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INFLUENCES OF SHADING AND ETHYLENE-RELEASING COMPOUNDS ON ANTHOCYANIN BIOSYNTHESIS AND MATURATION OF EARLY APPLE CULTIVARS

By

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ABSTRACT

INFLUENCES OF SHADING AND ETHYLENE-RELEASING COMPOUNDS ON ANTHOCYANIN BIOSYNTHESIS AND MATURATION OF EARLY APPLE CULTIVARS

By

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High temperatures and shading reduce anthocyanin biosynthesis in apple fruits. Ethephon (2-chloroethylphosphonic acid) enhances fruit coloration, but generally initiates ripening and shortens the shelf life of apples. The influences of shading levels and ethylene-releasing compounds on anthocyanin biosynthesis were investigated using early maturing apple cultivars.

'Paulared' apple trees previously treated with low concentrations of 2-chloroethylmethyl-bis-(phenylmethoxy)-silane (CGA 15281), were shaded at 0%, 51%, 73% or 92% of full sunlight two weeks before harvest. Fruit coloration and maturation were not affected by 0.5 mM or less CGA 15281. Shaded fruits were firmer but poorly colored and smaller than fully exposed fruit. Fruit maturation was not affected by shading.

'McIntosh' apple trees were treated with ethephon or CGA 15281 two or three weeks before harvest. Solvolytic fragmentation of ethephon was slow and constant, resulting in increased anthocyanin biosynthesis, autocatalytic ethylene production and flesh softening within six days of application. Solvolytic fragmentation of CGA 15281

occurred quickly, especially when applied three weeks before harvest. CGA 15281 at 1.0 mM increased anthocyanin biosynthesis without concomitant fruit ripening.

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Guidance Committee:

The paper format adopted for this thesis is in accordance with departmental and university regulations. Section II and Section III were written for publication in the *Journal of the American Society for Horticultural Science*.

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INTRODUCTION

High quality, early season apple cultivars like 'Paulared' and 'McIntosh' potentially command high market returns. Since most Michigan fruit is marketed as U. S. Extra Fancy, a fifty percent red color requirement must be met. In poor coloring years, a significant portion of the crop may not meet minimum grade requirements for color. Grower returns are substantially reduced as a result.

Apple producers take several approaches to improving coloration. Dormant pruning, followed by summer pruning, improves light penetration through the trees' canopies. Mineral nutrition, i.e., nitrogen and potassium, is monitored and adjusted accordingly. Growth regulators, mainly daminozide (Alar) and ethephon, are used extensively. Consistently poor coloring strains may be replanted or top worked with high coloring strains. Some growers delay harvest until fruit meets minimum color standards.

Although such approaches improve color significantly, the postharvest integrity of the fruit is sometimes adversely affected. Internal fruit ethylene levels rise when ethephon is used or harvest is delayed. Consequently, this fruit softens rapidly and is suitable only for short term storage. If the fruit is not sold promptly, consumer dissatisfaction, hence, wholesale buyer resistance, occurs.

Further reductions in grower returns may result not only for these varieties, but for later season varieties as well. A better understanding of anthocyanin biosynthesis in apples is necessary to prevent quality problems resulting from these chemical and cultural practices.

SECTION I.

LITERATURE REVIEW

Mechanics of Anthocyanin Biosynthesis

The anthocyanin pigment found in Malus sylvestris cv. `Jonathan' and `Stayman' is 3-B-galactosidylcyanidin, or idaein (49). Idaein develops late in the growing season, concomitant with other metabolic changes. Anthocyanin development in `McIntosh' apples is correlated to an increase in metabolic activity of the pentose-phosphate pathway (21). By inhibiting glycolysis and diverting glucose metabolism to the pentose-phosphate pathway, anthocyanin biosynthesis increases. Faust (21) reports that anthocyanin development in apple is enhanced under these conditions because the amount of shikimic acid is increased.

Anthocyanins are derived from shikimic acid, the direct precursor of benzene ring B of idaein (21). The conversion of shikimic acid to cinnamic acid requires the intermediate formation of l-phenylalanine. Phenylalanine ammonia-lyase (PAL) catalyzes the deamination of l-phenylalanine to trans-cinnamic acid, thus diverting the amino acid from protein synthesis to the phenylpropanoid pathway (31). The hydroxylation of trans-cinnamic acid to p-coumaric acid is next catalyzed by cinnamic acid hydroxylase or CAH (26). Ring A is the condensation product of three acetic acid units (24), which are attached one by one to ring B (3). Lastly, galactose is attached to the cyanidin molecule (3).

Factors Affecting Anthocyanin Biosynthesis in Apple Fruits

Light In apple skin, anthocyanin biosynthesis has two distinct radiation dependent phases (54). The first is a 12 to 20 hour induction period during which no visible anthocyanin development occurs (40, 54). A linear increase in anthocyanin follows which is a function of irradation time at 600 to 750 nm (54). Faragher and Chalmers (20) report similar kinetics, and in agreement with others (12, 14, 17, 25), correlate light induction or activation of the PAL enzyme to anthocyanin biosynthesis in plant tissues.

De novo synthesis of PAL, the rate-limiting enzyme involved in anthocyanin biosynthesis, has been observed using radioactive labelling techniques in Xanthium (61), mustard (50), and potato tuber discs (48) during light stimulation. In Xanthium, however, the "induction" of PAL is due to a decrease in the rate of PAL inactivation (62). Camm and Towers (5) suggest that there is a continuous production of a PAL-inactivator and that changes in PAL levels may reflect changes in the rate of PAL inactivator synthesis, not an increase in de novo synthesis of PAL.

Zucker (59) and Engelsma (16) report that cycloheximide (CHI) will inhibit both the appearance and disappearance of PAL in potato tuber discs and gherkin seedlings, depending on specific conditions. Zucker (60) reports that PAL synthesis can be inhibited by 50% in potato tuber discs cultured on 5 uM CHI and by almost 100% in discs cultured on 10 uM CHI. Where the maximal level of PAL is allowed to form in discs cultured on water, i.e., after 24 hours of light induction, PAL disappearance is prevented by a 70 uM CHI treatment. The loss of PAL under dark conditions also requires de novo synthesis. From this work, it is suggested that 1) early phases of PAL induction involve _de novo_ synthesis of the enzyme in the absence of any turnover; 2) a system which degrades or inactivates the lyase subsequently forms in the tissue; and 3) the formation of this degrading or inactivation system also requires _de novo_ protein synthesis (60).

Faragher and Chalmers (20) confirm the light requirement in apple skin of whole fruit. Without light, no anthocyanin and virtually no PAL form. Light treatments which increase or decrease PAL levels cause comparable changes in the rate of anthocyanin accumulation. This indicates that PAL is the rate-limiting enzyme involved in anthocyanin biosynthesis in whole apple fruits (20).

Apple skin discs held under dark conditions develop PAL activity but without anthocyanin biosynthesis (20, 55). PAL, therefore, is not considered the factor responsible for the light requirement in apple fruits. Light may be required for the production of some other enzyme or factor involved. Once this light requirement is met, PAL activity appears to regulate anthocyanin biosynthesis in whole fruits.

Ethylene Once the light requirement is met, it is likely that ethylene controls PAL activity (8). Ethylene and treatments inducing ethylene, i.e., wounding, u.v. light, cycloheximide, gamma irradiation and disease increase _de novo_ PAL synthesis in several plant tissues (7, 9, 21, 27, 28, 36, 43, 44). Some tissues known to be unresponsive to white light treatments are responsive to ethylene treatments

(43, 44).

In studies of pea seedling response to ethylene treatments, Hyodo and Yang observed a marked increase in PAL in epicotyl segments exposed to 10 ul l^{-1} ethylene or more (27). The induction period of PAL is estimated at 6 hours with maximal activity at 30 hours. If ethylene is withdrawn, PAL increases for a short time, then stops. Upon reintroduction of ethylene, PAL increases. Early additions of cycloheximide or actinomycin-D almost completely inhibit PAL synthesis; however, when they are added late, they stimulate PAL synthesis. This is consistent with the view that PAL and PAL-inactivator systems exist, and that both may require de novo synthesis.

A similar increase in cinnamic acid hydroxylase (CAH) occurs in response to ethylene treatments in pea seedlings (26). This enzyme, which catalyzes the conversion of cinnamic acid to p-coumaric acid, is increased when exposed to 43 ul l^{-1} ethylene. Its induction period is about 14 hours, with maximum activity occurring at 30 hours. CAH inhibition requires more cycloheximide or actinomycin-D than does PAL inhibition.

In general, plant responses to ethylene are saturated at concentrations of 10 ul l^{-1} (1). Anthocyanin biosynthesis in apple fruits is saturated at 1 to 5 ul l^{-1} ethylene (8). Therefore, the rate of anthocyanin biosynthesis in ripe apples, which contain 25 to 2500 ul l^{-1} ethylene (4), is unaffected by additional ethylene treatments (7, 19). In unripe apples, ethylene increases the rate of anthocyanin accumulation 3 to 4 fold and PAL levels 3 to 6 fold (19). Final levels of anthocyanin are similar to those observed in ripe apples (19). In fig, ethephon similarly increases the rate of pigment formation but does not affect the total amount accumulated (42).

Temperature An inverse relationship exists between temperature and anthocyanin biosynthesis (13, 19, 55, 57). In `McIntosh' apples, rapid anthocyanin formation is correlated to the onset of cool weather (13). Creasy (13) suggests that the lower temperatures reduce sugar loss, thereby increasing the rate of anthocyanin biosynthesis. This hypothesis is suspect because: 1) his data do not reflect large differences in fruit sugar contents between temperature treatments (13), and 2) fruit from heavily cropped trees color and ripen later than fruit from lightly cropped trees subjected to the same temperature regimes. Under such circumstances, the stage of maturity may have had a greater effect on anthocyanin formation than low temperature (19).

High night temperatures may be more inhibitory to anthocyanin biosynthesis than high day temperatures (57). High temperature PAL inactivation, rather than low temperature PAL induction is probably dominant in regulating PAL levels (18). Tan (55) reports higher levels of the PAL-inactivator in apples held at 18 C than at 6 C. Reduced exposure of fruit to light leads to less induction of PAL; darkness leads to PAL inactivation (18). High, dark temperatures promote more PAL inactivation than low, dark temperatures. This further supports the hypothesis that anthocyanin biosynthesis is most inhibited due to high night temperatures (18).

