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Involvement of Wound and Climacteric Ethylene in Ripening Avocado Discs[1]

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ABSTRACT

Avocado (*Persea americana* Mill. cv Hass) discs (3 mm thick) ripened in approximately 72 hours when maintained in a flow of moist air and resembled ripe fruit in texture and taste. Ethylene evolution by discs of early and midseason fruit was characterized by two distinct components, *viz.* wound ethylene, peaking at approximately 18 hours, and climacteric ethylene, rising to a peak at approximately 72 hours. A commensurate respiratory stimulation accompanied each ethylene peak. Aminoethoxyvinyl glycine (AVG) given consecutively, at once and at 24 hours following disc preparation, prevented wound and climacteric respiration peaks, virtually all ethylene production, and ripening. When AVG was administered for the first 24 hours only, respiratory stimulation and softening (ripening) were retarded by at least a day. When AVG was added solely after the first 24 hours, ripening proceeded as in untreated discs, although climacteric ethylene and respiration were diminished. Propylene given together with AVG led to ripening under all circumstances. 2,5-Norbornadiene given continuously stimulated wound ethylene production, and it inhibited climacteric ethylene evolution, the augmentation of ethylene-forming enzyme activity normally associated with climacteric ethylene, and ripening. 2,5-Norbornadiene given at 24 hours fully inhibited ripening. When intact fruit were pulsed with ethylene for 24 hours before discs were prepared therefrom, the respiration rate, ethylene-forming enzyme activity buildup, and rate of ethylene production were all subsequently enhanced. The evidence suggests that ethylene is involved in all phases of disc ripening. In this view, wound ethylene in discs accelerates events that normally take place over an extended period throughout the lag phase in intact fruit, and climacteric ethylene serves the same ripening function in discs and intact fruit alike.

The ripening of avocado fruit (*Persea americana* Mill. cv Hass) following harvest is biphasic: a lag period characterized by low, steady respiration rates and vanishingly low levels of ethylene production is followed by the so-called climacteric, in which a sharp autocatalytic increase in ethylene production accompanies a peak of respiratory activity (2). The climacteric, in turn, is attended by a cohort of phenomena we know as ripening, encompassing changes in color, aroma, and texture that result in edibility. Ethylene has long been perceived as a causative agent in the ripening phenomena associated with the climacteric. On the other hand, participation of ethylene in lag period events has been less obvious, albeit convincingly established (15). Whereas the course and duration of the avocado climacteric is fairly constant (comprising 3 to 4 d from inception to peak), the lag period ranges from 1 to 2 d to weeks, depending on fruit maturity. Accordingly, it is considered that the lag period involves a progressive increase in sensitivity, or responsiveness (23), to ethylene to the point where the endogenous ethylene level triggers the climacteric (27). As noted, ethylene itself is involved in implementing the change in sensitivity, although it cannot be wholly responsible.

Thus, when early-season, preclimacteric fruit are pulsed with ethylene for 24 h, the lag period is shortened but not abolished (4). Following a pulse, respiration rises to a peak at 24 h and falls away when exogenous ethylene is withdrawn, only to rise again with the onset of the climacteric (4). In early-season fruit, the pulse-induced respiration peak is well separated in time from the climacteric peak, whereas in late-season fruit the pulse peak coalesces with, or is subsumed within, the climacteric peak (19, *cf.* ref. 23). Furthermore, the concentration of ethylene required to trigger the climacteric in early preclimacteric fruit is considerably higher than that which triggers late-season preclimacteric fruit (2). In short, sensitivity to ethylene increases with fruit maturity as well as with the course of the lag period.

Ripening studies of intact avocado fruit are restricted in scope by limitations on experimental intervention. On the other hand, slices or discs of avocado mesocarp are readily manipulated and ripen under proper conditions in moist air (1). Although discs submerged in air-equilibrated solution (22), embedded in solid medium (14), or wetted with lens paper wicks (to provide exogenous metabolites and inhibitors)(22) have been purported to ripen, we have found that avocado discs truly ripen only in a humid flow-through system. Discs ripen in a matter of days and display a climacteric much as do fruit, as will be shown below (*cf.* ref. 1). Because the interval from onset to peak of the climacteric in discs is much the same as in fruit, it would appear that slicing, hence wounding, shortens or supersedes the lag period of intact fruit.

Inasmuch as slicing elicits a prompt flush of wound ethylene—both widespread in origin (17) and similar in biosynthetic path to ripening-related climacteric ethylene (28), the question we examine here is whether the shortening of the lag period and early triggering of the climacteric in discs is attributable to wound ethylene, wounding *per se*, or both. Wound-related gene expression not attributable to wound

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ethylene has been demonstrated in tomato fruit (8). Furthermore, the use of AVG,[3] an inhibitor of ACC synthase and thus ethylene production, and NBD, an inhibitor of ethylene action, allows for the investigation of the specific role of wound and climacteric ethylene in the ripening behavior of discs. Although fresh discs of bulky storage organs or fruits are metabolic anomalies (7), a condition that applies to avocado discs (20), avocado discs ripened as described here resemble ripe fruit and, accordingly, offer a versatile object for the experimental analysis of the ripening process.

MATERIALS & METHODS

Plant Material

Avocado fruits (*Persea americana* Mill. cv Hass) were harvested from the University of California South Coast Field Station between 10 and 12 AM or from an orchard in Carpenteria, CA, between 11 AM and 1 PM. Within 2 to 3 h discs were prepared as follows: whole fruit were bathed for 5 min in bleach (5% NaOCl) diluted 1:20 in water, and rinsed in deionized water. Each fruit was subsequently halved longitudinally, the seed removed, and each half cut along its length into slices 3 mm thick with a slicer comprising an adjustable microtome knife fixed in an appropriate bed. Slices were punched with a No. 9 cork borer, yielding discs 14 mm in diameter. There is an inherent variability in the ripening behavior of different regions of the fruit; therefore, discs were punched only from the equatorial region of slices from tissue abutting the seed. In this way, 75 to 150 discs were obtained per fruit, depending on its size. Discs from all fruit were combined, randomized, quickly rinsed five times with distilled water, and blotted dry. Subsequently, discs were placed in tubs and treated as described below.

