# The Effect of Benzimidazole, Cholesterol, and a Steroid Inhibitor on Leaf Sterols and Ozone Resistance of Bean

R. A. Spotts, F. L. Lukezic, and N. L. Lacasse

Former Graduate Assistant, Associate Professor, and Former Associate Professor, respectively, Department of Plant Pathology, The Pennsylvania State University, University Park 16802. Present address of senior author, Department of Plant Pathology, Ohio Agricultural Research and Development Center, Wooster, Ohio 44691.

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ABSTRACT

Experiments were performed to measure the effect of ozone and benzimidazole on the cholesterol, campesterol, stigmasterol, and β-sitosterol content of pinto bean leaves and to determine if ozone resistance in bean could be correlated with the level of individual sterols. Ozone at 0.25 μliters/liter caused a decrease in the concentration of cholesterol but not campesterol, stigmasterol, or β-sitosterol. Pretreatment with benzimidazole did not prevent the cholesterol decrease. Benzimidazole did increase the cholesterol content of nonozonated plants and of ozonated plants 48 hours after exposure. Pretreatment with cholesterol protected plants against ozone damage. Pretreatment with a steroid inhibitor increased the susceptibility of plants to ozone. This evidence suggests that cholesterol may be a factor in ozone resistance of bean.

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*Additional key words:* campesterol, stigmasterol, β-sitosterol.

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Ozone, a major phytotoxic air pollutant, has been shown to cause several physiological changes in plants which are closely related to altered membrane function. Ozonation caused a decrease in the reflection coefficient of bean leaves (4), shrinkage of chloroplasts in tobacco leaves (13) and reduction of the reflection coefficient of mesoerythritol and glycerol in isolated pea chloroplasts (10).

An understanding of the function of sterols in plant cell membranes is of fundamental importance to those concerned with membrane permeability alteration. Sterols have been reported to be more effective than $CaCl_2$, a classic membrane stabilizer, in inhibiting methanol-induced leakage of betacyanin from beet root disks (5). Cholesterol was more effective than stigmasterol or β-sitosterol in reducing the leakage. However, sterols had no effect on hydrogen peroxide-induced leakage of betacyanin. Cholesterol and campesterol reduced the ethanol-induced electrolyte leakage of barley roots (6); however, campesterol was less effective than cholesterol. Stigmasterol, β-sitosterol, cholesteryl glucoside, and cholesteryl palmitate did not influence leakage. Treatment with 10 μM cholesterol was most effective in reducing electrolyte leakage, while 100 μM cholesterol stimulated leakage.

Ozone exposure has been shown to cause a decrease in total free sterol and an increase in sterol glycoside content in pinto bean leaves (14) and in the leaves and extracted chloroplasts of bean and spinach (15). Treatment with benzimidazole, at levels that protected plants from ozone damage, prevented this ozone-induced decrease in free sterol. Benzimidazole has also been shown to prevent an ozone-induced increase in water potential, increase in electrolyte leakage, and decrease in osmotic potential in pinto bean (11).

To learn more about the nature of ozone toxicity and benzimidazole-induced resistance to ozone, experiments were performed to measure ozone and benzimidazole-induced changes in the cholesterol, campesterol, stigmasterol, and β-sitosterol content of bean leaves. Experiments were also conducted to determine if ozone resistance could be correlated with the level of individual sterols in bean leaves.

MATERIALS AND METHODS.—*Phaseolus vulgaris* L. 'Pinto 111' seeds were germinated in vermiculite and grown hydroponically under controlled conditions as described previously (11).

*Chemical treatments.*—1) Benzimidazole.—12 pots (two plants/pot) each received 2.5 mg benzimidazole in 400 ml nutrient solution 72 hours prior to ozonation. Twenty-four nontreated plants were maintained as controls.

2) Cholesterol.—8 pots (two plants/pot) containing 400 ml nutrient solution received 10.3 or 103.0 mg cholesterol per pot as an aqueous suspension 72 hours before ozonation. Nontreated plants were maintained as controls.

3) Steroid inhibitor.—SK & F 7997-$A_3$ [tris-(2-diethylaminoethyl)-phosphate-trihydrochloride] (Smith, Kline, and French Laboratories, Philadelphia, Pa.) was dissolved in distilled water and adjusted to pH 7.2 with 1.0M NaOH. Twenty-four hours prior to ozonation, the leaves of 12 plants were sprayed on the abaxial and adaxial surfaces until runoff with 0, 2, 20, or 200 $\mu$g per ml of freshly prepared solution.

