Effect of Scar Skin and Dapple Apple Diseases on Certain Groups of Phenolic Compounds in Apple

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ABSTRACT

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Scar skin and dapple apple are caused by graft-transmissible agents presumed but not yet proved to be viruses. In Red Delicious apple, scar skin results in consistently higher amounts of total phenols, flavonols, and chlorogenic acids but in lower amounts of anthocyanins. In Hyslop Crab apple, dapple apple appeared to have no significant effect on the levels of total phenols, flavonols, or chlorogenic acids, but resulted in markedly lower levels of anthocyanins. Comparison of the effects of these two diseases on phenolic metabolism with the symptoms of the diseases on the fruits indicates that changes in phenolic metabolism are associated with the events observed in development of symptoms.

Additional key words: Anthocyanins, flavonols, chlorogenic acid, total phenolics.

Scar skin and dapple apple are diseases of apple caused by graft-transmissible agents presumed but not yet proved to be viruses. Scar skin results in corky, scarred, necrotic areas on the surface of fruits of Red Delicious apples; brown necrotic tissue may cover up to 50% or more of the fruit surface (6). On the other hand, dapple apple results in discoloration but no necrosis of the surface of Hyslop Crab apples (12). Browning of apples in response to injury (18) and development of necrotic symptoms in plants in response to infection are generally thought to result from accumulation of phenolic compounds (8,10,13), which are subsequently oxidized and polymerized to form the brown substances observed after injury or infection. This implies that development of symptoms is associated with altered phenolic metabolism due to injury resulting from infection. The purpose of our study was to determine the effects of scar skin and dapple apple on the quantity and quality of certain groups of phenolic compounds in the peel of apple fruits during the various stages of fruit development and to determine if these changes were correlated with appearance and development of symptoms in the infected fruit.

MATERIALS AND METHODS

The apple fruits used in this study were taken from trees of the cultivars Hyslop Crab and Red Delicious growing in the University's orchard in Belchertown, MA. Hyslop Crab trees had been graft inoculated with buds from dapple apple-affected trees and Red Delicious with buds from scar skin-affected trees in 1968. Fruits of healthy and infected apples were collected every 20 days from 10 June through 8 October, 1975 and 1976. In the first collection, owing to the smallness of the fruit, the outer portion was used for analysis after removal of the core, but in subsequent collections the fruit was peeled with a potato peeler and only the peel was analyzed. Each replication used 10-g samples from 10 fruits, and three replications were used for each assay.

Extraction and quantitative determination. *Total phenolics.* Immediately after the apples were peeled, 10 g of peel was placed in 150 ml of methanol and boiled for 20 min. The methanol extract then was decanted and 150 ml of 50% methanol was added to the tissues, which were fragmented at high speed in a Waring Blendor for 2 min. The mixture then was boiled for 20 min, after which the supernatant was decanted and the residue boiled again with 50% methanol. Finally, the last boiled preparation and decanted supernatants were pooled and filtered through Whatman No. 1 filter paper. The final volume of the extract was made to 150 ml with methanol. The amount of total phenols was determined colorimetrically according to the method of Swain and Hillis (16).

Chlorogenic acids. Ten grams of apple peel were blended in a Waring Blendor with 50 ml of 95% ethanol containing 200 mg of ascorbic acid to prevent browning. The extract then was filtered through Whatman No. 1 paper and the residue was washed with 95% ethanol until a total of 100 ml of filtrate was collected. Chlorogenic acids were estimated colorimetrically according to the method of Zucker and Ahrens (21).

Flavonols extracted for paper chromatography. Ten grams of apple peel were blended in 70% methanol, and the total volume of the flavonol-containing extract was made to 200 ml (3). Three extracts obtained by this procedure were combined and concentrated to 80 ml in a rotary evaporator. The concentrated extract was washed twice with equal volumes of petroleum ether and four times each with ethyl ether and ethyl acetate. Ethyl ether and ethyl acetate extracts were combined and dried in a rotary evaporator. The flavonols were taken up in 10 ml of 95% ethanol and stored at 4 C until they were analyzed by paper chromatography.

