Ethylene Intensifies but is not a Requirement for Methyl Jasmonate-Enhanced Anthocyanin Synthesis by ‘Fuji’ Apple Fruit

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Abstract

Exogenous methyl jasmonate (MJ) stimulates anthocyanin accumulation in apple (*Malus sylvestris* var. *domestica*) fruit peel. Anthocyanin synthesis in some apple cultivars reportedly is also stimulated by exogenous ethylene, however, the role of ethylene action in regulation of anthocyanin synthesis in apple fruit is unclear. MJ enhances ethylene sensitivity in various plants, therefore, studies were conducted to evaluate the role of ethylene and MJ in stimulation of anthocyanin synthesis in immature ‘Fuji’ apple fruit. Following ethylene and MJ treatments, fruit were exposed simultaneously to UV-B and visible light, then pigments were extracted and analyzed by reverse-phase high performance liquid chromatography. Treatment with MJ alone enhanced anthocyanin accumulation, including idaein, the major anthocyanin in apple fruit. Anthocyanin accumulation was further stimulated by treatment with MJ + ethylene. Treatment with the ethylene action inhibitor 1-MCP followed by MJ reduced red coloration over MJ alone. Treatment with ethylene or 1-MCP alone, or ethylene+1-MCP had little effect on anthocyanin accumulation. Chlorogenic acid synthesis was also enhanced by treatment with MJ or ethylene, however, treatment with 1-MCP alone or with MJ decreased chlorogenic acid content. MJ enhanced production of hyperin, the major quercetin glycoside in peel tissue, while ethylene and 1-MCP had no effect on quercetin glycoside content when applied alone or in any combination. β-carotene synthesis was enhanced following MJ treatment, stimulated further by MJ plus ethylene, but was not enhanced by ethylene alone. The results indicate a synergistic response between ethylene and MJ for stimulation of anthocyanin synthesis. Treatments with ethylene alone or 1-MCP indicate a limited role for ethylene action in regulation of red color development by immature ‘Fuji’ apple fruit.

INTRODUCTION

Methyl jasmonate [methyl 3-oxo-2-(2-pentenyl)cyclopentane-1-acetate] application elicits many wound related responses (Creelman and Mullet, 1997) including anthocyanin synthesis (Francheschi and Grimes, 1991). Exposure of apple fruit disks (Kondo et al., 2001) and whole apple fruit to MJ (Rudell et al., 2002) leads to increases in peel anthocyanin levels. As with the cyanidin glycosides, quercetin glycosides, chlorogenic acid, and β-carotene also increase with MJ exposure (Rudell et al., 2002). Additionally, MJ application to pre-climacteric fruit can promote increases in ethylene synthesis as well as increase ethylene sensitivity (Fan et al., 1997).

Anthocyanin synthesis in apple fruit can also be stimulated by treatment with ethylene or the ethylene releasing compound, ethephon (2-chloroethyl phosphonic acid) (Blanpied et al., 1975; Faragher and Brohier, 1984; Gomez-Cordovés et al., 1996). As would be expected, application of aminoethoxyvinylglycine (AVG), an ethylene synthesis inhibitor, leads to reduced anthocyanin accumulation (Wang and Dilley, 2001). While reducing degreening in ‘Golden Delicious’ (Fan and Mattheis, 1999), the effects of the ethylene action inhibitor 1-methylcyclopropene (1-MCP) on anthocyanin synthesis has

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not been previously investigated. The present studies were conducted to evaluate the individual role of ethylene and MJ in stimulation of anthocyanin synthesis in immature ‘Fuji’ apple fruit.

MATERIALS AND METHODS

Fruit Source
‘Fuji’ apple fruit were harvested at 135 days after full bloom at the U.S. Department of Agriculture, Agricultural Research Service experimental orchard located near Wenatchee, Wash. Fruit were treated the following day.