Disc-Aging System

Avocado discs submerged and shaken in an aqueous medium in air remain hard and fail to ripen (1, 22). On the other hand, when submerged discs are oxygenated, they become soft and flaccid and may mistakenly be taken as ripe (24). Softening in this case represents waterlogging and structure degeneration born of water injection; such discs are neither buttery in texture nor edible. Because fluid injection into the intercellular space limits oxygen diffusion, the ethylene-mediated, oxygen-dependent ripening process can be expected to be adversely affected by vacuum infiltration of discs (11; *cf.* ref. 21). Our system, described below, produces discs that resemble ripened fruit, and allows the presentation of substrates and inhibitors without vacuum infiltration or waterlogging.

Circular stainless steel screens, each with a central Lucite spacer sleeve, were stacked on a stainless steel rod mounted in a Lucite base. The entire assembly was set in a 500-mL Tupperware tub with air-tight lid, fitted with an inlet port at the base and an outlet port at the top. Sterile water (20 mL) was added to the bottom of the tub in contact with a circular piece of 3-mm blotting paper placed over each Lucite base to facilitate evaporation and maintain humidity. A circular piece of 3-mm paper was fitted beneath the lid to prevent condensate from dripping on discs. Short lengths of rubber tubing attached ports to connectors that facilitated easy union with the air lines. Inlet lines were fitted with a bacterial air filter. All components were sterilized with 95% ethanol before use.

Four screens, each bearing 25 discs, were placed in each tub (45–50 g fresh weight, depending on fruit maturity). Where indicated, AVG solution (4 mM) was applied to each disc as a 25-μL droplet at designated times. Droplets spread rapidly across the disc surface and down the edge to the bottom face of the disc. To compensate for any effect of the droplet *per se*, 25 μL deionized water was applied to control discs. Droplets were completely absorbed within 6 to 8 h. A continuous 30-mL/min flow of water-saturated air, ethylene at 10 μL/L in air, or propylene at 500 μL/L in air was passed through the tubs, held at 20°C in a constant temperature incubator. A system of control valves allowed for easy switching of gas mixtures during the course of an experiment. For experiments involving NBD (a caustic volatile compound), the inlet and outlet ports of the tub were sealed. The tub was flushed with air every 8 h, and NBD was replenished. NBD was applied as a liquid droplet to the blotting paper beneath the lid, volatilizing to a final gas concentration of 10,000 μL/L.

Because AVG was applied as an aqueous solution, whereas NBD and propylene were applied as gases, wet, dry, or both types of controls were used, depending on the experiment.

Respiration and Ethylene Determinations

Respiration and ethylene production were monitored as described previously (19). Outflow from tubs was directed to an IR gas analyzer (Anarad, model AR500) by solenoid-activated switches controlled by an IBM personal computer equipped with a customized software program (Sable Systems, Los Angeles, CA). CO_2 concentrations were measured by the analyzer and specific respiration rates, *i.e.* rates per unit weight, computed and recorded by the personal computer. Each tub was sampled once every hour. Respiratory values were depicted on the screen as a continuous respiration profile throughout the entire disc-aging period. At designated times 10-mL gas samples were manually withdrawn from each outlet port and injected into a backflush-fitted GC (Hach Carle 04254-C) with flame ionization detector and a column at 70°C packed with specially modified packing material for ethylene determination (Hach Carle application 254-C). For NBD experiments, 15 discs were removed from each tub and assayed for ethylene evolution and EFE activity as described in the following section. Sampling periodicity was usually every 8 to 12 h, as specified.

EFE Assays

At each time point, after ethylene production in the flow-through system was first measured, 30 discs were removed from one tub of each experimental treatment and assayed for EFE activity (19). Fifteen discs (average of 7.25 g fresh weight) transferred to a large Petri dish were each treated with a 25-

[3] Abbreviations: AVG, aminoethoxyvinyl glycine; NBD, 2,5-norbornadiene; EFE, ethylene-forming enzyme; ACC, 1-aminocyclopropane-1-carboxylic acid.

μL droplet of 100 mM ACC, and EFE activity was calculated from the rate of ethylene evolution. Fifteen discs treated with 25 μL of water served as the control. Parenthetically, in the first hour after disc preparation, ethylene evolution in the absence of added ACC, *i.e.* by discs treated with a water droplet, can be taken as a measure of ACC synthase activity because (a) ACC sharply increases the rate of ethylene production, (b) AVG totally inhibits endogenous ethylene production, and (c) the rate of 1-(malonylamino)cyclopropane-1-carboxylic acid augmentation is minimal (18). Because ethylene production is low in the early hours after cutting, it follows that ACC synthase is not significantly active during this period. After 24 h, the sharply rising high rate of ethylene production rules out the 1-(malonylamino)cyclopropane-1-carboxylic acid pool as the source of climacteric ethylene. Because NBD experiments require a sealed system, ethylene could not be measured directly in an effluent gas stream. Accordingly, for NBD experiments, 15 discs were assayed as above without added ACC or water to estimate normal ethylene production. Because ethylene production proved essentially the same whether or not discs were treated with a water droplet, data for ethylene production by untreated discs are shown in the figures.

