Changes in Protein and Isozyme Content of Apple Fruits Following Infection by Monochaetia mali

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ABSTRACT

Proteins and isozyme patterns of five different enzymes formed by *Monochaetia mali* during fruit rot of 3- and 5-month-old 'Golden Delicious' apple fruits, were examined (relative to the enzyme patterns obtained from noninfected fruits and from the culture-grown fungus) by polyacrylamide gel electrophoresis. There were no changes in total protein bands in gels containing the extracts from infected and noninfected fruits regardless of maturity. However, the number of protein bands decreased with fruit maturity irrespective of infection by the fungus. The isozyme study showed that the number of polyphenol oxidase and malate dehydrogenase bands increased in the infected mature fruit but not in the immature fruit. No polyphenol oxidase and peroxidase isozyme bands were detected in the fungal extract. New isozyme bands which were not found either in extracts from healthy mature fruits or from the fungus, were detected with polyphenol oxidase and malate dehydrogenase in inoculated fruits following infection. An additional isozyme band for both glucose-6-phosphate dehydrogenase and acid phosphatase was found in infected fruits.

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Monochaetia mali (Ell. & Ev.) Sacc. was isolated from a cankered apple limb (11) in 1967, and its pathogenicity to apple fruit was demonstrated (9). Later its etiology in apple fruit was reported (10). It has been shown by Wallace et al. (19) that the amount of protein in apple fruits decreased with maturity which was associated with susceptibility to fungal infection. It has been suggested that increased respiration was accompanied by changes in the chemical constitution of apple fruits during their maturation and these changes contributed to the loss of resistance to *Botryosphaeria ribis, Glomerella cingulata, Neofabrea malicorticis*, and *Physalospora obtusa* (14).

Various workers have demonstrated that qualitative changes in most proteins were accompanied by an alteration in isozyme patterns in a number of host-parasite combinations (16, 18, 20). Plant proteins, particularly enzymes, are associated with changes in the metabolism of diseased plants (6). Some have suggested that the increased enzyme activity in diseased tissue may be either due to enzyme protein synthesis stimulated by parasitic attack (17) or activation (solubilization) of inactive enzyme (5, 16).

Our purpose was to determine whether there were any changes in proteins and isozymes in immature and mature apple fruit either infected or noninfected with *Monochaetia mali*.

MATERIALS AND METHODS.—Three- and 5-month-old 'Golden Delicious' apple fruits, collected from the University orchard, were washed with 0.15% solution of sodium hypochlorite, rinsed thoroughly with distilled water and wounded once per fruit with a sterile dissecting needle. The inoculum was obtained from an isolate of *M. mali*, from infected apple twig (10), and maintained on lima bean agar (LBA). For inoculation, a 4 mm mycelium disk from the margin of a 6-day-old culture on LBA was inserted into the wound. Wounded, noninoculated fruits served as controls. The control and inoculated fruits were placed together in plastic vegetable crisper boxes (19 × 13 × 8.5 cm) with two paper towels in the bottom, 10 ml of sterile distilled water was added to each box, which was then incubated at room temperature (25 C) for 7 days.

Extraction of proteins from fruits.—Proteins were extracted from infected and noninfected fruits by scraping away the original inoculum from surrounding tissue 1.0 cm below the point of inoculation. One hundred twenty-five g of fruit tissue were removed and added in 25 g increments to 200 ml of acetone with gentle stirring. Following 10 min homogenization, the pulps were stored in cold room at −35 C for 12 hr to complete the solubilization of sugars, acids, phenols and other low molecular weight substances. The homogenates were then vacuum-filtered in 500-ml filter flask encased in dry ice. Two hundred mg of acetone powder was suspended in 5 ml of 0.1 M potassium phosphate buffer, pH 7.5, containing 10% sucrose and 50 μmole disodium ethylenediaminetetraacetate. The suspension was allowed to stand at 0 C for 1 hr, and then centrifuged at 20,000 *g* for 30 min. The supernatant was directly analyzed by electrophoresis.

Extraction of fungal proteins.—Mycelium for electrophoresis was obtained by growing the fungus in a basal medium (7). Cultures were harvested 7 days after inoculation by vacuum filtration. The filtered mycelium was washed three times with 500 ml of distilled water and stored at −35 C. Two hundred mg of the fungal tissue was ground in a prechilled pestle and mortar with 10 ml of 0.1 M phosphate buffer, pH 7.0, containing 0.5 M sucrose and 0.1% mercaptoethanol and acid washed sand for 10 min at 4 C. The homogenate material was centrifuged at 35,000 *g* for 30 min at 0 C. Supernatants were collected and again centrifuged at 105,000 *g* for 1 hr.

