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Acetaldehyde Is a Causal Agent Responsible for Ethanol-Induced Ripening Inhibition in Tomato Fruit[1]

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Inhibition of tomato (*Lycopersicon esculentum* Mill.) fruit ripening by exogenously applied ethanol was shown to be caused by elevated endogenous levels of acetaldehyde (AA). Exposure of excised pericarp discs of mature-green tomato fruit to ethanol or AA vapors produced elevated levels of both compounds in the tissue, but only the levels of AA were associated with ripening inhibition. Ripening inhibition was dependent on both the applied concentration and the duration of exposure. Discs treated with inhibitory levels of AA had levels of ethanol that were elevated but below that associated with inhibition of ripening. The in vivo activity of alcohol dehydrogenase was inhibited 40 to 60% by 4-methylpyrazole (4-MP), a competitive inhibitor of this enzyme. The inhibitory effect of ethanol on ripening was reduced by the simultaneous application of 4-MP. Tissue treated with 4-MP plus AA vapors had higher endogenous levels of AA and ripening was inhibited longer than in tissue without 4-MP. The tissue AA level resulting from ethanol or AA application appears to be the critical determinant of ripening inhibition.

Endogenous levels of both AA and ethanol increase in ripening fruit (Gustafson, 1934; Fidler, 1968). AA is also an aroma constituent in most plant tissues (Janes and Frenkel, 1978); exposure to AA vapors improves the organoleptic quality of table grapes (Pesis and Frenkel, 1989), strawberries (Morris et al., 1979), and blueberries and tomatoes (Paz et al., 1981). The ripening of blueberry, tomato, and pear fruit is promoted by AA vapors (Paz et al., 1981), and the application of AA is more effective than C_2H_4 in promoting ripening of figs (Hirai et al., 1968). Exposure to AA also stimulates respiration in apple, blueberry, orange, and strawberry fruit (Fidler, 1968; Janes et al., 1978). Furthermore, application of AA vapors inhibits C_2H_4 production in grapes without affecting respiration (Pesis and Marinansky, 1992), and it slightly inhibits the ripening of whole tomato fruit (Pesis and Marinansky, 1993).

Exogenously applied or endogenously synthesized ethanol suppresses C_2H_4 production (Saltveit, 1989) and inhibits the ripening of tomato fruit at various maturity stages without affecting subsequent quality (Kelly and Saltveit, 1988; Saltveit and Mencarelli, 1988; Saltveit and Sharaf, 1992). Ethanol is produced in many plants under anaerobic (Kimmerer and Kozlowski, 1982; Donaldson et al., 1985) or aerobic (Chang et al., 1983; MacDonald and Kimmerer, 1993) conditions and can be isolated from mature tomato fruit at all ripeness stages (Gustafson, 1934). Although ethanol is not readily metabolized by plants, it can be oxidized to AA under aerobic conditions in the cytosol by an NAD-dependent ADH, peroxidized to fatty acids in glyoxysomes, or oxidized in the ER by an NADPH-dependent reaction (Donaldson et al., 1985). Meigh et al. (1966) found that AA levels increased in tomato tissue exposed to ethanol during sterilization or when ethanol was introduced into the culture solution.

Many effects of ethanol on plants and animals are attributed to AA rather than to ethanol. The process of carrot somatic embryogenesis is almost completely arrested by the addition of ethanol to the culture medium (Perata et al., 1988). The concentration of AA that inhibits cell growth is 40-fold lower than the concentration of ethanol that has a similar affect. The correlation between AA concentration and the percentage decrease in cell growth is independent of the ethanol concentration applied. Accumulation of AA in carrot cultures receiving ethanol is almost completely inhibited by 4-MP, an inhibitor of ADH (Perata and Alpi, 1991). The decreased growth of carrot embryogenic cells is attributed to AA, not to ethanol.

The anaerobic pathway in plants decarboxylates pyruvate to produce AA, which is then reversibly reduced by ADH to ethanol. Given the interconversion of AA and ethanol by ADH, it is difficult to resolve how applied AA or ethanol can have such diverse effects as enhancing the organoleptic quality of fruits and inhibiting or promoting fruit ripening while increasing or decreasing C_2H_4 production. Further confusion arises when one considers that exposure to low concentrations of ethanol vapors stimulates tomato fruit ripening (Beaulieu and Saltveit, 1997), whereas exposure to high concentrations inhibits ripening and suppresses C_2H_4 action (Saltveit, 1989). Therefore, the objectives of this research were to determine whether inhibition of tomato fruit ripening was attributed to AA or to ethanol and to determine the relative importance of endogenous ethanol and AA in retarding fruit ripening by applying vapors to tomato tissue after previous applications

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Abbreviations: AA, acetaldehyde; ADH, alcohol dehydrogenase; AlDH, aldehyde dehydrogenase; CYN, cyanamide; DIS, disulfiram; 4-IP, 4-iodopyrazole; MG, mature green; 4-MP, 4-methylpyrazole.

of inhibitors of ADH and AlDH. We present evidence that ethanol-induced ripening inhibition in tomato results from AA produced from the applied ethanol.

MATERIALS AND METHODS

Tomato (*Lycopersicon esculentum* Mill. cv Castlemart) fruit were hand-harvested from plants grown under normal cultural practices at the University of California Vegetable Crops Field Facility. Only unblemished, uniformly shaped MG fruit with a fresh weight of approximately 160 g were used to prepare discs. MG fruit are full-sized fruit that do not show any external red color. Fruit were washed in dilute bleach (a 1:20, v/v, solution of 5.25% sodium hypochlorite), rinsed three times with sterile, deionized water, and air-dried under a sterile laminar flow hood.

Pericarp discs were cut from the equatorial regions with a 1.4-cm-diameter cork borer. Adhering locular tissue was trimmed away with a razor blade to produce 4-mm-thick discs (0.627 ± 0.048 g). Discs were rinsed three times with sterile, deionized water, carefully blotted dry, blocked by intensity of green color among treatments (Saltveit, 1989), and placed epidermis down into sterile Petri dishes or deep-well culture plates. Discs were kept at 20°C overnight (approximately 18 h) in a flow of humidified, C_2H_4-free air to allow dissipation of wound-induced C_2H_4 (Saltveit, 1989).

