Effect of Ethephon and Daminozide on the Respiration of Isolated Leaf Cells

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Abstract. A combined foliar application of ethephon (2-chloroethylphosphonic acid) at 0.8 kg/ha and daminozide (butanedioic acid mono (2,2 dimethylhydrazide) at 3.2 kg/ha inhibited the vegetative growth of Black Valentine bean (*Phaseolus vulgaris* L.) without the leaf chlorosis and necrosis caused by ethephon alone. This antagonistic interaction was further evaluated by examining the effect of ethephon and daminozide on respiration and lipid synthesis of isolated leaf cells. Ethephon (1.0 mM) promoted $^{14}CO_2$ evolution from cells incubated with ^{14}C-glucose for 14 h by approximately 75%. Characterization of this response with Black Valentine bean mitochondria indicated that the observed stimulation could not be attributed to the existence of a major cyanide insensitive pathway or the possibility of ethephon acting as an uncoupler, which supports the view that ethephon (or ethylene) acts in the cytosol rather than in mitochondria. Daminozide at 30.0 and 60.0 mM inhibited $^{14}CO_2$ evolution of isolated cells by 30 and 70%, respectively. Ethephon in combination with daminozide (1.0 + 60 mM) resulted in a 32% inhibition of respiration. Daminozide (60.0 mM) inhibited the incorporation of ^{14}C-glucose into chloroform-methanol soluble products by 47%, but did not affect the incorporation of ^{14}C-acetate. The results suggest that daminozide may reduce or overcome any stimulatory effect of ethephon on respiration and support an active inhibitory site for daminozide in mitochondria.

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Applications of ethephon[2] in combination with daminozide to red maple stump sprouts resulted in less defoliation, stem dieback, and shoot growth inhibition than ethephon alone (See and Foy 1983). Ethephon and ethylene have been shown to promote color change associated with an accelerated rate of senescence in numerous plant species (Domir and Foy 1976, Dozier and Barden 1971, Herrero and Hall 1960, Puech et al. 1976). The antagonistic interaction of daminozide and ethephon may involve several physiological and biochemical mechanisms. One factor common to the potential mechanisms of action for both compounds which may partially explain this response is the potentially opposing effects that ethephon (ethylene) and daminozide may have on respiration (Dozier and Barden 1971, Halvey et al. 1966, Herrero and Hall 1960, Ismail and Kender 1967). Daminozide has been shown to competitively inhibit the activity of membrane-bound succinate dehydrogenase (See and Foy 1982). As such, CO_2 evolution and incorporation of hexose-derived acetyl residues into chloroform-methanol soluble products might also be reduced (Lehninger 1976). The present investigation was undertaken to confirm (under greenhouse conditions) observations made in the field and to determine the effect of ethephon and daminozide on the respiration and incorporation of respiratory products into lipids by isolated leaf cells.

Materials and Methods

Plant Material

Bean (*Phaseolus vulgaris* L. Black Valentine) plants were grown under greenhouse conditions in a mixture of weblite (a commercially available expanded clay), peat, and vermiculite in a 2:1:1 (v/v/v) ratio. Natural light was supplemented with 14 h of light provided by 400 w high pressure sodium lamps (Lucalox, General Electric) at approximately 120 $\mu E/m^2/sec$. Minimum greenhouse temperature was maintained at 20°C.

Growth

Foliar applications of (commercial) daminozide (85% ai) and ethephon (21.3% ai) were made to 10-day-old bean seedlings. At the time of treatment, the length of the longest leaflet of the second trifoliolate leaf was less than 1 cm. Treatment rates were 3.2 kg/ha of daminozide and 0.8 kg/ha of ethephon, applied alone and in combination. Applications were made on a 0.2809 m^2 grid with a DeVilbiss atomizer. Spray volume was equivalent to 748 L/ha. Water containing 0.2% (v/v) oxysorbic 20 (Tween-20) was the diluent. The plants were harvested 10 days following treatment. Dry weights were obtained after drying the plant material in a forced draft oven for 48 h at approximately 38°C. Treatments were replicated 6 times in a randomized block design.

