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Effect of Orchard and Postharvest Application of Daminozide on Ethylene Synthesis by Apple Fruit

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Abstract. The effects of daminozide (butanedioic acid-2,2-dimethylhydrazide) on ethylene synthesis by apple fruits were investigated. The objective was to determine the effects of postharvest applications as compared to the standard application of diaminozide in the orchard. Immersion in a solution containing 4.25 g L^{-1} active ingredient for 5 min delayed the rise in ethylene production in individual "Cox" apples at 15°C by about 2 days, whereas orchard application of 0.85 g L^{-1} caused delays of about 3 days. Both modes of application depressed the maximal rate of ethylene production attained by ripe apples by about 30%. Daminozide did not affect the stimulation of respiration by ethylene treatment of "Gloster" apples, but it delayed the increase in ethylene synthesis. Daminozide applied immediately after harvest delayed the rise in ethylene synthesis in "Golden Delicious" held at 15°C, but it was less effective when applied 48 h after harvest or when apples were held at 5°C. Exposure to 1–2 μl L^{-1} ethylene for 48 h was less effective in promoting the rise in ethylene in daminozide-treated "Cox" and "Gloster" apples than in untreated fruit. High (100–1000 μl L^{-1}) concentrations of ethylene more or less overcame the daminozide effect. Apples absorbed about 40% of surface-applied [^{14}C]daminozide in 48 h, but more than 90% of the radioactivity in the fruit was recovered from the peel and outer 1 cm of the cortex. Daminozide was partly converted to carbon dioxide and other metabolites.

Daminozide has been mainly used on apples to control various aspects of tree growth and fruit development. One effect of daminozide is to delay the onset of the climacteric rise in fruit respiration; this effect can be reversed by ethylene treatment of fruit after harvest (Looney 1968). Daminozide and ethylene seemed to be antagonistic in their effects on pectin solubilization and softening of apples (Knee 1986). Daminozide also appeared to inhibit the induction of ethylene synthesis by either ethylene or cold treatment of apples (Knee 1988). Successful low ethylene storage in apples may depend upon the use of daminozide (Liu 1979). Without daminozide, most apple varieties begin to produce ethylene early in storage, and it is then impossible to maintain low ethylene concentrations by removal of the gas from the storage atmosphere (Knee 1985).

The mode of action of daminozide has not been established. Direct application of daminozide to fruits has not been tested, and, therefore, its effects on fruit could be secondary consequences of application to the tree. These experiments were designed to test the direct effects of daminozide on ethylene synthesis in apples, and to establish the extent of penetration of radioactively labeled daminozide applied to the surface of whole apples.

Materials and Methods

Apples (*Malus pumila* L.) were harvested from trees of the varieties "Cox's Orange Pippin" (September 7, 1987) and "Gloster 69" (October 5, 1987) at East Malling, and "Golden Delicious" (October 1, 1988) at Summerland. Some of the "Cox" trees had been sprayed on July 24, 1987 with daminozide (butanedioic acid-2,2-dimethylhydrazide, trade name "Alar 85," Uniroyal Chemical) at 0.85 g L^{-1} and 1100 Lha^{-1}. The apples were used immediately for experiments except the "Gloster 69" which were held in 2 kPa O_2 at 1.5°C with continuous ethylene removal for 4 months (Knee and Tsantili 1988a). All apples had internal ethylene concentrations below 0.05 μl L^{-1} and were presumed to be preclimacteric at the start of the experiments.

