The Effect of Ozone on Chloroplast Lamellae and Isolated Mesophyll Cells of Sensitive and Resistant Tobacco Selections

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ABSTRACT

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Ozone treatment of whole tobacco leaves affected chloroplast electron transport in proportion to the degree of visible leaf injury resulting on either the sensitive or resistant selection. When chloroplasts were isolated from Bel-W3 (ozone-sensitive) and Bel-B (ozone-resistant) tobacco leaves and exposed to identical ozone doses in vitro, however, inhibition of electron transport was similar in both. Likewise, enzymatically-isolated leaf mesophyll cells were equally sensitive to in vitro ozone treatment. The differential ozone response of Bel-W3 and Bel-B tobacco was not expressed by the isolated chloroplast lamellae or leaf mesophyll cells.

Although it is well documented that Bel-W3 tobacco is extremely sensitive to ozone (O_3) and Bel-B is relatively resistant, the reason for the differential response is not known. Studies have failed to demonstrate consistent stomatal responses that might explain resistance on the basis of exclusion of O_3 from the leaf (4, 6, 9, 18). In a previous report we demonstrated, by the use of reciprocal grafts, that, contrary to a current notion (15), the consistently smaller root system of Bel-B does not determine its resistance (12). Because excised leaves of Bel-W3 and Bel-B tobacco exhibited the differential response to O_3, we concluded that the resistance/susceptibility factor lies within the leaf itself. Thus, we conducted several experiments to determine whether resistance in the leaf to O_3 could be detected at the cellular or subcellular level of organization. These experiments involved three objectives: (i) to determine the electron-transport activity of chloroplast lamellae isolated from leaves of Bel-W3 and Bel-B; (ii) to determine the electron transport activity of chloroplast lamellae isolated and exposed to O_3 in vitro; and (iii) to isolate leaf mesophyll cells from Bel-W3 and Bel-B tobacco leaves and expose to O_3 in vitro, followed by assay of oxygen evolution rates.

MATERIALS AND METHODS

Plant material.—Two selections of cigar-wrapper tobacco were utilized in all experiments; *Nicotiana tabacum*, 'Bel-W3', which is sensitive to O_3 and *N. tabacum* 'Bel-B', which is resistant. The plants were grown from seed to maturity in a charcoal-filtered greenhouse. Seeds were germinated in finely ground sphagnum moss and transplanted after 4-5 wk into sterilized loam in 7.5-cm diameter clay pots. When the plants had four to six leaves, they were repotted in 25-cm diameter plastic pots. We applied 3.2 g/liter solution of 20-20-20 (N-P-K) fertilizer weekly to maintain lush, dark-green foliage. The leaves used for the isolation of chloroplast lamellae and mesophyll cells had just reached full expansion, the stage at which we observed maximum O_3 susceptibility.

Fumigation and harvest of intact plants.—Ozone fumigations were carried out in a 6-m^3, glass-enclosed, fumigation chamber located in a greenhouse. Air flow in the chamber was sufficient to cause a complete air change every 45 sec, temperature was maintained at 24-27 C, and relative humidity at 75-80%. A turntable to support the plants assured that all plants received equal exposure. Ozone, generated by passing pure oxygen through a commerical ozone generator, was fed into a charcoal-filtered air stream. Ozone levels in the chamber were monitored using a Mast Ozone Meter (Mast Development Co., Davenport, IA 52802) calibrated by the neutral buffered KI method (7).

The tobacco plants were fumigated with 287-382 $\mu g/m^3$ ozone for 2 to 4 hr. The longer exposures were required to visibly injure Bel-B plants. Half-leaf samples from ozonated and nonexposed plants were harvested immediately after each fumigation for chloroplast assay. Half-leaves with midribs attached were allowed to remain on the plants to permit evaluation of visible injury after 24 hr.

