The Effect of Benzimidazole on Some Membrane Properties Of Ozonated Pinto Bean

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ABSTRACT

The mechanism of benzimidazole-induced resistance against ozone damage was investigated using bean (cultivar Pinto 111) plants grown under controlled conditions. Water flux, water potential, electrolyte leakage, and osmotic potential measurements were made immediately and 48 hours after ozonation. Benzimidazole treatment alone increased water efflux of plants, water influx of ozonated plants, and appeared to increase the calculated hydraulic conductivity for these fluxes. Benzimidazole also decreased electrolyte leakage and had no effect on osmotic potential or water potential. Benzimidazole prevented the immediate ozone-induced electrolyte leakage, increased water potential, and decreased osmotic potential. It is proposed that benzimidazole changes some structural component of plant cell membranes.

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Additional key words: electrolyte leakage, osmotic potential, water flux, water potential.

The effect of ozone on plant membranes resulting in subsequent changes in ion flux and water relations has been observed by several researchers. Sublethal ozone concentrations caused a significant decrease in permeability of potato tuber disks and tobacco leaves to phosphate and rubidium ions (17). Ozonation of pinto beans with 0.5 μliters/liter for 0.5 to 1 hour resulted in a decrease in hydraulic conductivity and the water diffusional coefficient (8). Treatment of isolated pea chloroplasts with 50 μliters/liter ozone for 5 minutes reduced the reflection coefficient of meso-erythritol and glycerol, indicating a marked increase in permeability of the chloroplast limiting membrane (20). Ozonation of tobacco with 0.30 μliters/liter for 2 hours induced shrinkage of chloroplasts which was interpreted as dehydration due to an ozone-induced membrane permeability change (28). Thus, ozone altered the membrane permeability of several plant species under various exposure conditions; and a difference in effect between sublethal ozone levels and toxic levels was observed.

Benomyl [methyl-1-(butycarbamoyl)-2-benzimidazole carbamate], a systemic fungicide, is effective in reducing ozone damage to pinto bean (21) and tobacco (18, 29). In plants, benomyl rapidly breaks down to methyl-2-benzimidazole carbamate (3, 24). The benzimidazole moiety of the benomyl molecule is reported to be responsible for the reduction in ozone damage to pinto bean (22, 27, 30) and spinach (30).

It is known that benzimidazole (BZI) stimulates chlorophyll formation (31), increases the number of intergrana and grana chloroplast lamellae (32), and conserves chlorophyll and proteins (23). BZI increased the NADP and ATP content of senescing wheat leaves (19), and it was suggested that lamellar membrane permeability was decreased.

The effects of BZI on ion flux and water relations which may be related to alteration of plant membranes have been studied. BZI increased K^+, Na^+, and Ca^{2+} uptake in excised barley roots and K^+ uptake in intact barley roots (15). It was effective in decreasing the loss of previously absorbed K^+ when roots were subjected to low pH. A similar effect of BZI on K^+ uptake in tobacco was observed (7). BZI inhibited the auxin-induced elongation of etiolated pea epicotyl sections while simultaneously stimulating water uptake (9). However, no effect on water potential was detected.

In order to elucidate the mechanisms by which BZI protects plants from ozone and, more basically, to develop a better understanding of the mechanisms by which ozone damages plants, experiments were conducted concerning the effects of BZI on the water relations and electrolyte leakage of plants exposed to selected ozone concentrations which produce visible damage ranging from slight to severe.

MATERIALS AND METHODS.—*Phaseolus vulgaris* L. 'Pinto 111' seeds were planted in vermiculite moistened with one-fourth strength nutrient solution (6) and germinated under controlled conditions (day; 12 hours, 25 ± 2 C, 59 ± 4% relative humidity, fluorescent-incandescent light energy with wavelengths between 400 nm and 700 nm of 160 microeinsteins/m^2/sec, and light intensity of 10,700 lux; night; 16 ± 2 C, 73 ± 4% relative humidity). One week after planting, two seedlings were transferred to each 10-cm diameter plastic pot containing 400 ml of aerated full-strength nutrient solution. When the plants were 10 days old, one-half of the 24 pots in each experiment received 2.5 mg of BZI in the nutrient solution. The treated and control pots were randomly distributed in the growth chamber.

Two weeks after planting, six pots each of BZI-treated and control plants were exposed to ozone for 4 hours between 1000 hours and 1400 hours in a modified growth chamber at 22.2 ± 0.5 C, 68 ± 1% relative humidity, 300 microeinsteins/m^2/sec fluorescent-incandescent light, and light intensity of 21,000 lux while the remaining six pots each of BZI-treated and control plants were maintained under identical conditions in carbon-filtered air. Plants were exposed to 0.06 ± 0.01, 0.12 ± 0.02, or 0.25 ± 0.02 μliters/liter ozone as indicated in the separate experiments. Ozone concentration was monitored with a Mast oxidant meter calibrated by the 1% neutral buffered KI method (14).

