Pigment Changes Associated with Application of Ethephon ((2-Chloroethyl)phosphonic Acid) to Fig (Ficus carica L.) Fruits

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ABSTRACT

The application of (2-chloroethyl)phosphonic acid (Ethephon) to 'Mission' fig fruits (Ficus carica L.) during late period II of their development stimulated ripening and change in color from green to bluish black within 8 days. Chlorophylls a and b decreased rapidly within 4 days after Ethephon treatment, and degradation continued at a decreasing rate for an additional 4 days, at which time the fruits had attained their maximum diameter and were considered fully ripe. Levels of β-carotene, lutein, violaxanthin, and neoxanthin decreased in a pattern similar to that of chlorophylls a and b. The rates of β-carotene and lutein degradation were initially greater than those of the xanthophyll pigments. Degradation rates of the various carotenoids were comparable 4 to 8 days after treatment.

There was no measurable anthocyanin synthesis during a 2- to 4-day period following Ethephon treatment. Beyond this lag phase, anthocyanin accumulation was linear, and the amount of pigment synthesized was a function of both light intensity and duration. Although Ethephon promoted the rate of anthocyanin accumulation, it did not increase the total amount of pigment synthesized in treated fruits. Etiolation of fruits from the time of Ethephon treatment until maturity stimulated an increase in growth and completely inhibited anthocyanin production in the skin. Ethephon-treated fruits which ripened while etiolated were larger in diameter and higher in both fresh and dry weights than nonetiolated controls.

Ethylene or ethylenogenic chemicals are frequently used to promote ripening, together with color changes in several products such as cantaloupes (15), citrus (13), and tomatoes (28). There is always a net degradation of Chl during the ripening or senescence of green fruits and vegetables (2, 13). The stability of various carotenoid pigments is a function of their association with cellular proteins and other substances. Thus, β-carotene in carrots, lycopene in tomatoes, and zeaxanthin in corn are relatively stable pigments which persist through prolonged storage (2). In other tissues such as senescing leaves, there is ultimately a net degradation of carotenoids (11).

Although there are reports of anthocyanin decolorizing systems in certain species (22, 30), the predominant pattern during senescence of most fruits and leaves is a net accumulation of anthocyanin. Anthocyanin synthesis is influenced by high carbohydrate concentration, stress, exposure to prolonged high light intensity, and various chemical stimuli.

The application of Ethephon to 'Mission' fig fruits at or after a critical stage of development stimulated their ripening within 8 days (5). Dramatic pigment changes occurred during this period as the fruit skins turned from green to bluish black (23, 24). This study reports the nature of pigment changes during the course of ripening.

MATERIALS AND METHODS

To facilitate timely Ethephon application, a fruit growth-in-diameter curve was developed using 'Mission' figs (Ficus carica L.) growing at the Wolfskill Experimental Orchards, Winters, Calif. Cross diameters of 15 fruits representing the basal-most fruit on 5 separate branches from each of three trees were measured weekly, using a vernier caliper. During the second half of period II (the period of slow growth), the leaves and fruits of 3 whole trees and 45 separate branches were sprayed with a 500 µg/g aqueous solution of Ethephon ((2-chloroethyl)phosphonic acid (Amchem 68-240)). Fruit samples of about 100 basal fruits were collected at the same time of spraying and thereafter at 2-day intervals until the fruits had attained their maximum diameter and were considered fully ripe.