Total anthocyanins and flavonols. The method of Lees and Francis (3,4) was followed, with minor modifications. Ten grams of apple peel were ground in a mixture of 95% ethanol and 1.5 N HCl (85:15 v/v) to yield a total of 100 ml of acidified ethanolic extract. The absorptivity of this ethanolic extract containing flavonols and anthocyanins was measured colorimetrically at 374 nm for flavonols and at 535 nm for anthocyanins. The quantities of total anthocyanins and total flavonols per gram of tissue were determined by using the following equations (4):

$$\frac{\text{Total anthocyanins}}{\text{10 g of tissue}} = \frac{\text{Absorbance} \times \text{dilution factor}}{98.2}$$

and

$$\frac{\text{Total flavonols}}{\text{10g of tissue}} = \frac{\text{Absorbance} \times \text{dilution factor}}{76.6}$$

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in which 98.2 is an average extinction coefficient for anthocyanins and 76.6 a similar coefficient for flavonols.

Separation by paper chromatography. *Chlorogenic acids.* An aliquot of a crude 95% ethanol extract of apple peel containing chlorogenic acids was applied as a streak (about 2 cm long) across a Whatman No. 3 M paper. The chromatograms were irrigated with water for 8 hr. Appropriate bands on the chromatogram were cut and eluted with 95% ethanol and absorptivities of the resulting solutions were measured colorimetrically at 330 nm. Standard chlorogenic acid (Sigma Chemical Co., St. Louis, MO 63178) was run for comparison and to make possible quantitative determinations.

In addition, chlorogenic acid was eluted with 95% ethanol from bands cut from the chromatogram and was rechromatographed in butanol/acetic acid/water (BAW), 6:1:2. The bands from the BAW chromatograms then were eluted and the resulting solutions chromatographed once more in 3% acetic acid. The band of purified chlorogenic acid was eluted with 100% methanol (spectral grade). The absorption spectrum was determined over the range of 200–400 nm with a DBG Beckman spectrophotometer, using the eluate from a blank area cut from the same chromatogram as a control. Chlorogenic acid standards were treated in an identical manner.

R_f data for chromatographically purified chlorogenic acid were obtained on Whatman No. 1 paper. The following solvent systems were selected for chromatography: butanol/acetic acid/water (6:1:2); distilled water (H_2O); 3% acetic acid (HAc); formic acid (Formic)/concentrated HCl/H_2O (5:2:3).

Flavonols. Separation of flavonols from the ethyl ether and ethyl acetate extracts was done according to the method of Siegelman (11). Separated flavonol bands were detected under ultraviolet (UV) light and were identified as flavonols on the basis of spectral analysis. The favonol bands were cut from the chromatogram and eluted with 95% ethanol. The absorptivity of eluates was measured at 374 nm.

Anthocyanins. Anthocyanins were purified and separated on Whatman No. 3 M paper by the method of Sun and Francis (15), and eluted with a mixture of methanol/acetic acid/water (9:5:5). The eluate was quantitatively determined by the method described for total anthocyanins.

RESULTS

Typical symptoms developed in scar skin-affected Red Delicious apples and dapple apple-affected Hyslop Crab apples during the years (1975 and 1976) in which collections were made for phenolic compound analysis.

Total phenols. *Effect of scar skin on total phenols.* Consistently higher amounts of total phenols were found in infected than in healthy tissues (Fig. 1A). Scar skin-affected apples had from 9 to 73% more total phenols in 1975 and from 29 to 114% more total phenols in 1976 than did healthy apples (Table 1).

Effect of dapple apple on total phenols. No significant difference in the amount of total phenols was observed between healthy and dapple apple-affected Hyslop Crab apples (Fig. 1B). The pattern of total phenol changes during fruit development was similar for both healthy and diseased apples.

Chlorogenic acids. *Effect of scar skin on chlorogenic acids.* Scar skin resulted in concentrations of chlorogenic acids higher than those of healthy apples (Fig. 2A). Increased accumulation of chlorogenic acids was detected in infected tissues at the first collection, when symptoms were not yet visible.

At least eight bands were observed under UV light on one-way paper chromatograms of crude ethanolic extracts of apple peel. The bands were numbered 1 through 8, starting from the origin. Differences in the color intensity of certain blue fluorescent bands, particularly the bright blue band labeled Band 6, appeared on chromatograms for scar skin-affected tissues. For healthy apples, a sharp decrease in color intensity was associated with Band 6 as the apples enlarged, and the compound was not present in tissues harvested at later stages of fruit growth (Fig. 2B and C). For diseased fruit, Band 6 retained its bright blue color intensity throughout the period of fruit growth. Band 6 components, later identified as chlorogenic acids, were 1.6 to 15 times more concentrated in scar skin-affected fruit than in healthy apples (Fig. 2B) and were detected before symptoms were visible (30 June). Similar results were obtained from chromatography of chlorogenic acids from ethyl ether and ethyl acetate extracts obtained primarily for the determination of flavonols (Fig. 2C).