Cultivar Apple fruit coloration is dependent on a cultivar and light interaction (29, 32, 41, 47). `McIntosh' apples require 25 to 30% sky (32) or 50% full sunlight (29) to develop acceptable color. Anthocyanin biosynthesis is unaffected, however, in Miller Sturdeespur `Delicious' by 95% shading from 55 days postbloom through harvest (47). Another strain of `Delicious', Vance, has significantly less anthocyanin biosynthesis when shaded 85% from 30 days postbloom through harvest (41). Uncovering this fruit 20 to 30 days before harvest increases anthocyanin levels to those levels present in fruit exposed to full sunlight throughout the period (41). Similarly treated `McIntosh' have significantly higher levels of anthocyanin than those exposed to light continually (41). For both `McIntosh' and Vance strain of `Delicious', a one day (or about 13 hours sunlight) lag period is reported for anthocyanin formation following uncovering. This lag period is consistent with earlier findings (40, 54).

Growth Regulators Two types of growth regulators are used to promote anthocyanin biosynthesis in apple and other fruit. The first type delays fruit maturation, and thus extends the growing season into cooler weather. The second induces ethylene production or undergoes solvolytic fragmentation to ethylene (22).

Daminozide (Alar) belongs to the first category. When applied to apple trees 60 days before harvest, daminozide delays the onset of the ethylene climacteric by 5 to 11 days (15, 30, 33, 56). Fruit firmness and color are enhanced (15, 23, 30), but fruit weight or size may be decreased (34). Fruit drop is also reduced (15, 30).

Stop-drop sprays such as fenoprop (2,4,5-TP) or NAA may induce ethylene production via auxin-induced ethylene biosynthesis. Ethephon (2-chloroethylphosphonic acid) is an ethylene-releasing plant growth regulator. In general, ethylene-inducing and ethylene-releasing growth regulators shorten the growing season by enhancing ripening because they are applied 2 to 3 weeks preharvest (10, 23, 35, 39, 52, 53, 56). They may consequently limit fruit size (10) because the cell enlargement stage is shortened. Anthocyanins are generally increased by ethylene-inducing and ethylene-releasing compounds (15, 22), but because ripening is also advanced, flesh firmness and shelf life decrease (10, 15, 39). Ethephon causes fruit abscission, therefore, an accompanied stop-drop spray is needed (15).

The ripening response to ethephon can be partially counteracted by daminozide. Daminozide decreases the rate of loss of flesh firmness and storage life caused by ethephon treatments (15, 33, 34) and has an additive effect on anthocyanin development (34). Consequently, a preharvest daminozide application is recommended whenever ethephon is used.

Even when daminozide is used, response to ethephon is often erratic (56). Solvolytic fragmentation of ethephon is very temperature dependent because ethephon has an energy of activation of about 30 kcal mol^{-1} (2, 38). At 25 C, decomposition is slow, even in basic solutions (2). It is unlikely that the cell pH of apples is higher than pH 7.5 (45, 46). This below optimum pH, coupled with large temperature fluctuations characteristic of late summer and early fall, often results in ethephon-induced autocatalytic ethylene production. Fruit ripening processes are initiated concomitantly.

CGA 15281 (2-chloroethylmethyl-bis-phenylmethoxy-silane) is, in contrast to ethephon, an unstable ethylene-releasing plant growth regulator (51). It has an energy of activation of only 10 kcal mol^{-1} (37) and is therefore much less sensitive to temperature fluctuations. Since solvolytic fragmentation of CGA 15281 is rapid in both acidic and basic pH regimes (6), the cell vacuole may be a favorable fragmentation site. Recent research with CGA 15281 confirms that solvolytic fragmentation occurs rapidly in apple fruits; however, fruit ripening may also be enhanced when CGA 15281 is used at higher concentrations (11).

Objectives of Present Study

Previous research indicates that anthocyanin biosynthesis in apple fruits can be regulated in the orchard by light and ethylene treatments. The present study was designed to determine: 1) how ethylene and light interact in anthocyanin formation in apple fruits under field conditions; and 2) if fruit coloration can be enhanced using ethylene-releasing plant growth regulators without stimulating fruit ripening. The results of these studies are found in the following papers.

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SECTION II.

EFFECT OF SHADING AND LOW ETHYLENE CONCENTRATIONS ON ANTHOCYANIN BIOSYNTHESIS OF `PAULARED' APPLES

Abstract

Low concentrations of 2-chloroethylmethyl-bis-(phenylmethoxy)-silane (CGA 15281) were applied two weeks before estimated harvest maturity to `Paulared' trees. Black shading cloth which eliminated 51%, 73% or 92% of full sunlight was wrapped around spurs bearing individual fruits. At Ø, 5, 8 and 11 days after shading, fruit weight, size, flesh firmness, anthocyanin content and internal ethylene measurements were taken. CGA 15281 had no significant effect, but shading decreased fruit size, weight and anthocyanin, and increased flesh firmness relative to fully exposed fruit. These differences were not attributed to differences in fruit maturation as shading treatments had no significant effect on starch degradation nor internal ethylene concentrations. The role of high temperature, low light levels and ethylene in anthocyanin biosynthesis and fruit maturation is discussed.

High summer temperatures frequently suppress anthocyanin formation in early apple cultivars by inactivating phenylalanine ammonia-lyase (PAL), the rate limiting enzyme involved in anthocyanin biosynthesis (6). The cultivar `Paulared', an important late summer apple grown in Michigan, perhaps colors poorly for this reason. Because of this, ethephon (2-chloroethylphosphonic acid) is commonly used to enhance coloration, presumably via ethylene-enhanced PAL synthesis. This compound often promotes fruit ripening, however, as its degradation is very temperature dependent (1, 11). Furthermore, some growers apply it at rates above those recommended with false hopes that it will substitute for good pruning practices which enhance light penetration.

In the present work, the effects of ethylene and shading on anthocyanin formation in the late summer cultivar 'Paulared' were investigated. Because fluctuating temperatures are common near harvest, we chose to use a much less temperature dependent ethylene releasing compound, CGA 15281 (2-chloroethylmethyl-bis-(phenylmethyoxy)-silane) instead of ethephon.

Materials and Methods

Experimental design Twenty-four 8-year old 'Paulared' apple trees were selected at a commercial orchard in Greenville, Michigan. The trees were of uniform vigor, size and crop load. Four concentrations of CGA 15281 were assigned at random to six whole trees each. Each tree was further divided into quadrants and four shading treatments were assigned so that none would occur more than once per tree. The experimental design was a split-split plot with chemical concentration in the whole plot, shading level in the subplot and sampling time in the sub-subplot.

Harvest date prediction Before chemical treatment, we predicted the optimum harvest date of the fruit based on long term CA storage recommendations (4). Ten uniform, blemish-free apples were harvested at random from trees and placed into 5.8 l plastic containers. An envelope of hydrated lime was placed into each to remove respiratory CO_2, a competitive inhibitor of ethylene action. At daily intervals, 1 ml gas samples were removed from each sealed container through a rubber septum fitted into the lid. These were analyzed using a Varian Aerograph Series 1700 gas chromatograph (Varian Instruments, Walnut Creek CA) equipped with a 1 m activated alumina column and flame ionization detector. Upon induction of the ethylene climacteric, or when 0.5 ul l^{-1} ethylene had accumulated within the container, the number of hours from sealing were recorded and multiplied by a constant of 0.125 days per hour (3). The product is the number of days from sampling to the estimated optimum harvest date in Michigan for long-term CA storage. This method was used in addition to the standard prediction methods based on temperature and days from full bloom.

<u>Chemical treatment</u> Two weeks before the estimated harvest date (20 August 1984), the following concentrations of CGA 15281 were applied to the respective whole trees as designated: 0 mM, 0.125 mM, 0.250 mM and 0.500 mM CGA 15281. All tank mixtures (including 0 mM control) contained X-77 (alkyl aryl polyethoxy ethanol and free fatty acids, Chevron Chem. Corp., San Francisco CA) and Fruitone-T (2,4,5-TP, Union Carbide Agricultural Products Co., Inc., Ambler PA). Both were added at the recommended rate of 0.625 ml l^{-1}.

<u>Shading treatment</u> Black polypropylene fabric (Chicopee Manufacturing Co., Chicopee GA) which eliminates 51%, 73%

or 92% of full sunlight was cut into 25 x 36 cm pieces. One day after chemical application, shading cloth was wrapped and stapled around spurs of individual fruits within the proper quadrant. The remaining uncovered quadrant of each tree was the 0% shading (or full sunlight) treatment.

Quality and maturity evaluations A minimum of 10 fruits per treatment and replication were harvested at 0, 5, 8 and 11 days after fruit shading to assess fruit quality and maturity. In addition to fruit weight and diameter measurements, a visual estimation of percent red surface coloration was made to the nearest 10%. Skin discs (1.4 cm diameter) were taken from the most highly colored cheek of each fruit and total anthocyanins were extracted in 10 ml of 10% oxalic acid at 5 C. Upon extraction, total anthocyanins were measured at 530 nm spectrophotometrically. Due to some shade cloth induced fruit scalding, only six fruits per treatment and replication were used for these evaluations after the first sampling time. Flesh firmness was determined using an Effigi penetrometer fitted with a 1.11 cm tip. Using the starch-iodine staining technique outlined for 'McIntosh' apples, a starch index was assigned to each fruit (13). Five fruits per treatment and replication were used for these measurements at the first sampling time; three fruits per treatment and replication were used at the later sampling times. Internal fruit ethylene samples were taken with a 1 ml syringe from the cavities of fruit at each sampling time. Because the number of fruit sampled varied between sampling times, this measurement was used to determine differences in maturity between chemical and shading treatments within sampling time only.

Statistical analysis All maturity and quality data were subjected to an analysis of variance. Treatment effects were determined using least significant difference (lsd) values at the 5% level.

Results and Discussion

Chemical treatment Low concentrations (75 ppm and less) of ethephon have been reported to stimulate coloration and advance the harvest of \`McIntosh' apples without sacrificing fruit condition or storage life (2, 7, 14). Since degradation of this compound to ethylene is very temperature dependent, results are often unpredictable under field conditions. CGA 15281, another ethylene releasing compound, is an unstable silicon compound, having a much lower energy of activation in contrast to ethephon (10).

The CGA 15281 concentrations used in this experiment were approximately equimolar to ethephon concentrations used with success on \`McIntosh' earlier (2, 7, 14). In this study, no significant differences in fruit coloration or maturity were attributed to concentrations of 0.5 mM CGA 15281 or less when applied to \`Paulared' apples 11 to 14 days before the optimum harvest date. Perhaps, the \`Paulared' cultivar is less sensitive to ethylene than \`McIntosh' or field temperatures were significantly higher during the course of our experiment, leading to a net PAL inactivation.