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[2] Abbreviations or chemical names for the principal compounds examined are: ethephon (2-chloroethyl)phosphonic acid; daminozide (formerly SADH, B995, B9, and aminozide), butanedioic acid mono (2,2-dimethylhydrazide) (formerly succinic acid-2,2-dimethylhydrazide); oxysorbic 20 (Tween 20), nonionic surfactant containing polyoxyethylene sorbitan monolaurate as the primary active ingredient.

Carbohydrate and Ether Extract

Five-day-old bean seedlings were treated with daminozide and ethephon as previously described. At the time of treatment the average length, width, and fresh weight of the primary leaves were 4.9 cm, 4.6 cm, and 0.39 g, respectively. The longest leaflet of the first trifoliolate leaf was less than 1 cm in length. Seven days following treatment, the leaves were excised, freeze-dried for 24 h and ground to pass a 20 mesh screen. Total nonstructural carbohydrates were determined by the methods of Wolf and Elmore (1973, 1975). Ether extract was obtained following a 12-h soxhlet extraction of 0.5 g of tissue.

Cell Isolation

The procedures used to isolate cells from the primary leaves of 5-day-old bean seedlings and subsequent assay methods were similar to those described by Ashton et al. (1977) and DeVilliers and Ashton (1977). Cells were isolated by vacuum infiltrating 4 g of leaf tissue for 10 min in 30 ml of a macerating medium consisting of 0.7 M mannitol, 0.5% Macerase/TM (Calbiochem), and 0.3% potassium dextran sulfate adjusted to pH 5.8. The tissue was incubated for an additional 10 min, filtered on a 50 μm nylon net and incubated for 30 min with continuous stirring in 40 ml of fresh maceration medium. The tissue was filtered again and incubated for an additional 30 min in 30 ml of fresh maceration medium. Cells obtained from the second and third incubations were collected by centrifuging the filtered solution at 160 g for 3 min. The cells were washed by resuspension in 10 ml of assay medium, recentrifuged at 160 g for 3 min, and resuspended in assay medium. The assay medium consisted of 0.6 M mannitol, 0.05 M MES, 7.5 mM $KHCO_3$, 0.01 μM $CuSO_4$, 1.0 μM $MgSO_4$, 0.1 mM $CaCl_2$, 0.5 mM KCL, 0.1 mM KH_2PO_4, and 2.5 mM KNO_3 adjusted to pH 5.8. The cell preparation used in this investigation contained approximately 0.04 mg Chl/ml.

Assays were conducted with 0.1 ml of the cell preparation dispensed in 10-ml glass tubes containing 0.9 ml of previously mixed assay medium and appropriate radioactive substrate. With the exception of exposures to ethylene gas, each tube also contained 0.1 ml of an appropriate treatment solution. The tubes were stoppered and placed in a shaking water bath maintained at 25°C. Approximately 10 μE/m²/sec of light was provided by overhead fluorescent lamps. In routine assays, the effect of the treatments described below on the metabolic processes studied in this investigation were determined following a 14-h incubation period.

Treatments

Isolated cells were exposed to ethylene, technical ethephon (98%), and daminozide (99%). All treatment solutions were made with assay medium and adjusted to pH 5.8 with KOH. Exposures to ethylene were accomplished by injection into the gas phase of tubes prepared as previously described and stoppered with serum caps. An untreated control and a control containing an

ethylene trap were included in all experiments. Tubes containing the ethylene trap were prepared by placing a 3-ml vial containing 2 ml of mercuric perchlorate and a wick (0.5 × 2.0 cm strip of filter paper) with appropriate tubes.

Respiration

The effect of the various treatments on the respiration of isolated cells was determined by absorbing the $^{14}CO_2$ released from 0.23 µCi of D-(1,5,6-^{14}C) glucose on paper discs previously soaked in 3.0 M KOH. Dark conditions were obtained by covering each tube with aluminum foil. Following the incubation period, radioactivity was determined by placing the discs in counting vials, adding scintillation fluid, and detecting in a Beckman 250 liquid scintillation counter.