By using 3% acetic acid, the components of Band 6 could be resolved into compounds detected as two bands, which were designated Band 6_A and Band 6_B.

On the basis of R_f values in several solvents, the reaction with ammonia and basic Hoepfner reagents, and the color under UV

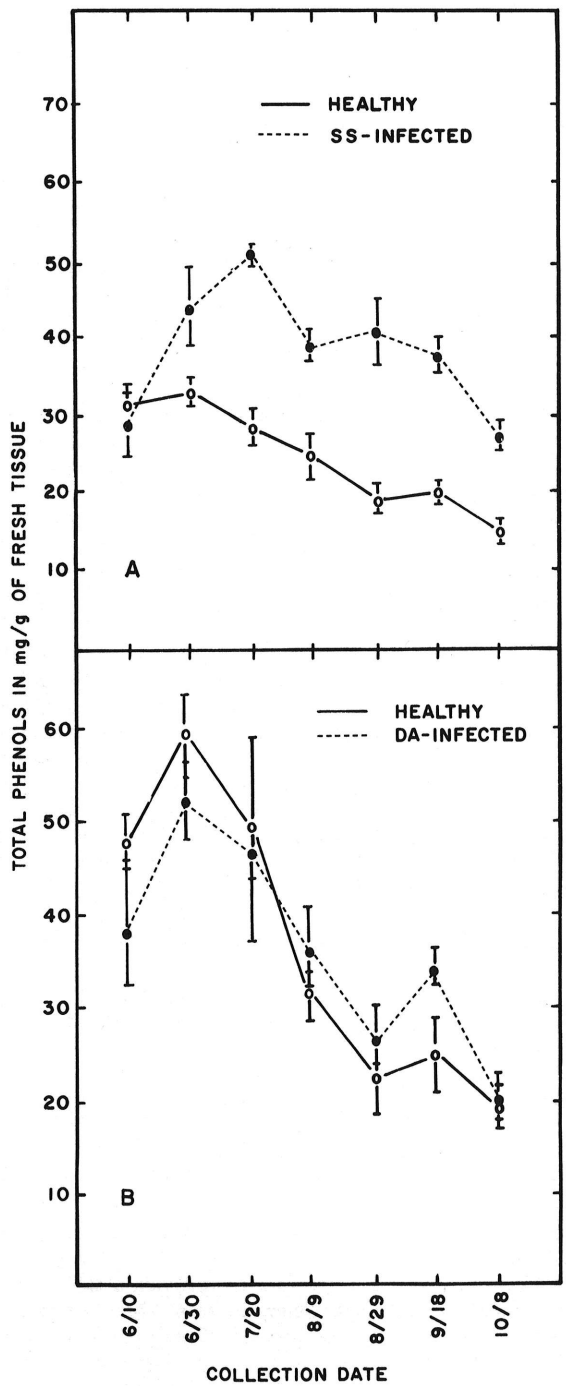

Fig. 1. Changes in concentration of total phenols in apple peel during development of healthy, and **A)** scar skin (SS)-affected Red Delicious, and **B)** dapple apple (DA)-affected Hyslop Crab fruits. Each point represents the average of three replications; the vertical bars represent standard deviations of those measurements.

light, the component of Band 6_A was indistinguishable from standard (Sigma Chemical Co.) chlorogenic acid. Also, the UV absorption spectrum for Band 6_A component was like that for chlorogenic acid. Band 6_B component had the same R_f values as did caffeic acid in 3% acetic acid and BAW solvent.

Effect of dapple apple on chlorogenic acid. No. significant difference in the quantity of chlorogenic acid or in the pattern of changes in chlorogenic acid content was observed for healthy and for dapple apple-affected apples (Fig. 3A).

Paper chromatography of crude extracts revealed seven bands but no significant difference in the major phenolic acid components determined for healthy and for dapple apple-affected apples. Figures 3B and C illustrate the pattern of changes for the brightest fluorescent band, Band 6, for infected and noninfected fruits. Band 6 component was resolved into Band 6_A, the component of which was identical to chlorogenic acid on the basis of R_f values in several solvent systems, the reaction with color reagents, and the color under UV light, and Band 6_B, which was not further characterized.

Flavonols. *Effect of scar skin on flavonols.* Scar skin-affected apples contained considerably higher levels of flavonols than did healthy apples (Fig. 4A). The flavonol content of diseased apples was higher than for healthy apples by 19–99% in 1975 and by 40–98% in 1976 (Table 1).