Ripeness Assay

Ripeness assays were done every 8 h. When EFE and ethylene production by discs transferred to Petri dishes as noted in the previous section were assayed, discs were checked for ripeness thereafter. For other time points, 15 discs were removed from tubs and assayed for ripeness (30 discs after softening was initiated). Ripening was assessed by softness, texture, and taste. Discs compressed at the perimeter between thumb and forefinger were assigned to one of three categories: unripe, hard and crisp; intermediate, folding when compressed but remaining intact; ripe, soft, extruding when compressed. Discs aged in air develop a thin, seemingly suberized skin that makes mechanical penetrometer measurements meaningless, as does the fact that discs aged in oxygenated solution become soft due to swelling and tissue disintegration, a condition to be distinguished from ripe. Thus, texture and taste must serve as criteria of ripeness in addition to softness. Ripe tissue is buttery in consistency and tastes like ripe fruit; by contrast, discs that soften in solution are mushy in texture and tasteless.

From the first sign of softening of a sample of discs until the time all discs are completely soft, the percentage of soft discs in the population rises linearly with time. The latter phenomenon reflects disc heterogeneity, presumably due to the variability among fruits and positional heterogeneity within a fruit. The reproducibility of the phenomenon speaks to the effectiveness of the randomization of the disc population before apportionment to individual screens and containers. Thus, the time taken for the full ripening of a disc population as set out in the tables reflects the end point of a series of measurements rather than a single measurement.

Materials

AVG and high purity ACC were purchased from Sigma. NBD was purchased from Aldrich Chemical Co. Ethylene and propylene (both 99.9% pure) used to make gas mixtures were purchased from Scott Specialty Gases, and compressed air was from Liquid Air Corporation.

RESULTS

Disc Behavior

After avocado discs are cut and subsequently incubated, they exhibit a ripening profile that compared with whole fruit is accelerated, has a characteristic elevated respiration above that seen in whole fruit, and exhibits dual ethylene and respiratory peaks (Fig. 1). Within hours of slicing, a sharp CO_2 spike is evidenced (Figs. 1, 2, and 5) that is virtually eliminated by washing the discs in pH 5.0 phosphate buffer for 10 min immediately after cutting. This suggests that the spike arises from CO_2 released on cutting from dissolved HCO_3^- in the cytosol and/or cell wall (6). A true short-lived respiratory peak follows, coincident with an early transient rise in ethylene production, the wound-induced ethylene. A subsequent increase in respiration, the respiratory climacteric, occurs at the onset of marked climacteric-related autocatalytic ethylene production. EFE activity is induced soon after cutting and continues to rise, peaking soon after the climacteric (Fig. 1, *cf.* Fig. 3C).

The climacteric is accompanied by typical avocado ripening

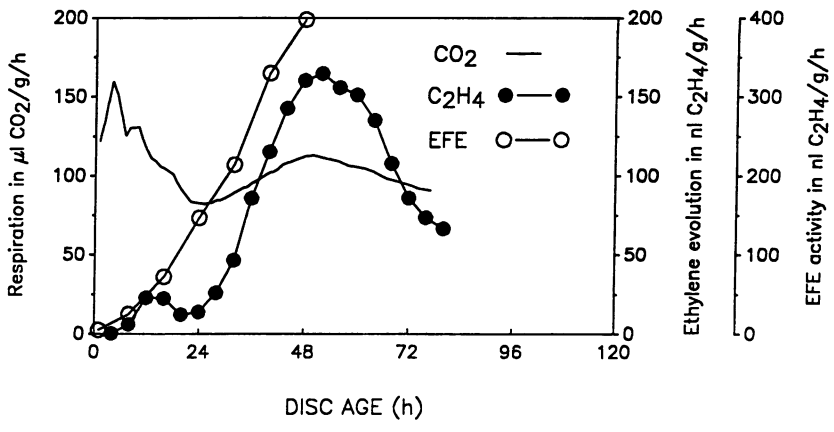

Figure 1. Respiration, ethylene production, and EFE activity of avocado discs as a function of time. CO_2 production, ethylene production, and EFE activity of discs from freshly harvested mid-season fruit. CO_2 production was monitored every hour, ethylene production every 4 h, and EFE activity approximately every 8 h. Disc age is from the time of slicing.

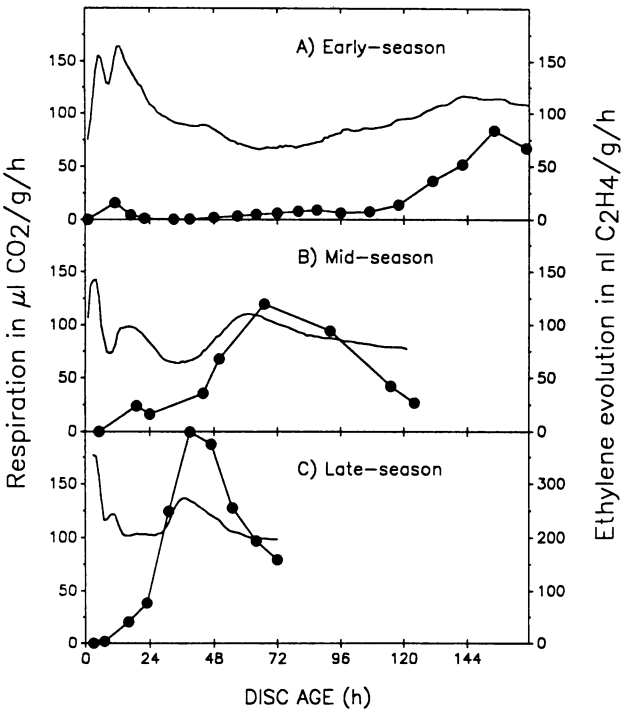

Figure 2. Respiration and ethylene production of avocado discs as a function of fruit maturity. CO_2 production (——) and ethylene production (●) of discs from freshly harvested early-season (A), midseason (B), and late-season (C) avocado fruit. CO_2 production was monitored every hour and ethylene production every 8 to 12 h. Disc age is from the time of slicing. Note the different scale for ethylene production in late-season discs (C).