Extraction and Isolation of Plastid Pigments. To minimize pigment degradation, all experimental manipulations were done at 1 to 4°C in reduced light. Strips of skin (consisting of epidermis and a few hypodermal cell layers) were peeled randomly from 5 fruits, weighed, and immediately homogenized in a VirTis 45 homogenizer with chilled acetone and 0.1 M NH₄OH (9:1, v/v). The homogenate was centrifuged at 10,000g for 10 min at −10°C in a Sorvall RC2-B centrifuge and then decanted. Using a 1:1 (v/v) mixture of basic acetone and diethyl ether, the pellet was re-extracted and centrifuged 3 times, or until no further pigment was extracted. Sufficient diethyl ether was added to the combined supernatant fluids to produce a final 1:1 (v/v) mixture of basic acetone and diethyl ether in the extract. The extract was partially purified by washing in a 10-fold volume of MgCO₃ saturated water. The apolar plastid pigments were retained in the ether supernatant fluid, whereas the acetone and water-soluble substances collected in the aqueous hypophase. The ether supernatant fluid was separated from the hypophase and centrifuged at 10,000g for 5 min to remove any traces of water. Then the pigment-containing ether extract was adjusted to a known volume, and Chl absorbance was determined on a Zeiss PMQ11 spectrophotometer. A sample of the pigment extract was concentrated under a N₂ stream, streaked on silica Gel H 500-µm thin layer plates, and developed in benzene-ethyl alcohol-H₂O (80:20:5.0, v/v/v). After their chromatographic separation, the xanthophyll-containing bands were scraped separately into ethyl alcohol, and those containing β-carotene into hexane, and each slurry was centrifuged at 20,000g for 10 min. The absorbance of the pigment-containing supernatant fluids was then determined spectrophotometrically.

Spectrophotometric Determinations. The concentrations of Chl a and b in diethyl ether were calculated using simultaneous equations derived by Comar and Zscheile (3). All absorbances were corrected for any light scattering by referring to a wave length-dependent scatter calibration curve as described previously (25).

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The individual carotenoids were estimated using specific absorption coefficients reported by Moster and Quackenbush (20) and recovery factors from Silica Gel H reported by Rebeiz et al. (25). All absorbances were corrected for light scattering as described earlier (25). The results were expressed as µg of pigment/g of fresh skin.

**Extraction of Anthocyanins.** The skin from treated and control fruits was peeled, weighed, and immediately submerged in 1% methanolic HCl to minimize oxidative degradation. Skin samples from unripe fruits were homogenized in a VirTis 45 homogenizer with 1% methanolic HCl. The homogenate was centrifuged at 20,000g for 10 min, after which the supernatant fluid was analyzed spectrophotometrically.

It was not necessary to homogenize ripening fruits since as fruit softening progressed (2–3 days after treatment), the anthocyanins were leached completely by overnight immersion of the skin at 5°C in 1% methanolic HCl. The extract was diluted with methyl alcohol to yield absorbance values with a range of 0.30 to 0.70, and was acidified with HCl to pH 1 to allow equilibration of the various colorless and colored forms of anthocyanin. After a 2-hr equilibration, spectrophotometric determinations were made on a Bausch and Lomb Spectronic 20 colorimeterspectrophotometer.

**Calculation of Total Anthocyanin.** Since cyanidin glycosides comprise more than 95% of the total anthocyanin (23), a single determination of absorbance was made at 530 nm, the absorption maximum of cyanidin 3-rhamnoglucoside. The results were expressed in total A units/g fresh skin as follows:

\[
\text{Total } A = A_{530\text{nm}} \times dv \times vf
\]

where \(A\) = absorbance of diluted volume in a 1-cm cell; \(dv\) = dilution volume, \(i.e., the volume in ml of the dilute extract on which the absorbance is determined; \(vf\) = volume factor, \(i.e., the volume of original extract from which a sample was removed divided by the volume of the sample.

\[
\text{total } A/\text{wt of skin (g)} = \text{total } A/\text{g fresh wt}
\]

**Etiolation Procedure.** Thirty individual fig fruits were etiolated before or after Ethephon treatment by enclosing them in double-walled bags (12 × 18 cm) made from 6-mil black polyethylene. To avoid excessive heat accumulation, fruits which did not receive any direct sunlight throughout the day were selected for bagging. Each bag was vented by attachment of an opaque rubber tube 28 cm long with an inside diameter of 0.4 cm. The bags were sealed with Scotch 88 vinyl plastic electrical tape. Previous work with figs had shown that Ethephon treatment of a subtending leaf was sufficient to induce normal ripening of its auxiliary fruit (5). Accordingly, the leaves subtending each etiolated fruit were sprayed with a 500 µg/g solution of Ethephon to stimulate fruit ripening.

Dry weight was determined by slicing six fruits and desiccating them at 60°C for 3 weeks, or until there was no further weight change.

