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Daminozide inhibits ethylene production in apple fruit by blocking the conversion of methionine to aminocyclopropane-1-carboxylic acid (ACC)

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Abstract

At harvest, fruit from apple trees sprayed with daminozide (+daminozide) had lower levels of aminocyclopropane-1-carboxylic acid (ACC) and produced significantly lower amounts of ethylene than untreated (−daminozide) fruit. Flesh discs from the fruit of +daminozide and −daminozide trees were fed precursors of ethylene to determine how daminozide inhibits ethylene production. ACC was metabolized to ethylene regardless of treatment. Methionine (MET), however, was only converted to ethylene by −daminozide fruit, and only after the fruit had been maintained at 4 °C for 5 months. +Daminozide fruit failed to convert MET to ethylene at harvest, as well as after cold storage. When daminozide was added to the incubation media of flesh discs it did not inhibit ethylene production or the conversion of ACC to ethylene. The addition of daminozide did, however, inhibit the metabolism of exogenous MET to ethylene. Aminooxyacetate acid (AOA) blocked both the endogenous production of ethylene and that from MET feeds. Daminozide inhibits ethylene production by preventing the conversion of MET to ACC, but it does not appear to act as a simple competitive inhibitor of ACC synthase activity.

Abbreviations: ACC, aminocyclopropane-1-carboxylic acid; AVG, aminoethoxyvinylglycine; AOA, aminooxyacetic acid; CH, cycloheximide; MET, methionine; PUT, putrescine

1. Introduction

One of the most useful compounds for prolonging storage life and fruit quality in apple was the growth regulator daminozide (butanedioic acid mono 2,2-dimethylhydrazide). While the compound has been removed from registration for food crop use in the US, it remains an important chemical tool for studying the ripening process in apple. Daminozide is unique in that it is capable of uncoupling many of the processes associated with ripening, for example, daminozide prolongs storage life by inhibiting fruit softening, yet it has no effect on the development of flavor or aroma, and in many cases actually increases fruit red color [3,4,10]. Fruit abscission is also reduced, lessening pre-harvest yield loss, and certain post-harvest storage disorders may be reduced in severity. Daminozide must be applied to immature fruit some 60-90 days before harvest to be most effective. The compound is known to inhibit the onset of ethylene production [7,12] and this inhibition is maintained for at least 9 months in simple cold storage. The biochemical basis for the effect of daminozide on ethylene and fruit softening is not known. The following research elucidates the step in the ethylene synthesis pathway blocked by daminozide and provides some preliminary information on its mechanism of action, which appears to be different from the inhibitors of ACC synthase activity such as aminoethoxyvinylglycine (AVG) or aminooxyacetic acid (AOA).

2. Materials and methods

Apple trees cv. 'Rome Beauty' were sprayed twice with 2500 ppm daminozide (Alar-85), Uniroyal Chemical, Bethany, CT) once at approximately 60, and again at 90 days before harvest in 1989 and 1990. Fruit were harvested at commercial maturity and stored at 4 °C for up to 9 months. Ethylene was determined by incubating individual fruit in 1.85 l jars at 25 °C for 1 h. Three 1-ml gas samples from the headspace were analyzed for ethylene by gas chromatography using a flame ionization detector and a stainless steel Porapak Q column (1.83 m length × 3.18 mm width) at 100 °C. Aminocyclopropane-1-carboxylic acid (ACC) was extracted from flesh tissue (10 g) at 4 °C in 5% (w/v) sulfosalicylic acid. The extract was passed through a Dowex 50 column (1 × 6 cm), washed with water, and eluted with 2N NH_4OH. ACC was analyzed according to the method of Lizada and Yang [11].

Feeding experiments were conducted with flesh discs using the methodology of Lieberman et al [9]. Ethylene production by whole fruit was first determined after which the fruit was sliced crosswise into circular slabs approximately 1 cm thick, and cylinders (discs) of tissue were removed using an 8 mm diameter cork borer. Discs (4, approximately 3 g) were weighed and placed in a 25 ml Erlenmeyer flask with 5 ml of test solution. Cutting and weighing of discs was done rapidly, and the flask was sealed with a serum stopper and shaken at 150–200 rpm for 1 h at 25 °C. Two 1-ml gas samples from the flask headspace were analyzed for ethylene. All test solutions contained 0.4 M sucrose and 0.1 M $NaHCO_3$ buffer, pH 8. One or more of the following was added to make the final concentration as indicated: L-methionine (MET), 1 mM; ACC, 100 µM; aminooxyacetic acid (AOA), 2.5 mM; cycloheximide (CH), 100 µM; putrescine (PUT), 50 mM; or daminozide, 10 mM. Treatments containing CH were pre-incubated in CH for 1 h after which the flasks were flushed with air. Other treatments were handled similarly only without CH. There were 3 replicate flasks per treatment, and each experiment was repeated twice. Fruit were sampled for ethylene measurements and feeding experiments once a month for nine months after harvest. The entire experiment was repeated the following year with similar results.