Preparation and assay of chloroplast membranes.—Chloroplast membranes were prepared by homogenizing half-leaves minus midribs for 1 min in 0.05 M potassium phosphate buffer pH 7.2, with 0.01 M

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sodium chloride and 0.4 M sucrose. The homogenate was centrifuged at 1,000 *g* and the pellet was resuspended in the same buffer minus sucrose to rupture the chloroplast envelopes. After a final centrifugation, the membranes were resuspended in the original homogenization medium. Chloroplast electron-transport activity was measured by the Hill reaction using 2, 6-dichlorophenol indophenol (DCPIP) as the electron acceptor. The assay medium contained 0.05 M potassium phosphate buffer pH 6.8, 0.01 M sodium chloride, and approximately 5×10^{-5} M DCPIP. Ammonium chloride (0.01 M) was added to the assay medium to obtain uncoupled rates of electron transport. Assays were carried out in a Beckman DB Spectrophotometer, and calculated on a $DCPIPH_2$/mg chlorophyll/hr basis. Five separate readings were taken for each sample. Results were subjected to an analysis of variance.

Ozonation of isolated chloroplasts.—Ozonation of chloroplast membranes was accomplished by bubbling (478 $\mu g/m^3$) O_3 in oxygen through a 1-ml sample of the chloroplast suspension for 1 min. Oxygen and nitrogen also were bubbled through selected samples to determine the effect of the bubbling per se. Since the tests were conducted on four different days, the results were treated as replicates.

Preparation, assay, and ozonation of mesophyll cells.—Suspensions of tobacco-leaf cells were prepared by enzymatic maceration of leaf tissue. The maceration medium contained 0.45 M mannitol and 0.5% Macerozyme (Kinki Yakult Mfg. Co., Ltd. 8-21 Shingikan-Cho, Nishinomiya, Japan), and was adjusted to pH 5.5. Approximately 2 g of leaf tissue with the lower epidermis removed was placed in 20 ml of the maceration medium and vacuum infiltrated for 3 min prior to incubation. Incubation was for 75-90 min at room temperature and with continuous agitation at 70 cycles/min. Cells were harvested after gentle swirling to shake them loose from the network of vascular tissue, washed in 0.45 M mannitol, and resuspended in 0.05 M potassium phosphate buffer (pH 7.0) containing 0.45 M mannitol and 8 mM sodium bicarbonate. Cell activity was assayed polarographically for oxygen evolution in the light using a Biological Oxygen Monitor (Yellow Springs Instrument Co., Yellow Springs, OH 45387). Light was provided by two 150-W, cool-beam spotlights focused on the sample chamber through a 9-cm water filter, which prevented excessive heating. Oxygen evolution was expressed as μmoles O_2/mg chlorophyll. Although each sample could be assayed only one time, each experiment was repeated up to 12 times. The results, expressed as percent of control values, were subjected to analysis of variance.

Ozone treatment of cell suspensions was accomplished by introducing air containing 11.4 mg/m^3 O_3 into a 125-ml Erlenmeyer flask containing 5 ml of cell suspension. The flask was shaken gently during the exposure period which lasted 5, 10, or 20 min. Ozone treatments were terminated by bubbling nitrogen through the suspensions for 1 min. Control flasks also were shaken and bubbled with nitrogen.

RESULTS

Experiment 1.—Reduction rates of DCPIP by chloroplast lamellae isolated from ozonated plants were depressed in proportion to the amount of injury that developed after 24 hr on the half-leaves remaining on plants (Table 1). Ozone concentration of 343 $\mu g/m^3$ for 1.5 hr was not great enough to cause visible injury to either Bel-W3 or Bel-B, and also there was no significant effect on chloroplast electron-transport rates in either cultivar. Bel-W3 plants exposed to 477 $\mu g/m^3$ for 2 hr developed slight to severe visible injury, and Bel-B had at most only slight foliar injury. Only Bel-W3 chloroplast lameallae had significantly lower rates of DCPIP reduction than the controls. Both selections exposed to 573 $\mu g/m^3$ O_3 for 2 hr were severely injured and both showed decreases in DCPIP reduction rates in isolated chloroplast membranes. The stimulating effect of the uncoupler on DCPIP reduction rates was similar in the two selections (Table 2), both in the presence or absence of ozone. Uncoupled electron-transport rates were significantly greater than coupled rates in all cases.