*Ozonation.*—Two weeks after planting, 12 benzimidazole treated and 12 control plants were exposed to $0.06 \pm 0.01$, $0.12 \pm 0.02$, or $.25 \pm 0.02$ $\mu$liters/liter ozone for 4 hours in separate fumigations under conditions previously described (11). Twenty-four cholesterol or steroid inhibitor treated plants and their controls were exposed to $0.25 \pm 0.02$ $\mu$liters/liter ozone for 4 hours. Twenty-four additional plants with and without chemical treatments in the benzimidazole (BZI), cholesterol, and steroid inhibitor experiments were maintained in carbon-filtered air under identical conditions. Ozone concentration was monitored with a Mast oxidant meter calibrated by the 1.0% neutral buffered KI method (8).

Immediately after ozonation the primary leaves of 12 ozonated and 12 nonozonated plants in the BZI experiment and all 24 nonozonated plants in the cholesterol and the steroid inhibitor experiments were collected and maintained at −20 C until analysis. Forty-eight hours after ozonation the remaining 24 plants in the BZI, cholesterol, and steroid inhibitor experiments were visually assessed for ozone damage to primary leaves and then harvested and stored at −20 C. Damage was assessed on a continuous scale from 0 to 100 with 0 representing no visible damage and 100 representing complete necrosis.

*Sterol analysis.*—The cholesterol, campesterol, stigmasterol, and $\beta$-sitosterol content of leaf tissue was determined by a modified method of Bae and Mercer (1). Four frozen leaves were ground with a mortar and pestle, extracted with 20 ml ethyl acetate, and filtered through a sintered glass funnel. The residue was re-extracted twice with 20 ml boiling ethanol, and twice with 20 ml ethyl acetate. In preliminary experiments, no sterols were found in additional extracts of the residue. The extracts were combined and reduced to dryness in a rotary evaporator at 55 C. The residue was dissolved in 40 ml of a petroleum ether (b.p. 30-60 C): ethanol mixture (2:1, v/v); 20 ml distilled water was added, and the sterol glycosides were partitioned into the aqueous phase. The aqueous phase was extracted once with 20 ml of the 2:1 petroleum ether:ethanol mixture. The combined ether extracts were dried and chromatographed on a pressurized (60 mm Hg) column of 10 g neutral alumina (water content adjusted to 5.5%) using 100-ml volumes of 1%, 6%, and 20% ether in petroleum ether (v/v) for development. Free sterols were eluted in the 20% fraction, steryl esters in the 1% fraction.

The free sterol fraction was reduced to dryness, redissolved in 0.1 ml ethyl acetate; and 5 $\mu$l was subjected to gas-liquid chromatography using a Varian Aerograph 1520B with a flame ionization detector. A 243 cm, 3.5 mm (i.d.) glass column packed with 3% OV-17 on 149/131 $\mu$m (100/120-mesh) Gas Chrom Q (Applied Science, State College, Pa.) was used. Operating temperatures were 268, 295, and 300 C for the column oven, injector block, and detector oven, respectively. Helium was used as the carrier gas at a flow rate of 70 cc/min. On-column injection was used. Standard mixtures of cholesterol, campesterol, stigmasterol, and $\beta$-sitosterol containing 1, 3, 5, 10, 20, and 30 $\mu$g of each sterol were chromatographed daily in order to quantify extracted sterols.

The above extraction and analytical procedure proved 95% efficient when tested with a mixture of pure sterols of known concentration. A preliminary study showed that ozone, BZI, and cholesterol had no effect on the fresh weight/dry weight ratio of bean leaves, and results were thus reported as micrograms free sterol per gram fresh weight of leaf tissue. Data from the BZI experiment were subjected to analysis of variance with a random experimental design and a factorial treatment design using time of analysis, ozone, and BZI as factors. Data from the cholesterol and inhibitor experiments were subjected to Duncan's new multiple range test mean separation procedures (12).