After disc extraction, the fruit was carefully scrutinized for its exact stage of development and only discs from MG2 or MG3 fruit were used. Fruit were considered to be at the MG2 stage at 6 to 10 d from the breaker stage with little or no locular jelly; seeds were not cut with a knife because of the mature and softened locular tissue. The MG3 stage fruit were 2 to 5 d from the breaker stage with well-developed locular jelly and no visible red color within the columella. Five to 10 percent of the surface of breaker fruit was red.

Measurements of Fruit Ripening

Lycopene concentrations were determined in discs varying from green to red by measuring the A_{503} of an acetone extract of individual discs. This wavelength is best for tomato lycopene because the influence of carotenoids (473 nm) is negligible (Beerh and Siddappa, 1959). Nondestructive a/b chromameter (Minolta, Tokyo, Japan) measurements were also taken of the discs before homogenization, and the a/b ratio was correlated with measured lycopene concentration. The best-fit exponential equation (i.e. highest r^2) varied with disc thickness (Beaulieu and Saltveit, 1996) and the r^2 was highest for 4-mm-thick discs; therefore, a standard thickness of 4 mm was used in all subsequent experiments.

Measurements of Ion Leakage

Three discs were placed into 50-mL conical bottom test tubes containing 30 mL of 0.3 M mannitol, gently shaken for 1 h at 100 rpm, and the conductivity was measured. The total conductivity was measured after two freeze/thaw (−20/20°C) cycles, and the average percentage conductivity was calculated for the three replicates after readings for the mannitol blank were subtracted.

Inhibitor Applications

Sterile, aqueous solutions of 4-MP, 4-IP, DIS, and CYN or water (control) were applied as 40- to 50-μL aliquots directly to the cut pericarp surface. In some experiments a 3.0-mL syringe affixed with a 0.2-μm sterile filter (Acrodisc, Gelman, Ann Arbor, MI) was used to deliver 2 mL of inhibitor solutions to bathe four discs in a 10- × 60-mm plastic Petri dish. The discs were positioned epidermis up in Petri dishes and gently shaken at 50 rpm for specified times. Discs were blotted dry immediately following incubation in the solutions and held overnight in a flow of humidified air before other treatments were applied. The ripening behavior of bathed discs was similar to controls receiving no water or inhibitor.

Kinetic Analysis of Inhibitors

Three grams of pericarp tissue from red-ripe tomato fruit was ground in 18 mL of 1 M Tris-Cl, 5 mM DTT, and 1 mM EDTA buffer at pH 7.4 (Longhurst et al., 1990). The supernatant after centrifugation for 15 min at 12,000*g* was used as the crude ADH enzyme extract. The 1-mL assay solution that was used to determine the competitive natures of 4-MP and 4-IP contained 1 mM Gly-KOH, 0.6 mM NAD^+, 200 μL of crude ADH enzyme extract, and various ethanol concentrations at pH 9.6. Spectrophotometric assays for ADH (A_{340} at 20°C) measured the increase in NADH after the addition of ethanol. One unit of ethanol-dependent ADH activity corresponded to the production of 1 μmol NADH min^{-1}.

Volatile Treatments

Absolute ethanol or reagent grade AA was used for all treatments and standards. After dissipation of wound-induced C_2H_4, culture plates of discs were transferred to a sterile hood, uncovered, and placed in 4-L glass jars. A folded 7-cm filter paper (Whatman) set up on half of a 15- × 60-mm plastic Petri dish was placed beside the culture plate in the jar. Water or ethanol was pipetted onto the filter paper, and the jar was then immediately capped with a Teflon-covered rubber stopper and sealed tightly with filament tape for 4 h of exposure at 20°C. Alternatively, Petri dishes or culture plates were placed inside 1-L glass jars and sealed with Teflon septa lids. Water, ethanol, or AA was injected through the septa lid onto a 4.25-cm-diameter filter paper (Whatman), which was taped inside the rim of the jar; the jar remained sealed for the 4-h treatment. Water, ethanol, and AA were applied on a tissue fresh weight basis. At the end of all treatments, culture plates or Petri dishes containing discs were transferred to 20-L glass jars at 20°C and ventilated with a 20-L h^{-1} flow of humidified air. All amounts of ethanol and AA used in treatments were visually determined to have volatilized before the end of the 4-h static treatment, and all filter papers that had been dampened with ethanol were dry to the touch when the treatment jars were opened.

AA vapors were also applied to fruit discs in a humidified flow-through system, as previously described (Janes et al., 1978). Treatment levels of AA were verified by injecting 1-mL headspace gas samples into the gas chromatograph, which was calibrated against a standard curve derived from known serially diluted AA-containing atmospheres. After treatment, culture plates or Petri dishes containing discs were transferred to 20-L glass jars at 20°C and ventilated with a 20-L h^{-1} flow of humidified air.

Volatile Determinations

The GC headspace method of Davis and Chance (1969) for volatile analysis of juice extracts was modified for tomato discs. Individual discs were placed in 10-mL glass syringes, which were drawn to 7 mL. The frosted tip was sealed with a red rubber serum stopper, frozen at −20°C, and heated for 1 h at 75°C in a water bath. A 1-mL gas sample was removed and analyzed by GC. The gas chromatograph was equipped with a 183-cm-long column (5% Carbowax 20M 60/80 Carbopack, Alltech Associates Inc., Deerfield, IL) held at 85°C, with the injection port at 150°C and the detector at 250°C. This modified method allowed for large groups of samples to be calibrated against a series of known serially diluted standards. The method was evaluated using discs spiked with ethanol at 0 to 15 $\mu L\ g^{-1}$. Measurements of headspace gas samples were linear with concentration ($r^2 = 0.97$), and approximately 100% of the added ethanol could be accounted for (data not shown).

Ethylene Determinations

The C_2H_4 concentration of extracted 1-mL headspace gas samples was measured by injecting them into a gas chromatograph (Carle Instruments, EG&G Chandler Engineering Tulsa, OK) equipped with a sodium chloride-modified alumina F1 60–80 column (Saltveit and Yang, 1987). Carbon dioxide was determined with an IR analyzer (model PIR-2000, Horiba, Irvine, CA) as previously described (Saltveit and Strike, 1989).

RESULTS AND DISCUSSION

Color Evaluations

Objective, nondestructive chromameter readings are more precise than subjective evaluations of ripening because they can be taken from specific regions of a given fruit or disc, can be used to follow ripening of the same tissue over time, and can accurately predict the lycopene concentration of the measured tissue (Fig. 1). The ability to repeatedly evaluate the ripeness of small areas of the fruit is important because the location from which the discs are excised can influence the timing of ripening and color development (Brecht, 1995). The equation $A_{503} = 0.14 + 0.60x + 1.121x^2$ ($r^2 = 0.96$) was used to calculate the lycopene concentration of 4-mm-thick discs from chromameter (Minolta) readings using the molar extinction coefficient of 17.2×10^4 mol cm^{-1} at 503 nm (Fig. 1).