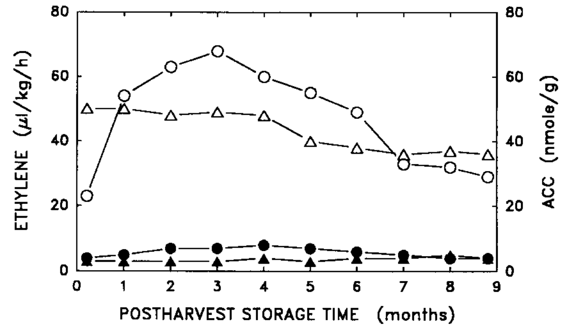

Fig. 1. Changes in ethylene production and aminocyclopropane-1-carboxylic acid (ACC) content of 'Rome Beauty' apple fruit from daminozide-treated (+daminozide) and untreated (−daminozide) trees, at harvest, and during cold storage at 4 °C. Ethylene: −daminozide O-O ($LSD_{.05}$ 10.6), +daminozide ●-● ($LSD_{.05}$ 1.8) ACC: −daminozide △-△ ($LSD_{.05}$ 4.4), +daminozide △-△ ($LSD_{.05}$ 1.1).

3. Results

At harvest, ethylene production was greater than 20 µl/kg/h for control fruit (−daminozide) (Fig. 1). This rate increased to about 60 µl/kg/h during several months in cold storage. Fruit stored for as long as 9 months continued to produce ethylene but at a lower rate. In contrast, daminozide-treated fruit (+daminozide) produced significantly lower amounts of ethylene at harvest (< 10 µl/kg/h), which did not change throughout the cold storage period.

The ACC content of −daminozide fruit ranged from about 50 nmoles/g at harvest to less than 40 nmoles/g after 9 months postharvest storage. There was little relationship between changes in ethylene production and ACC content, although after 7 months both ACC content and ethylene production rates had declined. In +daminozide fruit, ACC content was < 5 nmoles/g at harvest, and throughout the storage period there was little change in the amount of ACC.

Flesh discs taken from both +daminozide and −daminozide fruit produced ethylene at similar rates to whole fruit taken from each treatment (Table 1). The addition of daminozide to the buffer solution did not inhibit ethylene production by flesh discs from either the −daminozide or +daminozide fruit.

When ACC was fed to flesh discs from both −daminozide and +daminozide-treated fruit (Table 2), it was readily metabolized to ethylene

Table 1. Effect of daminozide, sprayed onto fruit at 2500 ppm 60 and 90 days before harvest, and/or added to the incubation media at 10 mM, on ethylene production by flesh discs of 'Rome Beauty' apple, after post-harvest storage at 4 °C for 1 month (n = 15)

Pre-harvest Treatment	Daminozide in incubation media (−)	(+)
	Ethylene (nl/g/h)	
None	41.6a^z	42.2a
Daminozide	8.8b	9.4b

^{z}Numbers followed by similar letters in rows and columns are not significantly different by $LSD_{.05}$.

regardless of treatment. In fact, the percentage increase in ethylene production as a result of feeding ACC was greater in +daminozide fruit as compared to −daminozide. The addition of daminozide to the incubation media had relatively little effect upon the conversion of exogenous ACC to ethylene.

MET was metabolized only when fed to −daminozide fruit (Table 3). +Daminozide-treated fruit did not convert exogenous MET to ethylene. The ability to metabolize MET in −daminozide flesh discs was, however, only apparent after 5 months in cold storage, and the rate of ethylene production from MET feeds was greater for fruit kept for even longer cold storage periods. The addition of daminozide to the incubation media significantly reduced the metabolism of exogenous MET to ethylene, in contrast to the results obtained with ACC. Daminozide feeds to flesh discs from either −daminozide or +daminozide-treated fruit, however, had no effect on the endogenous production of ethylene.

