Anti-Senescent Compounds Reduce Injury and Steroid Changes in Ozonated Leaves and Their Chloroplasts

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ABSTRACT

Whole tissue and chloroplasts from leaves exposed to ozone (0.5 μliters/liter) for 1 hr, contained less free sterol and more sterol glycoside and acylated sterol glycoside than did tissue and chloroplasts from nonozonated leaves. Chloroplasts from bean leaves contained 40% of the total free sterol in whole leaf tissue. Ozone decreased the free sterol content of leaves and chloroplasts 25 and 21%, respectively. Chloroplasts from spinach leaves contained 37% of the total free sterol. The free sterol content of whole tissue and chloroplasts of spinach was decreased 44 and 39%, respectively, after ozonation. Intact bean plants and leaves treated with the anti-senescence compounds benzimidazole, *N*-6-benzyladenine, and kinetin, were resistant to ozone injury and did not lose free sterol. Resistance to ozone injury was induced only by those concentrations of chemical treatments that inhibited the degradation of chlorophyll.

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It has long been known that ozone and other photo-oxidants induce premature senescence and degradation of chloroplasts in leaves (2, 5, 6, 14). A more recent development in air pollution research has been that plants can be protected against ozone injury by anti-senescence compounds that have the ability to inhibit yellowing and chloroplast degradation (10, 13, 19). Since there is evidence that these compounds inhibit senescence by preventing changes in membrane permeability (11, 12), it may be suggested that they also inhibit ozone injury by inhibiting changes in membrane permeability. One way that ozone may increase permeability is to induce the conversion of free sterol (FS) to sterol glycoside(SG) and acylated sterol glycoside (ASG) causing a net decrease in the FS content of membranes (3, 4, 16). In this paper we report that benzimidazole (Bd), *N*-6-benzyladenine (*N*-6-BA), and kinetin (K) retard senescence of bean leaves, inhibit ozone injury, and inhibit the loss of FS in whole tissue and chloroplasts of leaves exposed to ozone.

MATERIALS AND METHODS.—All plants were grown in 355-cc (12-oz) cups of sand in a greenhouse receiving charcoal-filtered air. In each experiment, six plants of either bean (*Phaseolus vulgaris* L.) 'Pinto' age 2 weeks, or spinach (*Spinacia oleracea*) L. 'Viroflay' age 1 month, of uniform size and appearance were selected, placed in a plexiglass fumigation chamber, and illuminated at 370 lux for 3 hr. At this time leaf samples were observed under the microscope to make sure stomates were open. Then half of the plants were removed from the chamber and immediately used for the preparation of control samples. Plants remaining in the chamber were exposed to ozone (0.5 μliters/liter) for 1 hr then immediately removed and used for the preparation of ozonated samples. Each experiment included two replicates and was repeated three times.

Chloroplasts were isolated in 0.35 M NaCl solution buffered to pH 8.0 to assure the least amount of enzymatic damage to lipids (7). Leaf tissue (1.5 g from two plants) was ground with a mortar and pestle previously cooled in an ice bath. Chloroplasts were filtered through a double layer of Kleenex tissue and pelleted by centrifuging at 2000 *g* for 10 min at 0 C.

All lipids, either in leaf tissue or chloroplast pellets, were extracted in a mixture of chloroform and methanol (1:1) and concentrated by evaporation under reduced pressure at 45 C. The FS, SG, and ASG were separated by thin-layer chromatography (TLC), recovered and measured as previously described (16) with one modification. The SG and ASG were not measured separately; they were pooled and hydrolyzed in 80% ethanol with 6 N HCl at 80 C for 4 hr. The hydrolyzed sterol was extracted in chloroform, purified by TLC and recovered in the usual way (16). Lipid extracts from tissue and chloroplasts containing 3-4 mg of chlorophyll were measured by Arnon's method (1). Sterol content measured by the Lieberman-Burchard reaction is reported as μmoles/3 mg chlorophyll.