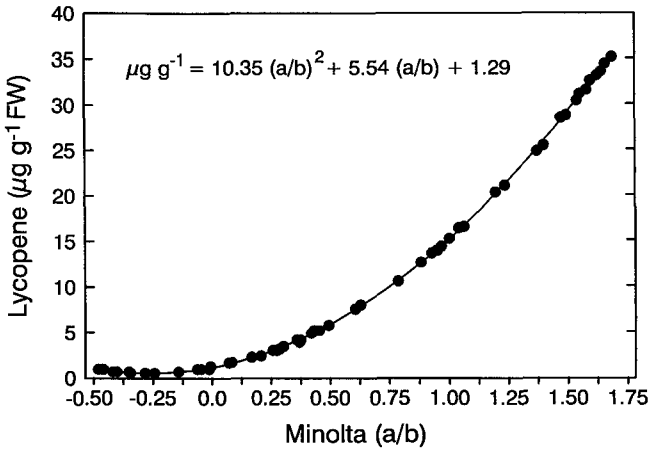

Figure 1. Relationship between Minolta a/b chromameter values and lycopene concentration of 1.4-cm-diameter, 4-mm-thick tomato discs. Discs were excised from tomato fruit at various stages of ripeness, trimmed to 4 mm, measured with a Minolta chromameter, and then assayed for lycopene as described in "Materials and Methods." Data points are from individual discs ($n = 68$). FW, Fresh weight.

Ethanol Applications

When tomato discs were exposed to ethanol vapor (0.83 μL/12.5 g fresh weight) in a closed 4-L jar, headspace AA rapidly accumulated for 6 h to 20 $\mu g\ g^{-1}$ and then held constant for 12 h before declining slowly (Fig. 2A). After the static exposure for 4 h, 98% of the applied ethanol remained in the headspace atmosphere in jars with dry filter paper, yet only 34% was measured in the headspace of jars containing 12.5 g of tomato discs or filter paper wetted with 12.5 mL of water. Ethanol measured in control jars declined slightly over time, possibly because of absorption by the rubber stoppers. Detectable levels of methanol and ethyl acetate (Fig. 2B) and other undetermined volatiles accumulated in atmospheres containing ethanol-treated tomato discs. Similar results were also obtained with whole fruit (data not shown). Thus, tomato fruit tissue exposed to exogenous ethanol was stimulated to produce endogenous volatiles or the supplied ethanol was metabolized via ADH to AA and other compounds.

The maximum tissue concentration of AA, ethanol, ethyl acetate, and methanol in discs held in a flow of humidified air after 4 h of static exposure to ethanol at 8 $\mu L\ g^{-1}$ varied considerably (57 $\mu g\ g^{-1}$ fresh weight for AA; 1626 $\mu g\ g^{-1}$ for ethanol; 0.62 $\mu g\ g^{-1}$ for ethyl acetate; and 276 $\mu g\ g^{-1}$ for methanol). The elevated tissue concentrations of ethanol and AA started to immediately decline after ethanol exposure was discontinued, reaching control levels within 40 and 52 h, respectively (Fig. 2B). Tissue levels of ethyl acetate continued to increase after ethanol exposure, reaching a maximum at 6 h before declining to control levels at 28 h. When the concentrations of AA, ethanol, and ethyl acetate started to decline, their rates of decline were similar. Unlike the other volatiles, the tissue concentration of methanol remained at approximately 80% of its maximum value for 40 h before showing a consistent decline.

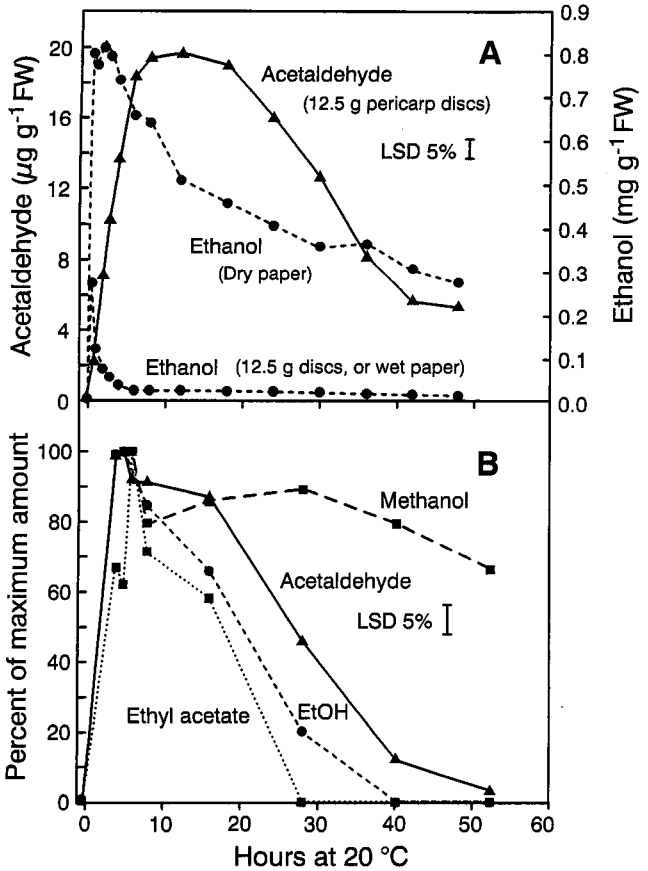

Figure 2. Effect of exogenous ethanol application on the headspace and tissue concentration of AA, ethanol, and other volatiles in tomato discs. Discs were continuously exposed to ethanol at 0.83 μL/12.5 g fresh weight in a 4-L jar at 20°C (A) or exposed to 8 μL g^{-1} for 4 h and then to a flow of air at 20°C (B). Jars (A) contained dry filter paper, filter paper moistened with 12.5 g of water, or 12.5 g of tomato discs. Headspace gas samples (A) or gas samples from individual discs (B) were taken and analyzed as described in "Materials and Methods." Maximum values in μg g^{-1} fresh weight are 57 for AA, 1630 for ethanol, 0.62 for ethyl acetate, and 276 for methanol. Data points are the means of four replicates. FW, Fresh weight; EtOH (in B), ethanol.

