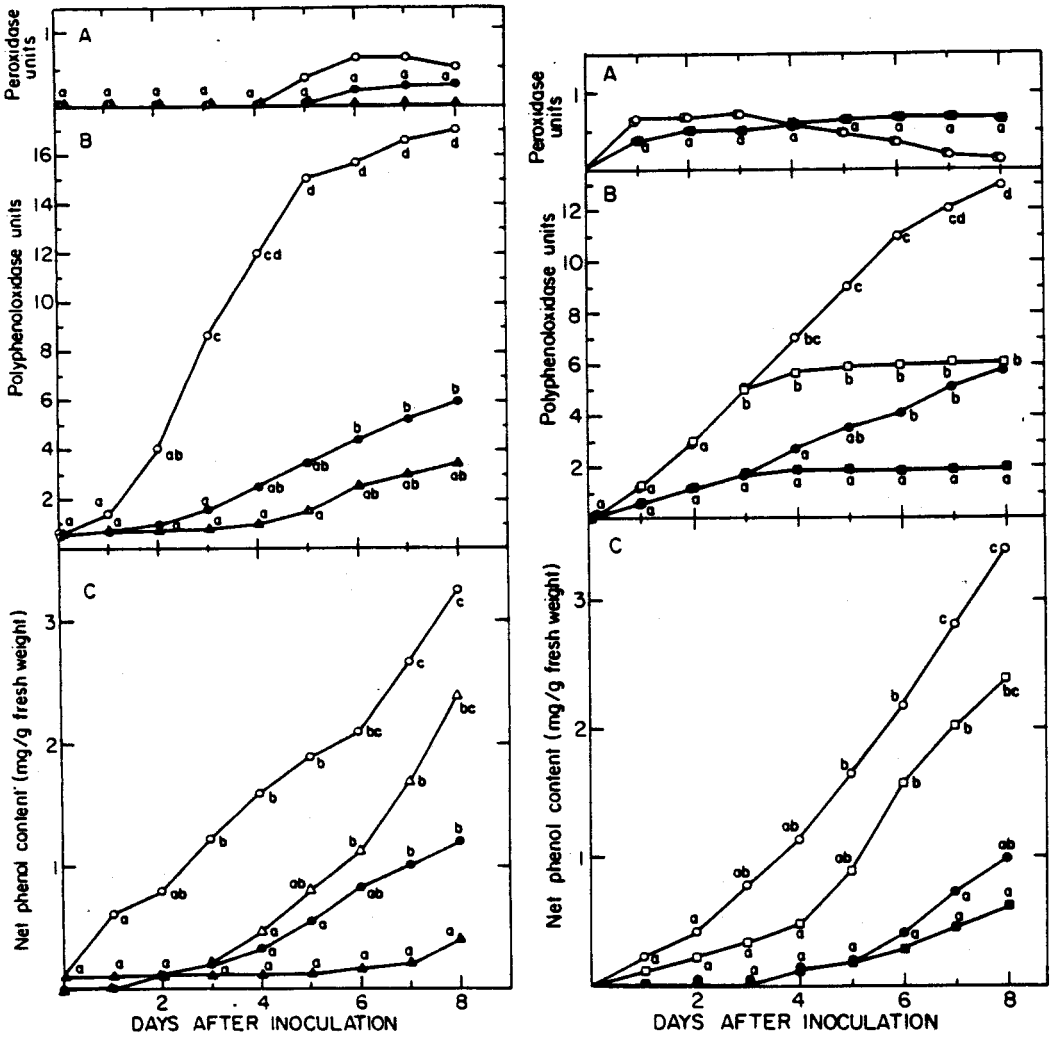

Fig. 2. (left) (A) PO and (B) PPO specific activity and (C) net phenol content of cotton plants during disease development in seedlings. ● — susceptible leaves; 0 — resistant leaves; ▲ — susceptible stems; Δ — resistant stems. Points in each sub-figure followed by different letters differ significantly at $P \leq 0.05$

Fig. 3. (right) Changes in (A) PO and (B) PPO specific activity and (C) net phenol accumulation in injured cotton plants with and without inoculation. ● — injured, inoculated susceptible plants; ■ — injured uninoculated susceptible plants; ○ — injured, inoculated resitant plants; □ — injured uninoculated resistant plants. Points in each sub-figure followed by different letters differ significantly at $P \leq 0.05$

both cultivars, no difference was found between uninoculated injured plants and those later inoculated with the pathogen (Fig. 3a).