RESULTS.—*Benzimidazole experiment.*—Visible damage was not observed on plants exposed to 0.06, 0.12 or 0.25 $\mu$liters/liter ozone immediately after ozonation, but in the absence of BZI, necrotic flecks developed on primary leaves after 48 hours at the two higher ozone concentrations. Average leaf damage due to ozone exposure was 21% and 50% for 0.12 and 0.25 $\mu$liters/liter ozone, respectively. Ozone at 0.06 $\mu$liters/liter did not cause visible symptoms. Ozonated plants pretreated with BZI did not develop ozone toxicity symptoms.

Among plants not treated with BZI (−BZI) the cholesterol content of those exposed to 0.25 $\mu$liters/liter ozone was significantly less than that of control (nonozonated) plants when measured immediately after ozonation (Table 1, 2). Pretreatment of plants with BZI did not prevent this decrease in cholesterol. Nonozonated plants pretreated with BZI contained significantly more cholesterol than −BZI plants at both times of analysis (Table 1). Within the 0.25 $\mu$liters/liter ozone level, +BZI plants contained significantly more cholesterol 48 hours after ozone than immediately after ozonation while the cholesterol content of −BZI plants did not change significantly with time.

Ozone at 0.25 $\mu$liters/liter appeared to cause an immediate decrease in the campesterol, stigmasterol, and $\beta$-sitosterol content of −BZI plants; but these decreases were not statistically significant, $P = 0.05$ (Table 1, 2). BZI pretreatment did not affect the campesterol, stigmasterol, or $\beta$-sitosterol content of ozonated or nonozonated plants.

No consistent significant effects of 0.06 and 0.12 $\mu$liters/liter ozone or of BZI at these ozone levels on sterol content were observed. Similarly, no consistent significant differences were observed between sterol content measured immediately after ozonation and sterol content determined 48 hours later.

*Cholesterol treatment experiment.*—While cholesterol at 10.3 mg per pot had no effect on leaf sterol levels, at 103.0 mg per pot it appeared to increase the cholesterol and campesterol content; but due to experimental

TABLE 1. Average cholesterol, campesterol, stigmasterol, and β-sitosterol content (μg per gram fresh weight) of primary pinto bean leaves as affected by ozone concentration, benzimidazole treatment, and time of analysis after ozonation

| Free sterol | Ozone concentration (μliters/liters) | Time of analysis (hr) 0 — Benzimidazole concentration (mg/liter) 0 | 0 — 6.25 | 48 — 0 | 48 — 6.25 |
|---|---|---|---|---|---|
| Cholesterol | 0 | 3.1 ± 0.16[a] | 4.1 ± 0.17 | 2.8 ± 1.01 | 3.8 ± 0.99 |
| | 0.06 | 2.9 ± 0.26 | 2.3 ± 0.38 | 2.3 ± 0.23 | 1.4 ± 0.06 |
| | 0.12 | 3.6 ± 0.25 | 2.8 ± 0.22 | 2.3 ± 0.39 | 2.5 ± 0.31 |
| | 0.25 | 1.8 ± 0.27 | 1.8 ± 0.26 | 2.0 ± 0.29 | 3.5 ± 0.51 |
| Campesterol | 0 | 11.0 ± 0.70 | 10.8 ± 1.19 | 11.8 ± 1.23 | 11.4 ± 1.12 |
| | 0.06 | 10.2 ± 1.54 | 9.3 ± 0.91 | 9.7 ± 1.29 | 9.0 ± 0.79 |
| | 0.12 | 12.1 ± 0.65 | 11.0 ± 1.09 | 14.7 ± 1.92 | 13.1 ± 0.19 |
| | 0.25 | 8.3 ± 0.35 | 9.0 ± 0.74 | 12.7 ± 1.13 | 9.0 ± 0.05 |
| Stigmasterol | 0 | 41.8 ± 3.22 | 40.0 ± 3.58 | 44.1 ± 4.26 | 50.5 ± 2.80 |
| | 0.06 | 46.5 ± 1.95 | 35.2 ± 3.57 | 36.0 ± 5.61 | 33.6 ± 3.11 |
| | 0.12 | 49.6 ± 1.94 | 44.0 ± 6.08 | 49.7 ± 4.70 | 52.4 ± 1.46 |
| | 0.25 | 37.3 ± 1.57 | 40.4 ± 0.75 | 49.3 ± 2.63 | 46.2 ± 4.88 |
| β-sitosterol | 0 | 75.6 ± 7.16 | 75.2 ± 4.57 | 79.0 ± 8.22 | 87.3 ± 5.50 |
| | 0.06 | 80.3 ± 7.40 | 59.2 ± 5.32 | 58.9 ± 10.37 | 55.7 ± 7.76 |
| | 0.12 | 97.0 ± 4.22 | 102102.7 ± 2.35 | 89.6 ± 7.78 | 96.8 ± 0.20 |
| | 0.25 | 61.3 ± 5.21 | 75.1 ± 1.16 | 75.3 ± 5.18 | 81.4 ± 6.92 |