The endogenous concentration of both AA and ethanol increased and ripening was inhibited in tissue exposed to various levels of ethanol for 4 h and then ripened for 6 d. After 6 d, discs exposed to ethanol at concentrations $\leq$2.6 mg g^{-1} were significantly riper (higher lycopene concentration) and had significantly lower AA levels than tissue exposed to ethanol at concentrations $\geq$3.4 mg g^{-1} (Fig. 3). The AA concentration in discs exposed to ethanol at $\geq$4.3 mg g^{-1} was similar (approximately 25–30 μg g^{-1}), as was the extent of ripening inhibition (approximately 90%). Although exposure to ethanol at $\geq$4.3 mg g^{-1} produced a linear increase in ethanol concentrations in the discs, the level of ripening inhibition was similar for these levels of ethanol exposure, even though the endogenous ethanol level varied more than 7.4-fold (from 90 to 670 μg g^{-1} fresh weight). A similar trend was observed with MG2-treated discs held at 20°C for 10 d (data not shown). A low level of

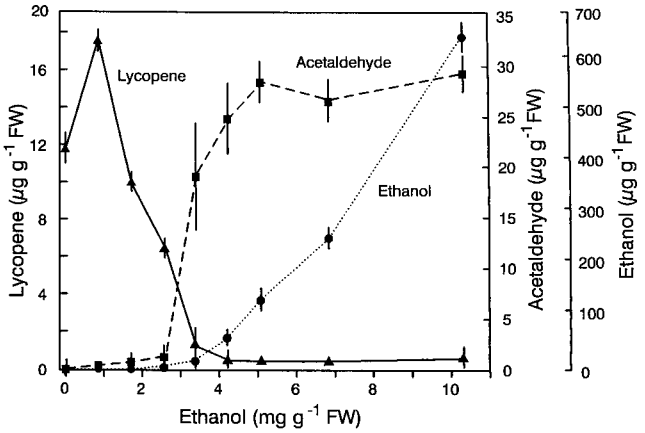

Figure 3. Effect of a 4-h exposure to increasing concentrations of ethanol vapor at 20°C on ripening (lycopene content) and endogenous AA and ethanol concentrations in MG tomato pericarp discs. Lycopene values were measured after 6 d of ripening in a flow of humidified air at 20°C. AA and ethanol data points are the means of six replicates, and lycopene data points are the means of 12 replicates. Error bars indicate the SD. FW, Fresh weight.

AA (0.44 μg g^{-1}) actually promoted ripening over lower (0.09 μg g^{-1}) or higher (0.74 μg g^{-1}) concentrations. Tissue levels of AA greater than 0.74 μg g^{-1} reduced lycopene synthesis to less than that of the control.

AA Applications

Ripening of MG tomato discs was not inhibited by exposure to AA vapors in the range of 25 to 1500 μg g^{-1} for 4 h in a closed 1-L jar; in fact, these AA concentrations promoted ripening (Fig. 4). However, a 2-d delay in ripening was seen after a 4-h exposure to approximately 2500 μg g^{-1}, whereas 4000 μg g^{-1} was deleterious and produced water-soaked, translucent discs. Ion conductivity measurements taken after 10 d of ripening confirmed that tissue necrosis occurred upon exposure to high levels of AA (3.4

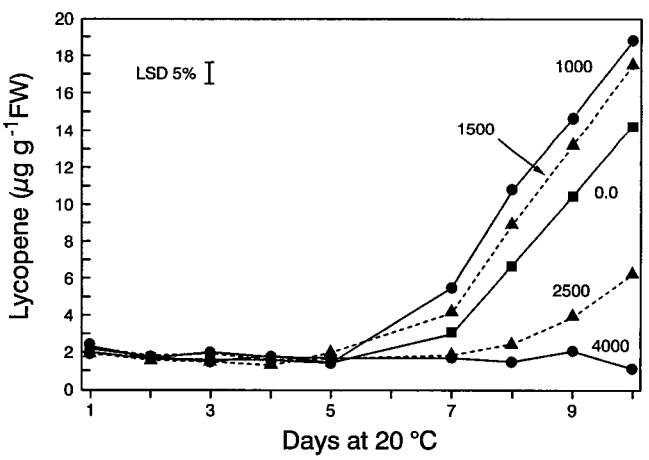

Figure 4. Effect of a 4-h exposure to increasing concentrations (μg g^{-1}) of AA vapor on subsequent ripening (lycopene content) of MG tomato pericarp discs. Data points are the means of 12 replicates. FW, Fresh weight.

versus 49.3% leakage for 0 versus 4000 μg AA g^{-1}, respectively). In contrast to the high levels of AA needed to inhibit the ripening of MG discs, exposure to AA at 780 μg g^{-1} produced a 1-d inhibition of ripening in discs excised from breaker tomato fruit (data not shown). After 6 d of ripening, the AA levels in MG discs exposed to AA at 235, 780, and 2350 μg g^{-1} were 0.8, 1.3, and 2.8 μg g^{-1}, respectively. Only trace levels of ethanol were detected in the discs (data not shown).

Reversible ripening inhibition of MG discs exposed to a continuous flow of humidified AA vapors was dependent on applied concentration and on duration of the exposure (Fig. 5). Exposure to a flow of AA in air at approximately 150 μL L^{-1} (176 ± 5 μL L^{-1} for the first 3 d and then 124 ± 7 μL L^{-1} through d 9) inhibited lycopene synthesis during exposure to AA and for 2 d after transfer to air (Fig. 5A). Ethylene production, which was generally suppressed during AA treatments, recovered in a normal climacteric fashion upon transfer to air (Fig. 5B). Tissue treated for 3 or 6 d had C_2H_4 climacterics 2-fold greater than the controls, whereas tissue treated for 9 d had C_2H_4 climacterics that were similar in magnitude to the controls. The increase in C_2H_4 production on d 7 for discs in the 9-d treatment suggests that the inhibitory effect of AA on C_2H_4 production was eventually overcome. However, the inhibitory effect of AA on ripening (i.e. lycopene synthesis) remained in effect for the duration of AA exposure (plus 2 d), even though C_2H_4 production had reached the climacteric maximum on d 11 (Fig. 5B).