In the resistant plants, PPO specific activity increased sharply, within 36 h as a result of injury. However, further increase in PPO activity occurring later than three days after inoculation was detected only in inoculated plants. Similar results, but at a lower magnitude, were observed in susceptible plants (Fig. 3b). Phenol accumulation in the two cultivars resembled PPO activity with only one difference — at the beginning of disease development phenol accumulation was slower than PPO activity, but increased sharply later (Fig. 3c).

PPO isozymes in healthy and diseased plants

Of the seven different PPO isozymes detected in diseased plants, two (Fig. 4, bands 3 and 7) were found in healthy plants too, two others (Fig. 4, bands 1 and 2) were induced in the plant during disease development and the remaining three (Fig. 4, bands 4, 5 and 6) were of fungal origin. One isozyme (Fig. 4, band 8), which was produced in culture by the pathogen, did not appear in diseased plants.

Relationship between disease severity, PPO activity and phenol content in *A. macrospora* infected resistant and susceptible cotton plants

Diseased plants showing most ranges of disease index (0 to 5) were sampled. PPO activity and phenol content, both 10 times higher in resistant plants than in the susceptible ones (Fig. 5a, b), decreased with the increase in disease index.

Table 1

Location of polyphenoloxidase activity and phenol content in cotton leaf tissues

Leaf site	Plant type	PPO activity (units)	Phenol content mg/g fresh weight
Average activity of all plant leaves from diseased plants	S[a]	3.47 b[b]	1.18 b
	R	12.68 d	3.23 c
Area surrounding the necrotic spot	S	5.18 c	2.88 c
	R	18.11 f	5.46 e
1 cm in diameter away from the necrotic spot	S	4.78 bc	2.71 c
	R	16.46 e	5.08 e
2 cm in diameter away from the necrotic spot	S	4.12 b	2.11 bc
	R	15.86 e	4.68 de
3 cm in diameter away from the necrotic spot	S	3.88 b	1.58 b
	R	14.79 e	4.17 d
Apparently healthy leaflets on diseased plants	S	1.08 a	0.61 a
	R	1.26 a	0.82 ab
Leaflets from healthy plants	S	0.41 a	0.11 a
	R	0.68 a	0.15 a

[a] S = susceptible cv. 'Pima'; R = resistant cv. 'Acala'

[b] = numbers followed by different letters in the same column differ significantly at $P \leq 0.05$

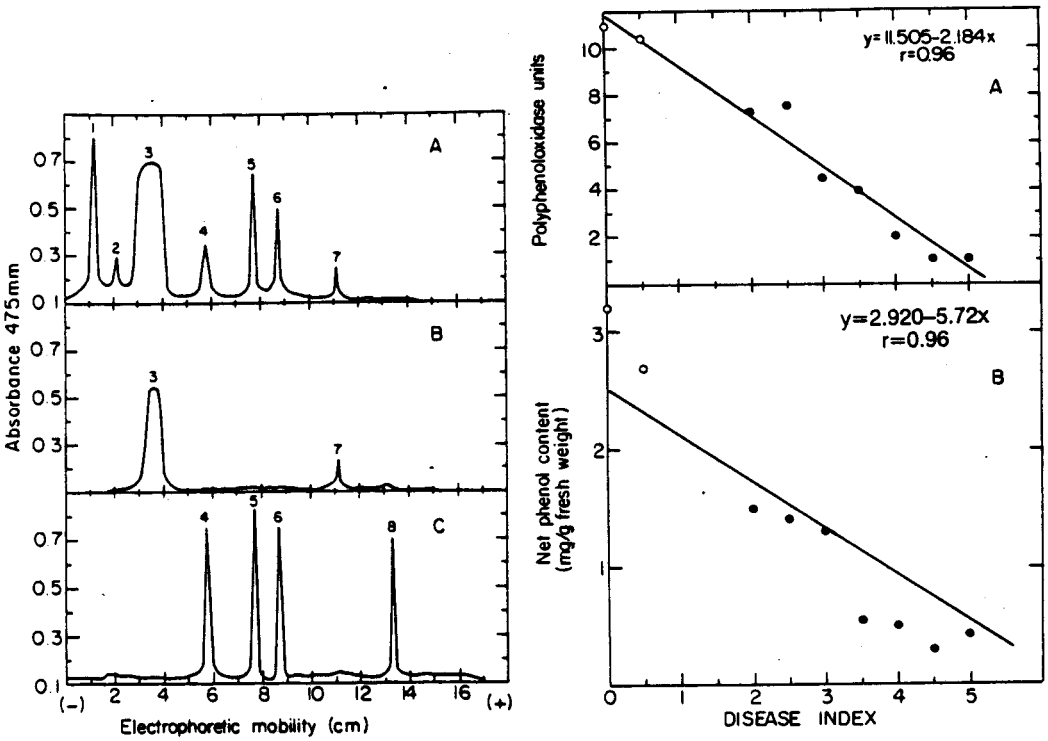

Fig. 4. (left) Isozymes and/or multiple forms of polyphenoloxidase in (A) diseased, (B) healthy, and (C) fungal liquid culture supernatant. Peaks followed by the same number are identical