Table I. *Disc Ripening as a Function of Fruit Maturity*

All fruit (from Carpenteria, CA) was sliced immediately after harvest. Ripening times were measured from time of slicing. Onset of softening is defined as the time at which discs first reach the intermediate stage of softening (see text). The second column represents the time at which all discs reached the soft stage of ripening. A minimum of 15 discs was assayed for softness every 8 h or less as described in "Materials and Methods."

Season and Date	Onset of Softening	All Discs 100% Soft
	h from slicing	
Early		
10/16/89	120	192
11/15/89	90	190
11/28/89	72	120
Middle		
2/5/90	56	96
2/27/90	56	90
3/12/90	56	82
4/12/90	48	78
5/6/90	48	76
Late		
6/19/90	40	70
7/6/90	40	64
7/14/90	32	64
7/31/90	32	56

Table II. *Effect of Fruit Pulsing and Disc Treatment with Ethylene on Disc Ripening*

All conditions as for Table I.

Conditions	Onset of Softening	All Discs 100% Soft
	h from slicing	
Wet *versus* dry discs		
Midseason: March 1990		
Dry	56	90
Wet	56	104
Late-midseason: May 1990		
Dry	48	64
Wet	48	80
Ethylene-treated discs		
Midseason: February 1990		
C_2H_4 treated	48	88
Air control	56	92
Late-season: July 1990		
C_2H_4 treated	32	54
Air control	40	56
Discs from pulsed fruit[a]		
Midseason: April 1989		
Discs cut 24 h after harvest		
Pulsed	[b]	72
Air control	[b]	80
Late-season: July 1989		
Discs cut 24 h after harvest		
Pulsed	28	48
Air control	32	64
Discs cut 72 h after harvest		
Pulsed	28	56
Air control	32	64
Aged fruit discs		
Late-season: July 1990		
96-h aged fruit	36	68
Freshly harvested fruit	36	68

[a] Fruit were pulsed with ethylene for 24 h immediately after harvest. [b] Onset of softening was not recorded.

phenomena. The tissue becomes soft, buttery, and edible, just as in ripe whole fruit. Discs from fruit of different stages of maturity reveal a seasonal trend in ripening behavior (Fig. 2). Early-season discs show distinct wound ethylene and respiration peaks separated from their climacteric counterparts by a few days (Fig. 2A). Midseason discs show the wound and autocatalytic ethylene peaks approaching each other, as do the respective ethylene-associated respiratory rises (Fig. 2, *cf.* A with B). Discs from late-season fruit exhibit a combined ethylene peak with a concomitant respiratory climacteric (Fig. 2C) and a remnant wound respiration peak. The magnitude of the autocatalytic ethylene rise increases with maturity (Fig. 2C, note scale of the ethylene y axis). As the season progresses, the time it takes for discs to soften becomes shorter (Table I). Treating discs with ethylene or propylene has little effect on their ripening behavior. At most, discs soften 2 to 4 h sooner when treated with ethylene (Table II) and show only a slight additional increase in respiration, EFE activity, and ethylene production compared with untreated discs (data not shown).

At least three or four replicates of each disc population define the time courses depicted in Fig. 2. Although respira-

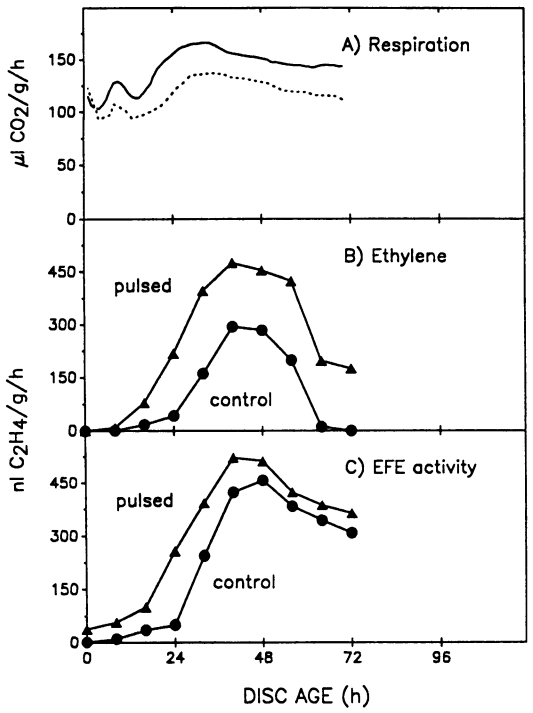

Figure 3. Respiration, ethylene production, and EFE activity of avocado discs from 24 h ethylene-pulsed and untreated late-season fruit. A, CO_2 production of discs cut from 24-h pulsed (——) and 24-h untreated (- - - -) fruit; B, ethylene production of discs cut from 24-h pulsed (▲) and 24-h untreated (●) fruit; C, EFE activity of discs cut from 24-h pulsed (▲) and 24-h untreated (●) fruit. CO_2 production was monitored every hour, ethylene production every 8 h, and EFE activity every 8 h. Disc age is from time of slicing.

tory and ethylene peaks—both wound and climacteric related—are consistently reproducible, replicate profiles are frequently slightly offset from one another, precluding formal statistical treatment. As noted in "Materials and Methods," assessment of the time taken for all discs of a sample to ripen (Table II) reflects consecutive measurements through the ripening time course rather than a single end point measurement. Whereas in replicate experiments the hours required for all discs to ripen in response to a given treatment may vary, the difference in full ripening time owing to differences in experimental treatment within an experiment is consistent and reproducible.