Shading treatment All shading treatments affected fruit size, weight, color and firmness. Fruit exposed to full sunlight continued to grow at a nearly linear rate during the 11 days observed (Figure 1). Shaded fruit ceased growth during the first five days, but appeared to adapt and increase in weight thereafter at a rate similar to the fully exposed fruit. These results support the findings of others (8, 9, 12,16) that shading reduces fruit size. However, nonsignificant differences in fruit weights of several artificially shaded cultivars have also been reported (15).

Firmness increased or remained the same during the first five days, depending on the shade treatment (Figure 2). This lack of softening has been reported elsewhere (8, 16) and was attributed to a shade-induced cessation of fruit growth and delayed maturation (8). Fully exposed fruit continued to grow at a linear rate and did not differ significantly from shaded fruit in ethylene concentration or starch index. Firmness decreased as fruit weight increased and shading affected both to about the same extent. Flesh softening during growth is largely due to the increase in intercellular airspace.

Anthocyanin content, expressed as absorbance at 530 nm decreased significantly during the first five days in all treatments except the non-shaded and 51% shaded treatments (Figure 3). Significant decreases, which probably reflected anthocyanin turnover, occurred during the last 3 days in fruit shaded 73% and 92%. Similar trends are seen in Figure 4 where percent red surface coloration was estimated

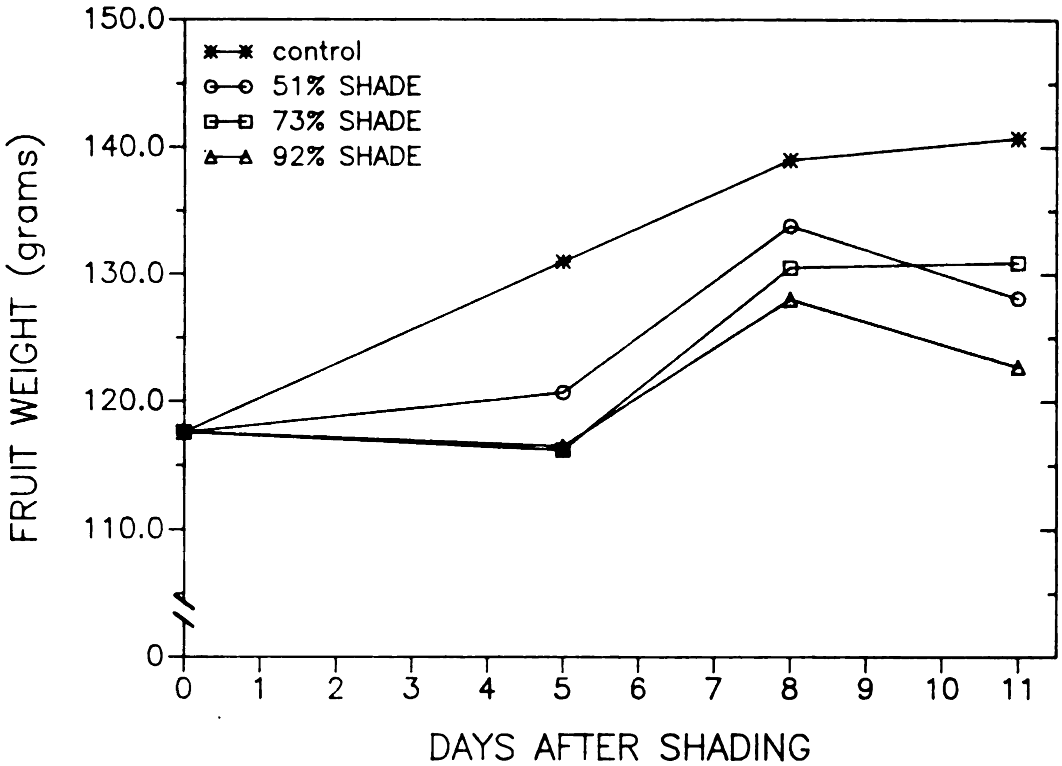

Figure 1. Fruit weight Ø, 5, 8 and 11 days after shading (n=144 except at day Ø where n=96Ø); lsd .Ø5 = 6.8Ø for comparing within same sampling time, and 5.87 within same shade level.

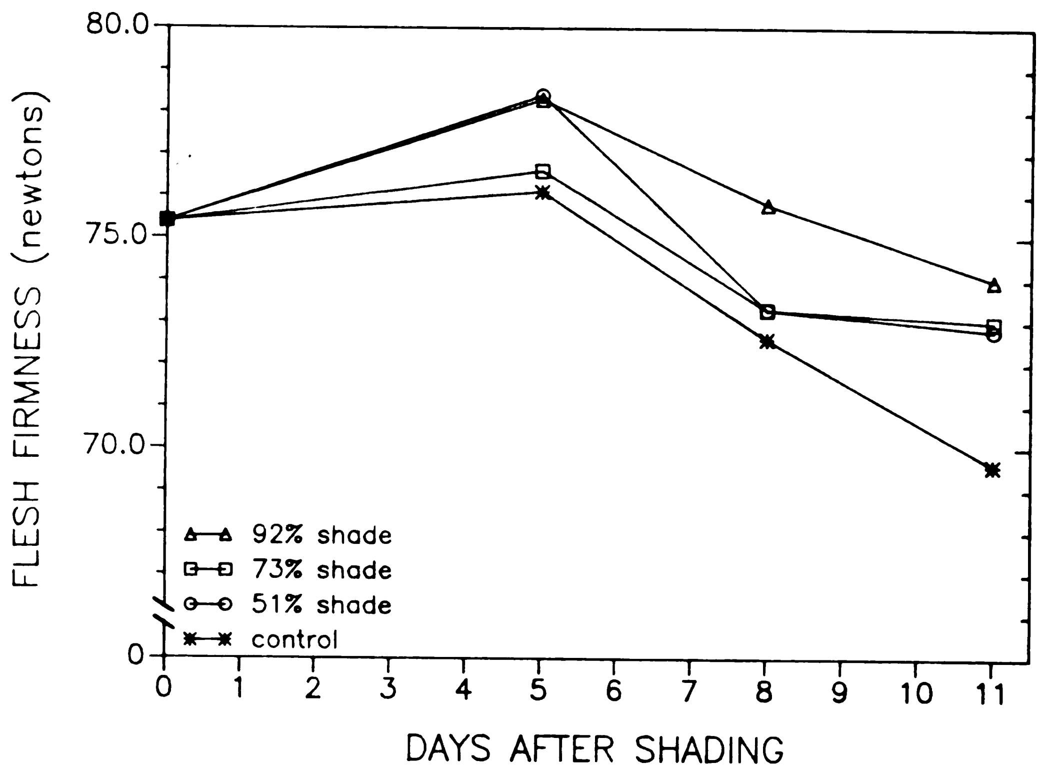

Figure 2. Flesh firmness of 'Paulared' apples 0, 5, 8 and 11 days after shading (n=72 except at day 0 where n=480); lsd .05 = 2.00 for comparing within the same sampling time, and 1.88 within same shade level.

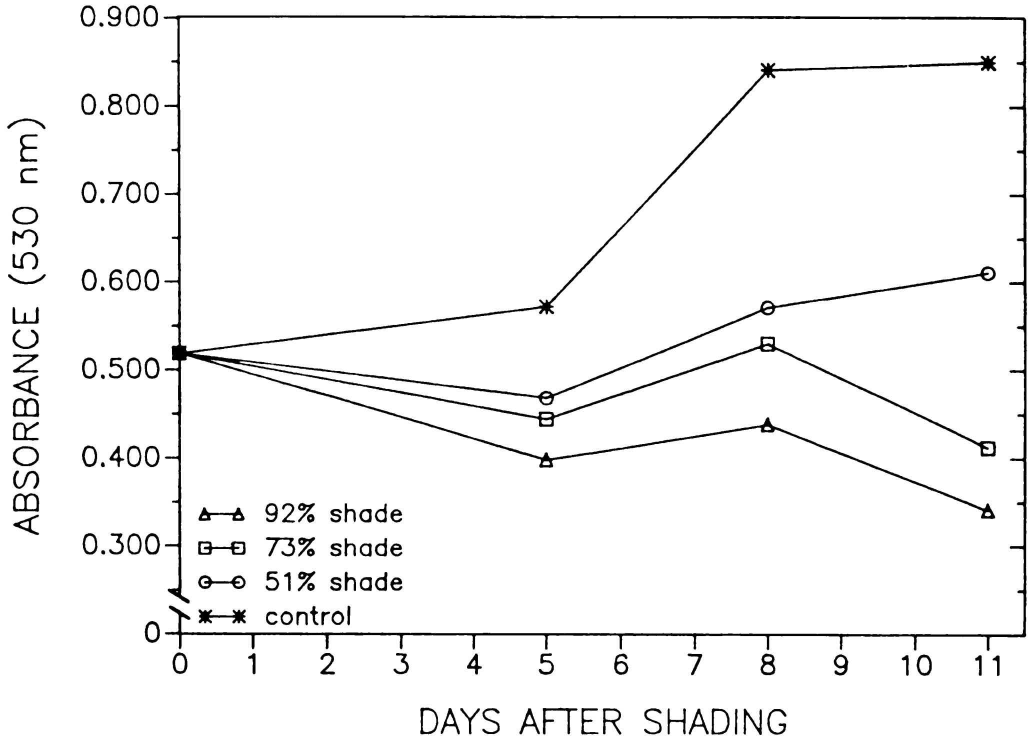

Figure 3. Absorbance at 530 nm of anthocyanin extracted from 'Paulared' apples 0, 5, 8 and 11 days after shading (n=144 except at day 0 where n=480); lsd .05 = .079 for comparing within same sampling time, and .076 within same shade level.