Table 2. Effect of aminocyclopropane-1-carboxylic acid (ACC) and daminozide, sprayed onto fruit at 2500 ppm 60 and 90 days before harvest, and/or added to the incubation media at 10 mM, on ethylene production by flesh discs of 'Rome Beauty' apple after post-harvest storage at 4 °C for 1, 3, 5, 8, and 9 months (n = 5)

Pre-harvest Treatment	Date	Control	+ACC	+ACC+daminozide
		Ethylene (nl/g/h)		
None	November	36.6b^z	50.4a	49.7a
	January	30.4b	51.5a	42.8ab
	March	35.0b	48.4a	43.8ab
	June	30.0b	49.2a	45.4a
	July	28.0c	52.2a	43.2b
Significance^y (date)		NS	NS	NS
Daminozide	November	7.1b	46.6a	45.2a
	January	6.8c	43.5a	39.6b
	March	7.4c	46.2a	41.6b
	June	4.0b	37.4a	34.6a
	July	5.2b	35.2a	34.2a
Significance^y (date)		*	*	*
(control vs. daminozide)		*	*	*

^{z}Numbers followed by similar letters within rows are not significantly different according to Duncan's Multiple Range test.
^{y}NS, *, not significant and significant within columns by $LSD_{.05}$.

Table 3. Effect of methionine (MET) and daminozide, sprayed onto fruit at 2500 ppm 60 and 90 days before harvest, and/or added to the incubation media at 10 mM, on ethylene production by flesh discs of 'Rome Beauty' apple after post-harvest storage at 4 °C for 1, 3, 5, 8, and 9 months (n = 5)

Pre-harvest Treatment	Date	Control	+MET	+MET+daminozide
		Ethylene (nl/g/h)		
None	November	30.0a^z	28.7a	29.8a
	January	35.2a	37.8a	33.6a
	March	33.6b	55.0a	46.6ab
	June	31.6b	60.4a	34.6b
	July	28.2b	69.6a	33.2b
Significance^y (date)		NS	*	*
Daminozide	November	7.7a	8.0a	7.3a
	January	8.4a	9.4a	8.4a
	March	8.8a	10.1a	9.4a
	June	4.8a	5.4a	5.6a
	July	5.8a	5.6a	5.4a
Significance^y (date)		*	*	*
(control vs. daminozide)		*	*	*

^{z}Numbers followed by similar letters within rows are not significantly different according to Duncan's Multiple Range test.
^{y}NS, *, not significant and significant within columns by $LSD_{.05}$.

The addition of AOA to the incubation media of flesh discs from −daminozide fruit significantly reduced ethylene production (Table 4). In addition, AOA prevented the conversion of exogenous MET to ethylene, but had no effect on the metabolism of ACC to ethylene. In contrast, PUT had no effect on ethylene.

When flesh discs from −daminozide fruit were pre-incubated in CH, ethylene production, was significantly reduced (Table 5). The conversion of exogenous MET to ethylene was also inhibited, however, CH had no effect on the conversion of ACC to ethylene. The low level of ethylene production exhibited by flesh discs from +daminozide fruit was not affected by CH.

4. Discussion

The results demonstrate that daminozide reduces ethylene production both at harvest and after at

Table 4. Effect of aminooxyacetic acid (AOA), putrescine (PUT), and daminozide, sprayed onto fruit at 2500 ppm 60 and 90 days before harvest, on ethylene production by flesh discs of 'Rome Beauty' apple (n = 6)

Treatment	Pre-harvest spray (−)	Pre-harvest spray (+)	Significance[z] (−) vs. (+)
	Ethylene (nl/g/h)		
None	24.7c[y]	4.5b	*
+AOA	3.0d	2.2b	NS
+PUT	24.5c	4.7b	*
+ACC	37.2ab	32.7a	NS
+AOA+ACC	38.0a	32.5a	NS
+PUT+ACC	33.7ab	31.8a	NS
+MET	30.1bc	4.7a	*
+AOA+MET	3.0d	2.8b	NS
+PUT+MET	31.8ab	4.3b	*

[z]NS, *, not significant within rows by $LSD_{.05}$.
[y]Numbers followed by similar letters within columns are not significantly different according to Duncan's Multiple Range Test.