One-way descending paper chromatography of ethyl ether and ethyl acetate apple extracts, which contain the flavonols, resulted in at least nine bands that fluoresced under UV light; these were numbered 1 through 9 from the top of the chromatogram. The amount of fluorescence associated with Band 2 was noticeably higher for diseased than for healthy apples. The pattern of changes noted for Band 2 was similar to that determined for total flavonols. Components of Band 2 were separated by paper chromatography into compounds that were located as three bands and designated as Band 2_A, 2_B, and 2_C; these materials had UV spectra apparently identical to that of quercetin-3-galactoside. Band 3 apparently contained one component; it had a UV spectrum identical to quercetin-3-rhamnoside and was at a slightly lower concentration

TABLE 1. Concentration (mg/g of fresh tissues) ranges of four groups of phenolic compounds from healthy and from scar skin-affected Red Delicious apples and dapple apple-affected Hyslop Crab apples during fruit development in 1975 and 1976

Phenolic compounds	Red Delicious					
	1975			1976		
	Healthy	Diseased	% Difference	Healthy	Diseased	% Difference
Total phenols	21.2–45.2	26.7–67.7	9–73	14.9–33.4	27.4–42.9	29–114
Chlorogenic acids	...	...	...	5.7–12.27	8.38–17.5	16–79
Flavonols	0.39–1.15	0.46–1.6	19–99	0.6–1.08	0.6–1.78	40–98
Anthocyanins	0.015–0.58	0.015–0.34	0–41	0.02–0.78	0.02–0.29	0–63
	Hyslop Crab					
Total phenols	18.9–59.3	18.6–51.3	±0	16.7–44.2	19.8–44.0	±0
Chlorogenic acids	...	...	...	4.8–13.9	4.6–15.5	±0
Flavonols	0.33–0.87	0.41–0.91	±0	0.53–0.94	0.53–0.92	±0
Anthocyanins	0.017–0.9	0.017–0.34	0–62	0.02–1.19	0.02–0.65	0–46