**RESULTS AND DISCUSSION**

Fruits treated with Ethephon attained maximum diameter and were mature 8 days after treatment and 18 days earlier than controls (Fig. 1). There was a drastic reduction in the levels of Chl \(a\) and \(b\) within 4 days after Ethephon application (Fig. 2). The rate of destruction of Chl \(a\) was about twice that of Chl \(b\) during the first 2 days after treatment (Fig. 3), causing the ratio of Chl \(a\) to \(b\) to change from 3:1 to 2.6:1. During the 3rd and 4th days after spraying, the rate of Chl \(b\) degradation exceeded that of Chl \(a\); and at the end of 4 days the original 3:1 ratio had been re-established. The degradation rates of Chl \(a\) and \(b\) were similar (6.2%) between the 5th and 8th days after treatment, and the 3:1 ratio remained unchanged in fully ripe fruits. Differences in the comparative degradation of Chl \(a\) and \(b\) were examined previously by Jeffrey and Griffith (14) and Wolf (32), who reported that Chl \(a\) degraded more rapidly than Chl \(b\) in senescing leaves. Schanderl et al. (27) found that in vitro the conversion of Chl \(a\) to phaeophytin \(a\) was 5 times that of Chl \(b\) to phaeophytin \(b\).

The catabolism of β-carotene was rapid, and had essentially ceased within 2 days after Ethephon treatment (Fig. 4A). During this time, 65% of the pigment originally present was degraded. Between the 2nd and 8th days, additional pigment destruction was slow and totaled 9%.
The overall pattern of xanthophyll breakdown was similar to that of Chl (Fig. 4, B, C, and D). The most abundant xanthophyll was lutein, followed in decreasing amounts by violaxanthin and neoxanthin. The degradation rates of violaxanthin and neoxanthin were comparable during the first 2 days after Ethephon application; that of lutein was 30% higher (Fig. 5). Between 2 and 4 days, the rate of neoxanthin degradation exceeded that of violaxanthin, but was lower than that of lutein. Between 4 and 8 days after treatment, there was a further 6% degradation of lutein, 5% of neoxanthin, and 4% of violaxanthin.

The growth and ripening responses of the fig to ethylene treatment include changes in fresh weight and in several constituents such as hormones (18), carbohydrates (6), RNA (19), and nitrogen (12). However, as the various plastid pigments degraded at different rates (Figs. 3 and 5), the influence of dilution on pigment changes during ripening appears negligible. Since the rate of pigment degradation declined between 5 and 8 days, the period of greatest increase in fresh weight, catabolism of the plastid pigments rather than dilution was further substantiated.

The pigment content of nontreated controls had begun to decline at the end of 8 days due to the onset of ripening. When, after an additional 10 days, the control fruits were fully ripe, their plastid pigment content was comparable to that of Ethephon-treated fruits (Table 1). Pigment degradation in ripe fruits continued during senescence. Overripe control fruits harvested prior to their abscission had about 50% less plastid pigments than either fully ripe Ethephon-treated figs or controls picked when fully ripe.

Ethephon decomposes at a pH greater than 2 to produce ethylene and other decomposition products (7). Ethylene production which originated from Ethephon applied to figs, was verified during the course of this investigation (unpublished). Thus, the effect of Ethephon in initiating fig ripening and pigment catabolism can be characterized as an ethylene response. The fig pigment most rapidly degraded was β-carotene. The maximum rate of Chl destruction was attained after catabolism of β-carotene was essentially completed. As β-carotene is believed to have a protective function, degradation of Chl and
ETHEPHON-INDUCED PIGMENT CHANGES IN FIGS

Table I. Comparison of Plastid Pigment Levels in Ethephon-treated and Control Mission Fig Fruits