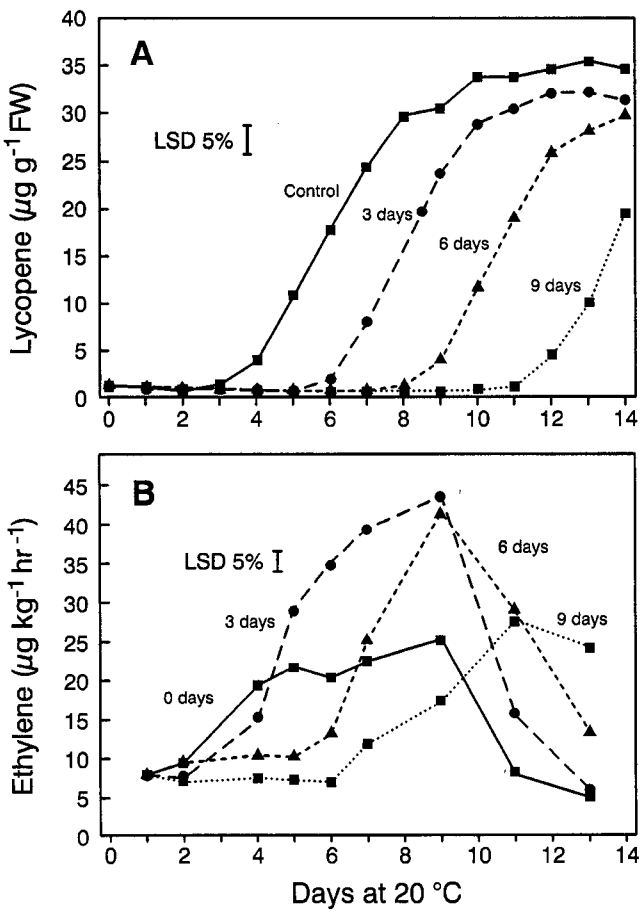

Figure 5. Effect of prolonged exposure to AA vapor on subsequent ripening (lycopene content; A) and C_2H_4 production (B) of MG tomato pericarp discs. AA was applied in a flow of humidified air at 20°C (176 ± 5 for the first 3 d and then 124 ± 7 μL L^{-1} through d 9). Data points are the means of 12 replicates (A) or three replicates (B). FW, Fresh weight.

Exposure to AA vapors (210 ± 13 μL L^{-1}) for 3 d inhibited the ripening of the three tomato cultivars Castelmart, Cobra, and Trust (data not shown). Although the rate of ripening differed among the cultivars, the AA treatment prevented ripening during the 3 d of application and for an additional 3 d after transfer to air. Tissue levels of AA and ethanol decreased to control levels after 2 d (data not shown).

Tomato discs provide an ideal system to study ripening because many homogeneous samples can be excised from the equatorial region of one fruit, and ripening of the discs is similar to that found in whole fruit (Saltveit and Mencarelli, 1988; Campbell et al., 1990). Furthermore, experiments with pericarp discs produce results that are not confounded by the physical barriers found in whole fruit, such as the waxy epidermis, which restricts gas diffusion into the fruit, and by physiological problems such as hollow areas around the stem scar, where AA presumably enters the tomato fruit (Pesis and Marinansky, 1993). Eighty to 90 percent of the C_2H_4 produced by cherry tomatoes is released through the calyx scar (Cameron and Yang, 1982; de Vries et al., 1995). Browning around the stem end has been observed in grape berries exposed to 8300 parts per million of AA (Pesis and Frenkel, 1989).

Suppression of C_2H_4 production in tomato discs by AA exposure was therefore not caused by alterations in a physical barrier to diffusion, since the natural barriers were removed during disc preparation, but may have been caused by biochemical alterations occurring at the site of C_2H_4 biosynthesis. Applied AA may also act as a fungistat; samples remained red-ripe for 2 to 3 weeks and were seldom lost to the fungal (*Alternaria*) decay that occasionally affected control fruit (data not shown).

Physiological Responses to Inhibitors of ADH and AlDH

Both in vivo and in vitro oxidation of ethanol by horse liver ADH was inhibited by pyrazole and the more active 4-substituted pyrazole, 4-MP (Theorell et al., 1969). Although these inhibitors are effective on isolated ADH, their in vivo effectiveness under anaerobic conditions in plants has been questioned (Pryor and Huppatz, 1982; Small et al., 1989). Nonetheless, simple 4-substituted pyrazoles have been reported to be competitive inhibitors of ethanol in the maize ADH reaction (Pryor and Huppatz, 1982), and they almost completely inhibit conversion of ethanol to AA in carrot cultures (Perata and Alpi, 1991). Ripening (lycopene production) of MG tomato discs was unaffected by the application of 20 μL of an aqueous solution of up to 100 mM 4-MP or 20 mM 4-IP to their cut surface. Discs were also not injured by treatment with up to 4 mM 4-MP in conjunction with AA at 3 μL g^{-1} or ethanol at 8 μL g^{-1}.

DIS or tetraethylthiuram disulfide inhibits AlDH by preventing AA oxidation via modification of protein-SH groups (Deitrich and Erwin, 1971), and CYN is a potent in vivo inhibitor of AlDH (Marchner and Tottmar, 1978). CYN has been used to examine the effects of alcohol and AA in the fermentative metabolic pathway in laboratory animals. Previously published (Deitrich et al., 1976; Marchner and Tottmar, 1978) physiologically active levels of CYN or DIS were applied to block AlDH activity in tomato discs. Ripening was slightly stimulated by CYN ± AA, whereas DIS ± AA slightly inhibited ripening. After ripening for 6 d, CYN-treated tissue had ethanol levels approximately 3-fold higher than the controls, whereas AA levels were no different from the controls. However, tissue AA or ethanol levels were no different between DIS-treated and control tissue (data not shown). It remains unclear why these AlDH inhibitors produced different ripening responses; therefore, their use for studying the aerobically active fermentative pathway in vivo is questionable.

Ripening Analysis with Inhibitors of ADH

Ethanol

Treatment with inhibitors (4-IP and 4-MP) caused ripening inhibition in discs. Although ripening of 4-IP-treated discs was always slower than that of the controls, it was always faster than in discs exposed to an inhibitory ethanol concentration (e.g. 8 μL g^{-1}; Fig. 6). Treatment with 4-IP had no effect on the rate of ripening of discs treated with ethanol at 8 μL g^{-1} fresh weight for up to 7 d. Discs treated with 4-IP plus ethanol had started to ripen by d 8 and were significantly more ripe than the ethanol-treated discs by d 9. Tissue AA levels were consistently lower in the 4-IP plus ethanol treatment (approximately 80 μg g^{-1} fresh weight) throughout ripening compared with the ethanol treatment alone (approximately 150 μg g^{-1}), whereas ethanol levels were similar (approximately 7–8 mg g^{-1} fresh weight) in both treatments (Fig. 7).