[a]Average and standard error of the mean of 3 replications.

variation, these differences were not significant, $P=0.05$. Cholesterol treatment had no effect on stigmasterol or β-sitosterol levels (Table 3). Cholesterol at 103 mg per pot resulted in a highly significant increase in resistance of bean to ozone (Table 3).

*Steroid inhibitor treatment experiment.*—Two μg per ml of the steroid inhibitor had no effect on leaf sterol levels or on percent leaf damage (Table 4). Twenty μg per ml of steroid inhibitor had no effect on leaf sterol levels, but caused a highly significant decrease in resistance of bean to ozone. Two hundred μg per ml steroid inhibitor had no significant effect on leaf sterol levels and significantly reduced resistance to ozone, $P = 0.05$.

DISCUSSION.—We found that stigmasterol and β-sitosterol accounted for 32 and 59% of the free sterols analyzed in primary bean leaves, respectively. Cholesterol and campesterol accounted for 2 and 7% of the free sterols, respectively. These values agree closely with those reported by Brandt and Benveniste (3).

TABLE 2. Average individual and total free sterol decrease measured immediately after ozonation in primary pinto bean leaves treated with two benzimidazole levels and exposed to 0.25 μliters/liter ozone

| Sterol | Sterol decrease (% of control[a]) Benzimidazole concentration (mg/l) 0.0 | 6.25 |
|---|---|---|
| Cholesterol | 42.2[b] | 57.0 |
| Campesterol | 24.3 | 16.7 |
| Stigmasterol | 10.9 | − 0.1 |
| β-sitosterol | 18.9 | 0.2 |
| Total free sterol[c] | 17.3 | 3.1 |

[a]Nonozonated plants at the corresponding benzimidazole level.
[b]Each value is an average of three replications.
[c]Sum of the four individual free sterols analyzed herein and weighted by individual sterol concentration.

TABLE 3. Foliage sterol concentrations and ozone damage of primary pinto bean leaves as affected by cholesterol treatments

| Cholesterol (mg/pot) | μg per gram fresh wt[a] Cholesterol | Campesterol | Stigmasterol | β-sitosterol | Leaf damage (%) |
|---|---|---|---|---|---|
| 0 | 2.7 | 8.5 | 42.3 | 91.7 | 41x[c,d] |
| 10.3 | 2.7 | 9.1 | 51.1 | 82.6 | 40x |
| 103.0 | 3.3 | 12.7 | 45.1 | 96.2 | 28y |

[a]Values represent the mean of four replications.
[b]Plants exposed to 0.25 μliters/liter ozone.
[c]Values represent the mean of 16 replications.
[d]Numbers followed by the same letter in leaf damage column are not significantly different, $P = 0.01$.

TABLE 4. Foliage sterol concentration and ozone damage of primary pinto bean leaves as affected by steroid inhibitor treatments

| Inhibitor[b] ($\mu$g/ml) | $\mu$g per gram fresh wt[a] | | | | Leaf damage[c] (%) |
|---|---|---|---|---|---|
| | Cholesterol | Campesterol | Stigmasterol | $\beta$-sitosterol | |
| 0 | 4.4 | 10.7 | 45.6 | 72.7 | 27x[d,e] |
| 2 | 3.3 | 10.2 | 43.2 | 72.0 | 26x |
| 20 | 3.0 | 10.3 | 44.8 | 74.2 | 54y |
| 200 | 2.9 | 8.1 | 35.6 | 57.1 | 37x |

[a]Values represent the mean of three replications.
[b]tris-(2-diethylaminoethyl)-phosphate trihydrochloride.
[c]Plants exposed to 0.25 $\mu$liters/liter ozone.
[d]Values represent the mean of 12 replications.
[e]Numbers followed by the same letter in leaf damage column are not significantly different, $P = 0.01$.