Discs from Pulsed Fruit

Discs from 24-h ethylene-pulsed (10 μL/L) fruit exhibit slightly higher respiratory activity, accelerated softening, increased EFE activity, and elevated ethylene production compared with discs from untreated fruit (Fig. 3, Table II). EFE activity at the time of slicing is significantly higher in discs of pulsed fruit than in discs of control fruit (*cf.* ref. 10), whereas ethylene production is essentially nil and unaffected by the pulse (Fig. 3, B and C) (19). Within hours, however, respiration, ethylene production, and EFE activity in discs from pulsed fruit begin to rise sooner and peak at higher levels than in discs from control fruit, leading to an approximately 8- to 12-hour acceleration in softening. When fruit is aged in air for an additional 48 h after a 24-h pulse, discs therefrom still exhibit accelerated softening (Table II), increased EFE activity, and increased ethylene production as compared with discs from untreated fruit (data not shown). However, discs from 4-d-old fruit take the same time to ripen as discs from freshly harvested fruit (Table II), *i.e.* the levels of endogenous ethylene in 4-d-old fruit are not high enough to shorten disc-softening time significantly.

Effects of NBD

Fig. 4 depicts the effects of NBD on ethylene production and EFE activity in discs from midseason fruit. NBD increases wound ethylene (Fig. 4B, Table III), eliminates climacteric ethylene (Fig. 4, *cf.* B with A), and inhibits softening (Table IV). Increased wound ethylene is unaccompanied by increased EFE activity (Table III). In NBD-treated discs, wound ethylene increases to a maximum between 24 and 36 h and

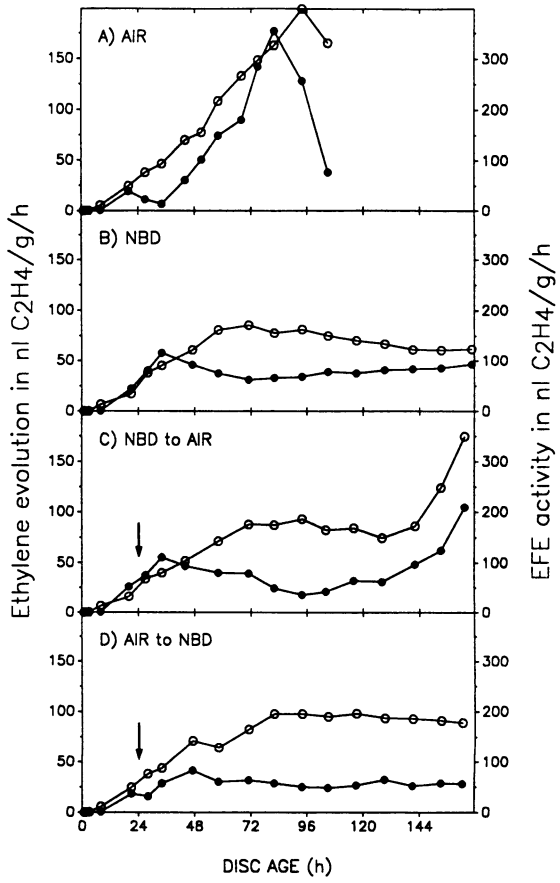

Figure 4. Ethylene production and EFE activity of NBD-treated avocado discs. Ethylene production (●) and EFE activity (○) of air control discs (A), continuously NBD-treated discs (B), discs switched from NBD to air at 24 h (C; arrow); and discs switched from air to NBD at 24 h (D; arrow). Discs were cut from freshly harvested midseason avocado fruit. NBD was maintained at a gas concentration of 10,000 μL/L during the times indicated. Ethylene production was monitored and EFE activity assayed every 6 to 12 h. Disc age is from time of slicing.

Table III. *Effect of NBD on Ethylene Production and EFE Activity in Avocado Discs*

Discs from freshly harvested avocado fruit were held in 10,000 μL/L NBD or in air. Ethylene evolution and EFE activity were measured at 24 and 30 h after slicing. All data are averages of samples from four separate containers, except late-season, which are for samples from a single container.

Season and Hour	Ethylene Production		EFE Activity	
	Air	NBD	Air	NBD
	nL C_2H_4/g/h			
Early				
24	4.8 ± 0.8	35.3 ± 4.0	78.2 ± 1.3	82.1 ± 3.3
30	2.8 ± 0.5	44.5 ± 3.8	108.3 ± 3.8	113.1 ± 6.0
Middle				
24	21.0 ± 2.35	21.6 ± 4.4	[a]	[a]
30	10.3 ± 0.8	33.0 ± 4.4	[a]	[a]
Mid-late				
24	13.7 ± 1.8	19.8 ± 2.5	49.9 ± 0	32.9 ± 0.8
30	14.1 ± 1.9	35.0 ± 5.3	76.3 ± 0.1	71.2 ± 3.8
Late				
24	18.3	68.2	[a]	[a]
30	51.9	85.1	[a]	[a]

[a] EFE data not recorded.

subsequently remains near wound levels without a climacteric rise. Similarly, EFE activity levels off at about 72 h (for midseason discs) with no climacteric rise (Fig. 4B). Delaying presentation of NBD for 24 h augments wound ethylene to a lesser degree but still eliminates the ethylene climacteric (Fig. 4D). If NBD is discontinued at 24 h, ethylene production and EFE activity remain as with NBD present, until rising in a climacteric, with final ripening, after a long delay (over 6 d in midseason fruit; Fig. 4C, Table IV). Discs treated with NBD continuously, or after a delay of 24 h, fail to reach the soft ripe stage but become flaccid and necrotic by 180 to 200 h.