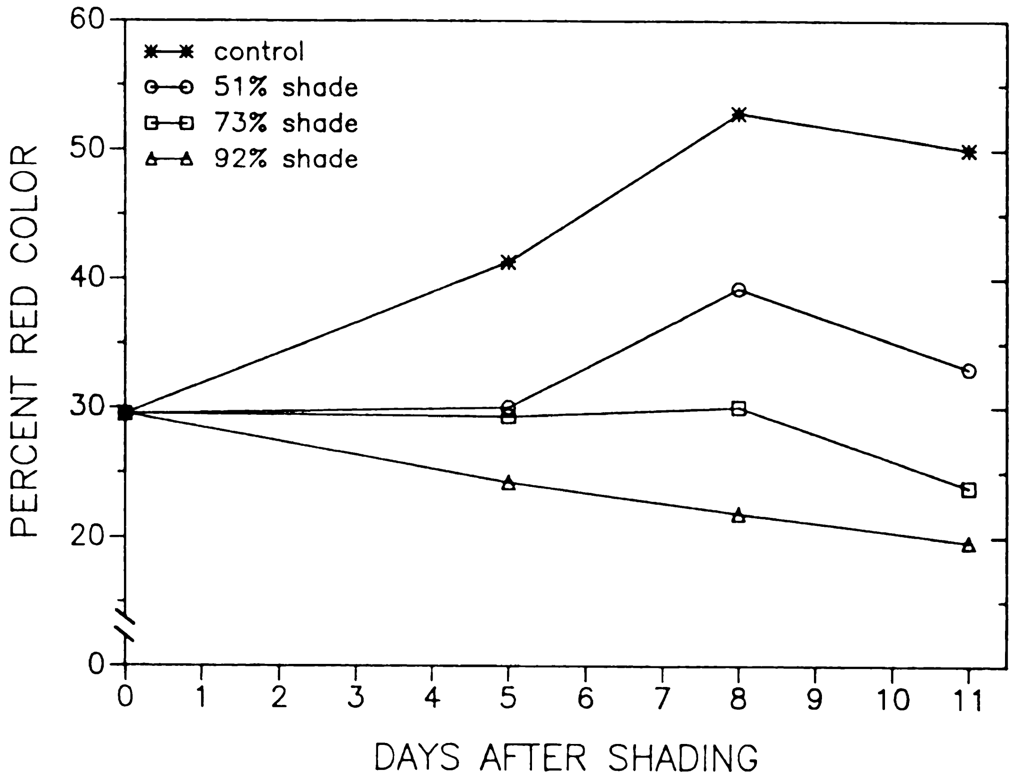

Figure 4. Visual estimation of percent surface coloration of 'Paulared' apples 0, 5, 8 and 11 days after shading (n=144 except at day 0 where n=480); lsd .05 = 4.4 for comparing within same sampling time, and 4.3 within same shade level.

visually at each sampling time.

Fruit maturation was unaffected by shading as no significant differences in starch indexes or internal ethylene concentrations were found at any sampling date. However, it was evident that the shading material not only reduced light penetration, but also increased temperatures surrounding the fruit as some fruit had to be discarded due to scalding. This is important since decreased light reduces PAL induction (5) and high temperatures inactivate PAL (17). High temperatures, combined with reduced light or darkness, are even more inhibitory as de novo PAL synthesis is reduced or inhibited and the amount of PAL-inactivator is increased concomitantly (5).

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SECTION III.

ANTHOCYANIN BIOSYNTHESIS AND MATURITY OF `MCINTOSH' APPLES AS INFLUENCED BY ETHYLENE-RELEASING COMPOUNDS

Abstract

Solvolytic fragmentation of 2-chloropropylphosphonic acid, 2-chloroethylphosphonic acid (ethephon), and 2-chloroethylmethyl-bis-(phenylmethoxy)-silane (CGA 15281) was studied. Based on laboratory results, ethephon and CGA 15281 were applied two or three weeks before the onset of autogenous ethylene production in 'McIntosh' apple. Solvolytic fragmentation of the compounds and autocatalytic ethylene production, anthocyanin biosynthesis, and flesh firmness changes of the fruit were monitored. 2.0 mM (290 ppm) of ethephon applied at either date degraded at a slow, constant rate resulting in a significant increase in anthocyanin biosynthesis within 6 days. However, autocatalytic ethylene production and flesh softening accompanied this increase. CGA 15281 generated ethylene within 3 hours of application. Ethylene levels of fruit treated with 1.0 mM CGA 15281 three weeks before harvest returned to low levels within 24 to 48 hours of application. Anthocyanin increased significantly within 6 days; this was not accompanied by autocatalytic ethylene production or accelerated flesh softening. Results indicate that anthocyanin biosynthesis can be enhanced in apples using ethylene-releasing compounds without inducing ripening.

The 'McIntosh' apple is one of the most important cultivars grown in the United States, ranking third in total production. Due to poor coloration, however, a significant percentage of the fruit harvested each year fail to meet the

U. S. Extra Fancy grade.

Cultivar, light, temperature and ethylene influence anthocyanin biosynthesis in apple skin (2, 3, 6, 9, 17). A light requirement must first be met, afterwhich anthocyanin biosynthesis is apparently limited by phenylalanine ammonia-lyase (PAL) (8). Ethylene enhances PAL activity in several tissues, including preclimacteric apples (7). High temperatures appear to inactivate PAL (18); however, the effect of temperature depends largely upon the stage of fruit maturity (6).

2-chloroethylphosphonic acid (ethephon), an ethylene-generating compound, is used commercially to enhance anthocyanin development in McIntosh and other early apple cultivars. Response to this compound is often erratic however, due to its relatively high energy of activation. At 25 C, decomposition is slow, even in basic solutions (1). Because of this, ethephon applied to trees with mature fruit often induces autocatalytic ethylene production. Ripening processes are initiated concomitantly, resulting in rapid cell wall breakdown and fruit softening.

Since development of the anthocyanin biosynthetic capacity requires only a brief exposure to ethylene or an olefin, an ethephon-like compound having a lower energy of activation is desirable. Anthocyanin biosynthesis could then be enhanced without sacrificing postharvest integrity. In a preliminary field study conducted in 1983, two unstable compounds, 2-chloropropylphosphonic acid and 2-chloroethyl-methyl-bis-(phenylmethoxy)-silane (CGA 15281) were compared to ethephon applied at similar rates. Degradation of the former (propylene-releasing) compound probably occurred prior to absorption as it did not affect anthocyanin biosynthesis. CGA 15281, however, stimulated anthocyanin development more quickly than ethephon and without any apparent stimulation of ripening.

In the present study, we followed the time course of ethylene generation from ethephon, 2-chloropropylphosphonic acid, and CGA 15281 under controlled pH and temperature regimes. A similar field study with McIntosh was conducted using only ethephon and CGA 15281. Autocatalytic ethylene production and postharvest changes of the fruit were monitored. We wished to determine if anthocyanin biosynthesis could be enhanced using ethylene-releasing compounds without stimulating fruit ripening.

Materials and Methods

Solvolytic fragmentation of ethylene- and propylene-generating compounds Twenty airtight polypropylene syringes (60 ml capacity) were used as vessels. Each was filled with 50 ml of ethylene-free air and immediately sealed with tightly fitting serum caps. Ten ml of citrate-phosphate buffer of pH 3.0, 4.0, 5.0, 6.0, or 7.0 was injected into quadruplicate vessels. The volume and pressure changes due to the addition of buffer were compensated for by adjusting the plunger to the 60 ml mark.

Ethephon, 2-chloropropylphosphonic acid, or CGA-15281 were injected via microsyringe from .1% stock solutions to one pH series each. The resulting concentration in 10 mls of buffer was 2.23 mM. Upon complete solvolytic fragmentation, 5 ul of ethylene or propylene would be released into the headspace of the vessels, disregarding the amount of gas dissolved in the buffer. The fourth pH series was used as a control.

The vessels were held at 20 C and gas samples were taken in duplicate with 1 ml syringes at 1.5, 3, 6, 12 and 24 hours. The headspace of each vessel was reduced by 2 ml to compensate for volume and pressure changes each time. Gas samples were analyzed using a Varian Aerograph Series 1700 gas chromatograph (Varian Instruments, Walnut Creek CA) equipped with a 1 m activated alumina column and flame ionization detector.

The experiment was a split-plot design with chemical and pH in the whole plot and time of sampling in the subplots. The experiment was replicated three times.

Field study Ten mature McIntosh trees bearing similar crop loads were selected at the Michigan State University Horticultural Research Center in East Lansing, Michigan. Each tree was divided into fifths and chemical treatments were assigned so that none would occur more than once per tree or location within the tree. The general experimental design was a split-split plot with treatment time in the whole plot, chemical treatment in the subplot, and sampling time in the sub-subplots.

Harvest date prediction Before chemical treatment, we predicted the optimum harvest date of the fruit based on long term CA storage recommendations(5). Ten uniform, blemish-free apples were harvested per tree at elbow depth and placed into 5.8 l plastic containers. An envelope of hydrated lime was placed into each to absorb respiratory CO_2, a competitive inhibitor of ethylene action. At daily intervals, 1 ml gas samples were removed from each sealed container through a rubber septum fitted into the lid. These were analyzed by gas chromatography. Upon induction of the ethylene climacteric, or when 0.5 ul l^{-1} ethylene had accumulated within the container, the number of hours from sealing were recorded and multiplied by a constant of 0.125 days per hour (4). The product is the number of days from sampling to the optimum harvest date in Michigan for long-term CA storage. This method was used in addition to the standard prediction methods based on temperature and days from full bloom.

Chemical treatment Three weeks before the estimated harvest date (19 September 1984), the following treatments were applied to the respective tree sections: 2.0 mM ethephon, 2.0 mM CGA 15281, 1.0 mM CGA 15281, 0.5 mM CGA 15281 and control. All tank mixtures (including control) contained both X-77 (alkyl aryl polyethoxy ethanol and free fatty acids, Chevron Chem. Corp., San Francisco CA) and Fruitone-T (2,4,5-TP); both were added at the recommended 0.625 ml l^{-1} rate. The same protocol was followed one week later when the second main plot was treated. Both chemical applications were completed between 8 and 9 am.

Solvolytic fragmentation of CGA 15281 and ethephon in apples At 0, 3, 6, 12, 24, 48, and 96 hours after application, internal ethylene samples were taken with a 1 ml syringe from the cavities of five fruits per replication and treatment. Solvolytic fragmentation of the ethylene-releasing chemicals was monitored by gas chromatography. Weather data recorded at the Research Center were used to interpret chemical absorption and degradation.

Quality and maturity evaluations A minimum of 15 fruits per treatment and replication were harvested at 0, 3, 6, 9, 12 and 15 days after the first application and through 12 days following the second. Five fruits from each sample were used to assess fruit quality and maturity at each time. In addition to fruit weight and diameter measurements, a visual estimation of the percent red surface coloration was made to the nearest 10%. Flesh firmness was determined using an Effigi penetrometer fitted with a 1.11 cm tip. Using the starch-iodine staining technique outlined for McIntosh apples (13), a starch index was assigned to each sample. Skin discs (1.4 cm diameter) were taken from the most highly colored cheek of each fruit and total anthocyanins were extracted in 10 ml of 10% oxalic acid at 5 C. Upon extraction, total anthocyanins were measured at 530 nm spectrophotometrically. The remaining ten or more fruit were stored in paper bags at approximately 20 C. Flesh firmness measurements were repeated in the same way at one and two weeks postharvest and the relative shelf life of fruit from each treatment was assessed.