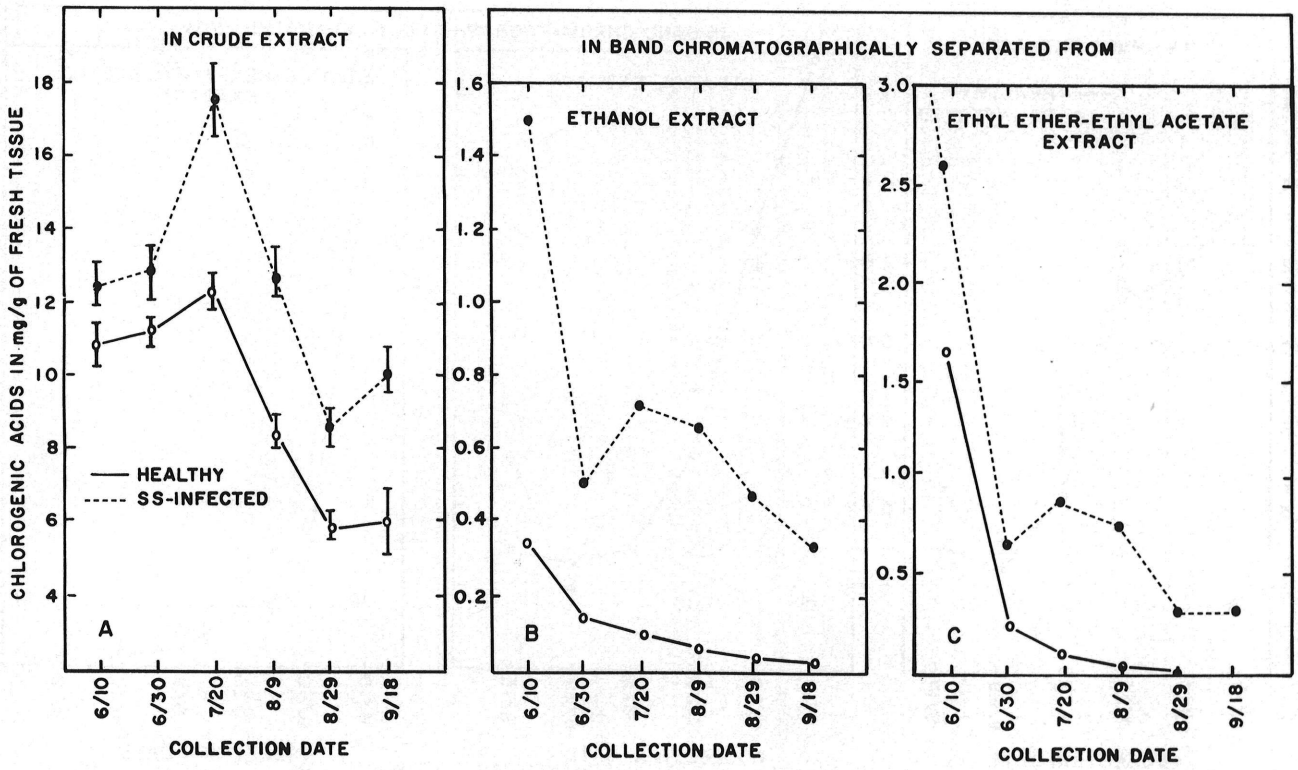

Fig. 2. Changes in concentration of chlorogenic acids in apple peel during the development of healthy and scar skin (SS)-affected Red Delicious fruits. The curves represent chlorogenic acid concentrations in **A)** crude extracts and in Band 6 chromatographically separated from **B)** ethanol extract or **C)** ethyl ether-ethyl acetate extract. Each point represents the average of three replications in graph A and two replications in graphs B and C.

in diseased than in healthy apples.

Effect of dapple apple on flavonols. Dapple apple appeared to have no significant effect on flavonol metabolism (Fig. 4B). No difference in flavonol composition was detected by paper chromatography for healthy and diseased apples.

Anthocyanins. *Effect of scar skin on anthocyanins.* A rapid synthesis of anthocyanins occurred both in infected and in uninfected fruits that remained after the fifth collection (August 29). Production of anthocyanins was reduced considerably by scar skin (Fig. 5A): anthocyanins in mature diseased fruit decreased by 41% in 1975 and 63% in 1976 from the concentration determined for healthy fruit of the same age (Table 1). Only a small portion of the reduction could be attributed to formation of corky surface tissue.

Crude extracts of anthocyanins from Red Delicious apples yielded three bands—one major and two minor bands designated as Bands 1, 2, and 3. Identical bands were obtained for extracts of healthy and diseased apples. As indicated by absorbance data, anthocyanin concentration was apparently higher in healthy than in diseased apples. Scar skin reduced the amount of Band 1 material by 62% and of the components of Bands 2 and 3 by 75% as compared with their concentration in extract from healthy tissues (Table 2). The patterns of changes for the three bands were similar to that for total anthocyanins.

Effect of dapple apple on anthocyanins. A rapid increase in anthocyanin content of Hyslop Crab apples was observed for apples remaining after the fourth collection (August 9). Anthocyanins were present in lower amounts in dapple apple-affected fruits than in healthy fruits (Fig. 5B). Mature dapple apple-affected fruits contained 62% less anthocyanins in 1975 and 46% less in 1976 than did mature healthy fruit (Table 1).

On paper chromatography, anthocyanin extracts from healthy or diseased Hyslop Crab apples usually resulted in three bands—one major and two minor ones, designated as Bands 1, 2, and 3. Prolonged irrigation of chromatograms of the extracts of mature fruit resulted in five bands. Qualitative changes in anthocyanins did not result from infection, but quantitative changes did occur. Band 1 material was decreased by 51%, Band 2 material by 53%, and Band 3 material by 30% from concentrations determined for these components in healthy apples (Table 2). The pattern of changes for the three bands was similar to that for total anthocyanins.

DISCUSSION

Little work has been done on the sequential changes in the levels of phenolic compounds during fruit development. Although some quantitative changes of phenolic compounds in whole apples have been reported (7,19), no information is available on phenolics in apple peel affected by diseases such as scar skin and dapple apple.

Amounts of apple phenolics seldom have been determined exactly (7). Different investigators (17) have reported marked differences in concentrations of phenolic compounds for various apple varieties. For Red Delicious apples, 2.2–3.45 mg of flavonols and 3.52–18.6 mg of anthocyanins per gram of fresh tissue have been reported (20). These figures are much higher than those that we determined for the same variety. Comparing the data for various studies is difficult, however, because of differences in extracting procedures, in methods used for quantitative determination, in varieties, and in stages and tissues of apple extracted and units in which the phenolic amounts were expressed (in milligrams per gram fresh weight, milligrams per gram dry weight, or milligrams per square centimeter of peel). In any case, our goal was to determine the relative amounts present in healthy and in symptomatic apple peel.

