Activity of *o*-Diphenol Oxidase in Postharvest Apple Decay by *Penicillium expansum* and *Physalospora obtusa*

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ABSTRACT

The decayed areas of 'McIntosh' apples inoculated with *Penicillium expansum* after controlled atmosphere (CA) storage were darker than the same areas of apples inoculated in the identical manner after refrigerated storage. *o*-Diphenol oxidase activity was lower in sound apples after CA storage than in apples from refrigerated storage.

There was little suppression of the *o*-diphenol oxidase system in extracts from CA-stored McIntosh apples when these were mixed with extracts from tissue decayed by *P. expansum*, but in refrigerated apples there was an 87% reduction in *o*-diphenol oxidase activity. *o*-Diphenol oxidase inhibitors were present in both cases, but the *o*-diphenol oxidase from CA-stored apples was not as sensitive to the inhibitors.

When 3,4-dihydroxyphenylalanine (DOPA) and catechol were used as substrates, *o*-diphenol oxidase activity was altered in all areas of apples inoculated with either *P. expansum* or *Physalospora obtusa*. No *o*-diphenol oxidase activity was found in rotted areas in refrigerated and CA-stored apples rotted by *P. expansum* and in the early stages of apples infected by *P. obtusa*. As *P. obtusa* decay progressed, the skin turned from brown to black and the pulp darkened, at which time *o*-diphenol oxidase activity was found.

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Penicillium expansum Link causes the most common rot of apples in refrigerated storage. A wound is generally necessary for infection to occur; fruits in contact with decaying fruits often do not rot unless the skin is broken or bruised (7). A break in the skin or a bruise stimulates the browning reaction. Oxidized phenols can inhibit pectolytic enzymes involved in tissue maceration by pathogens, and oxidized phenols are sometimes fungicidal (9). Since *P. expansum* is known to have pectolytic enzymes (2, 3), it seems reasonable that it may be to the pathogen's advantage to inhibit the apple's *o*-diphenol oxidase (*o*-diphenol: O_2 oxidoreductase, [EC 1.10.3.1] system. Walker (10) showed that in 'Granny Smith' apples the *o*-diphenol oxidase system was inhibited by *P. expansum*. We found that decayed tissue of 'McIntosh' apples inoculated after refrigerated storage was light colored; whereas, decayed tissue in fruit inoculated after CA storage was dark. This observation prompted an investigation on the browning reaction in infected apples.

Physalospora obtusa (Schw.) Cke. is the causal agent of black rot. There is no measurable *o*-diphenol oxidase activity in extracts from rotted tissue 3-7 days after inoculation. But 10-17 days after inoculation, the skin of

the infected apple turns from brown to black and the pulp also darkens. From this observation, the question arises on the enzymatic source of the coloration in black rot.

MATERIALS AND METHODS.—McIntosh, 'Greening', and Granny Smith apples stored no longer than 3 months in refrigerated storage at 0 C and McIntosh apples stored for 7-8 months in controlled-atmosphere storage were used in this study. The CA storage conditions were as follows: Temperature was 4 C, the CO_2 level was maintained at 5%, and the O_2 level at 2%.

Sound apples were inoculated after removal from storage with either *P. expansum* or *P. obtusa* originally isolated from apples. An apple was wounded with a sterilized glass rod and some infected tissue or a mycelial plug was placed in the wound. The inoculated apples were stored in perforated plastic bags at 25 C.

Samples were taken from four apple areas: (i) sound tissue from noninoculated apples; (ii) outer tissue 3 cm or more from visibly rotted tissue; (iii) edge tissue 0.5 mm away from visibly rotted tissue; and (iv) visibly rotted tissue. Rotted tissue was sampled up to 20 days after inoculation with *P. obtusa* decay.

All tissue was homogenized by hand with a mortar and pestle at 4 C. One g of tissue/ml of the test buffer was used. All the following buffers were used for tests to determine the pH optimum with CA-stored McIntosh apples: 0.4 M acetate buffer, pH 4.0, 4.6, and 5.0; 0.2 M citrate-0.4 M phosphate buffer, pH 5.4; 0.4 M phosphate buffer, pH 6.0, 6.5, and 7.0. For comparisons between refrigerated and CA-stored apples, 0.1 M phosphate buffer, pH 7.0, was used. The crude extract was filtered through Whatman No. 1 filter paper. One and one-half ml of crude extract was added to each of three test tubes, all of which contained 2.5 ml of the test buffer and 0.1 ml H_2O. Test tube 1 was the blank, test tube 2 contained 1 ml of 0.001 M catechol, and test tube 3 contained 1 ml of 0.001 M 3,4-dihydroxyphenylalanine (DOPA). The tubes were incubated at 25 C for 30 min, centrifuged for 1 min at 1,000 *g*, and the absorbance read at 475 nm (5). A minimum of three readings was taken for each test.