Experiment 2.—Chloroplast lamellae isolated from Bel-W3 and Bel-B tobacco plants and then exposed to ozone in vitro had significantly reduced rates of DCPIP reduction compared to those of nonexposed samples (Table 3). There was, however, no significant difference between the responses of the two selections. Reduction rates were decreased as much in chloroplasts from Bel-B (the ozone-resistant selection) as in Bel-W3 (the ozone-sensitive tobacco). In this experiment, the addition of an uncoupler stimulated DCPIP reduction rates to a similar degree regardless of selection or treatment (Table 4).

Experiment 3.—In vitro ozone treatment of enzymatically isolated leaf mesophyll cells from Bel-W3 and Bel-B tobacco resulted in decreased rates of oxygen evolution compared to control rates (Table 5). A significantly greater effect was obtained with increased exposure time. There was, however, no significant difference between the responses of cells of the two cultivars to in vitro ozone treatment.

TABLE 1. Rates of reduction of 2,6-dichlorophenol indophenol (DCPIP) by chloroplast membranes of ozonated Bel-B and Bel-W3 tobacco plants

Ozone dosage		Bel-B		Bel-W3	
Conc. ($\mu g/m^3$)	Time (hr)	Injury index[x]	DCPIP reduction (% of control)	Injury index[x]	DCPIP reduction (% of control)
343	1.5	0	104.0 a[z]	0	107.0 a
			95.3 a		103.0 a
477	2	0	100.0 a	1	94.3 a
			90.0 a		83.7 b
573	2	1	101.0 a	3	79.9 b
			85.6 b		75.2 b
669	2	3	77.7 b	4	...[y]

[x]Disease rating system 0-4; none to very severe injury.
[y]Tissue too severely damaged to permit extraction of chloroplast membranes.
[z]Values followed by the same letter are not significantly different, $P = 0.05$, by analysis of variance.

TABLE 2. Rates of reduction of 2,6-dichlorophenol indophenol (DCPIP) by chloroplast membranes from ozonated and control Bel-B and Bel-W3 tobacco plants in the presence of an uncoupler, expressed as a percentage of the coupled rates

Ozone dosage		Reduction of DCPIP (percentage of uncoupled rates)			
		Bel-B		Bel-W3	
Conc. ($\mu g/m^3$)	Time (hr)	Ozone-treated (%)	Control (%)	Ozone-treated (%)	Control (%)
343	1.5	167[z]	153	169	159
		158	160	166	178
477	2	162	165	169	189
		163	151	220	205
573	2	174	170	184	262
		237	238	266	240
669	2	192	148	...	...

[z]Value is the mean of five readings.

TABLE 3. Rates of reduction of 2,6-dichlorophenol indophenol (DCPIP) by chloroplast membranes from tobacco plants (cultivars Bel-B and Bel-W3) exposed to O_3 in vitro

Selection	Replicate	DCPIP reduction (% of control)
Bel-B	1	74.0 a[z]
	2	70.0 a
	3	86.9 a
	4	77.7 a
Bel-W3	1	84.0 a
	2	85.0 a
	3	86.0 a
	4	79.5 a

[z]Values followed by the same letter are not significantly different, $P = 0.05$, by analysis of variance.

TABLE 4. Stimulation of 2,6-dichlorophenol indophenol (DCPIP) reduction rates of ozonated and control tobacco plant (cultivars Bel-B and Bel-W3) chloroplast membranes in the presence of an uncoupler, expressed as percentage of coupled rates

Selection	Replicate	Reduction of DCPIP (percentage of the coupled rates)	
		Ozone-treated (%)	Control (%)
Bel-B	1	151[z]	131
	2	146	118
	3	123	139
	4	123	137
Bel-W3	1	185	178
	2	159	145
	3	173	143
	4	178	83

[z]Values are the means of five readings.