Lipid Synthesis

Treatment effects on lipid synthesis were determined by measuring the incorporation of 0.10 µCi of (1,2-^{14}C) sodium acetate and 0.23 µCi of D-(1,5,6-^{14}C) glucose into chloroform-methanol (2:1, v/v) soluble products. Following the incubation period, 2 ml of cold 10% trichloroacetic acid were added to each tube. The tubes were centrifuged at 2000 g for 10 min, and the supernatant was discarded. The pellet was mixed with 4 ml of chloroform-methanol (2:1, v/v) and maintained under room conditions for a minimum of 15 h. The chloroform-methanol mixture was washed twice with 2-ml volumes of distilled water and filtered through Whatman GF/C glass filter discs. Each tube and filter disc was rinsed with 4 ml of chloroform-methanol (2:1, v/v). The filtered solutions were collected in scintillation vials and allowed to evaporate until dry. Radioactivity was determined as previously described. The activity of each process was calculated as cpm/mg Chl. Average counting efficiency (±one standard deviation) for all experiments was 90.8 ± 1.6%. Data presented are the average values of 6 replications obtained from separate experiments, 3 replications each.

Mitochondria

The procedures used to isolate mitochondria from hypocotyl sections of 5-day-old Black Valentine bean plants and characterization of the preparation with regard to oxygen consumption and respiratory control were as previously described (See and Foy 1982). Determinations of mitochondria sensitivity to KCN were made during state 3 respiration at saturating concentrations of succinate, malate, or NADH. ADP concentration at final volume (2 ml) was 150 µM. DNP was used as an uncoupling standard. Treatment solutions of ethephon (98%) were made with reaction medium, adjusted to pH 7.5 and used immediately.

Table 1. Effect of foliar applications of ethephon and daminozide, alone and in combination, on the growth, carbohydrate content, and amount of other extractable materials of Black Valentine bean plants.[a]

Treatment	Rate (kg/ha)	% Inhibition[b] Dry wt	ht	Reducing sugars	Non-reducing sugars	Starch	Ether extract
				Total nonstructural carbohydrates[c]			
				(% dry wt)			
Control	0.0	—	—	3.1a	6.3a	6.6a	2.6a
Ethephon	0.8	51.6b	94.3b	2.9a	4.1c	8.1b	2.3a
Daminozide	3.2	21.6a	87.3a	3.5a	5.2b	6.8a	2.6a
Daminozide + ethephon	3.2 + 0.8	55.1b	99.4b	3.3a	4.2c	5.5c	2.8a

[a] Means in a column followed by a common letter are not significantly different at the 5% level according to Duncan's Multiple Range Test.
[b] Average dry weight and height values for control were 6.0 g and 49.9 cm, respectively.
[c] Data for growth (dry weight and height) and chemical composition (carbohydrates and ether extract) were obtained from separate experiments as described under **Materials and Methods.**

Results and Discussion

Greenhouse Studies

Both ethephon and daminozide were effective in reducing the growth of Black Valentine bean plants (Table 1). Ethephon caused a 30% greater reduction in dry weight than daminozide, but also caused 100% abscission of terminal and auxillary buds as well as interveinal chlorosis of treated leaves. Daminozide inhibited growth (height) without visual symptoms of injury. All plants treated with the combination of ethephon and daminozide were without the chlorosis caused by ethephon alone, but addition of daminozide to the treatment solution did not reduce bud abscission.

At the level of detection provided by the methods used in this investigation, ethephon and daminozide alone or in combination did not cause appreciable changes in the reducing sugar content or amount of ether extractable materials of treated leaves (Table 1). Applications of ethephon resulted in a slight decrease in nonreducing sugars and an increase in starch content. Daminozide caused a similar reduction in nonreducing sugars but did not appear to alter starch content. Ethephon in combination with daminozide resulted in a lowered content of nonreducing sugars similar to that caused by ethephon alone, but also reduced starch content.