The increased amounts of total phenolics, chlorogenic acids, and flavonols associated with scar skin or dapple apple might be attributed simply to the formation of necrotic tissue. Compounds of all three types were present, however, in high amounts in diseased fruit even before necrotic symptoms appeared (usually after June 30), and not only did they not increase in concentration but also the total amounts of phenols and chlorogenic acids actually decreased as necrosis increased. Moreover, in dapple apple in which no necrosis whatsoever occurred, none of the phenolic

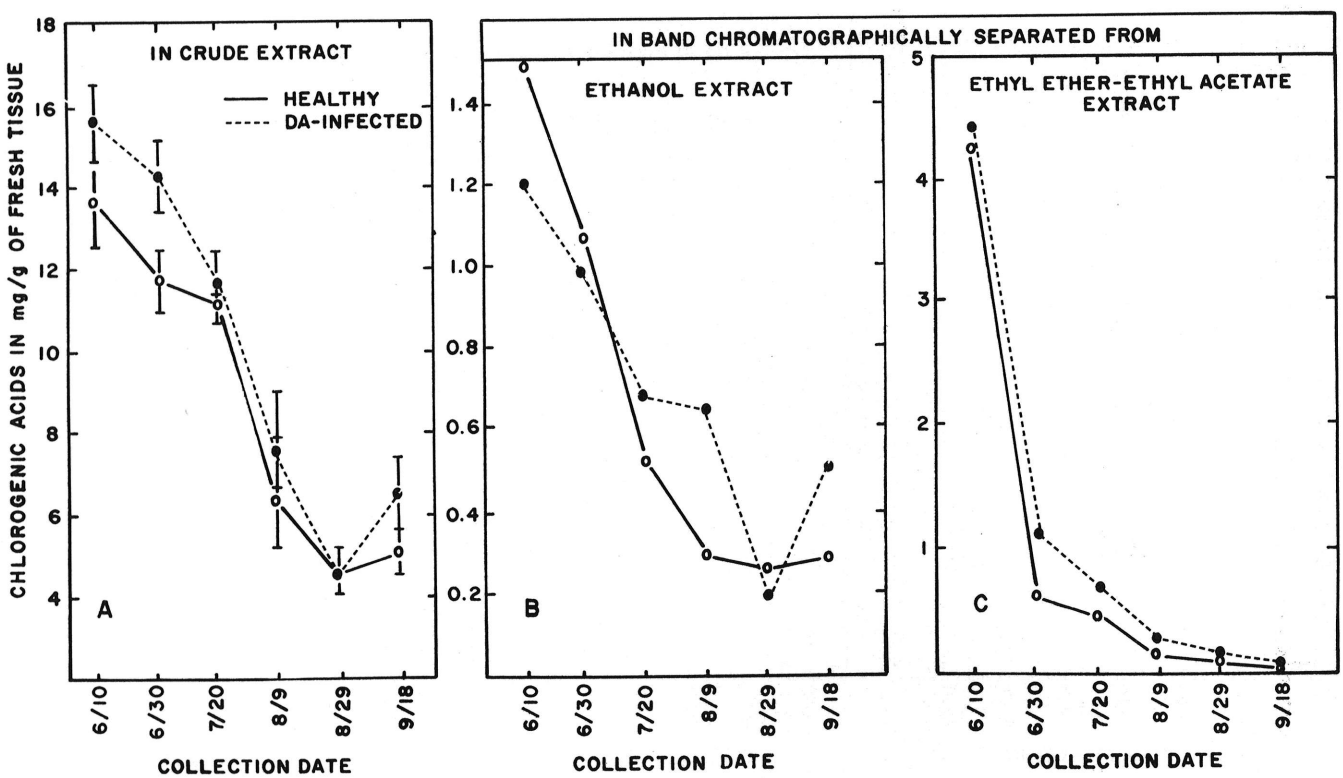

Fig. 3. Changes in concentration of chlorogenic acids in apple peel during development of healthy and dapple apple (DA)-affected Hyslop Crab fruits. The curves compare concentrations in **A)** crude extracts and in Band 6 chromatographically separated from **B)** ethanol extract or **C)** ethyl ether-ethyl acetate extract. Each point represents average of three replications in **A** and two replications in **B** and **C**.

compounds of any group increased above the levels found in healthy apples.

Accumulation of flavonols observed in scar skin-affected Red Delicious apples is in accordance with the findings described for other systemic infections, such as rusty mottle on cherry, western *X* disease on peach (2), and color-breaking virus on *Matthiola incana* (1).