<table>
<thead>
<tr>
<th>Pigment</th>
<th>At time of treatment</th>
<th>Control 8 days after treatment</th>
<th>Ethephon-treated mature</th>
<th>Mature control 18 days</th>
<th>Overripe control 23 days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chl a</td>
<td>144.7</td>
<td>85.6</td>
<td>44.1</td>
<td>50.1</td>
<td>23.1</td>
</tr>
<tr>
<td>Chl b</td>
<td>47.8</td>
<td>27.2</td>
<td>14.4</td>
<td>15.8</td>
<td>6.9</td>
</tr>
<tr>
<td>a-carotene</td>
<td>9.4</td>
<td>5.8</td>
<td>2.5</td>
<td>3.5</td>
<td>1.6</td>
</tr>
<tr>
<td>Lutein</td>
<td>29.1</td>
<td>14.7</td>
<td>8.3</td>
<td>9.6</td>
<td>5.1</td>
</tr>
<tr>
<td>Violaxanthin</td>
<td>19.4</td>
<td>12.8</td>
<td>8.9</td>
<td>8.3</td>
<td>3.2</td>
</tr>
<tr>
<td>Neoxanthin</td>
<td>10.1</td>
<td>5.3</td>
<td>3.5</td>
<td>2.1</td>
<td>1.7</td>
</tr>
</tbody>
</table>

Table II. Effect of Etiolation on Anthocyanin Synthesis in Ethephon-treated Fig Fruits

<table>
<thead>
<tr>
<th>Anthocyanins</th>
<th>total A mg/g fresh skin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exposed control</td>
<td>152.2</td>
</tr>
<tr>
<td>Etiolated at time of spray</td>
<td>1.3</td>
</tr>
<tr>
<td>Etiolated 3 days after spray</td>
<td>2.3</td>
</tr>
<tr>
<td>Etiolated 5 days after spray</td>
<td>84.4</td>
</tr>
<tr>
<td>De- etiolated 3 days after spray</td>
<td>133.9</td>
</tr>
<tr>
<td>De- etiolated 5 days after spray</td>
<td>65.0</td>
</tr>
<tr>
<td>De- etiolated 8 days after spray1</td>
<td>69.4</td>
</tr>
</tbody>
</table>

1 Harvested 11 days after spray, all others harvested 8 days after spray.

xanthophylls may be induced by photooxidative processes. Indeed Alschler (1) reported a promotive effect of ethylene on Chl synthesis in cucumber cotyledons. Thus, in the degradation of plastid pigments, the catabolic role of ethylene per se may be directed primarily at a-carotene.

Anthocyanin Synthesis. In addition to the marked increase in size (Fig. 1), anthocyanin synthesis was one of the most striking external changes observable in Ethephon-treated fig fruits. After treatment there was an initial period, or lag phase, of 2 to 4 days, during which there was no measurable anthocyanin synthesis (Fig. 6). Beyond this period, anthocyanin accumulation was linear, and the amount of pigment synthesized appeared to be related to light intensity. Fruits located in the shade had about 52% less pigment than those exposed to direct sunlight. Non-treated control fruits ripened 10 to 12 days later, but had a 32% higher anthocyanin content than comparably located Ethephon-treated fruits. Anthocyanin accumulation in treated fruits continued beyond the 8-day ripening period in fruits allowed to persist on the tree. At abscission, the anthocyanin content of Ethephon-ripened figs was 40% lower than that of comparably

Fig. 6. Anthocyanin synthesis in the skin of Ethephon-treated Mission fig fruits.

Fig. 7. Effect of etiolation on size and coloration of Mission figs sprayed with Ethephon. Left, etiolated; right, exposed.
mature control fruits. It appears that while Ethephon promoted the rate of anthocyanin elaboration, it did not increase the amount of pigment synthesized.

The 52% difference in anthocyanin content between exposed and shaded fruits treated with comparable amounts of Ethephon indicated that pigment synthesis was not exclusively dependent on ethylene production. To investigate the relative importance of light and ethylene in anthocyanin synthesis, some Ethephon-treated fruits were maintained in darkness. Etiolation stimulated an increase in fruit growth but completely inhibited anthocyanin production (Fig. 7). Prior to etiolation and Ethephon treatment, there was an initial anthocyanin level of 0.850 total A530/g fresh skin. Increments in A530 above this were considered as net anthocyanin synthesis. During an 8-day period after Ethephon treatment, no pigment was synthesized in etiolated fruits (Fig. 6), indicating that the anthocyanin-synthesizing system in figs has an obligatory light requirement. Between 9 and 10 days, however, fruits allowed to persist on the tree contained traces of anthocyanin (1.3–2 total A530/g fresh skin) around the ostiole.