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Table 1. Effect of orchard and postharvest applications of daminozide and ethylene treatment for 48 h on time of onset of ethylene production and peak ethylene production rates for individual "Cox" apples

(A) Back-transformed means for 16 fruit

	Time of onset (days) after ethylene treatment ($\mu l\ L^{-1}$)			Production ($\mu l\ kg^{-1}\ h^{-1}$) after ethylene treatment ($\mu l\ L^{-1}$)		
Daminozide	0	2	100	0	2	100
None	13.0	6.7	4.2	54.3	52.0	51.6
Orchard	17.1	13.6	9.4	33.0	37.3	41.7
Postharvest	15.4	12.7	5.6	39.9	35.8	45.5

(B) Probabilities of null effect of treatments

	Time of onset			Production		
Experimental comparison	D	E	D*E	D	E	D*E
Orchard/none	0.0001	0.0001	0.0072	0.0001	0.339	0.119
Postharvest/none	0.0001	0.0001	0.0080	0.0001	0.144	0.0716
Postharvest/orchard	0.0023	0.0001	0.0279	0.105	0.0126	0.381

D, daminozide effect; E, ethylene effect; and D*E, their interaction.

Before treatment, apples were equilibrated to 15°C for at least 3 h. Fruits were immersed in a solution of daminozide at 4.25 g L^{-1} for 5 min. The commercial formulation included an unknown surfactant, and control fruit were immersed in a solution of wetting agent (Agral, ICI) at 0.01 g L^{-1}.

Ethylene was applied to "Cox" and "Gloster" apples in sealed containers for 48 h with calcium hydroxide to absorb carbon dioxide for 48 h (Knee et al. 1987). "Golden Delicious" apples were held for 24 h in groups of 10 in containers ventilated with air or an ethylene–air mixture (1 $\mu l\ L^{-1}$) at 3 L h^{-1}. In all experiments, apples were transferred to single fruit containers which were continuously ventilated with ethylene-free air at 1 L h^{-1}. In the experiments with "Cox" and "Gloster" apples, ethylene was analyzed by gas chromatography of samples collected manually from 16 replicates every 2 to 3 days (Knee et al. 1987). In the experiment with "Golden Delicious," air passing over each of the 21 replicates was selected for analysis at 2-day intervals by a solenoid valve which allowed flow to an automatic sampling valve for injection into the chromatograph.

Ethylene was separated on alumina columns with the following dimensions, temperatures, carrier gas flow rates, and detection limits; 50 × 0.4 cm, 90°C, 60 ml min^{-1} nitrogen and 0.002 $\mu l\ L^{-1}$ at East Malling and 120 × 0.3 cm, 120°C, 30 ml min^{-1} of helium and 0.01 $\mu l\ L^{-1}$ at Summerland.

In some experiments, after ethylene records were complete, fruit firmness was measured with an automated penetrometer (Looney et al. 1981, Topping 1981) and peel reflectance was measured at 680 nm (Knee 1980).

In order to measure uptake of daminozide by apples, radioactively labeled material was prepared by condensation of 2,2 dimethylhydrazine with [1,4-^{14}C]succinic anhydride (4.1 GBq $mmol^{-1}$, from Amersham International) (Peiser 1971). The product was purified by TLC on cellulose, first with butanol–acetic acid–water (4:1:1, BAW), and second with propan-2-ol–ammonia-water (20:1:4, PNW) as developing solvents (Samaraweera and Hill-Cottingham 1980). Daminozide was localized by spraying unlabeled marker strips with a mixture of 1% ferric chloride and 1% potassium ferricyanide. The radioactive product was eluted from the cellulose with water. Assay by scintillation counting after chromatography in PNW showed that 92% of the activity was associated with daminozide and 8% with free succinate.

A 2 cm^2 area on the surface of an apple was outlined with petroleum jelly, the apple was immersed in the commercial daminozide formulation as described above and placed in a glass jar with the outlined area upward. Labeled daminozide (2.6 kBq) was applied within the square in 10 μl water. The apple was incubated at room temperature in the sealed jar; a vial containing 2 ml of 2 M sodium hydroxide was present in the jar to absorb carbon dioxide and was replaced at daily intervals. After incubation for 1, 2, or 7 days, three apples were cut in half parallel to the surface carrying the labeled daminozide. This surface was washed two times for 5 min with 20 ml of water. A block with parallel sides was cut to include the labeled area within its 6.25 cm^2 section. This was cut into a peel fraction (~2 g) and three equal flesh fractions (~8 g), outer cortex, inner cortex, and core. These fractions were each homogenized in 10 ml of 0.1 M hydrochloric acid. Radioactivity in the sodium hydroxide solution, water washes, and tissue homogenates was estimated after mixing with Liquiscint (National Diagnostics); if necessary water was added to create a stable gel and Beckman counters 3801 or 7800 were used with quench correction by "H" number.