All maturity and quality data were subjected to an analysis of variance and trend analysis. Treatment effects were determined using least significant difference (lsd) values at the 5% level. Rates of anthocyanin biosynthesis and fruit maturation were examined using linear regression; t values were computed and used to compare differences between treatment slopes.

Results and Discussion

Solvolytic fragmentation of ethylene- and propylene-generating compounds *in vitro* Solvolytic fragmentation was highly dependent on solution pH for all compounds studied. 2-chloropropylphosphonic acid buffered at pH 5.0, 6.0 and 7.0 degraded completely within 1.5 hours (see Figure 1A). These results explain, at least in part, our preliminary field results which showed no stimulation of anthocyanin biosynthesis by this compound. Complete degradation probably occurred before chemical absorption by the fruit, or possibly in the mixing tank. Because this compound appeared to be too unstable, it was not included in the 1984 field study.

CGA 15281 released ethylene more readily as acidity increased from pH 7.0 to 3.0. In contrast, ethephon was stable at pH 3.0, 4.0 and 5.0 (see Figures 1B and 1C). At pH 7.0, the amount of ethylene generated by both compounds was similar at each sampling time. However, neither compound underwent more than 30% solvolytic fragmentation at

Figure 1. Solvolytic fragmentation of A) 2-chloropropylphosphonic acid, B) CGA 15281, and C) ethephon in citrate-phosphate buffers adjusted to pH 3, 4, 5, 6 and 7. Incubation was at 20 C (n=6).

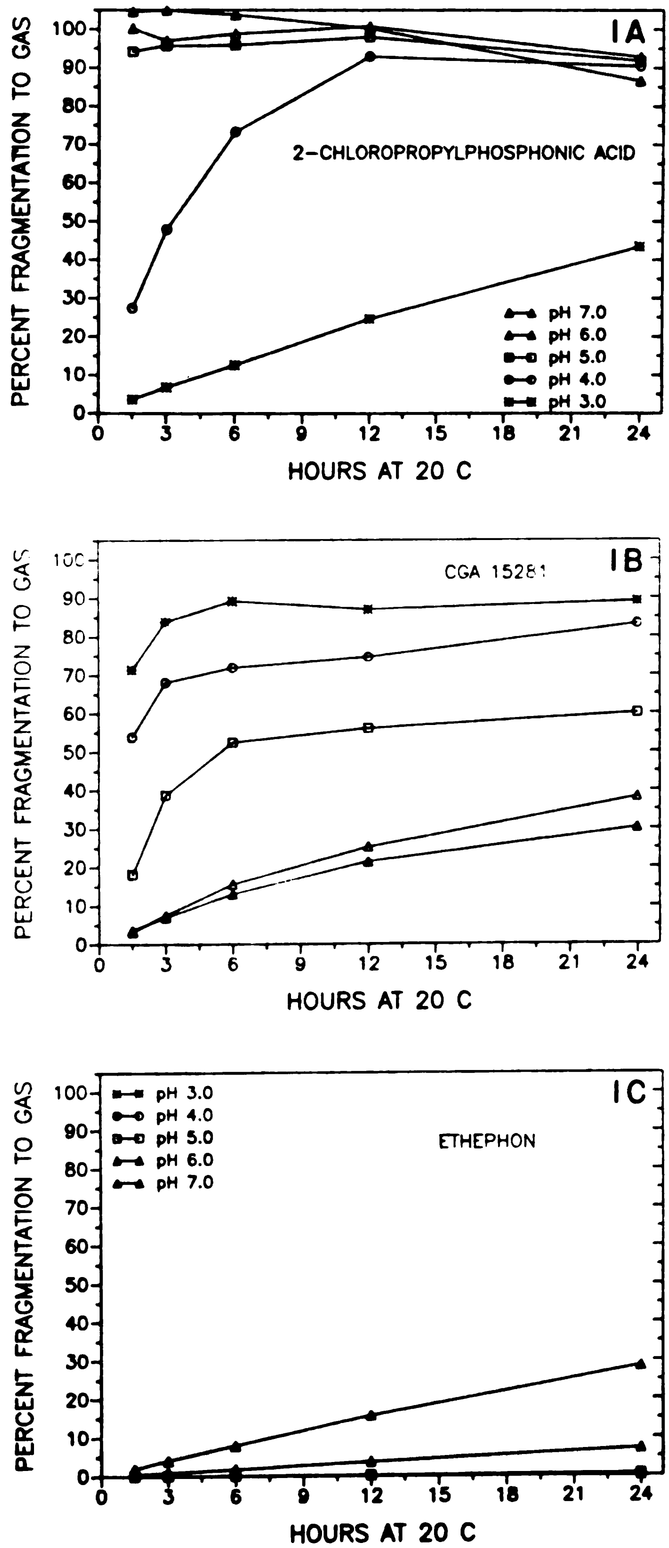

IA
2-CHLOROPROPYLPHOSPHONIC ACID
PERCENT FRAGMENTATION TO GAS
HOURS AT 20 C
pH 7.0
pH 6.0
pH 5.0
pH 4.0
pH 3.0
IB
CGA 15281
PERCENT FRAGMENTATION TO GAS
HOURS AT 20 C
IC
ETHEPHON
PERCENT FRAGMENTATION TO GAS
HOURS AT 20 C
pH 3.0
pH 4.0
pH 5.0
pH 6.0
pH 7.0

Figure 1.

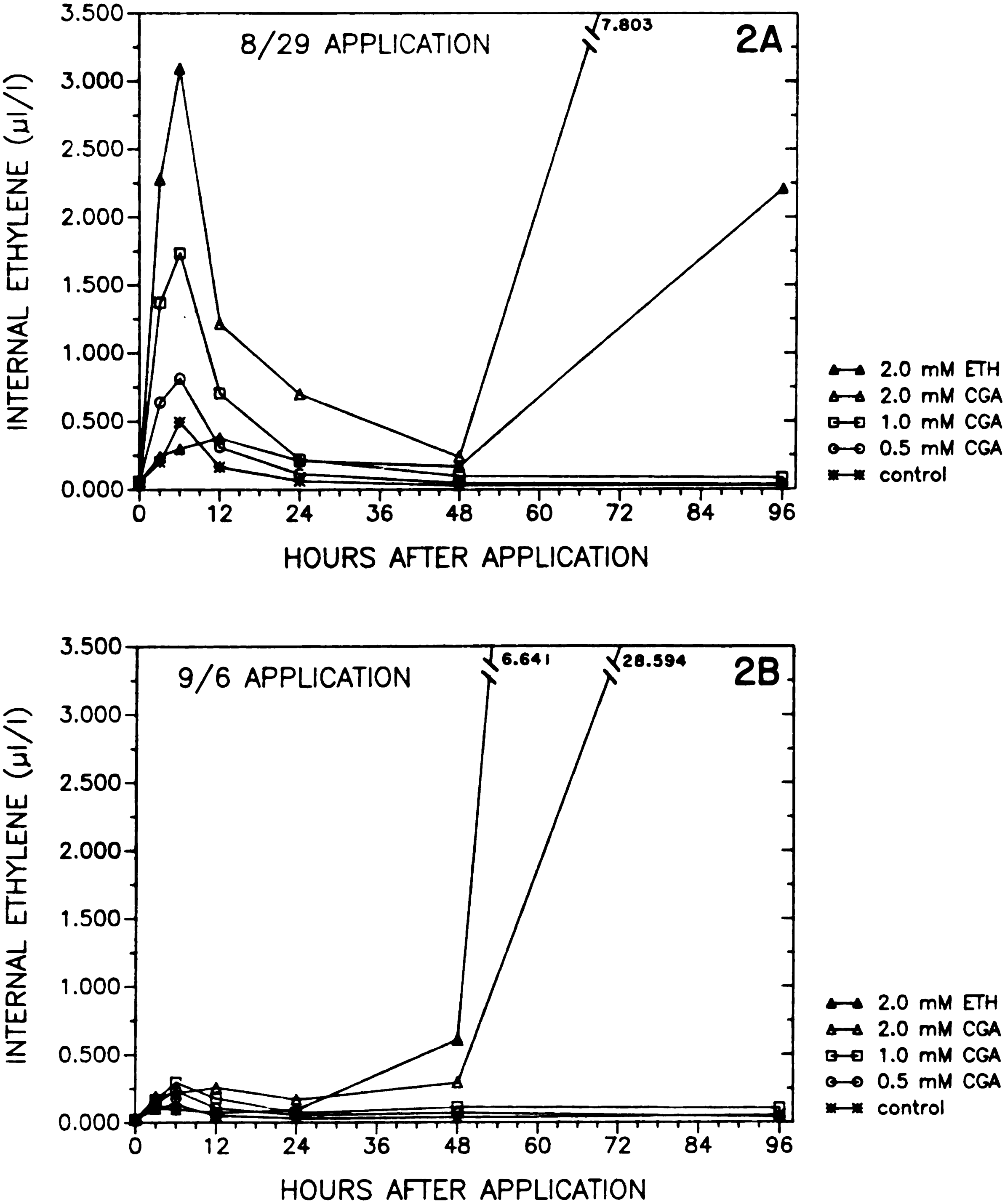
pH 7.0 when held at 20 C.

Solvolytic fragmentation of CGA 15281 and ethephon in apples

Several pH regimes which would affect the rate of solvolytic fragmentation would be encountered as these compounds are absorbed into the cell. In maize, the cytoplasmic pH is in the range of 7.0 to 7.5, and the pH of the vacuole about pH 5.0 to 5.5 (14,15). The cell wall probably has an intermediate pH. Due to the high concentration of malic acid found in apple fruits, however, we would expect the pH of these cell compartments to be more acidic. Therefore, the expected site of ethephon fragmentation would be largely cytoplasmic while fragmentation of CGA 15281 would be more favorable in the cell wall and vacuole. The rate of solvolytic fragmentation in the cytoplasm would be approximately equal for both compounds. Overall, the rate of ethylene release from CGA 15281 would probably be more rapid than that from ethephon in apple fruit tissue.

Internal ethylene concentrations of treated fruit are shown in Figures 2A and 2B. Solvolytic fragmentation of CGA 15281 to ethylene peaked about 6 hours after application on August 29, or three weeks before the predicted optimum harvest date. Internal ethylene concentrations were proportional to the concentration of CGA 15281 applied on this date. With the exception of the 2.0 mM treatment, internal ethylene concentrations returned to low levels within 24 to 48 hours of application. Ethephon degradation was slow, having no obvious "peak" time of ethylene release. Apparently, the internal ethylene concentrations of these

Figure 2. Solvolytic fragmentation to ethylene of CGA 15281 and ethephon applied to mature `McIntosh' trees on A) 29 August 1984 or B) 6 September 1984. Solvolytic fragmentation was measured as internal fruit ethylene by gas chromatography (n=25).