Electrolyte leakage, water uptake (F_i), water loss (F_e), water potential (ψ_w), and osmotic potential (ψ_s), of the bean leaves were determined from separate ozonations immediately and 48 hours after ozonation. Data were subjected to analysis of variance with a completely random experimental design and a factorial treatment design using ozone, BZI, and time of analysis as factors.

Electrolyte leakage.—One-and-one-half grams of 10.5-mm diameter disks free of major veins from four primary leaves were rinsed and placed in 20 ml of glass-distilled water. Nitrogen was continuously bubbled through the water to provide mixing. Conductance (mmhos/cm at 25 C) of the solution was measured 15, 30, 60, 120, and 180 minutes after treatment with a Beckman RD 15 Solubridge. Distilled water was added prior to each measurement to replace evaporative losses. The samples were frozen overnight at −20 C, thawed, and final conductance measurements were taken. All conductance values were expressed as percentages of the final values, which represented complete membrane disruption and maximum leakage (12).

Osmotic potential.—Primary bean leaves were frozen at −20 C, thawed, and the sap expressed with a mortar and pestle. Osmotic potential of the cell sap from four leaves was measured cryoscopically with a Fiske osmometer.

Water fluxes.—The rates of water uptake (F_i) and water loss (F_e) were determined by a method similar to that used by Hancock (12). Five disks (10.5-mm diameter) were removed at random from interveinal areas of four primary leaves (two plants), rinsed, and equilibrated in glass-distilled water for 15 minutes (F_i) or 45 minutes (F_e). For the F_i determinations the blotted disks were weighed (W_i) and incubated in 0.44 M mannitol solution for 40 minutes. After plasmolysis, disks were reweighed (W_o) and placed in distilled water. Weights (W_t) were taken at 20 minute intervals for 1 hour. Water uptake (F_i) was expressed as a percentage of the initial weight.

$$F_i = \frac{W_t - W_o}{W_i} \times 100.$$

The blotted and tared disks (W_i) used to determine F_e

were weighed after 20, 40, and 74 minutes in the 0.44 M mannitol solution. Water loss was similarly calculated as:

$$F_e = \frac{W_i - W_t}{W_i} \times 100.$$

Water potential.—Bean stems were cut between the cotyledons and primary leaves, and ψ_w of the upper portion was determined by the pressure bomb technique (2). Pressure was applied to the chamber with nitrogen until the xylem sap appeared at the cut petiole surface projecting from the chamber.

Hydraulic conductivity.—Relative hydraulic conductivity (L_p) values were calculated from the following equation:

$$L_p = \frac{J_v}{\Delta\psi_w}$$

where J_v represents water flux in cm^3 per cm^2 of leaf disk surface per sec, and $\Delta\psi_w$ represents the water potential difference in atmospheres between leaf tissue and 0.44 M mannitol solution.

L_p calculations for influx were based on the rate of water gain (F_i) during the first 20 minutes of the water uptake period. L_p calculations for efflux were based on the rate of water loss (F_e) during the 74-minute efflux period. The time intervals for L_p determination were selected to avoid saturation effects.

RESULTS.—Bean plants exposed to 0.12 and 0.25 μliters/liter ozone were not visibly damaged at the end of the 4 hr exposure; but in the absence of BZI, small necrotic flecks developed on the adaxial surface of primary leaves by 48 hours. Ozone at 0.06 μliters/liter did not cause visible symptoms. Ozonated plants pretreated with BZI never developed ozone toxicity symptoms. The percent leaf damage is indicated in the tables for the separate experiments. Because ozone sensitivity exhibits seasonal variation (13), ozone concentration used in the ψ_w and ψ_s experiments caused different amounts of damage than similar ozone levels used earlier in the electrolyte leakage and water flux studies.