When data are expressed as frequencies (Tables 2 and 3) the significance of treatment effects was analyzed by a χ^2 procedure. Other data were subjected to analysis of variance after logarithmic transformation. Coefficients of variation (*cv*) were calculated by back-transformation of the square root of the error variance and are expressed as percent of the mean at the appropriate level of replication.

Results

A postharvest application of daminozide delayed the onset of rapid ethylene production in "Cox"

Table 2. Effect of 4.25 g L^{-1} daminozide and 24-h exposure (starting 24 h after harvest) to 1 μl L^{-1} ethylene on frequency distribution of time of onset of ethylene production in individual "Golden Delicious" apples

Ethylene (μl L^{-1})	Daminozide applied	Frequency of onset (%)			Probability of null effect
		<4 days	4–8 days	>8 days	
0	None	57	0	43	
0	At 24 h	14	14	72	0.004
0	At 48 h	48	9	43	0.539
1	None	76	24	0	
1	At 24 h	57	38	5	0.190
1	At 48 h	71	19	10	0.726

The probabilities were derived from a χ^2 analysis for comparisons of daminozide-treated and untreated fruits.

apples (Table 1). An orchard spray application was more effective, but with both methods of treatment the largest effect was seen in apples exposed to 2 μl L^{-1} ethylene (Table 1). A higher concentration (100 μl L^{-1}) overcame this effect of daminozide. At the end of the experiment ethylene production by individual apples had attained stable rates, which were unrelated ($p > 0.05$) to their times of onset of ethylene production. Both daminozide treatments resulted in lower final rates of ethylene production than in control apples. Ethylene treatment did not affect the final rate in control apples, but ethylene at 100 μl L^{-1} partially reversed the effect of daminozide (Table 1).

In the experiment with "Golden Delicious" apples, the times when replicate individual apples in one treatment began to produce ethylene rapidly were not normally distributed. In all treatments at least a proportion of the apples were producing ethylene at the first measurement (4 days). In nonethylene-treated fruits held at 15°C, the mean time of onset for the remaining apples was 14.3 days, and there was no effect of daminozide ($p = 0.05$) on the mean. However, when daminozide was applied at the beginning of the experiment, a higher proportion of fruits was found in the group showing delayed ethylene production (Table 2). When application of the chemical was delayed by 24 h, there was no effect ($p > 0.05$). When apples were treated with 1 μl L^{-1} ethylene and held at 15°C, a higher proportion showed ethylene production at the first reading than among nonethylene-treated fruit (Table 2). The remaining apples among the ethylene-treated began to produce ethylene at a mean time of 7.2 days again without an effect of daminozide treatment. Daminozide applied at the beginning of the experiment increased the proportion of apples showing some delay of ethylene production, but the inhibitor was ineffective when applied after the ethylene treatment. When fruits were transferred to 5°C after 2 days at 15°C, ethylene production was detected in virtually every apple 2 days later, irrespective of daminozide or ethylene treatment (data not shown).