Although several significant effects of 0.06 and 0.12 $\mu$liters/liter ozone on the cholesterol, stigmasterol, and $\beta$-sitosterol leaf contents were observed, these changes were not consistently related to BZI treatment or time of analysis after ozonation; and a logical interpretation is not presently apparent. Ozone at 0.06 and 0.12 $\mu$liters/liter represents concentrations immediately on either side of the visible damage threshhold level for our conditions. McFarland (9) observed that sublethal ozone levels decreased the permeability of plant tissue. At ozone levels which produced visible damage, an increase in membrane permeability has been observed (4, 13). Thus, the interpretation of analyses of molecular constituents of plants exposed to near-threshhold ozone levels may be complicated by minor alterations in physiological and biochemical processes which may have a significant effect.

Several changes in leaf sterols were also observed herein between the two analysis times. Because the sterol concentration of plant tissue is relatively stable and changes slowly over a period of several weeks (1), the observed sterol changes were probably not caused by aging, but perhaps can be attributed to variable interactions between ozone and BZI.

Tomlinson and Rich (14, 15) reported that leaves of pinto bean exposed to 0.25 $\mu$liters/liter ozone for 3 hours lost 25% of their total free sterol. One-third of these sterols were lost from chloroplasts (15). We observed a similar apparent ozone-induced decrease in free sterols (Table 2) but due to experimental variation, only the cholesterol decrease was statistically significant.

The observed apparent decrease of free sterols due to ozone may result from decreased synthesis, increased degradation, or alteration of the balance between free sterol and sterol derivatives. No evidence is available concerning the effect of ozone on sterol synthesis or degradation. The effect of ozone on sterol derivatives, as reported by Tomlinson and Rich (14, 15) showed that a decrease in total free sterol was accompanied by an increase in sterol glycoside. We found that the sterol ester and sterol glycoside content of bean leaves were below the detection limits (0.3 $\mu$g per gram fresh weight expressed as free sterol) of our analytical method due to the small quantity of tissue available for extraction.

Although BZI did not prevent the immediate ozone-induced cholesterol decrease, the cholesterol content of ozonated +BZI plants was significantly higher 48 hours after ozonation than immediately after ozonation. No accompanying increase over time in the cholesterol content of −BZI plants was observed. BZI treatments also significantly increased the cholesterol content of nonozonated plants. These two effects associated with BZI treatment were observed only with cholesterol and not with campesterol, stigmasterol, or $\beta$-sitosterol. Thus, while cholesterol may not be associated with the initial BZI-induced protection mechanism against ozone, it may function later in the protection sequence prior to visible symptom expression. This hypothesis received indirect support from the cholesterol-feeding and sterol-inhibitor experiments. While addition of cholesterol to the root medium did not result in increased leaf cholesterol content, it did decrease ozone sensitivity of the leaves. Plants treated with the steroid inhibitor were more susceptible to ozone damage, but this decrease in resistance was not accompanied by a significant decrease in leaf cholesterol content. Grunwald (5, 6) found cholesterol more effective than other sterols in reducing methanol or ethanol-induced electrolyte leakage from beets and barley roots.

Interpretation of the steroid inhibitor study is impeded by a lack of knowledge about the effect of tris-(2-diethylaminoethyl)-phosphate trihydrochloride (TDPT) in plants. Preliminary studies indicated that an ozone-inhibitor surface reaction occurred in the 200 $\mu$g per ml steroid inhibitor treatment. TDPT has been reported to decrease stigmasterol and $\beta$-sitosterol in plants (2) and cholesterol in animals (7), but knowledge concerning the site of inhibition in plants, the sterol precursor which accumulates at the block, and the effect of this precursor on ozone resistance, is unavailable. Thus, percent leaf damage measurements probably do not accurately reflect changes in ozone resistance due to sterol changes alone.

Finally, no distinct threshhold sterol concentration was found below which leaves were susceptible to ozone, and above which leaves were resistant. Conformational changes involving sterol bonding in the membrance must not be overlooked. As Vandenheuvel (16) points out, small changes in the position of the cholesterol molecule in the membrane can result in significant changes in pore size which may be reflected as altered membrane permeability.

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