Both scar skin and dapple apple caused significant reduction in fruit anthocyanins. The effect of both diseases on the levels of each of the three pigments is more or less similar. Reduction in anthocyanins by systemic infections is rather common in virus-infected and mycoplasma-infected flowers (5). The classical disease known as breaking of tulips involves localized inhibition of anthocyanin formation (14). Similar symptoms are caused by TMV on flowers of tobacco, by bean yellow mosaic on sweet pea (5), by color breaking virus on *Matthiola incana* (1), and by many mycoplasma diseases that result in virescence of flowers and poor fruit coloration (5).

Decreases in anthocyanins are unlikely to result from increases in flavonols, since dapple apple causes significant reduction of anthocyanins but has no effect on flavonols. A similar phenomenon was observed in virus-infected flowers (1) for which it was shown that the effects of virus on synthesis of anthocyanins and flavonols are almost essentially independent.

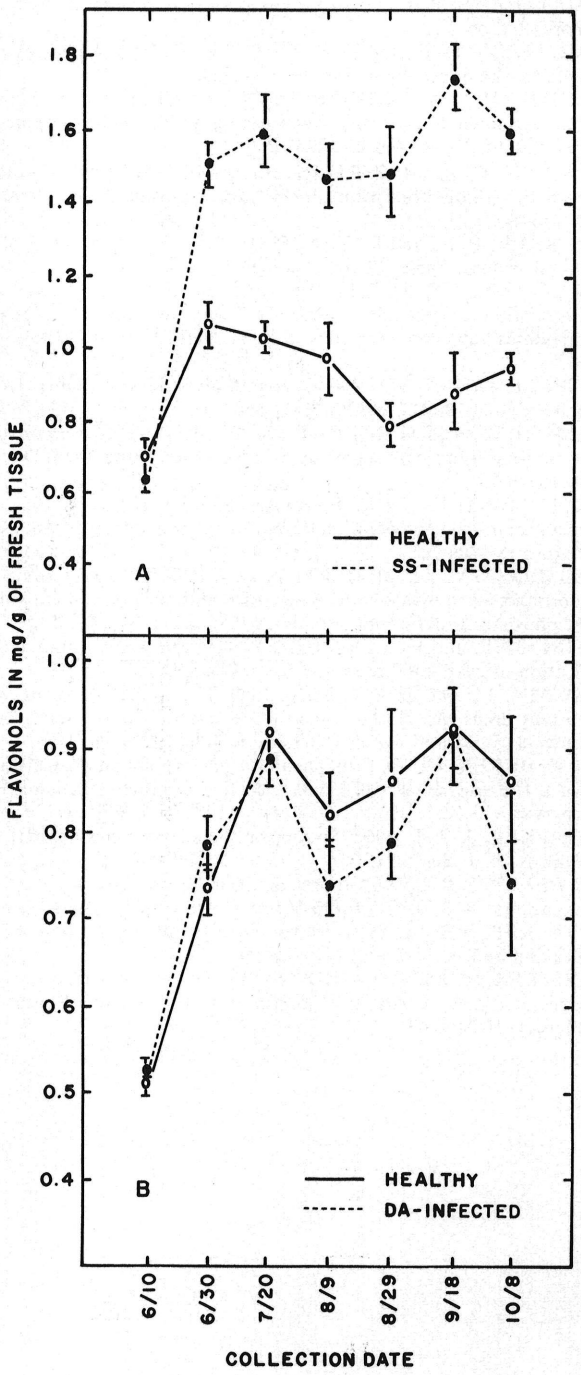

Fig. 4. Changes in concentration of flavonols in apple peel during development of healthy, and of **A)** scar skin (SS)-affected Red Delicious and of **B)** dapple apple (DA)-affected Hyslop Crab fruits. Each point represents the average of three replications; vertical bars represent the standard deviations of these measurements.