In an experiment with "Gloster 69" apples, the respiration rates during ethylene treatment for daminozide-treated apples were identical to those of untreated fruit (see Fig. 2 in Knee and Tsantili 1988b). The time of onset of ethylene production in this variety is not normally distributed (Knee and Tsantili 1988b) and results are presented as frequency distributions (Table 3). Daminozide decreased the proportion of apples in which onset occurred within 10 days and increased the proportion in which it did not occur for 45 days. Ethylene partially overcame this effect but even 1000 μl L^{-1} was not fully effective (Table 3). The effects on firmness and reflectance were less consistent, but inspection of the data showed that 53% of daminozide-treated fruit retained a firmness above 30 N and 75% retained a reflectance at 680 nm below 35% by comparison with 33% above 30 N and 63% below 35% in controls. Ethylene tended to overcome these effects.

"Gloster 69" apples with an average weight of 170 g gained 0.47 g after immersion in the daminozide solution. Thus, about 2 mg of daminozide should have been deposited on each apple. About 60% of the radioactive daminozide could be recovered by washing the fruit surface after 2 days, but by 7 days, only 5% could be recovered in this way (Fig. 1). At first most of the absorbed radioactivity was confined to the peel, even after 7 days only 5% of the activity had penetrated the inner cortex and almost none to the core. Activity was recovered in carbon dioxide, especially in the first 3 days (Fig. 1). The possibility of lateral spread of the compound was investigated by cutting slices (~8 g) adjacent to the sides of the block which was originally cut. At 7 days, a total of 8.9% of the applied activity was found in these adjacent slices. Also the proportion of radioactivity, remaining as daminozide was in-

Table 3. Effect of 4.25 g L^{-1} daminozide and 48-h exposure to ethylene on frequency distribution of time of onset of ethylene production in individual "Gloster 69" apples

Ethylene ($\mu l\ L^{-1}$)	Frequency (%) of onset before 10 days			Frequency (%) of onset after 45 days		
	Water	Daminozide	p	Water	Daminozide	p
0	19	0	0.069	50	94	0.006
1	25	19	0.669	62	66	0.719
10	69	38	0.077	25	50	0.144
100	63	25	0.033	25	63	0.033
1000	81	69	0.414	12	21	0.200

The probability (p) of a null effect of daminozide was calculated from a χ^2 analysis of the daminozide effect on the frequency in one class and the sum of the other classes. (The 15–45 class was combined with another class because low frequencies can invalidate this method of analysis.)

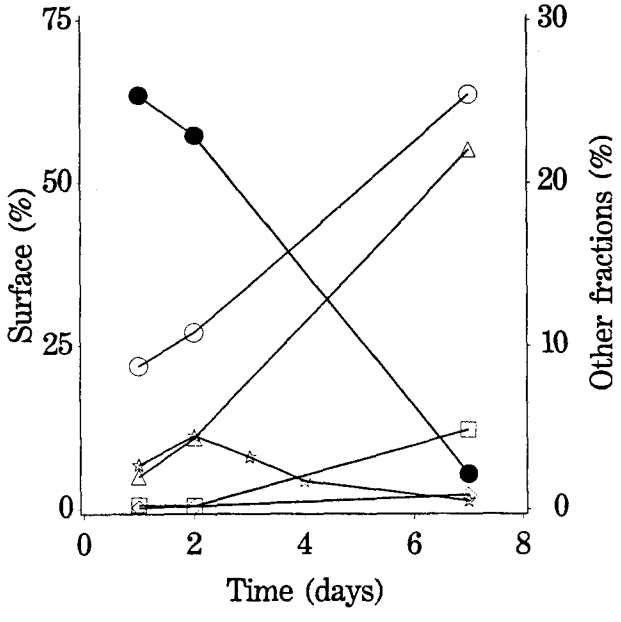

Fig. 1. Recovery of [^{14}C]daminozide as carbon dioxide, from fruit surface and tissue of "Gloster 69" apples. ●, surface (cv = 16%); ○, peel (cv = 15%); △, outer cortex (cv = 11.6%); □, inner cortex (cv = 38%); ◇, core (cv > 50%); ☆, carbon dioxide (cv = 18%). Coefficients of variation (cv) were calculated as percent of the means for four observations.

vestigated by chromatography of the extracts from apples after 7 days. About 70% of the activity recovered from the chromatogram coincided with daminozide, about 7% coincided with succinic acid, and 12% was found in an unknown compound.