The electrolyte leakage of plants exposed to 0.25 μliters/liter ozone and not treated with BZI (−BZI) was significantly greater than leakage from nonozonated plants when measured immediately after ozonation

TABLE 1. Visible ozone damage to attached leaves and the effect of ozone concentration, benzimidazole concentration, and time following ozonation on electrolyte leakage from pinto bean leaf disks

Ozone concentration (μ liters/liter)	Average electrolyte leakage[a] from leaf disks (% of total)				Visible leaf damage[b] (%)
	0 hours after ozonation		48 hours after ozonation		
	Benzimidazole concentration (mg/liter)				
	0	6.25	0	6.25	
0	18.4 ± 1.10[c]	16.0 ± 1.37	14.3 ± 0.82	13.1 ± 0.84	0
0.12	19.5 ± 2.61	17.7 ± 0.46	11.6 ± 0.31	13.4 ± 0.68	5
0.25	22.9 ± 1.20	16.5 ± 0.63	13.1 ± 0.73	10.8 ± 0.54	16

[a]Electrolyte leakage (mmhos/cm at 25 C) from 1.5 g of 10.5-mm-diameter leaf disks after 180 minutes of extraction in 20 ml of nitrogen-bubbled distilled water, expressed as a percentage of the maximum conductivity of the same sample frozen overnight at −20 C, thawed, and similarly extracted the following day.

[b]Values represent the mean percent of primary leaf affected by adaxial fleck, 48 hours after ozonation, estimated in increments of 5 percent, of 16 replications of plants not treated with benzimidazole.

[c]Average and standard error of the mean of four replications of the indicated treatment combinations.

TABLE 2. Visible ozone damage to attached leaves and the effect of ozone concentration, benzimidazole concentration, and time following ozonation on osmotic potential (ψ_s) of sap expressed from primary pinto bean leaves

Ozone concentration (μ liters/liter)	Average osmotic potential[a] of leaf sap (bars)				Visible leaf damage[b] (%)
	0 hours after ozonation		48 hours after ozonation		
	Benzimidazole concentration (mg/liter)				
	0	6.25	0	6.25	
0	−7.8 ± 0.15[c]	−7.8 ± 0.29	−8.4 ± 0.26	−7.9 ± 0.17	0
0.25	−9.2 ± 0.46	−7.7 ± 0.17	−8.9 ± 0.37	−8.6 ± 0.12	40

[a]Osmotic potential (bars) of sap expressed from primary pinto bean leaves following freezing at −20 C and measured cryoscopically.

[b]Values represent the mean percent of primary leaf affected by adaxial fleck 48 hours after ozonation, estimated in increments of 5 percent, of 12 replications of plants not treated with benzimidazole.

[c]Average and standard error of the mean of three replications of the indicated treatment combinations.

(Table 1). BZI pretreatment prevented this ozone-induced increase in electrolyte leakage and caused a significant overall reduction of electrolyte leakage. The electrolyte leakage of ozonated plants was significantly less 48 hours after ozonation than immediately after ozonation at both BZI levels.

The osmotic potential (ψ_s) of −BZI plants exposed to 0.25 μliters/liter ozone and measured immediately after ozonation was significantly less (more negative) than ψ_s in nonozonated plants (Table 2). BZI pretreatment prevented this ozone-induced decrease in ψ_s, but itself caused no significant overall increase or decrease in ψ_s.

The F_i of −BZI plants exposed to 0.25 μliters/liter ozone and measured immediately after ozonation was significantly less than F_i in nonozonated plants (Table 3). BZI pretreatment prevented this ozone-induced F_i decrease. The F_e was not significantly affected by ozone at any BZI-time combination (Table 3). BZI pretreatment significantly increased the overall F_e of plants and F_i of ozonated plants (Fig. 1 and Table 3).

The ψ_w of −BZI plants exposed to 0.12 and 0.25 μliters/liter ozone and measured immediately after ozonation was significantly greater (less negative) than ψ_w in nonozonated plants (Table 4). BZI pretreatment prevented this immediate ozone-induced increase in ψ_w and caused a significant overall decrease in ψ_w of ozonated plants.

Ozone appeared to cause a decrease in the influx and efflux L_p values of −BZI plants immediately after ozonation (Table 5). The L_p values of +BZI plants appeared greater than those of −BZI plants at all ozone-time combinations. The L_p values for conditions 48 hours after ozonation appeared less than those immediately after ozonation under all combinations except the −BZI, + ozone combination in which the influx L_p appeared less immediately after ozonation.