3.500
3.000
2.500
2.000
1.500
1.000
0.500
0.000
INTERNAL ETHYLENE (µl/l)
8/29 APPLICATION
7.803
2A
0
12
24
36
48
60
72
84
96
HOURS AFTER APPLICATION
2.0 mM ETH
2.0 mM CGA
1.0 mM CGA
0.5 mM CGA
control
9/6 APPLICATION
6.641
28.594
2B

Figure 2.

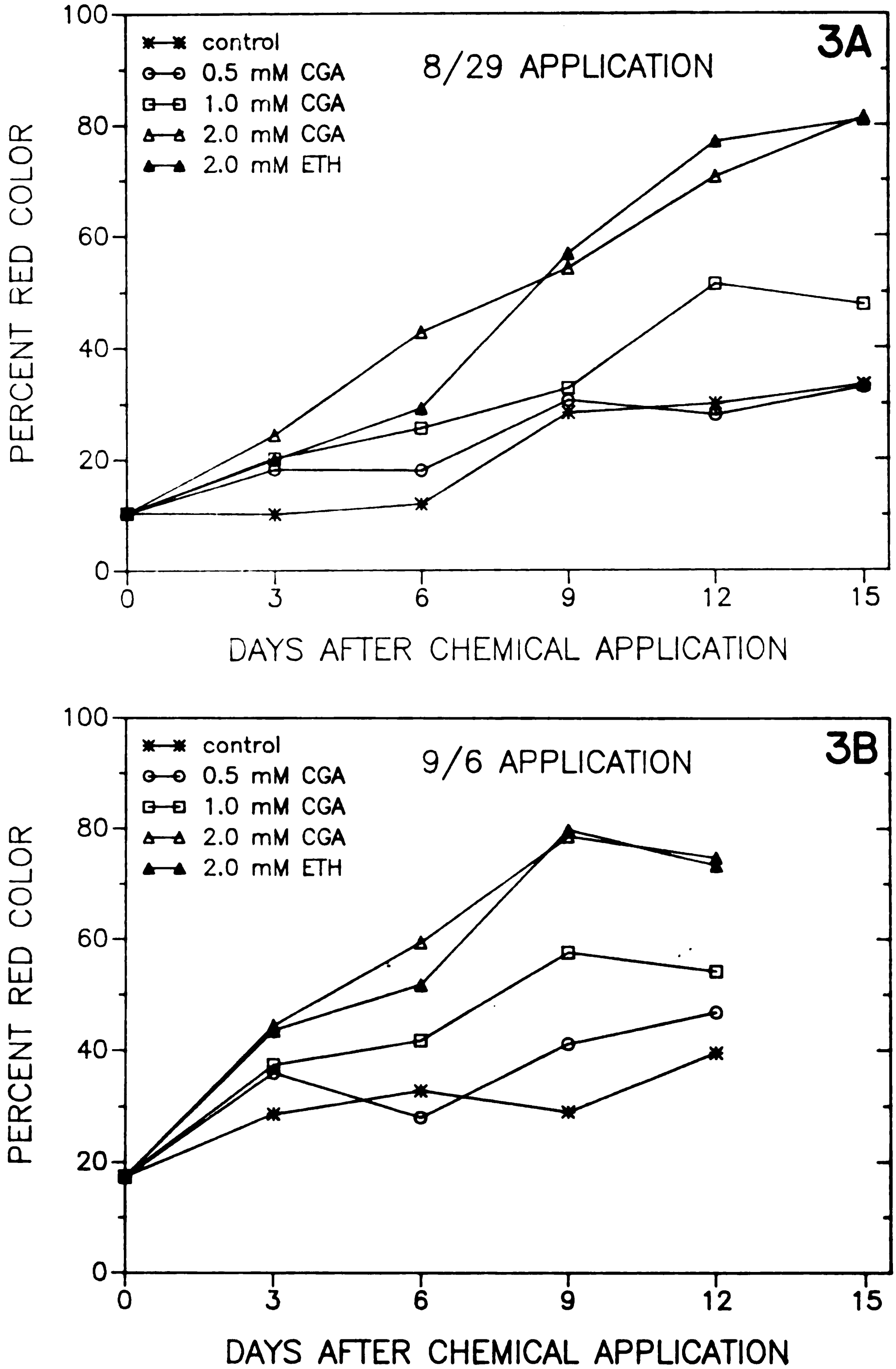
fruit, as well as those treated with 2.0 mM CGA, remained above the ambient level characteristic of preclimacteric fruit; within 2 to 4 days of application, autocatalytic ethylene production was induced by both treatments. Autocatalytic ethylene production did not occur until 12 to 15 days following treatment with the lower CGA concentrations (data not shown).

When applied to trees on 6 September, or two weeks before the predicted harvest date, we saw no well defined ethylene peaks due to chemical degradation (see Figure 2B). This difference in response between application dates is most likely due to the much cooler temperatures, i.e. a maximum of 32.2 C on 29 August vs. 18.3 C on September 6. Lower temperatures would account for slower rates in chemical degradation (1,11,12), and chemical absorption (10). With temperatures rising slightly during the following days, internal ethylene concentrations of fruit treated with 2.0 mM CGA 15281 or ethephon also rose. From the data, we cannot postulate whether the increase in internal ethylene observed at 48 hours after application was due to a delay of solvolytic fragmentation or if this was autocatalytic ethylene production. At 96 hours, however, it was apparent that the fruit had entered the ethylene climacteric.

Quality and maturity evaluations No significant differences in fruit weight or diameter were attributed to chemical treatment. Anthocyanin content, expressed as percent surface coloration and total anthocyanins extracted, increased over time but also depended on chemical treatment (see Figures 3A, 3B, 4A, and 4B). Regardless of the date applied, anthocyanin increased significantly in fruits treated with 1.0 mM or 2.0 mM CGA 15281, and 2.0 mM ethephon. Significant increases occurred within 6 days of the 29 August application. Fruit treated one week later responded similarly, except that the 1.0 mM CGA 15281 treatment did not promote a significant increase in anthocyanin until 9 days after application. Anthocyanin increases over time were generally nonsignificant in control fruit and fruit treated with 0.5 mM CGA 15281. Since highly significant linear trends characterized anthocyanin biosynthesis over time, we used linear regression to define the rate increases. At both application dates, a t test indicated that anthocyanin biosynthesis occurred at significantly greater rates in fruit treated with 1.0 mM CGA 15281 than control or 0.5 mM treatments. Both the 2.0 mM ethephon and CGA 15281 treatments promoted higher rates of anthocyanin biosynthesis than the 1.0 mM CGA 15281 treatment, however.

Flesh firmness also depended on an interaction of concentration and sampling time (see Figures 5A and 5B). Fruit treated with either 2.0 mM ethephon or 2.0 mM CGA 15281 three weeks prior to optimum harvest were significantly softer than control fruit after 9 and 12 days, respectively. Fruit treated with the same rates on September 6 were significantly softer within only 6 days. Twelve days after this application, fruit treated with 1.0

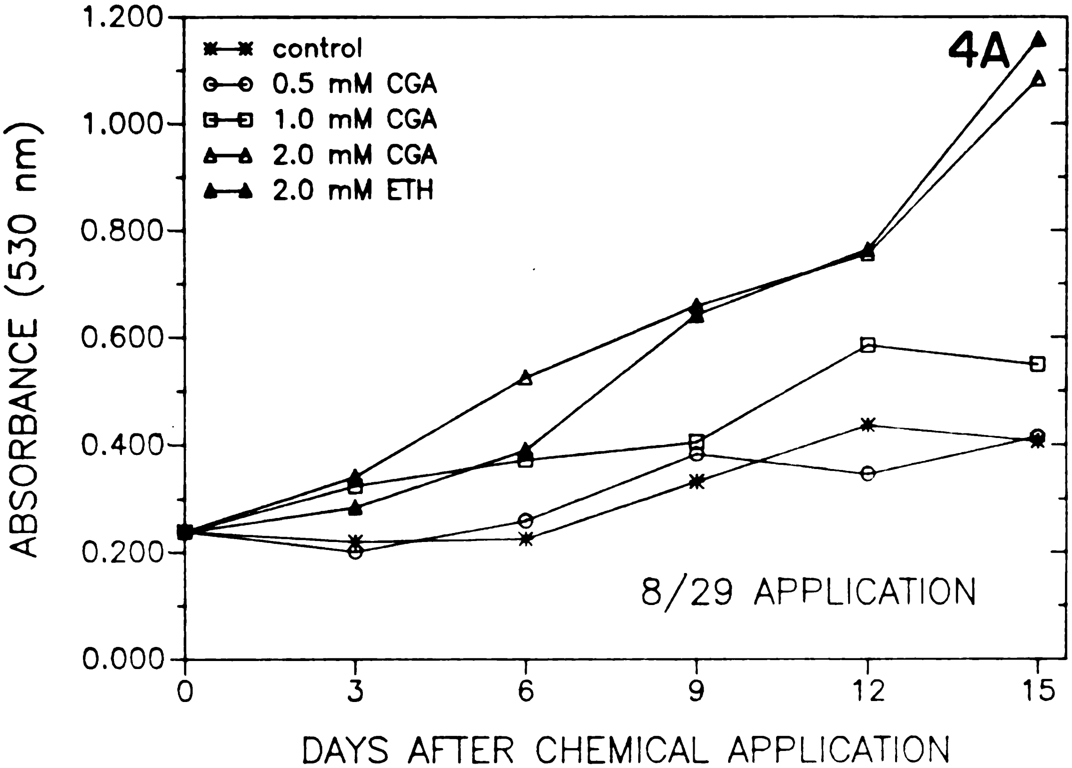
Figure 3. Percent red coloration of `McIntosh' apples treated with CGA 15281 or ethephon on A) 29 August 1984 orB) 6 September 1984. Red surface coloration was estimated visually to the nearest 10% (n=25); lsd .05 withinsampling date = 12.939 (A) and 13.450 (B), and within concentration = 11.260 (A) and 13.133 (B).



3A
8/29 APPLICATION
control
0.5 mM CGA
1.0 mM CGA
2.0 mM CGA
2.0 mM ETH
PERCENT RED COLOR
0
20
40
60
80
100
0
3
6
9
12
15
DAYS AFTER CHEMICAL APPLICATION
3B
9/6 APPLICATION
control
0.5 mM CGA
1.0 mM CGA
2.0 mM CGA
2.0 mM ETH
PERCENT RED COLOR
0
20
40
60
80
100
0
3
6
9
12
15
DAYS AFTER CHEMICAL APPLICATION

Figure 3.