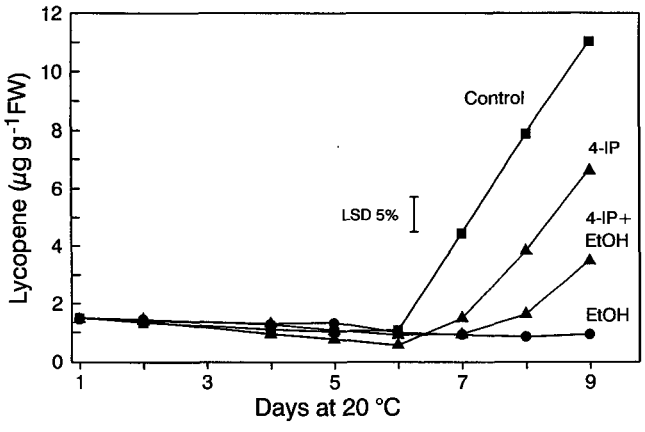

Figure 6. Influence of 4-IP on ethanol-induced ripening inhibition (lycopene content) of MG tomato pericarp discs. Discs were treated at 20°C with 50 μL of 20 mM 4-IP and then exposed to an inhibitory concentration of ethanol (EtOH) vapors (8 μL g^{-1} fresh weight) for 4 h the following day. Lycopene data points are the means of 18 replicates. FW, Fresh weight.

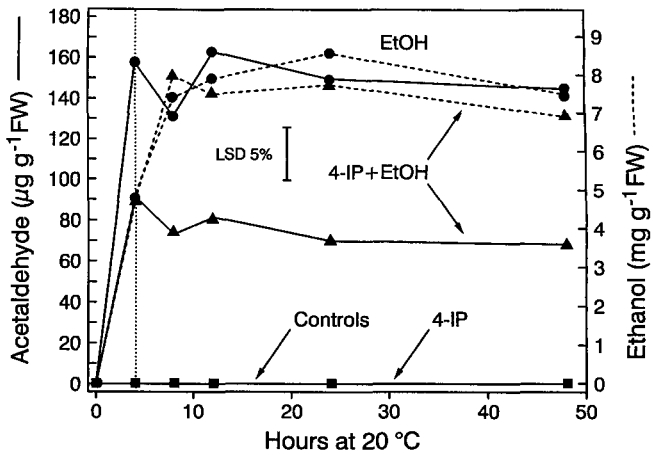

Figure 7. Influence of 4-IP on endogenous levels of ethanol (EtOH) and AA in MG tomato pericarp discs. Discs were treated at 20°C with 50 μL of 20 mM 4-IP and then exposed to an inhibitory concentration of ethanol vapors (8 μL g^{-1} fresh weight) for 4 h the following day. The vertical dashed line represents the termination of the ethanol treatment. AA and ethanol data points are the means of three replicates. FW, Fresh weight.

After 24 h, measurement of AA from 4-MP-treated tissue receiving a 4-h static exposure to ethanol at 2, 4, or 8 μL g^{-1} was significantly reduced by 93, 88, and 85%, respectively. Tissue AA levels in the ethanol plus 4-MP-treated discs were at least 25% lower than levels in the ethanol-treated tissue for 72 h. During this time, ethanol levels were usually higher in discs previously treated with 4-MP, but these discs ripened faster than discs treated with ethanol alone (data not shown).

If ethanol was the effective agent inducing ripening inhibition, then increasing tissue ethanol levels would further delay ripening, as is the case when fruit are treated exogenously with increasing levels of ethanol (Kelly and Saltveit, 1988; Saltveit and Mencarelli, 1988; Saltveit, 1989). However, even in the presence of excessive tissue ethanol, tissue ripened faster when ADH inhibitors reduced the conversion of ethanol to AA. This increase in ripening of the ethanol plus inhibitor-treated discs over the ethanol-treated discs occurred even though the ADH inhibitors alone caused some ripening inhibition. These data support our assumption that AA, not ethanol, is the agent responsible for ethanol-induced ripening inhibition.

AA

To determine whether ripening inhibition in ethanol- or AA-treated tissue could be attributed to elevated AA levels, ADH inhibitors were applied in concert with AA. Discs of MG2 tomato fruit were bathed in 2 mL of 4 mM 4-MP for 10 min and exposed to 1 μL g^{-1} AA vapors the following day. The discs were frozen immediately following removal from the 4-h static treatments or after being ventilated an additional 2 or 4 h. There was no appreciable production or accumulation of AA or ethanol in control tissue not exposed to AA (Fig. 8). Tissue AA concentration was highest immediately after exposure to AA, and 4-MP blocked in

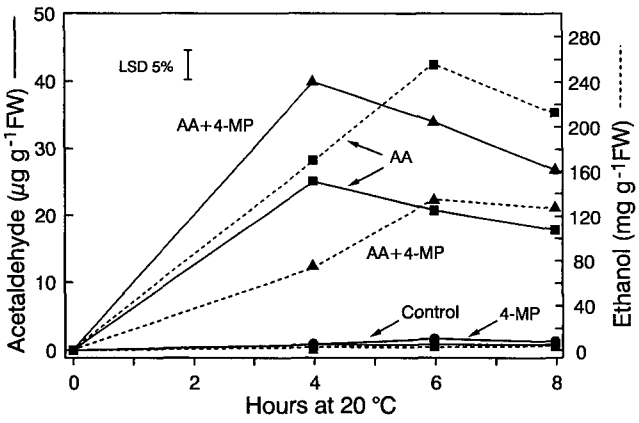

Figure 8. Influence of 4-MP on endogenous levels of AA and ethanol in MG tomato pericarp discs. Discs were soaked for 10 min in 4 mM 4-MP and then exposed for 4 h at 20°C to 1 μL g^{-1} AA vapor the next day. Samples were taken immediately after the AA exposure and after ventilation in humidified air for an additional 2 or 4 h at 20°C. Data points are means of three replicates. FW, Fresh weight.

vivo AA loss by 61, 64, and 52% after 0, 2, and 4 h, respectively (these times correspond to 4, 6, and 8 h in Fig. 8). Measured amounts of ethanol from AA-treated tissue was higher than from tissue treated with AA plus 4-MP by 57, 47, and 40% at 0, 2, and 4 h, respectively. Therefore, it appears that 4-MP inhibited in vivo ADH activity and metabolism of AA by approximately 40 to 60%.