DISCUSSION.—An increase in electrolyte leakage of ozonated plants not pretreated with BZI was measured

TABLE 3. Visible ozone damage to attached leaves and the effect of ozone concentration, benzimidazole concentration, and time following ozonation on water uptake (F_i) and water loss (F_e) of pinto bean leaf disks

Water uptake[a] or loss[b]	Ozone concentration (μ liters/liter)	Time of analaysis after ozonation (hours): 0 — Benzimidazole concentration (mg/liter): 0	0 — 6.25	48 — 0	48 — 6.25	Visible leaf damage[c] (%)
Uptake	0	18.1 ± 1.84	18.3 ± 0.57	16.5 ± 1.03	16.3 ± 0.32	0
Uptake	0.12	14.2 ± 1.05	21.7 ± 2.14	18.2 ± 2.60	17.6 ± 1.00	5
Uptake	0.25	12.4 ± 0.68	19.7 ± 2.22	17.3 ± 1.30	18.5 ± 1.46	16
Loss	0	17.7 ± 0.89	18.8 ± 0.95	16.5 ± 0.70	17.5 ± 0.99	0
Loss	0.12	15.2 ± 1.51	18.6 ± 0.55	17.5 ± 2.25	18.2 ± 0.90	5
Loss	0.25	14.9 ± 3.09	18.6 ± 1.14	13.3 ± 1.46	15.6 ± 0.87	16

[a]Average and standard error of the mean of four replications of 5, 10.5-mm-diameter leaf disks plasmolyzed 40 minutes in 0.44 M mannitol followed by a 20-minute rehydration in distilled water and expressed as a percent of the initial weight (prior to plasmolysis).

[b]Average and standard error of the mean of four replications of 5, 10.5-mm-diameter leaf disks in which weight loss during a 74-minute dehydration in 0.44 M mannitol is expressed as a percent of the initial weight.

[c]Values represent the mean percent of primary leaf affected by adaxial fleck 48 hours after ozonation, estimated in increments of 5 percent, of 16 replications of plants not treated with benzimidazole.

TABLE 4. Visible ozone damage to attached leaves and the effect of ozone concentration, benzimidazole concentration, and time following ozonation on water potential (ψ_w) of pinto bean shoots

Ozone concentration (μ liters/liter)	Average water potential[a] of bean shoots (bars): 0 hours after ozonation — Benzimidazole concentration (mg/liter): 0	0 — 6.25	48 hours after ozonation — 0	48 — 6.25	Visible leaf damage[b] (%)
0	−5.2 ± 0.12[c]	−5.3 ± 0.08	−5.1 ± 0.11	−4.9 ± 0.11	0
0.06	−5.2 ± 0.27	−5.5 ± 0.30	−4.4 ± 0.21	−4.9 ± 0.16	0
0.12	−4.5 ± 0.23	−5.1 ± 0.12	−5.2 ± 0.22	−5.4 ± 0.31	21
0.25	−4.6 ± 0.38	−5.3 ± 0.20	−5.0 ± 0.17	−5.7 ± 0.14	50

[a]Water potential (bars) of pinto bean shoots cut between the cotyledons and primary leaves and measured by the pressure bomb technique.

[b]Values represent the mean percent of primary leaf affected by adaxial fleck 48 hours after ozonation, estimated in increments of 5 percent, of six replications of plants not treated with benzimidazole.

[c]Average and standard error of the mean of six replications of the indicated treatment combinations.

TABLE 5. Effect of ozone concentration, benzimidazole concentration, and time following ozonation on relative hydraulic conductivity (L_p) of pinto bean primary leaf tissue

Water influx or efflux	Ozone level[b]	Average relative hydraulic conductivity[a]			
		0 hours after ozonation		48 hours after ozonation	
		Benzimidazole concentration (mg/liter)			
		0	6.25	0	6.25
Efflux	0	2.3	2.6	2.0	2.1
Influx	0	8.5	9.2	7.5	8.2
Efflux	+	2.1	2.8	1.9	2.2
Influx	+	6.0	10.2	8.3	9.8

[a] Relative hydraulic conductivity (cm/sec/atm $\times 10^{-7}$) calculated from water flux and water potential values (see equation in text).

[b] L_p calculations were based on a 0.25 μ liters/liter ozone level for water flux and a 0.12 μ liters/liter ozone level for water potential due to similar degrees of ozone damage in these two ozonations done at different times of the year. Because combination of experiments was done, ozone is considered either absent (0) or present (+).