Etiolation at 3 or 5 days after Ethephon treatment disrupted further pigment synthesis (Table II), thereby indicating a continuous light requirement for anthocyanin production. De-etiolation at 3, 5, or 8 days after treatment, initiated anthocyanin synthesis within 12 hr. The amount of anthocyanin formed in de-etiolated fruits was proportional to the length of irradiation before harvest. Prolonged de-etiolation resulted in proportionately high pigment synthesis. Thus, the lower anthocyanin content of Ethephon-treated fruits appeared to be a consequence of their accelerated growth and protracted light exposure, compared to the slower ripening control fruits.

At maturity, etiolated fruits were larger in diameter and had higher fresh and dry weights than did light grown controls (Table III). The increase in growth could be induced in some fruits by etiolating them 2 to 4 days after Ethephon treatment; however, the results were not consistent compared to etiolation at the time of treatment. The effect of an artificial environment on growth of enclosed fruits was also evaluated using vented, clear polyethylene bags similar in size and thickness to those used for etiolation. Fruits enclosed in the translucent bags had only a slight increase in growth compared to that of exposed control fruits. As humidity and ethylene accumulation were presumably comparable in both the translucent and opaque bags, it was apparent that the enhanced growth of etiolated fruits was a result of etiolation per se.

Since the fleshy portion of the fig fruit consists of peduncular rather than ovarian tissue, the growth response of the etiolated collective fruit may be considered parallel to that of an etiolated stem. An inhibitory effect of light on fruit (26) and stem (17, 31) growth of some species has been reported previously, and the phenomenon is well documented in the case of maize and dwarf peas (16, 21). Since application of gibberellin acid to period III fig fruits promoted shoot growth but failed to increase fruit size (4), it is unlikely that endogenous GA is a limiting factor in fruit growth. Rather, a photomediating increase or activation of endogenous antigibberellin-like substances may influence the ultimate size attained.

Light may also regulate fruit growth through the production of phenols (9). The in vitro activity of IAA oxidase can be enhanced by certain substituted phenols (10). A decreased production of monophenols in etiolated fruits may depress the activity of IAA oxidase and thereby increase the level of endogenous auxins. Higher auxin levels in etiolated fruits may have promoted their growth compared to that of the controls.

The role of ethylene in stimulation of anthocyanin synthesis in fig skin may be confined to mobilization of carbohydrates and the synthesis of anthocyanin precursors in treated fruits. The accumulation of these substances requires 2 to 4 days, after which pigment synthesis is initiated. Stimulation of phenylalanine ammonia-lyase by light (29) and ethylene (8) has been reported previously. Phenylalanine ammonia-lyase catalyzes the deamination of L-phenylalanine to trans-cinnamic acid, which can then be incorporated into numerous phenolic compounds, including the anthocyanidins. Induced phenylalanine ammonia-lyase undergoes rapid decay in many tissues upon transfer from light to dark (33). Since ethylene was not limiting in etiolated fruits and anthocyanin synthesis was inhibited, it appears that in this instance phenylalanine ammonia-lyase induction may have been light-dependent. Etiolation of entire fruits 2 to 3 days after Ethephon treatment may have inactivated the induced enzyme and thereby precluded anthocyanin synthesis in the skin.

**LITERATURE CITED**


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**Table III. Effect of Etiolation on Growth of Mission Figs during Period III**

<table>
<thead>
<tr>
<th>Time</th>
<th>Diameter</th>
<th>Fresh weight</th>
<th>Dry weight</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(mm)</td>
<td>(g)</td>
<td>(g)</td>
</tr>
<tr>
<td><strong>Exposed</strong></td>
<td><strong>Etiolated</strong></td>
<td><strong>Exposed</strong></td>
<td><strong>Etiolated</strong></td>
</tr>
<tr>
<td>At spray</td>
<td>31.7</td>
<td>31.6</td>
<td>13.8</td>
</tr>
<tr>
<td>At full maturity</td>
<td>39.4</td>
<td>47.6</td>
<td>36.7</td>
</tr>
<tr>
<td>% increase due to etiolation</td>
<td>20.8</td>
<td>67.0</td>
<td>22.6</td>
</tr>
</tbody>
</table>

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**Note:** The data in Table III represent the means of three replications. Standard errors are not shown, but they are typically small (less than 10%).
ETHEPHON-INDUCED PIGMENT CHANGES IN FIGS