Effects of AVG

Treatment of late-season discs with AVG (immediately after cutting and again at 24 h) reduces both wound and climacteric ethylene production to low levels (<3 nL C_2H_4 g^{-1} h^{-1}; Fig. 5, *cf.* B with A). Ethylene production eventually begins a gradual rise after 72 h (Fig. 5B) which is prevented by further addition of AVG (data not shown). The inhibition of wound ethylene production by AVG (Fig. 5B) reduces the associated wound-related respiration peak (the peak normally being visible at 18 to 24 h; Fig 5A), as well as virtually eliminating the climacteric respiration rise. Two-fold treatment with AVG at 0 and 24 h delays complete disc softening to 132 h (Table IV), whereas a third AVG treatment at 48 h gives discs that soften only upon necrosis.

When AVG is applied at 24 h, the normal climacteric ethylene production rate of discs in air is reduced to a few nl g^{-1} h^{-1} before beginning gradually to rise again (Fig. 5D). Wound respiration appears as in the control, peaking at about 18 h, and a somewhat reduced respiratory climacteric occurs at the expected time. Treatment with AVG at 48 h slightly reduces climacteric ethylene production without effect on respiration (data not shown). Delaying AVG treatment until 24 or 48 h produces no perceptible difference in softening times compared with control discs (Table IV; see "Discussion").

Fig. 5C depicts the behavior of discs treated at 0 h with AVG, followed by 500 μL/L of propylene at 24 h. Propylene, an active analogue of ethylene, is used in its place when it is desired to monitor endogenous ethylene production (12). Although ethylene production remains at very low levels through 72 h, a slightly reduced and somewhat drawn out climacteric respiratory rise occurs only a few hours later than in air-treated discs. Application of propylene to discs after 48 h of treatment with AVG (*i.e.* at 0 and 24 h) yields discs that exhibit a respiratory pattern similar to that of discs treated with AVG only (data not shown). AVG-treated discs exposed to propylene at 24 h soften completely with a delay of only 1 d, whereas propylene addition at 48 h to AVG-treated discs gives completely soft discs by 72 h thereafter (Table IV). Simultaneous treatment of discs with AVG (at 0 and 24 h) and propylene given continuously beginning at zero h gives a respiratory and ethylene production pattern similar to propylene-treated controls, which in turn resemble water-treated controls (data not shown). Furthermore, whereas discs treated simultaneously with AVG and propylene softened sooner than AVG-treated discs but not as soon as controls (Table IV), discs treated simultaneously with AVG and ethylene softened in approximately the same time as controls (data not shown).

The appearance of a small respiration spike in about 6 to 8 h after initial AVG or water (control) treatment (appearing within a few hours after the bicarbonate-derived CO_2 spike in Fig. 5) varies in intensity from sample to sample and is apparently an artifact due to the application of the liquid droplet.

Effects of AVG and NBD Combined

Treating discs with both AVG and NBD virtually eliminates ethylene production during the first 72 h, after which only a gradual rise occurs (Fig. 6B, note reduced y axis scale as compared with Fig. 5). Discs so treated stay as hard and crisp as freshly cut discs, showing little of the normal color changes associated with aging discs and no signs of necrosis in the 200+ h of the experiment. Treatment with AVG and NBD at 24 h temporarily knocks back ethylene production, which rises slowly after 72 h (but less than does AVG treatment alone; *cf.* Fig. 6D with Fig. 5D), but delays softening; only 75% of the discs soften at 168 h (Table IV). To limit the effects of AVG and NBD to the first 24 h, propylene was applied to discs at 24 h in the absence of NBD with no further AVG addition. Discs treated with AVG and NBD for 24 h before rescue with propylene exhibit greatly reduced ethylene production which rises only after 60 h (Fig. 6C). Such discs did not begin to soften until almost 90 h and did not completely soften until 172 h (Table IV).

Because a drop of solution added to discs increases the time for discs to soften (Table II), control discs in AVG experiments were treated with water, as were control discs and NBD-treated discs in experiments combining AVG and NBD.

Table IV. *Effect of NBD and/or AVG on Disc Ripening*

All conditions as for Table I. Experiments with NBD alone were terminated at 200 h. Water and AVG in water were applied as 25-μL droplets; AVG concentration, 4 mM; NBD final gas concentration 10,000 μL/L and renewed every 8 h; propylene concentration, 500 μL/L. For further details see "Materials and Methods."

	Treatments in First 48 h			Ripening Time	
	0 h	24 h	48 h	Onset of softening	All discs 100% soft
				h from slicing	
NBD-treated discs[a]					
Early season: December 1989					
Air	[b]	[b]	[b]	150	168
NBD	NBD	NBD	NBD	[c]	[c]
Air to NBD at 24 h	[b]	NBD	NBD	[c]	[c]
NBD to Air at 24 h	NBD	[b]	[b]	[c]	[c]
Midseason: March 1989					
Air	[b]	[b]	[b]	72	108
NBD	NBD	NBD	NBD	[c]	[c]
Air to NBD at 24 h	[b]	NBD	NBD	[c]	[c]
NBD to Air at 24 h	NBD	[b]	[b]	150	[c]
Late season: June 1989					
Air	[b]	[b]	[b]	72	96
NBD	NBD	NBD	NBD	[c]	[c]
Air to NBD at 24 h	[b]	NBD	NBD	[c]	[c]
NBD to Air at 24 h	NBD	[b]	[b]	108	192
AVG-treated discs					
Late season: July 1990					
Control dry	[b]	[b]	[b]	48	64
Control wet	H_2O	H_2O	[b]	48	80
AVG	AVG	AVG	[b]	72	132
24 h AVG	H_2O	AVG	[b]	48	80
48 h AVG	H_2O	H_2O	AVG	48	80
AVG, 24 h C_3H_6	AVG	H_2O/C_3H_6	C_3H_6	56	108
AVG, 48 h C_3H_6	AVG	AVG	C_3H_6	64	116
NBD + AVG-treated discs					
Late season: July 1990					
H_2O control	H_2O	[b]	[b]	36	68
AVG + continuous C_3H_6	AVG/C_3H_6	AVG/C_3H_6	C_3H_6	40	112
AVG	AVG	AVG	[b]	72	156
NBD[a]	H_2O/NBD	NBD	NBD	90	168[d]
AVG + NBD, 24 h C_3H_6[a]	AVG/NBD	C_3H_6	C_3H_6	88	172
AVG + NBD[a]	AVG/NBD	AVG/NBD	NBD	[e]	[e]
24 h AVG + NBD[a]	H_2O	AVG/NBD	NBD	90	168[f]
Air control[a]	H_2O	[b]	[b]	48	68