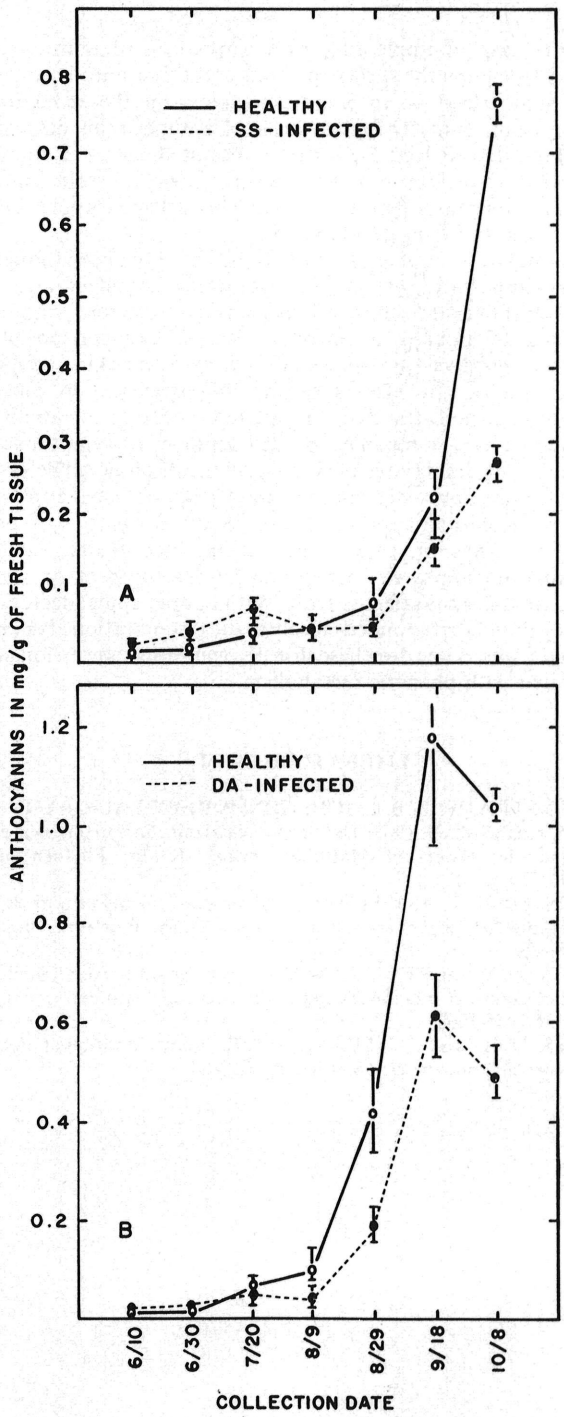

Fig. 5 Changes in concentration of anthocyanins in apple peel during development of healthy, and of **A)** scar skin (SS)-affected Red Delicious, and **B)** dapple apple (DA)-affected Hyslop Crab fruits. Each point represents the average of three replications; the vertical bars represent the standard deviations of these measurements.

TABLE 2. Concentration (mg/g of fresh tissue)[a] of chromatographically separated anthocyanins from apple peel of healthy and of scar skin-affected Red Delicious and dapple apple-affected Hyslop Crab apples at maturity

	Band[b] 1			Band 2			Band 3		
	Healthy	Diseased	Decrease (%)	Healthy	Diseased	Decrease (%)	Healthy	Diseased	Decrease (%)
Scar skin	0.24[c]	0.092	61.7	0.033	0.008	75.8	0.012	0.003	75
Dapple apple	0.43	0.21	51.2	0.045	0.021	53.3	0.01	0.007	30

[a]Measured according to method of Lees and Francis (3,4).
[b]Obtained from acidified ethanol extract of apple peel applied as streak across top of Whatman No. 3 paper and developed with mixture of butanol/acetic acid/water (6:1:2) for 24 hr.
[c]Each number represents average of two replications.

In the case of apple scar skin, formation of nonfunctional necrotic tissues on the surface of fruits at the time anthocyanins are being synthesized is an important factor in the reduction in anthocyanins. Slightly higher levels of anthocyanins detected in scar skin-affected Red Delicious apples at the second and third collection confirm earlier findings (9), ie, red pigment accumulation was observed in scar skin-affected apple fruit at similar stages of fruit development.

Accumulation of phenolic compounds has long been thought to play an important role in plant resistance to infection, in the formation of local lesions in hypersensitive reactions, and in the browning of injured or infected tissues. Comparison of the symptoms produced on apples affected with scar skin and dapple apple, and of the effects of the two diseases on phenolic metabolism, indicates that the altered phenolic metabolism is followed or is accompanied by development of symptoms. The significant accumulations of chlorogenic acids observed before and after the appearance of symptoms in scar skin-affected apples may be a particularly important factor in the formation of brown necrotic tissues on the surface of infected fruits. Flavonol accumulation appeared to parallel the development of necrotic tissues in scar skin-affected fruit. With dapple apple, decreases in anthocyanins corresponded closely to the discoloration observed in infected fruits. Thus, for these diseases symptom expression also is associated with phenolic metabolism.

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