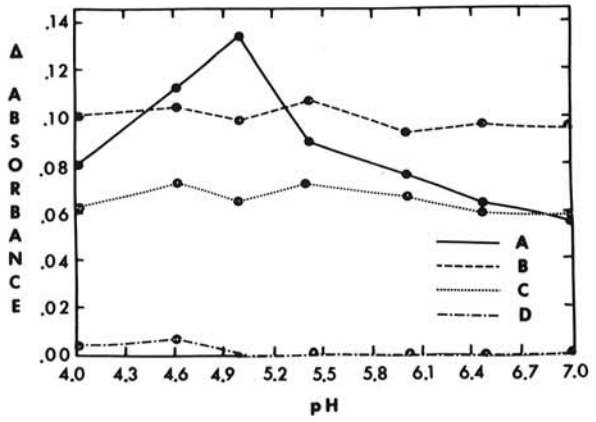

Fig. 1. Effects of *Penicillium expansum* decay on *o*-diphenol oxidase from A = noninfected tissue, B = outer tissue (tissue taken 3 cm or more away from visibly rotted tissue), C = edge tissue (tissue taken 0-5 mm away from visibly rotted tissue) and D = rotted tissue of CA-stored McIntosh apples. One g of tissue/ml of buffer was homogenized at 4 C, then filtered. All tubes contained 2.5 ml of buffer, 0.1 ml H_2O, 1.5 ml of crude extract, and 1 ml of 0.001 M catechol. The tubes were incubated for 30 min at 25 C, centrifuged for 1 min at 1,000 *g* and the absorbance read at 475 nm. The following buffers were used: 0.4 M acetate pH 4.0, 4.6, and 5.0; 0.2 M citrate-0.4 M phosphate pH 5.4; 0.4 M phosphate pH 6.0, 6.5, and 7.0. Each point is the average of a minimum of six readings.

RESULTS.—*Effect of storage conditions upon o-diphenol oxidase activity in sound McIntosh apples.*—*o*-Diphenol oxidase activity was 60% lower in CA-stored McIntosh apple tissue compared to refrigerated McIntosh apple tissue at pH 7.0 using catechol as the substrate. A pH optimum of 5.0 was observed for *o*-diphenol oxidase in noninfected CA-stored McIntosh apple tissue (Fig. 1).

Effect of P. expansum on o-diphenol oxidase of CA-stored and refrigerated McIntosh apples.—In both refrigerated and CA apples no *o*-diphenol oxidase activity was found in tissue decayed by *P. expansum* (Fig. 1). Edge tissue taken 0 to 5 mm and outer tissue taken at least 3 cm away from the visibly rotted tissue were also tested. For the CA-stored McIntosh apple tissue, enzyme activity for the outer tissue was higher than for the sound tissue at pH 4.0, 5.4, 6.0, and 7.0; at pH 4.6 and 5.0 it was lower (Fig. 1). Activity was less in the edge tissue at all pH values (Fig. 1); also, there was no optimum pH for the outer or edge tissue (Fig. 1). *o*-Diphenol oxidase activity at pH 7.0 from refrigerated McIntosh outer tissue was higher than from the noninfected tissue (an absorbance of 0.28 compared to 0.21 using DOPA as a substrate), whereas the activity from the edge tissue was less than for the noninfected tissue (an absorbance of 0.09 as compared to 0.21 using DOPA as a substrate). Walker (10) did not compare outer tissue with noninfected tissue. Our results indicate that outer tissue as well as rotted tissue and edge tissue had been physiologically altered.

Inhibitor study with CA-stored and refrigerated McIntosh apples rotted by P. expansum.—Equal weights of sound tissue and tissue decayed by *P. expansum* were extracted with equal volumes of buffer. The enzyme activity of this extract was compared with an extract of one gram of sound tissue per ml of buffer (Fig. 2). Fifty percent of the *o*-diphenol oxidase activity was still present in the mixed extracts using sound CA-stored tissue. In extracts from refrigerated apples, an 87% reduction in activity was seen at pH 7.0. The inhibitory substances were removed by running the rotted tissue extract through a 10-ml Sephadex column (G-50 medium). These inhibitors were more effective on refrigerated tissue extracts than on CA tissue extracts. The inhibitors probably included ferulic acid and *p*-coumaric acid, two *o*-diphenol oxidase inhibitors reported by Walker (10) to be associated with *P. expansum* decay.