[a] Discs aged in sealed non-flow-through tubs with air renewed every 8 h. [b] No treatment. [c] Some discs showed nonripening-related softening leading to necrosis. [d] Only 50% of discs reached the soft stage. [e] Discs did not soften. [f] Only 75% of discs reached the soft stage.

DISCUSSION

The inherent duration of the lag period in avocado fruit, *i.e.* the time from harvest until the onset of the climacteric, depends, among other factors, on the internal ethylene concentration (2). The lag period can be shortened with an exogenous pulse of ethylene (or propylene) (4) and, in the extreme case, can be reduced from weeks to a matter of days by continuous treatment with ethylene (19). Discs of avocado fruit, on the other hand, ripen in days without exogenous ethylene administration, there being an extensive curtailment of the lag period. Whereas in endogenous ripening of intact fruit a considerable lag period is followed by a burst of respiratory activity and autocatalytic ethylene production, the climacteric, discs show an early peak of wound respiration concomitant with a transient evolution of wound ethylene, followed in short order by a true climacteric. We wish to know how wounding (*i.e.* disc preparation) shortens the lag period and expedites ripening and, in particular, whether it does so by way of wound ethylene, in consequence of wounding *per se*, or both.

When whole fruit is pulsed with ethylene or propylene (24 h), a discrete pulse-induced respiration peak is followed by a respiratory climacteric. Analogously, when whole fruit is cored, *i.e.* when one or more pericarp plugs are removed, a transient wound respiration is accompanied by a brief production of wound ethylene that acts like an exogenous ethylene pulse in shortening the lag period (19). When fruit is given ethylene continuously, the pulse peak merges with the

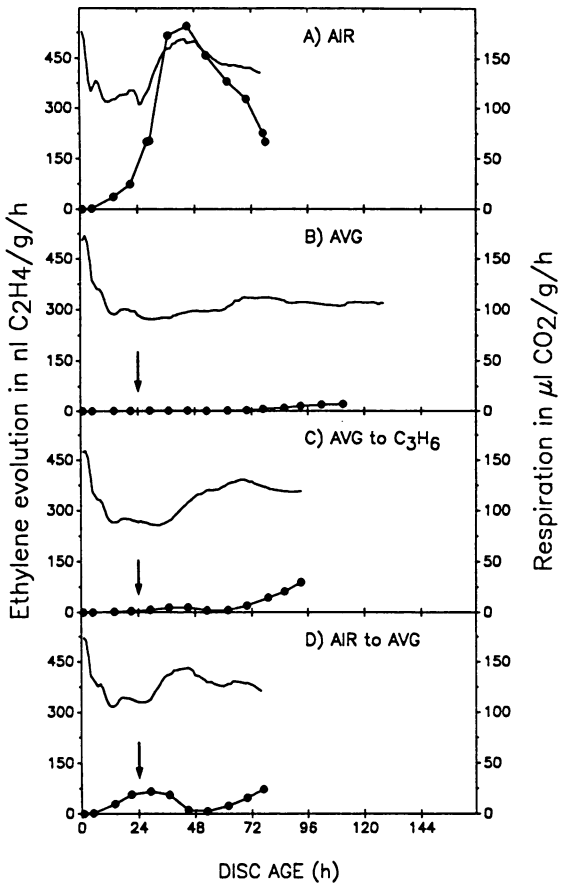

Figure 5. Respiration and ethylene production of AVG-treated avocado discs. Respiration (——) and ethylene production (●) of air control discs (A), AVG at 0 and 24 h (B; arrow), AVG at 0 h and propylene (500 µL/L) at 24 h (C; arrow), and AVG at 24 h (D; arrow). Discs are from freshly harvested late-season fruit. AVG, 4 mM in 25-µL droplets. All discs received 25-µL droplets of either AVG or water at 0 and 24 h. Respiration was monitored every hour and ethylene production every 8 h. Disc age is from time of slicing.

climacteric peak (4, 19) in late-season fruit, creating a so-called shoulder on its leading edge (4, 24), whereas in early or midseason fruit the respiration profile is bimodal (19). In our view, the wound-induced and climacteric-associated respiratory peaks in discs (Figs. 1 and 2) are analogous to the dual peaks in intact early and midseason fruit given continuous exogenous ethylene or propylene (19).

Within hours of disc preparation, EFE activity and ethylene production begin to increase. As explained earlier (see "Materials and Methods"), under these circumstances ethylene production can be taken as a measure of ACC synthase activity. Whether wound ethylene peaks in a transient rise before reaching the climacteric (early-season fruit) or leads directly to autocatalytic ethylene production (mid- or late-season fruit), disc tissue experiences endogenous ethylene concentrations substantially higher than in preclimacteric whole fruit (3). Together, wound and climacteric ethylene in discs may serve, in effect, as a continuous ethylene treatment, causing discs to ripen much as continuous ethylene treatment ripens whole fruit. In fact, discs ripen and reach the soft edible state in the same time as do continuously ethylene-treated whole fruit (data not shown).