Figure 4. Absorbance of anthocyanin extracted from \`McIntosh' apples treated with CGA 15281 or ethephon on A) 29 August 1984 or B) 6 September 1984 (n=25); lsd .05 within sampling date = .126 (A) and .145 (B), and within concentration = .119 (A) and .136 (B).


4A
control
0.5 mM CGA
1.0 mM CGA
2.0 mM CGA
2.0 mM ETH
8/29 APPLICATION
ABSORBANCE (530 nm)
DAYS AFTER CHEMICAL APPLICATION
1.200
1.000
0.800
0.600
0.400
0.200
0.000
0
3
6
9
12
15

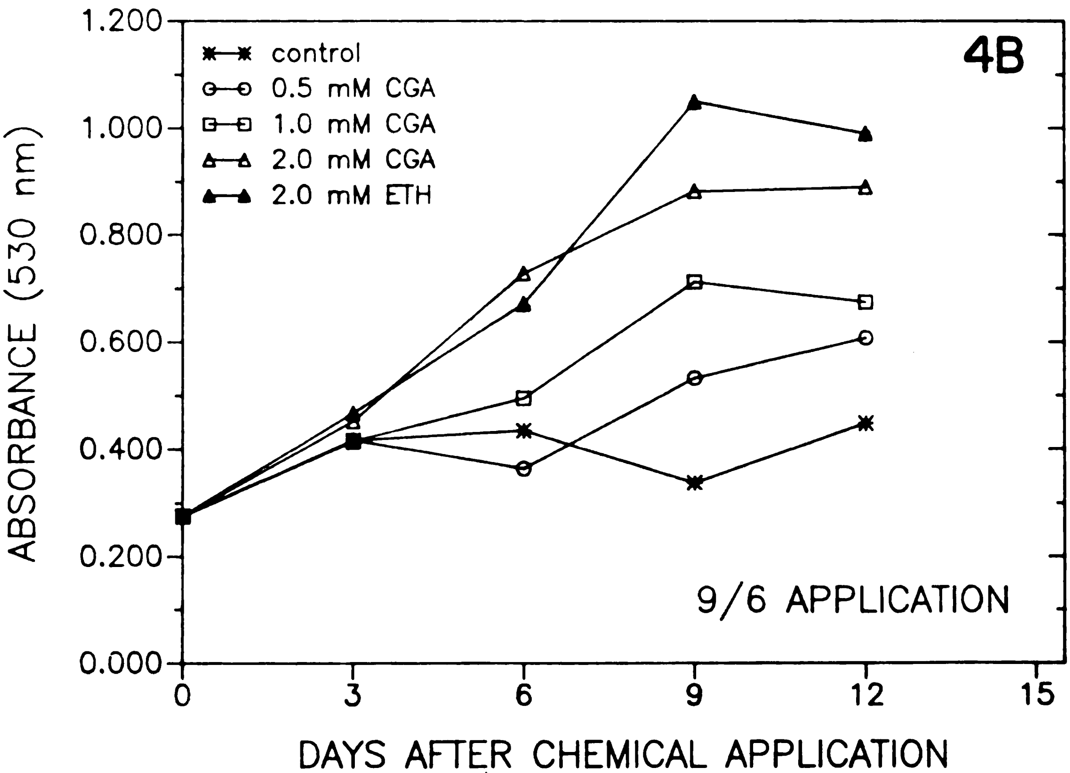

4B
control
0.5 mM CGA
1.0 mM CGA
2.0 mM CGA
2.0 mM ETH
9/6 APPLICATION
ABSORBANCE (530 nm)
DAYS AFTER CHEMICAL APPLICATION
1.200
1.000
0.800
0.600
0.400
0.200
0.000
0
3
6
9
12
15

Figure 4.

Figure 5. Flesh firmness of `McIntosh' apples at each sampling date treated with CGA 15281 or ethephon on A) 29 August 1984 or B) 6 September 1984 (n=25); lsd .05 within sampling date = 3.893 (A) and 3.060 (B), and within concentration = 3.597 (A) and 3.169 (B).

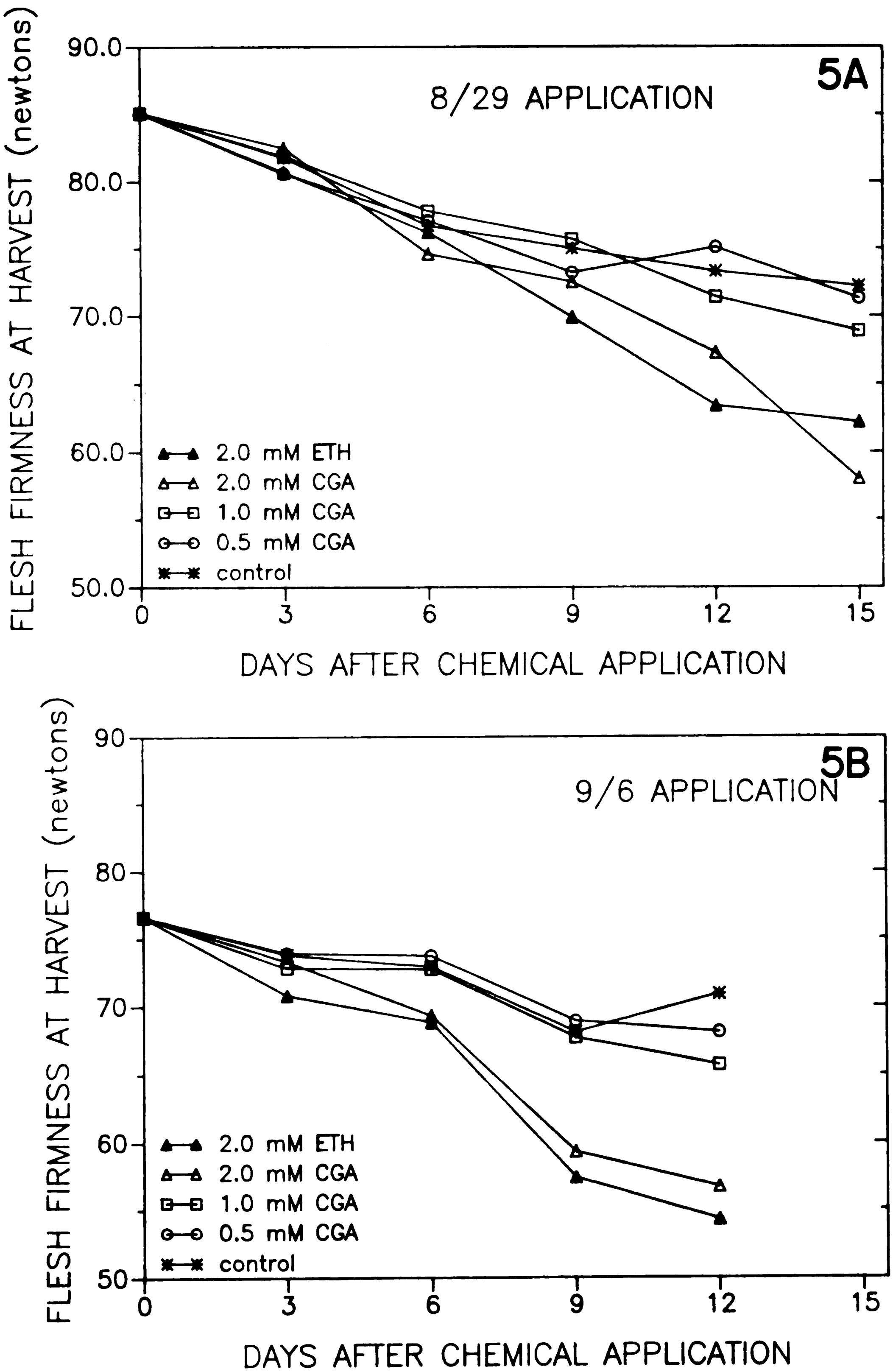

5A
8/29 APPLICATION
FLESH FIRMNESS AT HARVEST (newtons)
90.0
80.0
70.0
60.0
50.0
0
3
6
9
12
15
DAYS AFTER CHEMICAL APPLICATION
2.0 mM ETH
2.0 mM CGA
1.0 mM CGA
0.5 mM CGA
control
5B
9/6 APPLICATION
FLESH FIRMNESS AT HARVEST (newtons)
90
80
70
60
50
0
3
6
9
12
15
DAYS AFTER CHEMICAL APPLICATION
2.0 mM ETH
2.0 mM CGA
1.0 mM CGA
0.5 mM CGA
control

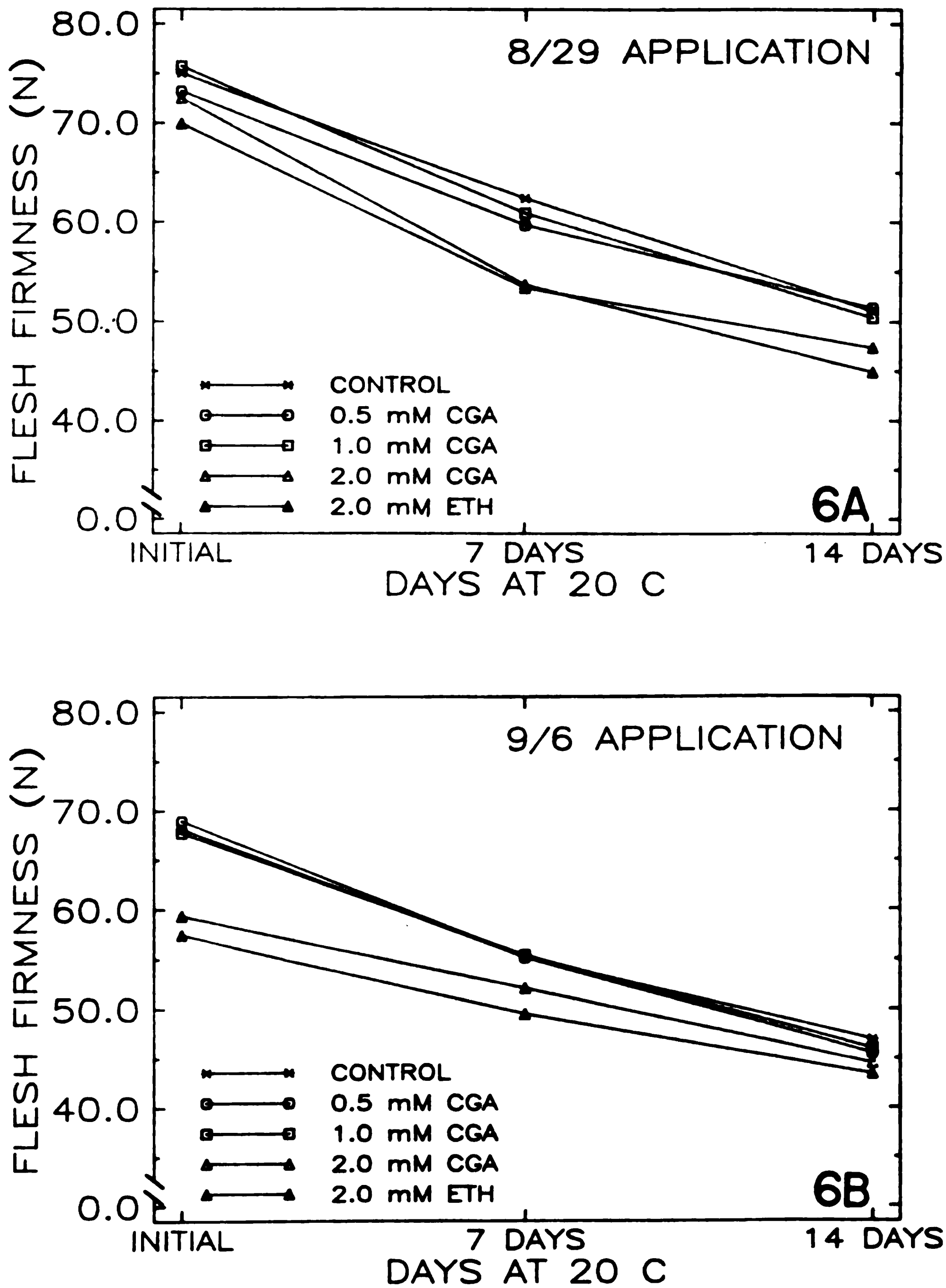
Figure 5.