Chemicals
Ethephon (21.7% w/v, Ethrel® formulation) was obtained from Rhône-Poulenc Ag Company, Research Triangle Park, N.C., USA. 1-MCP (0.14% w/v, EthylBloc® formulation) was obtained from Floralife®, Inc., Walterboro, S.C., USA. MJ and Tween® 20 (polyethylenesorbitan monolaurate) was obtained from Aldrich, Milwaukee, Wis., USA.

Chemical Treatment
1-MCP (10 ppm) treatments were performed as described (Fan and Mattheis, 1999). Following 1-MCP treatment, apples (1-MCP treated and untreated controls) were immersed for 2 min in deionized water with 0.177% (v/v) Tween® 20 alone or containing 400 µL L⁻¹ ethephon. MJ was applied as an emulsion with the same concentration of Tween 20 at a rate of 1.12 g L⁻¹ or in combination with 400 ppm ethephon where appropriate. A summary of the chemical treatments includes: control, ethephon, MJ, ethephon+1-MCP, 1-MCP, MJ+1-MCP, and MJ+ethephon. After treatment, apples were placed on pressed paper trays and air-dried for ≈15 min at 21°C.

Exposure to Artificial Light
Following the MJ treatments, fruit were held in the dark (control, ethephon, MJ) or placed ≈15 cm (apple surface to lights) under two- outlet 1.22 m (length) fluorescent light banks, each containing one- 40W Sylvania (Versailles, Ky.) Cool White Deluxe fluorescent bulb and one- 40W Phillips (Somerset, N.J.) fluorescent UV lamp. The irradiated area was delimited with aluminum foil. The marked side of each apple faced towards the light source. The intensity of key wavelength ranges were measured at various points within the chamber using a PMA2100 light meter (Solar Light Co., Philadelphia, Pa.) for UV-A and UV-B irradiance and a LI-250 light meter (LI-COR, Inc., Lincoln, Nebr.) for visible light irradiance (400-700 nm). The temperature in the light treatment chamber was 25 °C. Apples were exposed to this irradiation for 40 h. “Dark” treated apples were treated then kept in the dark simultaneously for an equal period of time.

Peel Color Analysis
Following harvest, the peel of each apple was marked at two sites on the shaded side (not facing the sun on the tree or non-colored side) and color at each site determined using a Minolta CR-200 colorimeter (Minolta Corp., Osaka, Japan). Measurements were obtained using the CIE L* (light to dark), a* (green to red), b* (blue to yellow) color space, then a*b* values were converted to hue angle (b°, tan⁻¹ b/a; McGuire, 1992). Color measurements were performed prior to and following light treatments.

Immediately following exposure to artificial light, peel on the exposed sides of irradiated fruit or fruit held in the dark was removed with a fruit peeler, flash frozen in liquid N₂, and then stored under N₂ gas at -80°C.

Anthocyanin Flavonoid Analysis
Frozen, crushed peel tissue (0.5 g) was extracted in 2 mL 74:25:1 methanol/
tetrahydrofuran/HCl (v/v) for 5 h in an ice-water bath covered with aluminum foil. The bath was sonicated for the first and last hour of the extraction period. The extracts were partitioned with 3 mL hexanes. The hexanes phase was discarded and the remaining fraction was centrifuged and filtered.

Pigment composition of the filtered extract was analyzed by reversed-phase high-performance liquid chromatography (HPLC). A 20 μL sample was injected into a HP Series 1100 HPLC system (Hewlett Packard) equipped with a 5 μm HP Hypersil ODS (4.0 x 125 mm) column and a Waters 996 photo-diode array (PDA) detector (Waters, Millford, Mass.). Solvents used for the elution were (A) 1:10:89 H₃PO₄/methanol/deionized water (v/v) and (B) 1:70:29 H₃PO₄/methanol/deionized water (v/v). The column temperature was 25 °C and the solvent flow-rate was 1 mL·min⁻¹. Solvent A was used for the first 2 min, then a linear gradient of A plus B was initiated reaching 20% A and 80% B at 34 min. The rate then increased ending at 100% B after 36 min. Chromatograms from 280, 328, 357, and 519 nm were extracted and used for quantification.