The complex involvement of ethylene in disc ripening can be demonstrated in a number of ways and reveals two possible important roles: wound ethylene may prepare or sensitize the tissue for ripening initiation, whereas climacteric ethylene implements softening and other ripening-related events. If ethylene production or action is drastically reduced with AVG or NBD, respectively, disc softening is eliminated or markedly delayed. AVG and NBD applied concomitantly and continuously completely prevent ripening and even eliminate necrosis-related softening. If either inhibitor is present solely for the first 24 h (flushing NBD, or rescuing AVG-treated discs with propylene at 24 h), discs soften, but softening is delayed at least 24 h, and often much more in experiments in which NBD is removed, because NBD dissolved in tissue escapes slowly. The delay in softening suggests that ethylene-mediated

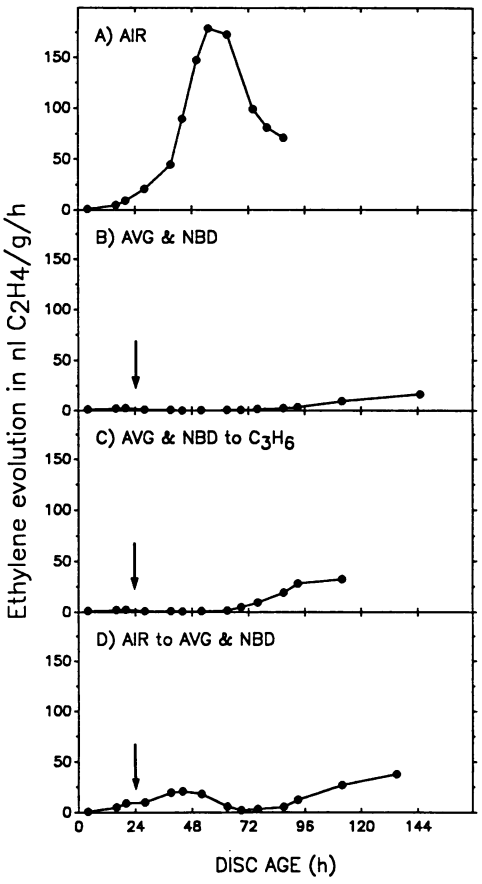

Figure 6. Ethylene production of avocado discs treated with AVG and NBD together. Ethylene production of air control discs (A), continuous AVG plus NBD (B), discs switched from AVG plus NBD in air to propylene (500 µL/L) at 24 h (C; arrow), and discs in air treated with AVG plus NBD in air at 24 h (D; arrow). Discs are from freshly harvested late-season fruit. NBD, 10,000 µL/L during the times indicated; AVG, 4 mM in 25-µL droplets at 0 and 24 h (B; arrow), at 0 h (C), and at 24 h (D; arrow). All discs received 25-µL droplets of either AVG or water at 0 and 24 h. Ethylene production was monitored every 6 to 12 h. Disc age is from time of slicing. Note the smaller scale for ethylene compared with Figure 6.

events that occur in the first 24 h are requisite for subsequent ripening phenomena.

Demonstration of the ongoing role of ethylene is revealed when NBD is applied 24 h after cutting. In this case, discs fail to soften, suggesting that even if ripening-related events of the first 24 h occur, ethylene is needed to implement later ripening. If AVG is applied after 24 h, discs soften in essentially the same time as do untreated discs. This suggests that wound ethylene-mediated events of the first 24 h sensitize the discs so that a persisting low level of ethylene (either produced in the presence of AVG or remaining in the tissue from the first 24 h) is sufficient to trigger and/or mediate ripening. It thus appears that ethylene, in sufficient concentrations, is required at all stages of disc ripening, much as in whole fruit.

Sensitization of the tissue can be taken a step further by pretreating intact fruit with ethylene or propylene for 24 h before cutting (19). Discs cut from pulsed fruit exhibit greater EFE activity immediately and produce greater amounts of ethylene eventually, with the result that softening is hastened compared with discs from 24-h untreated fruit. Even fruit held an additional 48 h after a 24-h ethylene pulse yield discs that ripen sooner. Hence, some of the preparatory events that take place during the initial phase of disc ripening either occur during the pulse or are subsequently accelerated by pretreating the fruit with ethylene.

When ethylene is applied continuously to AVG-treated discs, softening occurs in the same time as in untreated controls. On the other hand, continuous treatment of otherwise untreated discs with ethylene or propylene has little effect on ripening time. It would seem that discs produce saturating levels of ethylene within hours of cutting and that this ethylene is sufficient to induce full ripening in a matter of days. It remains an open question whether wounding *per se* synergizes the effect of wound ethylene.

Wound ethylene production (as well as climacteric ethylene production) in discs is inhibited by AVG, indicating it is ACC synthase dependent (*cf.* ref. 28). NBD not only fails to reduce wound ethylene but in fact increases it slightly (without increasing EFE activity), indicating that wound ethylene production is not modulated by a positive ethylene-mediated feedback loop. The stimulatory effect of NBD through 24 h or more (Fig. 4, Table III; *cf.* ref. 5) (thought to arise from the release of ethylene-mediated inhibition of ethylene production as seen in pulsed preclimacteric fruit [26]), is presumably due to the deinhibition of ACC synthase transcription (13, 16). Less likely, stimulation by NBD may to some extent be the result of inhibition of ethylene-mediated diversion of ACC to malonyl-ACC (9). By contrast, NBD eliminates climacteric ethylene production, maintaining ethylene at wound levels and reaffirming that climacteric ethylene production is mediated by a positive ethylene-mediated feedback loop.

There is evidence that in some plants more than one ACC synthase gene exists and that the different genes are regulated differentially and induced under different circumstances (13, 25). Our data suggest that wound ethylene production is mediated by one ACC synthase enzyme, wound ACC synthase, whereas climacteric ethylene production is mediated by ripening ACC synthase. The fact that wound ethylene production is not controlled by the positive ethylene feedback loop evident in climacteric ethylene production lends credence to this prospect.

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