Discussion

As apples approach commercial maturity they progress to a state in which endogenous ethylene production is rapidly induced by low concentrations (~0.1 $\mu l\ L^{-1}$) of exogenous ethylene. Before this time, high concentrations (>1 $\mu l\ L^{-1}$) of ethylene do not immediately induce ethylene production but advance the fruit toward the inducible state (Knee 1989, Knee et al. 1987); low concentrations are ineffective or even inhibitory at this time. In certain apple varieties, including "Golden Delicious," exposure to a low temperature also promotes induction of ethylene synthesis (Knee et al. 1983), although exogenous ethylene is less effective at low temperatures (Knee 1988).

Earlier work with daminozide (Knee 1988) suggested that treated fruit remained longer in the non-inducible state and that the chemical inhibited attainment of the inducible state in response to ethylene or low temperature. This work involved application of daminozide several weeks before harvest, so the effects observed after harvest could be indirect consequences of earlier events in the fruit or even in the tree. The ripening of apples and various other fruits is believed to be inhibited while they are attached to the plant (Adato and Gazit 1974). It is plausible that daminozide could enhance the effect of the tree on the fruit. However, the present demonstration of postharvest effects of daminozide excludes an indirect mode of action and implies that the chemical acts directly on the ethylene–response system.

There are several possible ways in which the induction of ethylene synthesis could be affected by daminozide. Known inhibitors act by inhibiting binding of ethylene to receptors (e.g., norbornadiene) and by inhibiting ethylene synthesis (e.g., aminoethoxyvinylglycine, AVG). Norbornadiene was shown to inhibit an immediate respiratory response to ethylene and the induction of ethylene synthesis in "Gloster" apples (Knee and Tsantili

1988b). The chemical structure of daminozide shows no similarity to ethylene analogues, even in the broad sense proposed by Sisler (1977). The lack of effect of daminozide on the stimulation of respiration by ethylene argues against an effect on the primary process of ethylene binding, unless different receptors are involved in this response and the induction of ethylene synthesis. Unlike AVG, daminozide does not inhibit ethylene synthesis in slices of postclimacteric apple tissue (Knee 1985). However, lower postclimacteric rates of synthesis are seen in daminozide-treated whole fruit. Ethylene synthesis in climacteric fruits has long been described as "autocatalytic." The observation that ACC synthase activity is inducible by ethylene treatment of AVG-treated apples (Bufler 1984) implies that there is indeed a positive feedback loop. There are likely to be other components in this control loop in addition to an ethylene receptor and the enzymes of ethylene synthesis. Daminozide could act on one of the intervening processes, perhaps at the level of signal transduction. Immature fruit seem to have the capacity for signal perception and response, in terms of ethylene production. Fruit development may involve the acquisition of the capacity for signal transduction, linking perception, and response (Knee 1989). Daminozide may return fruit to a state in which transduction is inhibited.

Radioactive daminozide was applied to "Gloster" apples immediately after they had been treated with unlabeled material in the same way as in the other experiments on postharvest application. Thus, the labeled compound should have been absorbed and metabolized by the fruit in the same way as the unlabeled. The observed pattern of penetration of daminozide implies that its action on the external tissue of the fruit was responsible for its physiological effects. This could be interpreted as showing that ripening is normally initiated in the peel. Earlier studies suggested that daminozide was stable after uptake by plants, particularly in fruit tissues (Samaraweera and Hill-Cottingham 1980). Metabolism during uptake might be a feature of the mode of application employed in this work. Although the intact molecule is thought to be biologically active, the activity of metabolites cannot be excluded until these are characterized. Release of labeled carbon dioxide implies that unlabeled metabolites should be present; these might be detected if daminozide labeled in other positions were supplied to fruit tissue.

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