Fig. 5. (right) Correlations between disease index, net polyphenoloxidase activity (A) and net phenol content (B). ○ — cv. 'Acala' (resistant); ● — cv. 'Pima' (susceptible)

Effect of phenols, extracted from plants, on pathogen growth and PPO activity

The addition of phenols, extracted from either resistant or susceptible healthy plants, to the growth medium, had only a minor effect on pathogen growth but it enhanced PPO activity in culture extracts. The addition of phenol, from diseased plants together with EDTA to the growth medium, inhibited *A. macrospora* growth and arrested PPO activity. These effects were more marked with phenols extracted from the resistant cultivar (cv. 'Acala'). The addition of PVP or PVP + EDTA did not affect pathogen growth and PPO activity, whereas the addition of phenols with EDTA and DTT, a treatment that maintained phenols as extracted without oxidation, did inhibit fungal growth. Addition of PVP, DTT or EDTA without extracted phenols neither affect fungal growth nor PPO activity (Table 2).

Discussion

One of the explanations for plant resistance, besides phytoalexin accumulation which is now the most accepted mechanism of plant resistance for necro-

Table 2
Effect of extracted phenolic compounds and various inhibitors on pathogen growth[a] and polyphenoloxidase activity

Source of extracts	Addition of extracts[b]	Inhibitors PVP[c]	DTT[d]	EDTA[e]	Fungal growth (mg dry weight)	PPO specific activity (units)[f]
Healthy	+	+	—	—	586 a[g]	0.27 d
resistant	+	—	+	+	416 b	0.0 d
or	+	—	—	+	502 a	3.18 c
susceptible	+	+	—	+	572 a	0.35 d
plants	+	—	—	—	519 a	10.98 b
Diseased	+	+	—	—	535 a	0.18 d
susceptible	+	—	+	+	116 c	0.21 d
plants	+	—	—	+	176 c	0.0 d
	+	+	—	+	519 a	0.24 d
	+	—	—	—	517 a	17.03 a
Diseased	+	+	—	—	549 a	0.31 c
resistant	+	—	+	+	84 c	0.24 d
plants	+	—	—	+	96 c	0.08 d
	+	+	—	+	526 a	0.38 d
	+	—	—	—	515 a	16.61 a
Control	—	—	—	—	543 a	0.12 d
	—	+	—	—	561 a	0.17 d
	—	—	+	—	513 a	0.11 d
	—	—	—	+	558 a	0.0 d

a = 10 replicates; b = 200 mg/ml; c = 3.5 %; d = 20 mg/l; e = 10^{-3} M; f = ΔA_{475}/min/mg protein — five replicates; g = numbers followed by different letters in each column differ significantly at $P \leq 0.05$.

trophic organisms, is the involvement of the phenol system and related oxidative enzymes (HARE 1966). Following infection by the phytopathogenic fungus, the plant releases several phenolic compounds, whose oxidation products, such as quinones, are toxic to invading fungi. Phenol accumulation is usually higher in resistant and incompatible pathological relationships than in susceptible plants. However, phenol involvement varies according to the pathogenic relationship. Activity of oxidative enzymes also changes in pathological interactions. Sometimes PO, PPO and catalase activities are detected simultaneously and sometimes only one enzyme operates (FARKAS and KIRÁLY 1962, FRIČ 1976, KOSUGE 1969, KUĆ 1966, SEQUEIRA 1983).

In the *Alternaria*-cotton interaction, only PPO activity was detected concomitantly with phenol accumulation. As expected, PPO activity was stronger and phenol accumulation higher in the resistant cultivar compared to the susceptible one. The lower PPO activity in the stems may explain their higher susceptibility to the disease. Though PPO activity was strongest near the developing necrosis, it was still considerable even far away from necrosis,

indicating a general defence mechanism. PPO activity is directly related to fungal attack because though also produced by mechanical injury, further increase in activity with time occurs only during disease development. This phenomenon is probably produced by the pathogen, since production of PPO activity and phenol accumulation by injury and later inoculation did not decrease but rather enhanced disease severity.

The phenol oxidation activity in diseased plants is caused by all participants in the pathogenic interaction, i.e., plant, pathogen and the interaction between them, as revealed by the isozyme pattern. In addition, oxidative phenols, especially those produced by resistant plants may, theoretically, inhibit fungal growth. Their inactivation may be explanied by their rapid oxidation, by fungal PPO, to polyphenols which are not toxic to the invading pathogen. This point, however, needs further study. The negative linear correlations between disease severity and PPO activity or phenol accumulation provides further evidence for the involvement of the oxidation system in the resistance mechanism of the plant.