Respiration and Lipid Synthesis

The pattern of $^{14}CO_2$ evolution by cells exposed to ethylene was not different from that observed for untreated controls. The ineffectiveness of ethylene in eliciting a response might be attributed to an inability of cells isolated from

Table 2. Effect of ethephon and daminozide on the $^{14}CO_2$ evolution of cells isolated from the primary leaves of 5-day-old Black Valentine bean plants.[a]

Treatment	Concentration (mM)	$^{14}CO_2$ evolved (cpm/mg Chl $\times$ 10^{-5})[b]	% Inhibition	% Stimulation
Control	0.0	25.20c	—	—
Control + trap	0.0	24.88c	—	—
Ethephon	1.0	44.08a	—	74.9
Ethephon	10.0	25.36bc	—	—
Ethephon	30.0	10.22e	59.4	—
Daminozide	10.0	27.68b	—	9.8
Daminozide	30.0	17.74d	29.6	—
Daminozide	60.0	7.18f	71.5	—
Ethephon + daminozide	1.0 + 60.0	17.19d	31.8	—

[a] Assay system as described under **Materials and Methods.**
[b] Means in a column followed by a common letter are not significantly different at the 5% level according to Duncan's Multiple Range Test.

vegetative tissue to respond to ethylene, or an insufficient ethylene concentration at the active site(s) of the isolated cells used in this investigation.

Ethephon at 1.0 mM stimulated $^{14}CO_2$ evolution from isolated cells by approximately 75% (Table 2). Increasing the concentration to 30 mM inhibited $^{14}CO_2$ evolution by 59%. Addition of daminozide (60 mM) to the incubation medium in combination with ethephon (1.0 mM) completely overcame the stimulatory effect of ethephon alone and resulted in a net inhibition of approximately 32%. These data suggest that, although the observed responses show a strong concentration dependency, daminozide may reduce or overcome any stimulatory effects of ethephon on respiration. This evidence is in agreement with investigations conducted with intact plant parts and supports the potentially opposing effect that ethephon and daminozide may have on respiration (Edgerton and Blanpeid 1970, Looney 1971).

Potential sites for respiratory stimulation by ethephon (ethylene) were further evaluated with Black Valentine bean mitochondria. The effects of 50 µM KCN on succinate, malate, and NADH dependent state-3 respirations are given in Table 3. These values were in close agreement with similar data obtained with mung bean (*Phaseolus aureus* Roxb.) mitochondria (Wilson and Bonner 1970). Black Valentine bean mitochondria are often considered to be intermediate in cyanide sensitivity between potato (*Solanum tuberosum* L.) and skunk cabbage (*Symplocarpus foetidus* L.) mitochondria (Bendall and Bonner 1971). Although the relative differences of KCN-induced inhibition between succinate- and malate-dependent respiration may suggest some degree of cyanide insensitivity, the difference might also be attributed to flux differences between the respective dehydrogenases (Solomos 1977). The nearly complete inhibition of NADH oxidation by mitochondria that appear to elicit a slight insensitivity to KCN may be due to poor electron flow from NADH to ubiquinone or low availability of ubiquinone to the alternate oxidase once reduced by exogenous NADH (Solomos 1977). Although it is difficult to assess any potential mitochondrial resistance to KCN that may be lost during isolation,

Table 3. Effect of KCN on the rate of oxygen consumption by Black Valentine bean mitochondria supplied with succinate, malate or NADH.[a]

Substrate	Rate of O_2 uptake (state 3) μmoles/mg protein/h		% Inhibition
	Control	KCN (50 μM)	
Succinate (10 mM)	5.59	1.86	67
	(0.12)[b]	(0.11)	
Malate (30 mM)	5.34	3.34	40
	(0.26)	(0.12)	
NADH (0.7 mM)	6.85	0.70	89
	(0.27)	(0.07)	

[a] Experimental conditions were as described under **Materials and Methods.**
[b] Values within parentheses are standard deviations.

the relatively high degree of inhibition caused by 50 μM KCN in this investigation precludes the existence of a major cyanide insensitive pathway to explain the stimulatory effect of ethephon (or ethylene) on the respiration of isolated cells.