The proteins in the extracts were concentrated by dialysis against a 0.1 M phosphate buffer, pH 7.0, containing 50% sucrose in cold room at 4 C, until the sample volume reduced to approximately 2.5 ml.

Electrophoresis.—Polyacrylamide gel electrophoresis was done as described by Davis (4) with some modifications. In place of sample gel, 20% sucrose was used. The gels were made in 0.5- X 7.6-cm diam pyrex glass tubes by using 1.5 ml small pore solution and 0.2 ml large pore solution. Protein samples were layered on the top of large pore gel and electrophoresis was carried out at 4 C. Initially, a current of 1 mA per gel was applied to avoid disturbance of the sample; then separation was conducted using a low current of 2 to 2.5 mA per gel. The protein separation was continued until the marker dye (bromophenol blue) had migrated about 35 to 40 mm into the separation gel.

Protein staining and isozyme localization.—Soluble proteins were stained with 1% (w/v) aniline blue in 7% (v/v) acetic acid and isozymes were visualized by histochemical staining procedures.

Localization of polyphenol oxidase isozyme bands was done according to the procedure described by Clements (3).

Staining procedure for glucose-6-phosphate dehydrogenase was the same as described by Baptist (1). The localization of isozyme bands for the other three enzymes was conducted according to the procedures outlined by Canalco (2).

RESULTS.—Nineteen and 16 protein bands were observed in extracts from immature and mature fruits, respectively. These were not altered by infection with *M. mali* (Table 1).

The extracts from control and infected immature fruits showed no changes in intensity, electrophoretic mobility, or number of isozyme bands of polyphenol oxidase (Table 1). In contrast, the extract from infected mature fruit yielded a new isozyme band (Fig. 1, 2B) having a greater mobility than band 3 (Fig. 1) compared to the control (Fig. 1A). The bands were numbered 1, 2, and 3 (Fig. 1B) in order of decreasing electrophoretic mobility. The fungal band did not contain a polyphenol oxidase isozyme band.

Three bands with peroxidase activity were resolved in fruit extracts, but none in fungal extracts. No new peroxidase bands were detected in the extracts of infected fruits, irrespective of maturity. Extracts from control immature and mature fruits showed the same two acid phosphatase isozyme bands. Fungal extract contained only one band. An additional acid phosphatase band having the same R_F value as the fungal band was found in all extracts from infected fruits.

Regardless of maturity, fruit tissue contained the same three glucose-6-phosphatase dehydrogenase bands. One additional band, with similar R_F value as one of the fungal bands, occurred in all infected fruits.

Several changes were found in the malate dehydrogenase with extract from infected mature fruit (Fig. 2B). Band 4 was more intensely stained. Bands 1 and 2 were frequently stained less intensely in comparison to the corresponding bands in the extract infected fruits (Fig. 2A). Band 5 (Fig. 2B) which was present in the diseased tissue, was not found in gel when it contained a mixture of the extracts from control fruit and the fungus. Band 1 of the fungal extract (Fig. 2C) was not found in infected mature fruit extract. In the extracts of control and infected immature fruit tissue, there were three bands in each (Table 1) and there were no differences in the intensity, electrophoretic mobility, or locations of isozyme bands of the enzyme.

DISCUSSION.—There were no changes in total protein bands present in gels containing the extracts from control and infected apple fruits regardless of maturity. Other reports vary depending upon host-pathogen combinations. Johnson et al. (8) have reported that, in a cell-free extract of barley plants infected by *Erysiphe polygoni*, there was no change in total protein bands. On the contrary, Staples & Stahmann (15) found changes in protein bands in bean leaves infected by *Uromyces phaseoli*.

The number of protein bands decreased with fruit maturity regardless of infection by *M. mali* indicating a decrease in the amount of proteins in fruits with age. This agrees with an earlier finding that the amount of protein in apple fruit decreases continually as the fruit matures (19).

TABLE 1. Multiple molecular forms of proteins and isozymes from the fungal extract and from immature and mature fruit control and infected with *Monochaetia mali*

Substance	Number of bands on gels with extract from				
	Fungal extract	Immature fruit		Mature fruit	
		Control	Infected[a]	Control	Infected[a]
Protein	9	19	19	16	16
Polyphenol oxidase	0	2	2	2	3
Peroxidase	0	3	3	3	3
Glucose-6-phosphate dehydrogenase	2	3	4	3	4
Acid phosphatase	1	2	3	2	3
Malate dehydrogenase	4	3	3	3	6

[a] Seven days after inoculation.