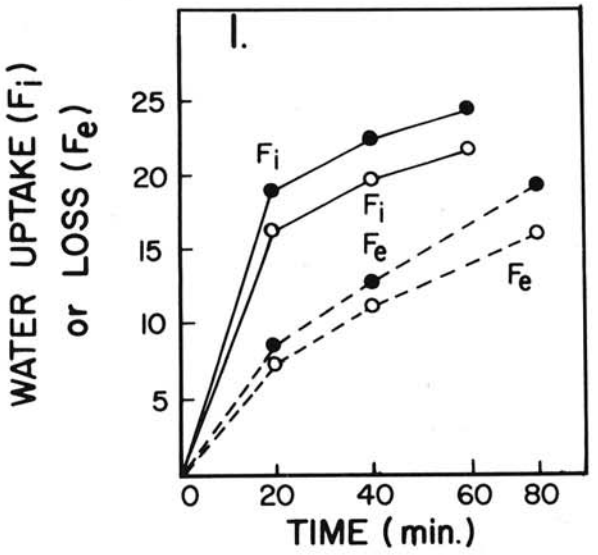

Fig. 1. Effect of benzimidazole (BZI) on water uptake (F_i) and water loss (F_e) of pinto bean leaf disks after various times in distilled water following plasmolysis (F_i), or in mannitol solution following equilibration (F_e). Disks from plants treated (●) and not treated (○) with BZI. Twenty-four determinations were averaged for each point. F_i and F_e are expressed as disk weight change per unit initial weight × 100 (see equations in text). Within both BZI levels, ozone concentration and time of analysis after ozonation were not partitioned but averaged to obtain the primary BZI effect.

immediately after ozonation. The electrolyte leakage of ozonated plants pretreated with BZI did not increase nor decrease. Similarly, BZI offset the ozone-induced increase in ψ_w. A decrease in ψ_s was also observed immediately following ozonation. When the plants were pretreated with BZI, the ψ_s was not changed by ozonation. The less negative ψ_w and more negative ψ_s are probably closely related to the observed electrolyte leakage and reflect ozone-induced membrane damage. Thus, as water is lost by damaged cells, the intracellular solutes become more concentrated (ψ_s decreases); and electrolytes will readily leak from this tissue when it is placed in distilled water. Recovery after 48 hours is apparent except for small groups of cells (necrotic flecks). The effects of BZI on ψ_w and water flux observed herein agree with those reported by Galston et al. (9).

The immediate ozone-induced changes in electrolyte leakage, water flux, ψ_s, and ψ_w were not observed 48 hours after ozonation. These physiological differences between recently ozonated leaves and leaves 2 days after ozonation may be related to a rapid decrease in ozone sensitivity which follows the brief period of maximum sensitivity (5).

Glinka and Reinhold (10) recommended that efflux measurements be made using a hypotonic external solution to establish whether water flux changes could be attributed to changes in L_p or to changes in the reflection coefficient, σ. This determination was based solely on water flux measurements. In the present work this would have restricted the mannitol to about 0.24 molal or less, making the weight changes smaller and less accurate. In addition both ψ_w and water flux were measured herein, and L_p was obtained through calculation. Following the efflux test, disks placed in distilled water weighed approximately 20 percent more than initially (before efflux). This indicated that membrane integrity was not destroyed during plasmolysis.

Recently, Amar and Reinhold (1) described an osmotic shock effect resulting in protein release and loss of membrane transport ability in leaf cells. This effect should be considered when water influx studies using osmotica are performed due to close similarity of the two techniques. In the present study F_i was large relative to F_e. This situation is encountered frequently in water flux studies and may be partially explained by the altered membrane transport caused by osmotic shock.

BZI appeared to increase L_p for both water efflux and influx. However, the calculated L_p values must be regarded as relative. In addition to flux measurement limitations two other assumptions must be used to calculate L_p. First, ψ_w was measured in the shoot system of the entire plant while water fluxes were determined for leaf disks where the ψ_w difference between the cells and the surrounding medium was different from what it was in the intact plant (4). Second, while water flux is theoretically based on flow per unit area of membrane, the above calculations were based on disk surface area.

Because leaf disks often exhibit substantial edge effects (26), L_p was probably underestimated.

In spite of the limitations of the methods used, it is clear that BZI treatment results in significant changes in leaf cell membrane function including increased water flux and L_p, and decreased solute leakage. Also such membranes are less subject to ozone-induced damage. Morphological observations of chloroplasts following BZI treatment support these conclusions. These include a closer spacing of grana and intergrana lamellae in wheat chloroplasts (32) and Elodea chloroplasts (33). Recently, an electron microscope study showed that benomyl affected pinto bean chloroplast structure. It was postulated that benomyl protected the leaf parenchyma plastids from ozone injury by maintaining the cellular membranes and the integrated lamellar structure (25). Functional membrane changes induced by BZI could be related to changes in the lipoprotein complex and the ice-like or polarized structure of the membrane water (16). In any case quantitative and/or qualitative biochemical changes in membrane components must be involved.

Several biochemical effects of BZI have been reported. These include an increase in the NADP/NAD ratio (11), an increase in the NADP and ATP levels in leaves (19), and the conservation of leaf protein and chlorophyll (23). Of more interest with regard to membrane function is the recent report that BZI protects against an ozone induced total free sterol decrease in bean leaves (30). Recent work in this laboratory concerns the individual sterol levels of bean leaves and the influence of BZI and ozone on these levels (27).

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