Carotenoid / Chlorophyll Analysis
Approximately 1 g of frozen, crushed peel tissue from each sample was washed repeatedly, in the presence of 56 mg CaCO₃, with cold 75:25 methanol/tetrahydrofuran (v/v), until colorless. The combined washes, on ice, were partitioned with hexanes until the hexanes phase was colorless. The hexanes phase was then dried under a stream of purified N₂ gas at room temperature. Pigments were dissolved in 75:25 methanol/tetrahydrofuran (v/v) and clarified using centrifugation and filtration prior to analysis. Light exposure was minimized throughout the entire procedure.

Samples were analyzed immediately following extraction using the same HPLC system described previously. Solvents used for elution were (A) 80:20 methanol/deionized water (v/v), and (B) ethyl acetate. The flow rate was 1.0 mL·min⁻¹. Solvent A was for the first 2 min, then solvent B increased linearly and reached 50% at 21 min. This mixture was maintained until the end of the analysis at 33 min. A chromatogram from 446 nm was extracted and used for quantification.

Peak Identification and Quantification
Specific peaks were identified using spectral and retention comparisons with authentic standards and quantified by response comparison with authentic standards. The wavelengths at which a peak had its greatest response and least interference were used for quantitation. Chlorogenic acid and β-carotene were purchased from Sigma (St. Louis). Hyperin and idaein were purchased from Indofine (Somerville, N.J.).

Statistical Design and Analyses
Experiments were conducted using a randomized complete block design with 10 treatments and 18 individual fruit replicates per treatment. Analyses of variance and Fisher’s least significant difference tests were performed on data collected using SAS statistical analysis software (SAS Inst., Inc., Cary, N.C.).

RESULTS AND DISCUSSION
Treatment with MJ alone resulted in enhanced red coloration and anthocyanin (idaein) production, as previously reported by Kondo et al. (2001) and Rudell et al. (2002), (Fig. 1; Table 1). MJ+ethephon enhanced and MJ+1-MCP reduced red coloration and idaein production when compared to MJ alone. Nevertheless, red coloration and idaein production by MJ+1-MCP treated fruit was still significantly higher than control fruit. Ethephon treatment did not affect red coloration whereas 1-MCP and ethephon+1-MCP treatments adversely affected it. Idaein production was not affected by ethephon or 1-MCP either alone or in combination. Treatments not exposed to light resulted in no changes in coloration (Fig. 1) or pigment constituents (data not shown) during this experiment.
Although previous evidence generally points to the involvement of ethylene in promoting red coloration and anthocyanin production in apples (Blanpied et al., 1975; Gomez-Cordovés et al., 1996; Awad and de Jager, 2002), possibly by increasing enzymatic activity in the phenylpropanoid pathway (Faragher and Brohier, 1984; Li et al., 2002), our data does not clearly demonstrate this.

While ethylene promoted and 1-MCP reduced red coloration in conjunction with MJ treatment, ethylene or 1-MCP alone had little effect similar to that reported with peel degreening by Fan and Mattheis (1999). This indicates ethylene and MJ may act synergistically, with ethylene enhancing the effects of MJ while it has little effect alone. As indicated in the present study, Kondo et al. (2001) found that treatment with AVG only had a limited effect on anthocyanin accumulation when coupled with MJ treatment suggesting MJ could promote anthocyanin formation without the presence of ethylene. The apparent impotence of ethylene in inducing coloration in ‘Fuji’ apple fruit in this study is corroborated by Fortes (1984, cited from Saure, 1990). However, this discrepancy is just as likely due to variations in experiment length, light environment, maturity, or other varietal differences between this and previous studies. Incidentally, Li et al. (2002) reported that field treatments of more mature ‘Fuji’ apples with ethephon did enhance red peel color.