DISCUSSION

Ozonation of intact tobacco plants affected chloroplast electron transport confirming Chang and Heggestad's earlier report (1) for spinach leaves. The inhibition of elctron transport was proportional to the degree of visible injury on leaves of Bel-W3 and Bel-B tobacco. A higher dosage was required to cause injury and to inhibit electron transport in Bel-B because of its partial resistance.

In isolated chloroplast lamellae, electron transport rates also were reduced by O_3 but Bel-B and Bel-W3 were inhibited to the same degree. It is possible that our system was too simplified to permit expression of the "resistant" response in the Bel-B selection.

In their work with spinach chloroplasts Coulson and Heath (2, 5) reported decreased electron transport with O_3 treatment. They hypothesized that O_3 probably never penetrated beyond the plasmalemma in an intact leaf, but, by altering the osmotic and ionic conditions in the cytoplasm, it indirectly affected the chloroplast. Applying Coulson and Heath's (2) reasoning to tobacco, we suspect that the plasmalemma of Bel-B was more resistant than Bel-W3, that the Bel-B chloroplasts were less sensitive to changes in the cytoplasm milieu, or that Bel-B was capable of repairing the damage up to some threshold level. Ting and Sutton (16) reported preliminary evidence for the operation of a repair mechanism following ozone damage in bean.

With isolated mesophyll cells, O_2 evolution was used as a criterion for evaluating ozone sensitivity because of the many reports relating reduced photosynthetic rates with visible damage in intact plants (3,6,11,14). Even at the lowest O_3 dose that caused a measurable change in O_2 evolution in Bel-W3 tobacco (11.4 mg/m^3 for 5 min) cells of Bel-B (the resistant selection) responded similarly. The differential response that was so distinct and consistent in intact plants was not expressed in isolated mesophyll cells, although they are the first to become injured in the intact leaf.

This disparity in the response of isolated cells and intact leaves is similar to results reported by Otsuki et al. (10)

TABLE 5. Effect of in vitro ozone (O_3) treatment on oxygen evolution by isolated mesophyll cells of Bel-W3 and Bel-B tobacco, expressed as percentage of control rates[w]

Ozone dosage			
Conc. (mg/m^3)	time (min)	Bel-W3	Bel-B cells
11.4	5	87.5[x] a	82.0[z] a
11.4	10	76.8 ± 3.2[y] b	69.9 ± 2.0 b
11.4	20	45.3 ± 6.5 c	44.4 ± 4.1 c

[w]Maximum O_3 evolution rate was 36.6 μmoles O_2/mg chlorophyll/hr.

[x]Values for 5-min O_3 treatment are the mean of two experiments.

[y]Values for 10- and 20-min treatments are the mean of 8 to 12.

[z]Values followed by the same letter are not significantly different.

with TMV and the expression of the local-lesion gene. Intact leaves of the local-lesion host inoculated with the virus developed local lesions, but those of the systemic host developed a mottle. The necrotic response resulted in restricted virus replication in the local-lesion host which is considered to be resistant because of restrictions of virus spread. However, with inoculation in vitro the virus replicated equally well in isolated cells or protoplasts from both hosts. The analogy to our results also is interesting in view of the similarity between the O_3 toxicity symptoms in tobacco and the hypersensitive response induced by TMV; the lesions are similar in appearance, and at the biochemical level, they also have much in common.

Phenylalanine ammonia lyase (PAL), a key enzyme in phenol metabolism, is associated with the development of the necrotic response. Although present in intact leaves of the local-lesion host, it was not detected in cell or protoplast suspensions (13). These same biochemical pathways have been associated with ozone toxicity. Kremer and Howell (8) reported that ozone-injured leaves had accumulations of various phenolic compounds and enhanced PAL activity. Tingey (17) also measured altered PAL and polyphenol-oxidase activity in soybean leaves exposed to ozone. As in the virus study (10), the failure of our cell suspensions to reflect differential ozone sensitivity shown by intact Bel-W3 and Bel-B tobacco plants may be due to the lack of enzymes or substrates that are involved in the necrotic reaction in the intact plant.

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