least 9 months in cold storage as has been previously reported [3,4,10]. ACC levels in +daminozide fruit were significantly reduced at harvest and did not change during cold storage. ACC content of −daminoxide fruit declined somewhat during post harvest storage but no clear pattern emerged in relation to ethylene production. For the variety 'Golden Delicious' [8] ACC levels peaked at 39 nmoles/g during the first month of cold storage then declined to less than 10 nmoles/g and remained at that level. For 'Delicious' [15] ACC levels peaked after 6 weeks cold storage at about 12 nmoles/g, and declined to about half this level for longer storage intervals. In both of these experiments fruit were harvested pre-climacteric, whereas in our experiments the fruit were havested in the early climacteric period after the onset of ethylene production. This may be in part responsible for the differences in ACC content. In other fruit such as avocado, the synthesis of ACC occurs prior to the increase in ethylene production, declines during the ethylene climateric, and then rises again as ethylene production decreases [5]. In 'Rome Beauty' apple (figure 1), ethylene production did not peak until 3 months in cold storage and remained as high as 50% of this level through 9 months storage. The high and persistent levels of ACC in apple fruit probably reflect these differences in ethylene production among fruit from different species.

Table 5. Effect of aminocyclopropane-1-carboxylic acid (ACC), methionine (MET), cycloheximide (CH), and daminozide, sprayed onto fruit at 2500 ppm 60 and 90 days before harvest, on ethylene production by flesh discs of 'Rome Beauty' apple (n = 5)

Treatment	Pre-harvest spray (−)	Pre-harvest spray (+)	Significance[z] (−) vs. (+)
	Ethylene (nl/g/h)		
None	51.4b[y]	17.2b	*
+MET	75.8a	19.2b	*
+CH	26.2c	16.4b	*
+MET+CH	26.0c	16.0b	*
+ACC	81.0a	60.2a	NS
+ACC+CH	74.8a	54.6a	*

[z]NS, *, not significant within rows by $LSD_{.05}$.
[y]Numbers followed by similar letters within columns are not significantly different according to Duncan's Multiple Range Test.

The low levels of ACC in +daminozide fruit at harvest and throughout the cold storage period suggest that the inability to produce ethylene may be due to a block in the production of ACC, unless of course ACC, or an earlier metabolite, is converted to another product instead of ethylene when treated with daminozide. It would be of interest to determine malonyl-ACC and perhaps polyamine content in +daminozide fruit, as malonyl-ACC is a well-known conjugate [13], and the polyamines are derived from the ACC precursor S-adenosylmethionine [2]. Nevertheless, when fruit discs were fed ACC it was readily converted to ethylene in both −daminozide and +diminozide-treated fruit (Table 2). +Daminozide fruit have, therefore, an active ACC oxidase, and unless exogenous ACC did not mix with internal pools, the low levels of ACC found in this study (Figure 1) probably reflect low rates of synthesis.

The results of MET feeds to +daminozide and −daminozide fruit were different (Table 3). +Daminozide fruit were unable to convert exogenous MET to ethylene at harvest and throughout the cold storage period. The block in the ethylene pathway therefore seems to be between MET and ACC. ACC synthase is of course a well-documented regulatory enzyme for the ethylene synthesis pathway [13].

The inability of −daminozide fruit, at harvest as well as early in the cold storage period, to metabolize exogenous MET to ethylene, when ethylene synthesis was occurring at a significant rate was surprising. It was not until the fruit had been stored for at least 5 months that feeds of MET produced significant ethylene, however, similar results have been reported before [9]. It is unclear what changes must have taken place in the fruit during cold storage which now allow MET feeds to produce additional ethylene.

AOA almost completely inhibited ethylene production (Table 4) as expected, since it inhibits the activity of ACC synthase [1]. Daminozide in contrast, had no effect on ethylene production when added to the discs (Table 1), suggesting that daminozide does not act in a similar manner as AOA, and is not likely therefore to be an inhibitor of ACC synthase activity. Daminozide inhibits the development of the ability to produce ethylene in pre-climacteric fruit, but is not able to block the existing production of ethylene once it has begun, at least in short term feeding experiments. Perhaps daminozide inhibits the transcription, translation, or processing of ACC synthase, and in this way reduces the ability of pre-climacteric fruit to produce ethylene. The results from the CH experiment (Table 5) certainly indicate that protein synthesis is required for ethylene production, and ACC synthase is known to be particularly sensitive to CH treatment in wounded [16] or chilling-injured [14] fruit tissue.