The exact rôle of PPO in the resistance of cotton plants to Alternaria blight is as yet unclear. On the one hand, inhibition of phenol oxidation *in vitro*, by EDTA, inhibited fungal growth in the presence of plant products, and on the other, a positive correlation was observed between PPO level and plant resistance. It is also possible that the increase in PPO activity, observed in infected resistant plants, is a secondary reaction of the plant to the accumulation of phenol substances. The exact nature of the composition of phenols present in extracts of susceptible and resistant cotton plants is still unknown. It is possible that the extracts contain quinones as a result of PPO activity. Thus, PPO may play a contradictory role in plant resistance. Increased fungal PPO activity may reflect an attempt to inactivate toxic plant phenols. The total PPO activity of an infected plant is the sum of these possible enzymatic sources.

By analyzing data from this and another study (BASHAN 1984), it can be concluded that resistance to *A. macrospora*, though not evident at the seedling stage, plays an important rôle in more mature plants, where a phenol oxidation system is activated.

This paper was written in memory of the late Mr. AVNER BASHAN for his constant encouragement and interest during this research.

Literature

BASHAN, Y., 1984: Transmission of *Alternaria macrospora* in cotton seeds. Phytopath. Z. 110, 110—118.

BASHI, E., Y. SACHS, and J. ROTEM, 1983: Relationships between disease and yield in cotton fields affected by *Alternaria macrospora*. Phytoparasitica 11, 89—98.

BEERS, R. F., Jr., and I. W. SIZER, 1952: A spectrophotometric method for measuring the breakdown of hydrogen-peroxide by catalase. J. biol. Chem. 195, 133—140.

DAVIS, B. J., 1964: Disc electrophoresis — II Method and application to human serum proteins. Ann. N.Y. Acad. Sci. 121, 409—427.

EBBELS, D. L., 1980: Cotton diseases. Outlook Agric. 10, 176—183.

FARKAS, G. L., and Z. KIRÁLY, 1962: Role of phenolic compounds in the physiology of plant diseases and disease resistance. Phytopath. Z. 44, 105—140.

FRIČ, F., 1976: Oxidative enzymes. In: Encyclopedia of Plant Physiology, Physiological Plant Pathology, New Series, Ed. by R. HEITEFUSS and P. H. WILLIAMS, **4**, 617—631. Springer-Verlag, Berlin, N.Y.

HADAS, S., and T. JAKOBY, 1981: The *Alternaria* disease of cotton. Phytoparasitica **9**, 252 (Abstr.).

HARE, R. C., 1966: Physiology of resistance to fungal diseases in plants. Bot. Rev. **32**, 95—137.

KUĆ, J., 1966: Resistance of plants to infectious agents. Annu. Rev. Microbiol. **20**, 337—370.

KOSUGE, T., 1969: The role of phenolics in host response to infection. Annu. Rev. Phytopathol. **7**, 195—222.

KRITZMAN, G., and I. CHET, 1980: The role of phenols in the pathogenicity of *Botrytis allii*. Phytoparasitica **8**, 27—37.

LEE, T. T., 1973: On extraction and quantification of plant peroxidase isozymes. Physiol. Plant. **29**, 198—203.

LEONARD, T. J., 1971: Phenoloxidase activity and fruiting body formation in *Schizophyllum commune*. J. Bact. **106**, 162—167.

SEDMAK, J. J., and S. E. GROSSBERG, 1977: A rapid, sensitive and versatile assay for protein using Coomassie brilliant blue G250. Anal. Biochem. **79**, 544—552.

SEQUEIRA, L., 1983: Mechanisms of induced resistance in plants. Annu. Rev. Microbiol. **37**, 51—79.

STAFFORD, H. A., and A. W. GALSTON, 1970: Ontogeny and hormonal control of polyphenoloxidase isozymes in tobacco pith. Plant Physiol. **46**, 763—767.

VASUDEVA, R. S., 1969: Diseases in Cotton in India. A monograph. Indian Central Cotton Committee, Bombay, pp. 164—216.

WALTER, W. M. Jr., and A. E. PURCELL, 1979: Evaluation of several methods for analysis of sweet-potato phenolics. J. Agric. Fd. Chem. **27**, 942—946.

WATKINS, G. M., 1981: *Alternaria* leaf spot. In: Compendium of cotton diseases. Ed. G. M. WATKINS. The American Phytopathological Society, Minn.