The isozyme study shows that there were no inherent differences in healthy control fruits regardless of maturity. This suggests that if any differences occur, it is due to differential responses by the host tissue to the pathogen.

No new peroxidase isozymes are stimulated as a result of infection in apple fruit by *M. mali* which is contrary to the finding of Stavely & Hanson (17) who detected an increase in number of peroxidase bands in *Erysiphe polygoni*-infected *Trifolium pratense*. The new acid phosphatase, and glucose-6-phosphate dehydrogenase isozyme bands found in the infected fruit appear to have been contributed by the fungus as indicated by electrophoretic mobilities of the fungal isozyme bands.

In polyphenol oxidase and malate dehydrogenase, a significant change could be found in mature fruits between the control and infected, whereas no such change was found in immature fruit.

New isozyme bands of polyphenol oxidase (Fig. 1, B2) and malate dehydrogenase (Fig. 2, B5) appeared in infected mature fruits, although they were not present either in the healthy control fruits or in the cultured fungus. In malate dehydrogenase, the intensities of isozyme bands 1 and 2 (Fig. 2B) in infected fruit decreased compared to the corresponding band in control fruit (Fig. 2A), but band 4 (Fig. 2B) was stained more intensely as compared to band 3 (Fig. 2C) from culture-grown fungus. Similar patterns of isozyme changes in some enzymes have been shown by Sako & Stahmann (13) in barley leaves infected with *Erysiphe graminis* and

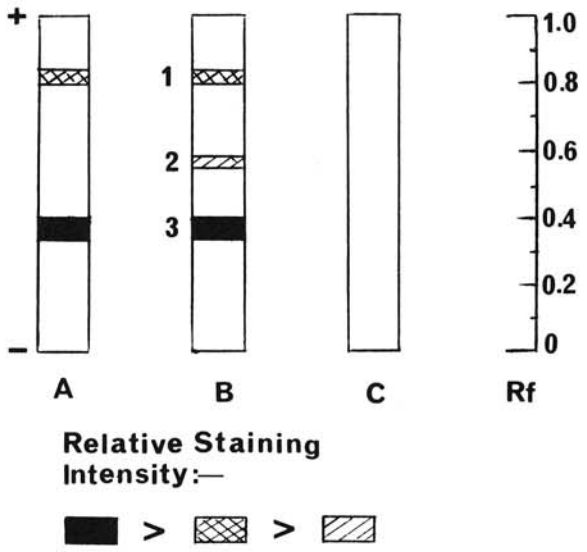

Fig. 1. Zymograms of polyphenol oxidase bands of gels prepared from extracts of **A)** noninoculated mature apple fruit; **B)** mature apple fruit at 7 days after inoculation; **C)** mycelia of the culture-grown fungus. 1,2,3 = polyphenol oxidase bands from infected fruit extract. Isozyme bands were numbered with the isozyme having the highest electrophoretic mobility towards the anode, being numbered "*one*".

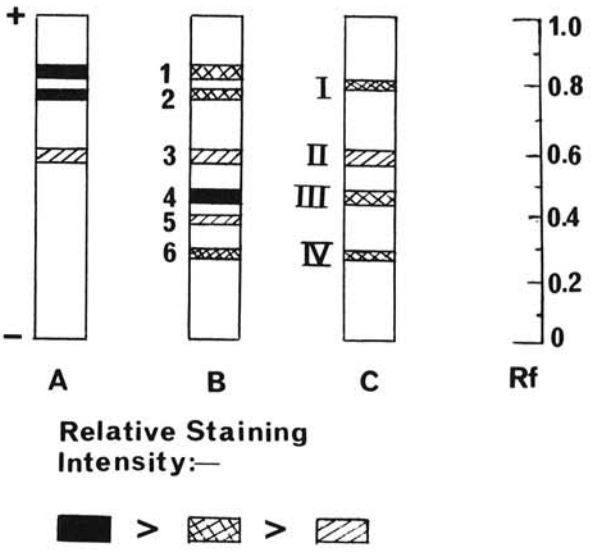

Fig. 2. Zymograms of malate dehydrogenase bands of gels prepared from extracts of **A)** noninoculated mature apple fruit; **B)** mature apple fruit at 7 days after inoculation; **C)** mycelia of the culture grown fungus. I,II,III,IV = Malate dehydrogenase bands from mycelial extract. 1,2,3,4,5,6 = Malate dehydrogenase bands from infected fruit extract. Isozyme bands were numbered with the isozyme having the highest electrophoretic mobility towards the anode, being numbered "*one*".

by Reddy & Stahmann (12) in peas infected with *Fusarium oxysporum*.