Both ethephon and MJ treatments resulted in increases in chlorogenic acid. Further increases were elicited by treatment with MJ+ethephon while 1-MCP alone or in concert with ethephon or MJ lead to significant decreases in chlorogenic acid when compared to the control, ethephon, or MJ (respectively). This indicates that chlorogenic acid production is regulated to a greater degree by ethylene (alone) in addition to MJ. This conflicts with evidence provided by Awad and de Jager (2002) who found that ethephon application did not lead to increases in chlorogenic acid. Differences in regulation eluded to by the present study may result from differential interaction of ethylene at various regulation points in the phenylpropanoid pathway. Chlorogenic acid synthesis results from a branch in this pathway many steps upstream of anthocyanin (Dixon and Paiva, 1995).

Hyperin, a prevalent quercetin glycoside that increases the most among other similar compounds following MJ treatment (Rudell et al., 2002), increased as a result of MJ treatment but was unaffected by MJ+ethephon and positively affected by MJ+1-MCP (Table 1). Treatment with ethephon and 1-MCP, alone or together, did not affect the hyperin content. β-carotene production increases with MJ treatment in dark conditions (Perez et al, 1993) and at a more rapid rate with MJ plus artificial irradiance (Rudell et al., 2002). In the present study, MJ slightly enhanced β-carotene levels while MJ+ethephon lead to significantly higher levels. β-carotene levels were not enhanced by MJ in the dark within the time-frame of this study (data not shown). This shows that the isoprenyl-derived β-carotene may also be enhanced by an interaction between MJ and ethylene like pigments from the phenylpropanoid pathway.

CONCLUSIONS

Anthocyanin synthesis in ‘Fuji’ apple fruit can be stimulated by MJ independently of ethylene. Ethylene appears to play an enhancing function when present with MJ although it exhibited no discernable effect on anthocyanin production when applied alone under these experimental conditions. Treatment with 1-MCP alone and in concert with MJ or ethephon provides further evidence for this. However, ethylene and MJ enhance chlorogenic acid levels when applied alone or in concert and 1-MCP has the reverse effect eluding to more separated roles for these growth regulators in different parts of the phenylpropanoid pathway. Because ethylene appears to augment MJ enhanced anthocyanin synthesis, using these compounds in concert could result in more viable treatments to promote apple red color and commercial value.

**Literature Cited**

Awad, M.A. and de Jager, A. 2002. Formation of flavonoids, especially anthocyanin and
### Tables

Table 1. Effects of MJ, ethylene, 1-MCP on peel content of prevalent pigment constituents in ‘Fuji’ apple peel for fruit exposed to light.

<table>
<thead>
<tr>
<th></th>
<th>idaein</th>
<th>hyperin</th>
<th>chlorogenic acid</th>
<th>beta-carotene</th>
</tr>
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<tr>
<td></td>
<td>µg·g⁻¹ fresh weight</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>initial</td>
<td>1.70 a¹</td>
<td>219 a</td>
<td>129 a</td>
<td>1.37 a</td>
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<tr>
<td>control</td>
<td>37.4 b</td>
<td>936 bcd</td>
<td>393 c</td>
<td>2.15 cd</td>
</tr>
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<td>ethephon</td>
<td>23.8 ab</td>
<td>722 b</td>
<td>471 d</td>
<td>1.65 ab</td>
</tr>
<tr>
<td>1-MCP</td>
<td>28.9 b</td>
<td>905 bc</td>
<td>254 b</td>
<td>1.85 bc</td>
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<tr>
<td>ethephon +1-MCP</td>
<td>22.1 ab</td>
<td>726 b</td>
<td>358 c</td>
<td>2.16 cd</td>
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<td>MJ</td>
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<td>1270 d</td>
<td>493 d</td>
<td>2.57 d</td>
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<td>1370 e</td>
<td>386 c</td>
<td>2.25 cd</td>
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<tr>
<td>MJ + ethephon</td>
<td>262 e</td>
<td>1140 cd</td>
<td>669 e</td>
<td>3.34 e</td>
</tr>
</tbody>
</table>

¹ different lower case letters denote significant differences as determined by ANOVA and Fisher’s least significant difference test ($P\leq0.05$; n=3).

### Figures

Fig. 1. Effects of MJ, ethephon, and 1-MCP treatment of whole ‘Fuji’ apple fruit on hue angle ($\Delta h^o$).