Effect of P. obtusa on o-diphenol oxidase of CA-stored and refrigerated McIntosh apples.—In both CA and refrigerated apples the *o*-diphenol oxidase activity from the outer and edge tissue was less than for the sound tissue. In the CA stored McIntosh apple tissue, a pH optimum of 5.0 was noted for both the outer and edge tissue (Fig. 3). Furthermore, in both cases no detectable *o*-diphenol oxidase activity was found in the tissue rotted by *P. obtusa* up to 8 days after inoculation. However, *o*-diphenol oxidase activity was found in the decayed area using DOPA as a substrate in both cases 10-17 days after inoculation when the apple's skin and pulp were

darkening. In CA-stored McIntosh apple tissue infected by *P. obtusa*, a 60% increase in enzyme activity over the noninfected tissue was noted 17 days after inoculation. For the refrigerated McIntosh apple tissue infected by *P. obtusa*, the absorbance increased from 0.02 to 0.12 when the apple's skin and pulp were darkening. Tests for *o*-diphenol oxidase inhibitors in apple tissue infected by *P. obtusa* proved negative.

Effect of P. expansum on Greening and Granny Smith apples.—The decayed area of Greening apples had two distinct color zones. The zone on the periphery was brown and soft, whereas the inner zone was white and firm. The darkness of the outer zone was pronounced, indicating that *o*-diphenol oxidase system of the apple tissue had been active. Decayed areas of Granny Smith apples were uniformly light colored.

Effect of bruising on o-diphenol oxidase of CA-stored and refrigerated McIntosh apples.—Apples were bruised by rubbing them firmly against a lab bench. In both CA and refrigerated apples a reduction of *o*-diphenol oxidase activity was noted. After 1 day, a 33 to 55% loss of *o*-diphenol oxidase activity at pH 7.0 was noted in the bruised tissue of CA-stored McIntosh apples, whereas a 65% loss of enzyme activity was noted in the bruised tissue of refrigerated McIntosh apples. After 4 days no *o*-diphenol oxidase activity was detected in either case.

DISCUSSION.—*o*-Diphenol oxidase activity is different in refrigerated and CA-stored McIntosh apples. After CA storage there is a net decrease in activity. Cheung & Henderson (1) found with potatoes that prolonged storage led to a loss of the more active forms of *o*-diphenol oxidase; only the less active forms were still present. Constantinides & Bedford (4) showed that *o*-diphenol oxidase isozymes from several plants differ in their susceptibility to certain inhibitors.

In CA-stored McIntosh apples there is little effect on the *o*-diphenol oxidase system by soluble inhibitors associated with *P. expansum* decay. Our data with refrigerated McIntosh apples and Walker's data with Granny Smith apples (10) indicate there is a dramatic reduction of the *o*-diphenol oxidase system in apple tissue rotted by *P. expansum*. The rotted areas were light colored in these apples. From this, one can postulate that the isozymes responsible for browning in CA-stored McIntosh apples are less susceptible to the inhibitors present in the rotten tissue, than the isozymes responsible for browning in refrigerated McIntosh apples. However, bruising also reduced *o*-diphenol oxidase activity. This may be due to inhibition by the products of the browning reaction (6, 8) and may account for a significant part of the reduction in *o*-diphenol oxidase activity in apple rotted by both *P. expansum* and *P. obtusa*.

The fact that *o*-diphenol oxidase activity is not greatly affected by soluble inhibitors present in Greening and CA-stored McIntosh apples rotted by *P. expansum* indicates that, though *P. expansum* may indirectly cause the formation of inhibitors of *o*-diphenol oxidase [by the hydrolysis of chlorogenic acid and *p*-coumarylquinic acid to yield caffeic acid and *p*-coumaric acid (respectively), and the subsequent O-methylation of caffeic acid to yield ferulic acid] (10), it has little to do with the ability of the fungus to invade the apple. This does not support the hypothesis that the *o*-diphenol oxidase system is acting as a defense against fungal invasion (6, 9), especially with pathogens that invade wounded tissue. But the question still remains: Why is there a difference in the browning