It is difficult to establish the source of the increase or decrease in the intensity and in the number of isozyme bands of these enzymes. The appearance of new isozyme bands is probably related to an alteration of metabolism in fruit tissue around the infection site.

The changes in the intensities of the bands may represent an increase or decrease in enzyme activity. The increased activity of malate dehydrogenase may be due to the activation of enzyme precursor or the synthesis of new enzyme protein. These changes may be also due to interaction of enzyme subunits of both host fruit tissue and the fungus to form hybrid enzyme with an increased or decreased activity.

The detection of changes only in mature apple fruit tissue suggests that more new synthesis or alteration of enzyme occurred there than in the immature apple fruit tissue.

LITERATURE CITED

1. BAPTIST, J. N., C. R. SHAW, & M. MANDEL. 1969. Zone electrophoresis of enzymes in bacteriology 99:180-188.
2. CANAL INDUSTRIAL CORPORATION. 1963. Special subject. Enzyme Analysis. Canal Industrial Corp., Bethesda, Md. 12 p.
3. CLEMENTS, R. L. 1965. Fruit proteins: Extraction and electrophoresis. Anal. Biochem. 13:390-401.

4. DAVIS, B. J. 1964. Disc electrophoresis. II. Method and application to human serum proteins. New York Acad. Sci. Ann. 121:404-427.
5. FARKAS, G. L., & L. LOVREKOVICH. 1965. Enzyme levels in tobacco leaf tissues affected by the wildfire toxin. Phytopathology 55:519-524.
6. FARKAS, G. L., & M. A. STAHMANN. 1966. On the nature of changes in peroxidase isozymes in bean leaves infected by southern bean mosaic virus. Phytopathology 56:669-677.
7. GILL, H. S., & D. POWELL. 1968. The use of polyacrylamide gel electrophoresis in delimiting three species of Phytophthora. Phytopathol. Z. 63:23-29.
8. JOHNSON, L. B., B. L. BRANNAMAN, & F. P. ZSCHEILE, JR. 1966. Protein and enzyme changes in barley leaf infected with Erysiphe graminis f. sp. hordei. Phytopathology 56:1405-1410.
9. NAIK, R. 1972. A study of apple canker and its incitant Monochaetia mali with some aspects of etiology and physiology of pathogenesis. Ph.D. Thesis, Univ. of Illinois, Urbana. 44 p.
10. NAIK, R., & D. POWELL. 1971. Etiology of Monochaetia mali in apple. Phytopathology 61:904 (Abstr.).
11. POWELL, D., & R. NAIK. 1971. An apple canker in Illinois. Plant Dis. Reptr. 55:96.
12. REDDY, M. N., & M. A. STAHMANN. 1972. Multiple molecular forms of enzymes in peas infected with Fusarium oxysporum f. sp. pisi race 1. Phytopathol. Z. 74:55-68.
13. SAKO, N., & M. A. STAHMANN. 1972. Multiple molecular forms of enzymes in barley leaves infected with Erysiphe graminis f. sp. hordei. Physiol. Plant Pathol. 2:217-226.
14. SITTERLY, W. R., & J. R. SHAY. 1960. Physiological factors affecting the onset of susceptibility of apple fruit to rotting by fungus pathogens. Phytopathology 50:91-93.
15. STAPLES, R. C., & M. A. STAHMANN. 1963. Malate dehydrogenase in the rusted bean leaf. Science 140:1320-1321.
16. STAPLES, R. C., & M. A. STAHMANN. 1964. Changes in proteins and several enzymes in susceptible bean leaves after infection by the bean rust fungus. Phytopathology 54:760-764.
17. STAVELY, J. R., & E. W. HANSON. 1967. Electrophoretic comparison of resistant and susceptible Trifolium pratense noninoculated and inoculated with Erysiphe polygoni. Phytopathology 57:482-485.
18. URITANI, I., & M. A. STAHMANN. 1961. Changes in nitrogen metabolism in sweet potato with black rot. Plant Physiol 36:770-782.
19. WALLACE, J., J. KUĆ, & H. N. DRAUDT. 1962. Biochemical changes in the water insoluble material of maturing apple fruit and their possible relationship to disease resistance. Phytopathology 52:1023-1027.
20. WEBER, D. J., B. CLARE, & M. A. STAHMANN. 1967. Enzymatic changes associated with induced and natural resistance of sweet potato to Ceratocystis fimbriata. Phytopathology 57:421-424.