Since 4-MP was only partially effective as an in vivo ADH inhibitor, we analyzed the in vitro effectiveness of 4-MP and 4-IP on ADH activity in tomato extracts. The ADH inhibitor 4-MP was determined to be a competitive inhibitor of ADH with respect to ethanol, with an apparent K_i = 6.0 μM, whereas the apparent K_m for ethanol was 5.5 mM (Fig. 9). 4-IP was also determined to be a competitive inhibitor of ADH with respect to ethanol, with an apparent K_i of 11 μM (data not shown). It appears that 4-MP and 4-IP are very effective in vitro inhibitors of tomato ADH. Their moderate in vivo inhibitory activity may result from poor uptake or metabolism of the inhibitors.

Since only about 4% of AA exogenously applied to discs was recovered after 4 h (Fig. 8), we evaluated whether tomato tissue metabolizes AA. Tomato discs and juice were prepared with or without boiling (to kill endogenous enzyme activities), allowed to equilibrate for 2 h, and treated with a range of AA concentrations. Recovery of applied AA was approximately 88% from extracts and from boiled extracts and discs over the range of AA at 2.5 to 50 μg g^{-1}. In contrast, recovery of AA from discs was 11% over the range of 2.5 to 25 μg g^{-1} and 27% for 50 μg g^{-1}. Loss of AA from the extracts and boiled samples was negligible. Therefore, it appears that metabolism of exogenously applied AA by excised tomato pericarp discs was significant, as it is in seeds (Oppenheim and Castelfranco, 1967). This rapid metabolism of AA by intact discs likely explains why a continuous supply of AA must be present to inhibit ripening.

Application of 4-MP to discs the day before a 4-h exposure to AA significantly increased the inhibitory effect of AA on ripening (Fig. 10). The additional inhibition of ripening caused by 4-MP increased as the concentration of AA increased from 500 to 2500 μg g^{-1} (for the sake of clarity, data for intermediate AA levels are not shown). For example, on d 10, the lycopene content of control discs was reduced by 9.5% (from 15.8 to 14.3 μg g^{-1}) by 4-MP, whereas ripening was reduced by 25% (from 17.3 to 12.9 μg g^{-1}) and 38% (from 7.1 to 4.4 μg g^{-1}) when 4-MP was used in conjunction with AA at concentrations of 500 and 2500 μg g^{-1}, respectively (Fig. 10). These results are consistent with the hypothesis that AA, not ethanol, is responsible for ripening inhibition. Results presented in Figure 10 are consistent with those in Figure 8, which shows that the appli-

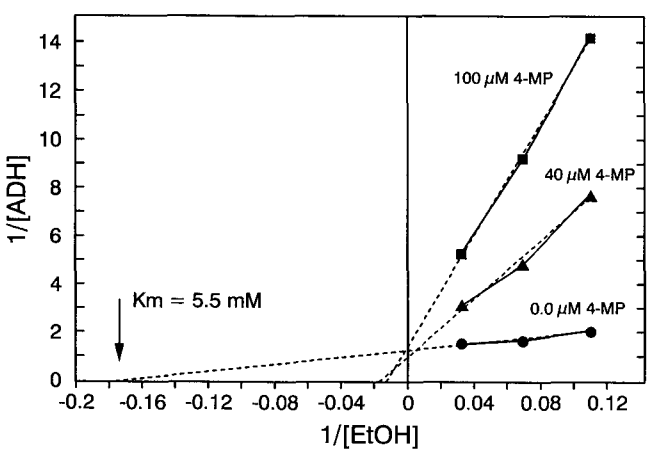

Figure 9. A Lineweaver-Burk plot of ADH activity (units mg^{-1} protein, where 1 unit of ADH activity corresponds to the production of 1 μmol NADH min^{-1}) at various substrate (ethanol [EtOH]) concentrations (mM) and with various levels of the ADH inhibitor 4-MP. Enzyme extracts were prepared and analyzed as described in "Materials and Methods." It was determined that 4-MP was a competitive inhibitor of ADH with respect to ethanol, with an apparent K_i = 6.0 μM and an apparent K_m for ethanol of 5.5 mM.

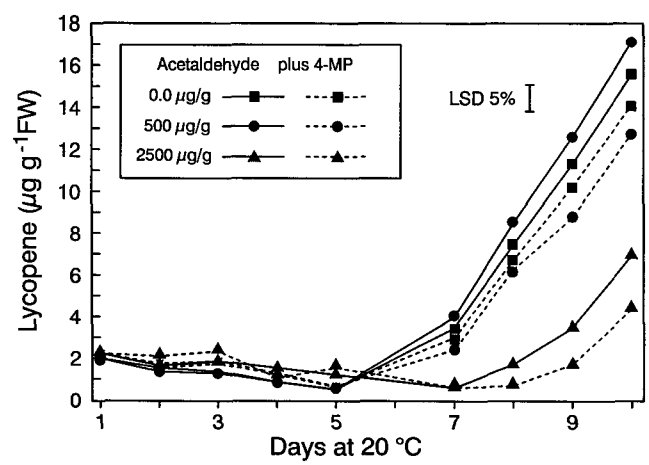

Figure 10. Effect of the ADH inhibitor 4-MP on the ripening of MG pericarp discs exposed to various levels of AA. Discs were treated with 40 μL of 50 mM 4-MP the day before they were exposed to 500 or 2500 μg g^{-1} for 4 h at 20°C. Lycopene data are means of 12 replicates. FW, Fresh weight.

cation of AA with 4-MP yields higher tissue AA levels and lower tissue levels of ethanol than does AA without 4-MP.

The ripening of pericarp discs was inhibited about 3 to 4 d by a 3-d exposure to a flow of AA vapors (280 ± 20 μg g^{-1}) in air with or without 4-MP (40 μL of 100 mM 4-MP) (Fig. 11). Again, 4-MP-treated discs ripened slower than the control but faster than the AA-treated discs. By d 14, 4-MP alone had reduced ripening (lycopene synthesis) by 14% (from 35.2 to 30.2 μg g^{-1}) compared with the controls, whereas both AA and AA plus 4-MP-treated discs had lycopene contents of 24 μg g^{-1} fresh weight. It appears that 4-MP does not significantly alter the ability of AA to retard ripening of tomato pericarp discs. If AA had to be converted to ethanol to exert its inhibitory effect on ripening, then an ADH inhibitor should reduce the effectiveness of applied AA. That this did not occur suggests that AA, not ethanol, is the active agent causing the inhibition of ripening.