In some cases (Table 3), however, ethylene produced by MET feeds was inhibited by the addition of daminozide. Perhaps the ability to metabolize exogenous MET in fruit results from the expression of a different ACC synthase [6] than the one responsible for endogenous ethylene production. It is possible that daminozide inhibits the synthesis of a substrate-inducible ACC synthase which develops in post-climacteric fruit after long term cold storage. Alternatively, the addition of daminozide to MET feeds could induce the synthesis of an enzyme that degrades an ACC synthase, which metabolizes exogenous MET. In this case the addition of CH in MET feeds might increase ethylene production in the presence of daminozide. In contrast, the addition of daminozide to ACC feeds had almost no effect on the conversion of ACC to ethylene (Table 1).

PUT had no effect on ethylene production in contrast to previous results [2]. This was probably due to the differences in incubation time. In their experiments PUT was effective only after a 6 h incubation, whereas our experiments were conducted for shorter time periods (1 h) and therefore prior to the time in which an effect of PUT might become apparent. Other evidence indicates that PUT may not influence ethylene production in apple. During cold storage of fruit, PUT levels increased concomitantly with ethylene production [15].

In conclusion, daminozide reduces ethylene production in fruit by inhibiting the metabolism of MET to ACC. Daminozide does not appear, however, to act as a simple competitive inhibitor of ACC synthase. Future work should focus on determining if daminozide blocks the production of the enzyme (ACC synthase) responsible for this step in the pathway.

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References

1 Amrhein N, Schneebeck D, Scoruka H, Tophof S and Stockigt J (1981) Indentification of a major metabolite of the ethylene precursor 1-aminocyclopropane-1-carboxylic acid in higher plants. Naturwissenschaften 68: 619–620
2 Apelbaum A, Burgoon AC, Anderson JD, Lieberman M, Ben-Aire R and Mattoo AK (1981) Polyamines inhibit biosynthesis of ethylene in higher plant tissue and fruit protoplasts. Plant Physiol 68: 453–456
3 Blanpied GD, Smock RM and Kollas DA (1966) Effect of daminozide on optimum harvest dates and keeping quality of apples. Proc Amer Soc Hort Sci 90: 467–474
4 Elfving DC, Lougheed EC and Cline RA (1991) Daminozide, root pruning, trunk scoring and trunk ringing effects on fruit ripening and storage behaviour of 'McIntosh' apple. J Amer Soc Hort Sci 116: 195–200
5 Hoffman NE and Yang SF (1980) Changes of 1-aminocyclopropane-1-carboxylic acid in ripening fruits in relation to their ethylene production rates. J Amer Soc Hort Sci 105: 492–495
6 Kim WT, Silverstone A, Yip WK, Dong JG and Yang SF (1992) Induction of 1-aminocyclopropane-1-carboxylic acid synthase mRNA by auxin in mung bean hypocotyls and cultured apple shoots. Plant Physiol 98: 465–471
7 Knee M (1988) Effect of temperature and daminozide on the induction of ethylene synthesis in two varieties of apple. J Plant Growth Regul 7: 111–119
8 Lau OL, Liu Y and Yang SF (1984) Influence of storage atmospheres and procedures on 1-aminocyclopropane-1-

carboxylic acid concentration in relation to flesh firmness in 'Golden Delicious' apple. HortScience 19: 425–426
9 Lieberman M, Kunishi A, Mapson LW and Wardale DA (1966) Stimulation of ethylene in apple tissue slices by methionine. Plant Physiol 41: 376–382
10 Liu FW (1979) Interaction of daminozide, harvest date, and ethylene in CA storage in 'McIntosh' apple quality. J Amer Soc Hort Sci 104: 599–601
11 Lizada CC and Yang SF (1979) A simple and sensitive assay for 1-aminocyclopropane-1-carboxylic acid. Biochemistry 100: 140–145
12 Looney NE (1968) Inhibition of apple ripening by succinic acid 2,2-dimenthylhydrazide. Plant Physiol 43: 1133–1137
13 Mattoo AK and White WB (1991) Regulation of ethylene biosynthesis. In: Mattoo AK and Suttle JC, eds. The Plant Hormone Ethylene, Chapter 2. Boca Ratan: CRC Press
14 Wang CY and Adams DO (1982) Chilling-induced ethylene production in cucumbers (*Cucumus sativus* L.) Plant Physiol 69: 424–427
15 Wang SY and Faust M (1992) Ethylene biosynthesis and polyamine accumulation in apples with watercore. J Amer Soc Hort Sci 117: 133–138
16 Yu YB and Yang SF (1980) Biosynthesis of wound ethylene. Plant Physiol 66: 281–285