Addition of DNP to mitochondria immediately after initiation of state-3 respiration by addition of ADP resulted in approximately 50% inhibition of oxygen uptake (Table 4). Adding DNP following utilization of ADP in state-3 and reversion to state-4 respiration stimulated oxygen uptake by approximately 66%. This unexpected response of Black Valentine bean mitochondria in state-3 respiration (inhibition instead of stimulation) has been previously reported for cauliflower (*Brassica oleracea* L.) and potato mitochondria (Jung and Hanson 1975, Laties 1973). The inability of DNP to stimulate respiration until after nearly complete utilization of ADP in the first state 3 has been attributed to

Table 4. Effect of 2,4-dinitrophenol (DNP) and ethephon on succinate dependent (10 mM) oxygen consumption and respiratory control of Black Valentine bean mitochondria.[a]

Respiratory state	Treatment	Oxygen uptake μmoles/mg protein/h	Respiratory control	% Inhibition	% Stimulation
3	Control	5.52 (0.11)[b]		—	—
4		2.34 (0.16)	2.32	—	—
3	+ DNP (0.5 mM)[c]	2.69 (0.12)		50.4	—
4	+ DNP (0.5 mM)	3.89 (0.23)		—	66.2
3	+ ethephon (1.0 mM)	5.41 (0.18)		—	—
4		2.42 (0.02)	2.23	—	—
4	+ ethephon (1.0 mM)	2.38 (0.05)		—	—

[a] Assay system as described under **Materials and Methods.**
[b] Values within parentheses are standard deviations.
[c] DNP and ethephon were added at the initiation of the first state 3. ADP concentration at final volume (2 ml) was 150 μM. Additions of DNP and ethephon to state 4 were made at approximately 1 minute after establishment of this condition.

Table 5. Effect of ethephon and daminozide on the incorporation of ^{14}C-glucose into lipids by cells isolated from the primary leaves of 5-day-old Black Valentine bean plants.[a]

Treatment	Concentration (mM)	Incorporation of ^{14}C-glucose into lipids (cpm/mg Chl $\times$ 10^{-5})[b]	% Inhibition
Control	0.0	20.1a	—
Ethephon	1.0	21.6a	—
Daminozide	60.0	10.5b	47.7
Ethephon + daminozide	1.0 + 60.0	15.1c	24.9

[a] Assay system as described under **Materials and Methods.**
[b] Means in a column followed by a common letter are not significantly different at the 5% level according to Duncan's Multiple Range Test.

initially low levels of endogenous ADP after isolation or somewhat leaky membranes and an associated inability to maintain an appropriate membrane charge gradient (Jung and Hanson 1975, Laties 1973).

Ethephon (1.0 mM) did not appear to alter oxygen consumption or respiratory control of Black Valentine beam mitochondria (Table 4). This lack of mitochondrial response to ethephon (or ethylene) is in support of published data (Ku and Leopold 1970). Collectively, these data support the view that ethephon (ethylene) acts in the cytosol and not in mitochondria.

Daminozide inhibited the incorporation of ^{14}C-glucose by isolated cells into chloroform-methanol soluble products by approximately 50% (Tables 5 and 6). Addition of ethephon to the incubation medium in combination with daminozide partially alleviated the inhibitory effect of daminozide. As shown in Table 6, daminozide did not cause a significant reduction in the amount of ^{14}C-acetate incorporated into the chloroform-methanol extract. Assuming that the depressed activity is a result of limited availability of ^{14}C-acetate derived from ^{14}C-glucose, these data support an active inhibitory site for daminozide in mitochondria.

Table 6. Effect of ethephon and daminozide on the incorporation of ^{14}C-acetate into lipids by cells isolated from the primary leaves of 5-day-old Black Valentine bean plants.[a]

Treatment	Concentration (mM)	Incorporation of ^{14}C-acetate into lipids (cpm/mg Chl $\times$ 10^{-5})[b]
Control	0.0	42.5ab
Ethephon	1.0	45.4a
Daminozide	60.0	38.6b
Ethephon + daminozide	1.0 + 60.0	39.9ab

[a] Assay system as described under **Materials and Methods.**
[b] Means in a column followed by a common letter are not significantly different at the 5% level according to Duncan's Multiple Range Test.

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