The cytokinins and Bd in water were added to sand and around roots of bean plants potted in sand at rates of 10, 20, and 30 μg/g sand when the plants were 10 days old. Each treatment was applied to six different plants. Four days later, ozonated and nonozonated samples were obtained as described above. Cytokinins and Bd were also applied directly to the leaves. When the stomata of primary bean leaves were completely closed, the upper surface was wetted with 95% methanol using a camel's-hair brush. After the 95% methanol evaporated, the leaf could be readily wetted with solutions of 50% methanol. In this way, one primary leaf of each plant was treated with 50% methanol for comparison with the opposite leaf treated with 25, 50, or 100 ppm of Bd, *N*-6-BA, or K in 50% methanol. After the 50% methanol had dried, the plants were saved for ozone treatment 2 days later or they were placed in the dark for 7 days to induce senescence. Each treatment was duplicated in four separate experiments.

In additional experiments, disks (3-cm diam) from opposite primary bean leaves were floated either on solutions of Bd or water in covered petri dishes in the

dark. The experiment contained six replications of each treatment. After 7 days, the chlorophyll content of the disks from each pair of leaves was measured spectrophotometrically by Arnon's method (1). The chlorophyll retention in disks treated with Bd compared to untreated disks determined the percent of relative senescence.

RESULTS.—Compared to chloroplasts from non-ozonated leaves, chloroplasts isolated from bean leaves exposed to ozone consistently contained less free sterol and more of the sterol derivatives SG and ASG (Table 1). These observations are consistent with results previously reported for sterol changes observed in extracts from ozonated leaves (16).

The effects of ozone on the free sterol content of leaves and chloroplasts of bean and spinach are summarized in Table 2. Based on equal amounts of chlorophyll, bean chloroplasts contained 40% and spinach chloroplasts 37% of the total free sterol found in leaf extracts. During the ozone fumigation, bean leaves and spinach leaves lost 25 and 44% of their total free sterol content, respectively. The chloroplasts from ozonated leaves of bean and spinach were similarly affected and lost 21 and 39%, respectively.

Primary leaves of bean plants treated with either Bd or *N*-6-BA at 10-20 μg/g sand were resistant to ozone injury and dark-induced senescence. Kinetin was most effective in treatments of 30 μg/g sand. At this higher concentration, Bd and *N*-6-BA were toxic to the plants (Table 3). In addition, we found that when leaves were resistant to ozone injury and senescence, their FS content was maintained at normal levels (0.8-0.9 μmoles/3 mg of chlorophyll) during ozonation.

Primary bean leaves treated directly with 95 or 50% methanol were not injured, and their susceptibility to ozone was not changed. However, 2 days after treatment with methanol solutions of 25-200 ppm of either Bd or *N*-6-BA, ozone resistance was evident. The same chemical treatments were also anti-senescent. As in the sand treatments, higher concentrations of K were required to protect the leaf, whereas higher concentrations of Bd and *N*-6-BA were toxic. The results summarized in Table 4 show that treatments which induced ozone resistance were anti-senescent; the only exception being the highest concentrations of *N*-6-BA which induced ozone resistance after 2 days but showed symptoms of phytotoxicity after 4 days. None of these chemical treatments inhibited stomatal opening.

Disks of bean leaf tissue floated in the dark for 7 days on solutions of Bd at 50-100 ppm consistently retained 3 to 5 times more chlorophyll than disks floated on water for the same length of time.

DISCUSSION.—Whole tissue and chloroplasts from leaves exposed to ozone, contain less FS and more sterol glycosides, resulting in a net loss of free sterol. These results indicate that ozone either inhibited the conversion of sterol glycosides to FS or increased the rate of sterol glycoside formation. We have suggested that ozone increases the conversion of FS to SG and ASG (16). This suggestion has been supported by evidence that the synthesis of SG and ASG was relatively resistant to inhibition by high concentrations of ozone and was, in

TABLE 1. Changes in free sterol and sterol derivatives of chloroplasts in bean leaves exposed to ozone (0.5 μliters/liter for 1 hr)

Steric complement	μmoles/3 mg chlorophyll[a]					
	Control			Ozonated		
Free sterol	0.38	0.39	0.35	0.24	0.25	0.26[b]
Sterol derivatives	0.10	0.11	0.09	0.14	0.15	0.17[c]

[a] Each value is the mean of duplicate readings made from 3 g of leaves.
[b] Significantly different beyond 1% level.
[c] Significantly different beyond 5% level.