The difference in results between the 4-h static and the 3-d flow-through exposures to AA vapors may be related to the endogenous levels of AA produced by both treatments. Discs treated with 4-MP and exposed to AA had the highest tissue levels of AA recovered during the 3-d AA exposure (Fig. 12). Endogenous AA levels declined in both treatments but decreased so rapidly to near 0 levels in 4-MP-treated discs that discs without 4-MP had significantly higher AA levels from d 4 to 7. The prolonged exposure to higher AA levels in discs without 4-MP after AA was discontinued could have retarded ripening in these discs and minimized the differences between the two treatments. If the inhibitory effect of AA on the ripening of climacteric fruit is ubiquitous, it may help explain why the application of ethanol to mango fruit (Burdon et al., 1994) has no effect on ripening, whereas applications of AA have profound ripening-related effects (Burdon et al., 1996).

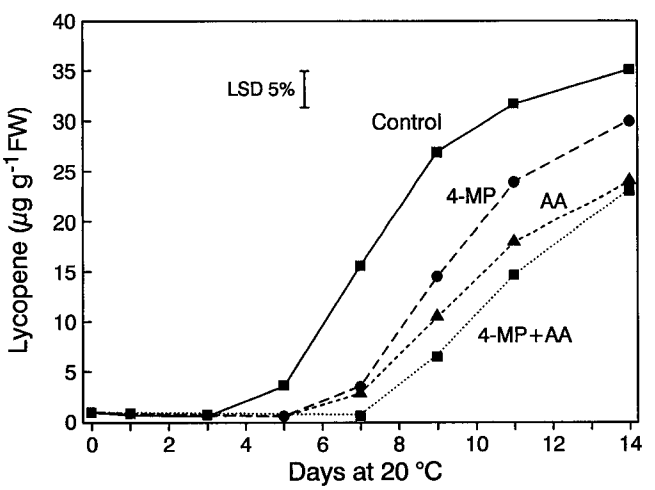

Figure 11. Effect of the ADH inhibitor 4-MP on the ripening of MG pericarp discs exposed to AA. Discs were treated with 40 μL of 100 mM 4-MP the day before they were exposed to 280 ± 20 μg g^{-1} AA in air for 3 d at 20°C. Measurements of lycopene content are described in "Materials and Methods." Lycopene data are means of 18 replicates. FW, Fresh weight.

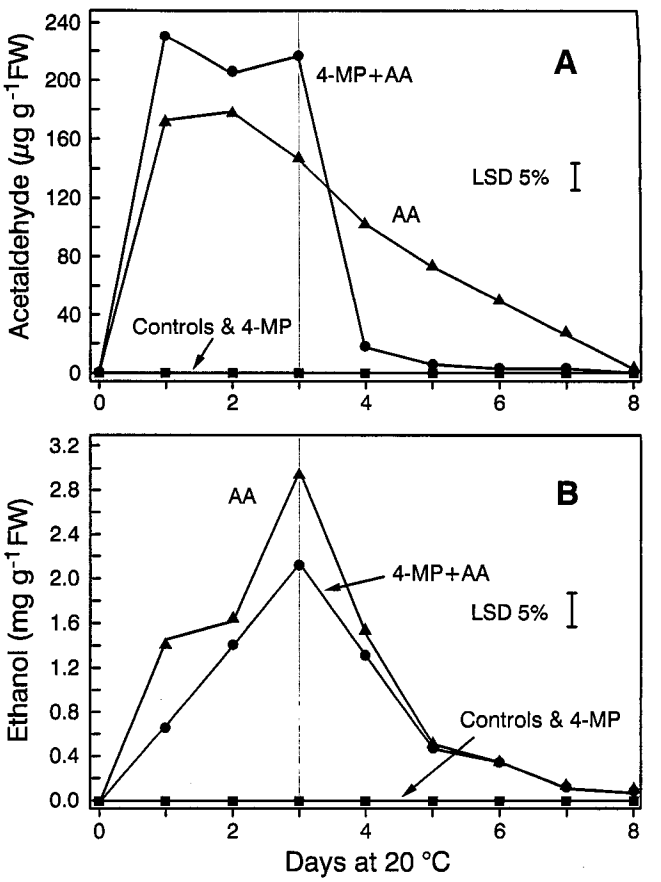

Figure 12. Effect of the ADH inhibitor 4-MP on the AA (A) and ethanol (B) content of MG pericarp discs exposed to AA. Discs were treated with 40 μL of 100 mM 4-MP the day before they were exposed to 280 ± 20 μg g^{-1} AA in air for 3 d at 20°C. Endogenous levels of AA and ethanol are the means of three replicates. The vertical dashed line represents the termination of the AA exposure. FW, Fresh weight.

Endogenous ethanol, whether induced by anaerobic fermentation (Kelly and Saltveit, 1988) or by exogenous application of ethanol (Saltveit, 1989), inhibits the ripening of tomato fruit (Saltveit and Sharaf, 1992; Pesis and Marinansky, 1993), as does the exogenous application of AA (Pesis and Marinansky, 1993). Applying exogenous AA and ethanol to excised discs of tomato pericarp tissue and monitoring endogenous levels of AA and ethanol as well as the synthesis of C_2H_4 and lycopene, has shown that ripening inhibition (i.e. reduction in lycopene synthesis) is more closely related to the endogenous concentration of AA than it is to ethanol. Two compounds that inhibit ADH and thereby reduce the interconversion between AA and ethanol showed that the inhibitory effectiveness of ethanol was reduced when its conversion to AA was reduced (as shown by reduced endogenous AA levels), whereas the effectiveness of AA was somewhat improved by these ADH inhibitors. The endogenous tissue concentration of AA that was associated with ripening inhibition was similar whether induced by exogenous exposure to AA or ethanol, whereas the endogenous ethanol concentration that was associated

with ripening inhibition was vastly different depending on whether it was induced by exogenous exposure to AA or ethanol. These lines of evidence strongly suggest that AA, not ethanol, is the active compound inhibiting tomato fruit ripening.

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