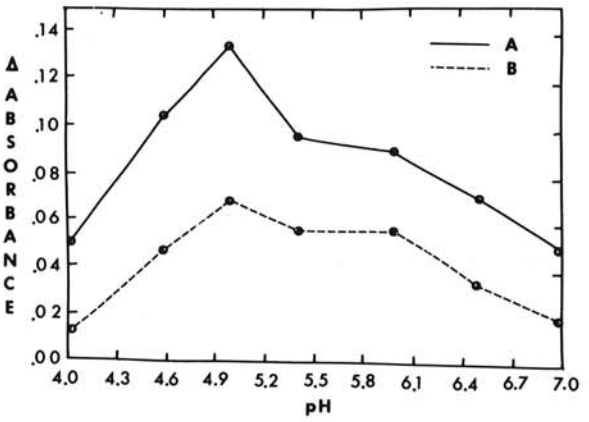

Fig. 2. Inhibitor study with CA-stored 'McIntosh' apples rotted by *Penicillium expansum*. A = 1 g of noninfected apple tissue/ml of buffer and B = 0.5 g of noninfected apple tissue and 0.5 g of tissue rotted by *P. expansum*/ml of buffer. The tissue was homogenized at 4 C, then filtered. All tubes contained 2.5 ml of buffer, 0.1 ml H_2O, 1.5 ml of crude extract, and 1 ml of 0.001 M catechol. The tubes were incubated for 30 min at 25 C, centrifuged for 1 min at 1,000 *g*, and the absorbance read at 475 nm. The following buffers were used: 0.4 M acetate pH 4.0, 4.6, and 5.0; 0.2 M citrate-0.4 M phosphate pH 5.4; 0.4 M phosphate pH 6.0, 6.5, and 7.0. Each point is the average of a minimum of three readings.

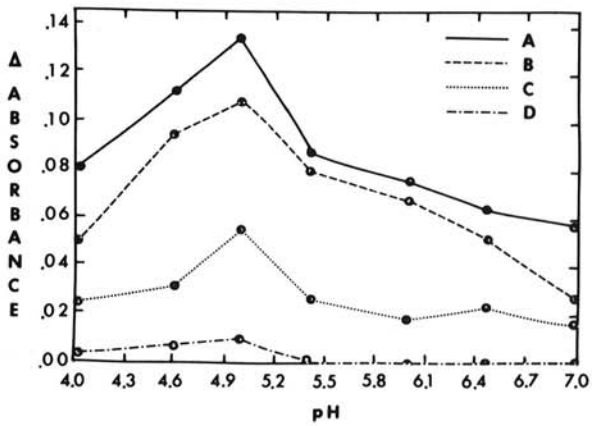

Fig. 3. Effects of *Physalospora obtusa* decay on *o*-diphenol oxidase from A = noninfected tissue, B = outer tissue (tissue taken 3 cm or more away from visibly rotted tissue), C = edge tissue (tissue taken 0-5 mm away from visibly rotted tissue), and D = rotted tissue of CA-stored 'McIntosh' apples. One g of tissue/ml of buffer was homogenized at 4 C, then filtered. All tubes contained 2.5 ml of buffer, 0.1 ml H_2O, 1.5 ml of crude extract, and 1 ml of 0.001 M catechol. The tubes were incubated for 30 min at 25 C, centrifuged for 1 min at 1,000 g and the absorbance read at 475 nm. The following buffers were used: 0.4 M acetate pH 4.0, 4.6, and 5.0; 0.2 M citrate-0.4 M phosphate pH 5.4; 0.4 M phosphate pH 6.0, 6.5, and 7.0. Each point is the average of a minimum of six readings.

reaction between Granny Smith and refrigerated McIntosh apples, where the inhibitors are effective, and Greening and CA-stored McIntosh apples, where the inhibitors are not effective?

In black rot, the increase in *o*-diphenol oxidase activity (with DOPA used as the assay substrate) as the apple is turning black indicates that the darkening is due to the pathogen's *o*-diphenol oxidase system. The tests with noninfected, CA-stored, McIntosh apples support this since the apple's *o*-diphenol oxidase system oxidized DOPA slowly. Also, microscopic examination revealed that the *P. obtusa* mycelia were darkly pigmented only at the time the skin and pulp were dark. Characterization of the isozymes of apples infected by *P. obtusa* and *P. expansum* by polyacrylamide disc-gel electrophoresis is pending.

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