TABLE 2. Changes in free sterol content of whole tissue and chloroplasts of bean and spinach leaves exposed to ozone (0.5 μliters/liter for 1 hr)

Plant part	Free sterol content[a] (μmoles/3 mg chlorophyll)	
	Control	Ozonated[b]
Bean		
Whole leaves	0.83	0.62
Chloroplasts	0.33	0.26
Spinach		
Whole leaves	1.16	0.65
Chloroplasts	0.43	0.27

[a] Each value represents the mean of three experiments. Each experiment consisted of duplicate readings on leaf extracts of two plants.
[b] Loss of free sterol significant beyond 1% level.

TABLE 3. The inhibition of ozone injury and senescence in sand-potted bean plants following the addition of anti-senescence compounds at rates of 10-30 μg/g of sand

Treatment[a] (ppm)	Ozone injury[b]	Relative senescence[c]
Benzimidazole		
10	10	50
20	10	25
30	90	Toxic
N-Benzyladenine		
10	20	40
20	50	40
30	90	Toxic
Kinetin		
10	90	100
20	50	50
30	30	50
Water only (control)	80	100

[a] Plants were exposed to ozone (0.5 μliters/liter) 4 days after chemical treatment or placed in the dark for 1 week.
[b] Ozone injury is the percent of leaf area visibly damaged.
[c] Relative senescence is the percent of chlorophyll lost by treated plants compared to controls. Treatments were duplicated in three separate experiments.

TABLE 4. Inhibition of ozone injury and senescence of primary bean leaves following direct application of anti-senescence compounds in solutions of 50% methanol

Leaf treatment[a] (ppm)	Ozone injury[b]	Relative senescence[c]
Benzimidazole		
300	80	Toxic
200	20	50
100	40	50
50	20	30
25	40	30
N-Benzyladenine		
300	20	Toxic
200	20	50
100	40	50
50	50	50
25	50	65
Kinetin		
400	50	50
300	80	100
200	90	100
100	80	100
Water only (control)	80	100

[a] Plants were exposed to ozone 2 days later (0.5 μliters/liter for 1 hr) or placed in the dark for 1 week.

[b] Ozone injury is the percent of leaf area damaged.

[c] Relative senescence is the percent of chlorophyll lost by a primary leaf receiving chemical treatment compared to its opposite leaf which received only 50% methanol. Each treatment was repeated three times.

fact, stimulated by low concentrations of sulfhydryl reagents (8). Evidence that FS is more effective than the sterol derivatives in controlling the permeability of plant cell membranes (4) indicates that a net loss of free sterol in membranes during ozonation may cause an increase in permeability. This is in agreement with previous results showing an increase in synthesis of γ-amino butyric acid (15). In addition, there are several reports of histological evidence that chloroplasts and membranes are the first to show symptoms of ozone and other photo-oxidant injury (2, 5, 14).

There is increasing evidence that chemical treatments may be useful in protecting plants against ozone injury. Many compounds known to protect plants against ozone injury are also known to increase greening and to inhibit senescence (10, 13, 17). We consistently found that when bean plants were treated with Bd, *N*-6-BA, and K that concentrations which inhibited ozone injury also inhibited senescence. Furthermore, these resistant plants did not lose FS from their membranes or show symptoms of cell leakage during ozonation. This is additional evidence for the correlation between FS content and the development of ozone injury, which indicates that these anti-senescence compounds inhibit ozone injury by inhibiting sudden changes in the structure and function of membranes. It has, in fact, already been reported that auxins inhibit senescence by maintaining the integrity of plant cell membranes (11, 12). Therefore, the factor in common among chemicals that have both anti-senescence and ozone-resistant properties may be their ability to maintain normal membrane structure and functions.

Although Wang et al. (18) were not able to inhibit the loss of chlorophyll from bean leaf tissue floated on solutions of Bd, we found a marked inhibition. Since the loss of chlorophyll from *Xanthium* leaves was also inhibited by Bd, it does have anti-senescent properties in dicotyledenous plants (9). There is, therefore, evidence that all three compounds used in this report have anti-senescent properties which are consistent with our suggestion that protection against ozone injury is correlated with the ability of these compounds to maintain membrane integrity.

We suggest that studies of mode of action of anti-senescence compounds which inhibit ozone-induced membrane senescence (i.e. the loss of free sterol) in plant tissue would improve our understanding of ozone injury and our ability to select methods of control.

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