mM CGA 15281 were significantly softer than the controls. Linear trends best characterized the response to chemical treatment over time. Comparing slopes of the regression lines, we found no significant differences in softening rates of fruit treated with 1.0 mM CGA 15281 or control. Fruit treated with 2.0 mM ethephon on August 29 and 6 September, respectively, softened at significantly greater rates than fruit treated with 1.0 mM CGA 15281, however.

Figures 6A and 6B illustrate the shelf life of fruit harvested 9 days after chemical treatment. With fruit treated on 29 August, no interaction was present between chemical treatment and sampling time, but both main effects were highly significant. The rates of softening were thus nonsignificant in fruit treated on this date. Fruit treated with either 2.0 mM CGA 15281 or ethephon on September 6 were also initially softer than fruit of other treatments 9 days after application, however, after 2 weeks at 20 C, firmness values were not significantly different between treatments. Rates of softening were significantly greater in fruit from control, 0.5 mM and 1.0 mM CGA 15281 treatments.

Starch conversion to sugar also increased significantly in fruit treated with 2 mM CGA 15281 and ethephon (see Figures 7A and 7B). Starch conversion was linear and occurred at rates dependent on an interaction of chemical treatment and time. Significant treatment differences were observed within 9 days of the 29 August application and 6 days of the 6 September application.

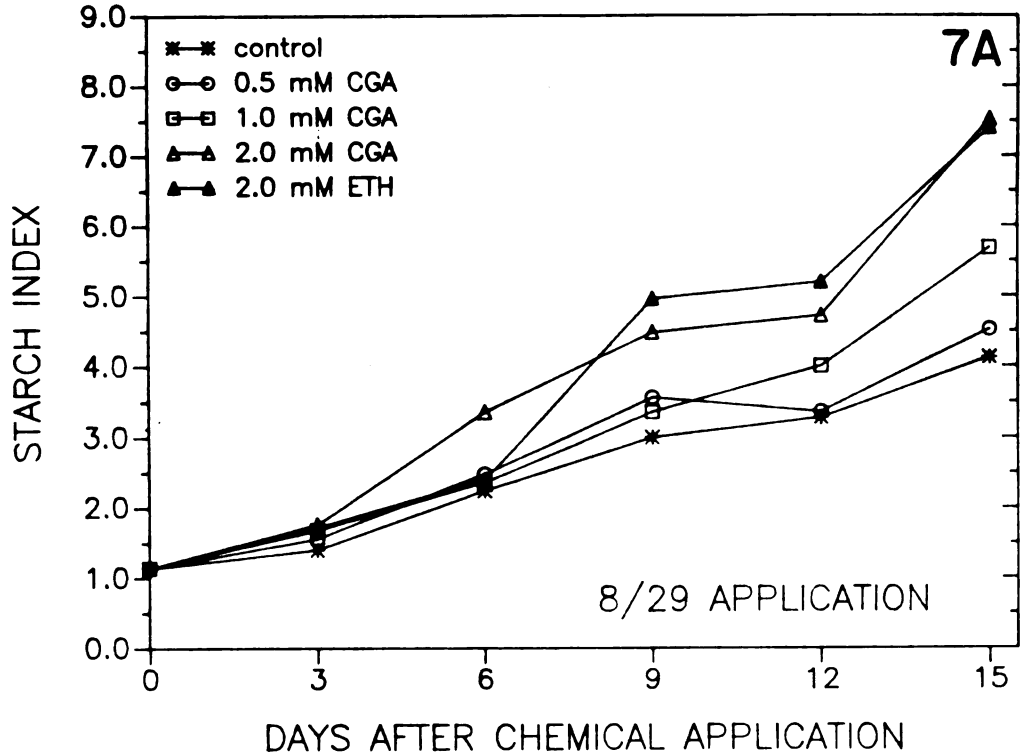
Figure 6. Flesh firmness at 0, 1, and 2 weeks of `McIntosh' apples harvested 9 days after treatment on A) 29 August 1984or B) 6 September 1984. Fruit was stored at 20 C (n=25); lsd .05 within sampling week = 3.737 (A) and 2.559 (B), and within concentration = 3.682 (A) and 2.199 (B).



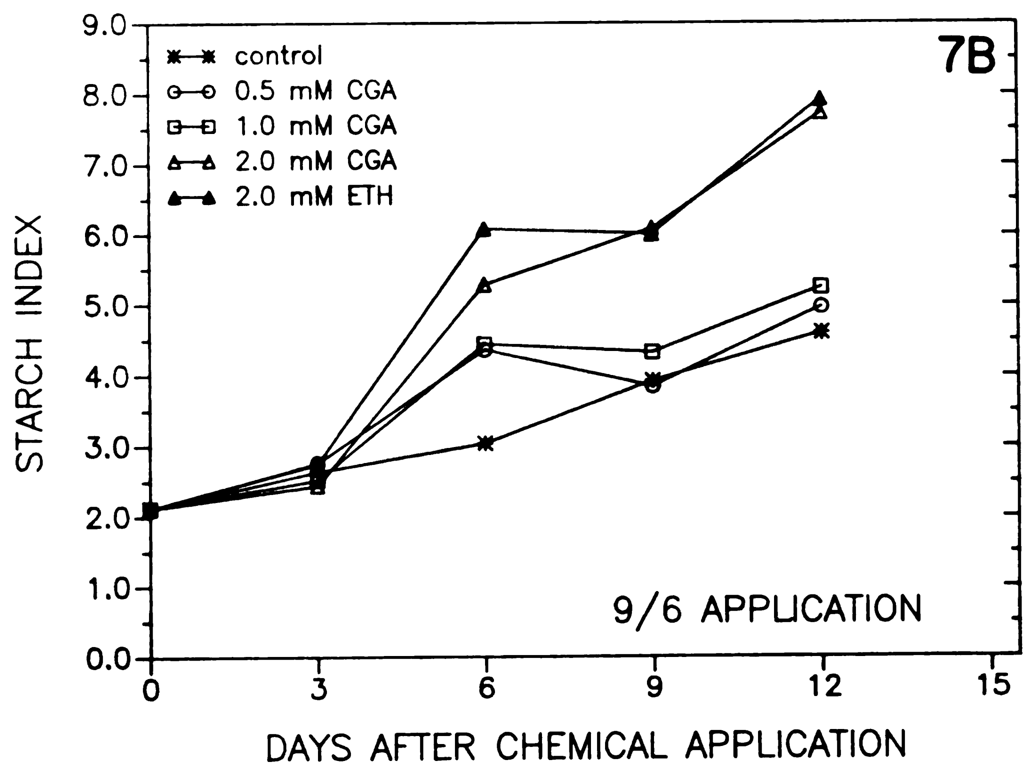
80.0
70.0
60.0
50.0
40.0
0.0
FLESH FIRMNESS (N)
8/29 APPLICATION
CONTROL
0.5 mM CGA
1.0 mM CGA
2.0 mM CGA
2.0 mM ETH
6A
INITIAL
7 DAYS
14 DAYS
DAYS AT 20 C
9/6 APPLICATION
6B

Figure 6.

Figure 7. Starch index of `McIntosh' apples treated with CGA 15281 or ethephon on A) 29 August 1984 or B) 6 September 1984 (n=25); lsd .05 within sampling date = .853 (A) and .840 (B), and within concentration = .763 (A) and .796 (B).



7A
control
0.5 mM CGA
1.0 mM CGA
2.0 mM CGA
2.0 mM ETH
8/29 APPLICATION
STARCH INDEX
DAYS AFTER CHEMICAL APPLICATION
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
0
3
6
9
12
15



7B
control
0.5 mM CGA
1.0 mM CGA
2.0 mM CGA
2.0 mM ETH
9/6 APPLICATION
STARCH INDEX
DAYS AFTER CHEMICAL APPLICATION
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
0
3
6
9
12
15

Figure 7.

These observations were consistent with others that climacteric fruits become more sensitive, and consequently, more responsive to ethylene as they mature. This creates a problem, therefore, when ethylene-releasing compounds such as ethephon are applied near harvest to enhance fruit coloration. Temperatures in the orchard at this time (late summer and early fall) fluctuate greatly. Having an energy of activation of about 30 kcal mol^{-1}, the solvolytic fragmentation of ethephon is highly dependent on temperature (1, 12). CGA 15281 is, in contrast, much less sensitive to changes in temperature and has an energy of activation of just 10 kcal mol^{-1} (11). In our study, solvolytic fragmentation of CGA 15281 occurred quickly within the fruit. This burst of ethylene apparently enhanced anthocyanin biosynthesis through the activation of the PAL enzyme, but was diffused from the tissue prior to synthesis of the ripening enzymes. Consequently, anthocyanin biosynthesis was enhanced in apple fruits without loss of postharvest integrity.

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