Effect of an Ozone Injury Retardant Chemical on Isozyme Profiles from Alfalfa Callus in Vitro

John P. Rier, Jr., Virendar K. Sood, Aaron Whitaker, and Colin Watson

Department of Botany, Howard University, Washington, DC 20059.
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ABSTRACT

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Plant ozone injury retardant *N*-[2-(2-oxo-1-imidazolidinyl)-ethyl]-*N'*-phenylurea (EDU or ethylenediurea) at 1.0 ppm inhibited growth of callus of alfalfa cultivars Williamsburg (ozone-sensitive) and MSB-CW5An2 (ozone-insensitive) germplasm of *Medicago sativa*. The presence of EDU (0.1 ppm) in the growth medium increased the number of protein and peroxidase isozyme bands in alfalfa cultivar Williamsburg stem callus and ozone modified their intensities. Protein profiles of MSB stem callus from media containing EDU or exposed to ozone were unchanged. Marked differences were observed between the peroxidase profiles of ozonated and control ozone-insensitive stem callus from media containing EDU. Protein profiles of ozonated ozone-insensitive leaf callus differed slightly from controls. The peroxidase profile of ozonated ozone-sensitive leaf callus was not altered when its growth medium contained EDU, but when it was absent, changes were observed in these profiles.

Atmospheric ozone at certain concentrations can cause visible injury in plants. The degree of injury produced under similar conditions of cultivation and ozone stress has been found to be species- (13,14,45) and strain- or cultivar-specific (22,26,31,34). Endogenous carbohydrates (15,17), ascorbic acid (18,20,28), nutrients (4,10,26,33), mixed-function oxidase inhibitors (23) or ozone pretreatment (37) can alter the severity of ozone injury in plants. Furthermore, visible ozone injury was found to be reduced in plants treated with benomyl (29) and *N*-[2-(2-oxo-1-imidazolidinyl)-ethyl]-*N'*-phenylurea (EDU) (6–9,25,44).

Postulations as to the basis for plant susceptibility to ozone have been derived primarily from data on whole plants grown in fields, greenhouses, or growth chambers. Among factors believed to be related to ozone injury and susceptibility in plants are stomatal function (2,16,21,24), photoperiod (17,19,30), temperature (16), and tissue age (2,16,24). There remains a need for a test system(s) that can produce the data necessary to fully understand the phenomenon of ozone susceptibility in plants.

Callus tissue in vitro could be used as experimental material in

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studies on ozone susceptibility in plants. These tissues carry germplasm of the parent plants and avoid certain physiological and morphological encumbrances found in whole plants, which could impede fully understanding the basis for ozone tissue injury. When grown in vitro, callus, a primarily parenchymatous tissue, exhibits morphogenetic and biochemical modifications in response to its culture conditions (40,41).

Furthermore, plant organs and callus tissue in vitro have shown promise in studies of ozone injury (35,36). Among the advantages of a callus-based system are: accessibility of cells for gaseous exposure without concerns for stomatal function and other structural barriers found in whole plants; capacity for maintaining in a small area under controlled conditions several tissue clones derived from stems, leaves, roots, and endosperm of a variety of plant species, strains, and cultivars; and facility for adding substances to the growth media that can be assayed for their effectiveness in modifying tissue biochemistry in relation to environmental stress such as that caused by ozone (29).

Peroxidases in many forms are distributed throughout the plant kingdom (39). They form characteristic banding patterns when assayed electrophoretically in starch or polyacrylamide gel systems (3). These patterns represent developmental, hormonal, and genetic activities at molecular levels, which can be modified by stresses in the environment, notably ozone (12,15,42,43). Moreover, variations in the electrophoretic patterns and activities of peroxidases have been found within genera and among cultivars and strains of species (12,31,43). Ozone has been shown to modify the patterns of peroxidase in ozone-sensitive plants more readily than in ozone-insensitive plants (12). Also, ozone-induced elevation in peroxidase activity has been demonstrated in plants that failed to show visible injury (11). Peroxidase is perhaps only one of several enzymes that is a target for ozone-induced plant tissue changes associated with visible injury (43).

Among the chemicals tested for the control of suppression of ozone injury in plants, EDU has shown significant promise. Foliar and root applications of EDU have reduced ozone injury in a variety of ozone-sensitive herbaceous and woody plants. It has been suggested that EDU can transform ozone-sensitive plants to ozone-insensitive ones (1,6–9). So far, there are no reports on the effect of EDU on ozone-sensitive and ozone-insensitive callus tissues.

The objective of this study was to examine the effects of EDU on the protein and peroxidase isozyme patterns of extracts from leaf and stem callus of ozone-sensitive and ozone-insensitive strains of alfalfa.

MATERIALS AND METHODS

Two strains of *Medicago sativa* L. were obtained from the U.S. Department of Agriculture, Beltsville, MD, courtesy of R. K. Howell, research plant pathologist. The strains were designated MSB-CW5An2 (ozone-insensitive) and Williamsburg (ozone-sensitive) (22). Callus tissue cultures from stem and leaf pieces of both germplasms were initiated and maintained on a medium formulated by Saunders and Bingham (38). Cultures were maintained under a 12-hr light and dark cycle in a growth chamber (M-32; Environmental Growth Chambers, Chagrin Falls, OH) at 25 ± 1 C under 14,644 lux (1,400 ft-c) of illumination provided by cool-white fluorescent tubes and incandescent lamps.

Leaf and stem calli of each strain, in their second subculture period (12–14 wk), were used in this study. Preliminary work had shown that gels of extracts from older cultures had poor peroxidase band definition. Tissues from each strain were subdivided into 60–70 mg pieces and cultured on the medium described above with and without EDU 0.1, 1.0, and 10.0 ppm. At the end of a culture period of 6 wk, tissues were harvested, ozonated, and analyzed electrophoretically according to the procedures given below.

Tissues were ozonated in a chamber (80 × 30 × 30 cm) that had a frame made of 1.25-cm-diameter polyvinyl tubing and was covered by a sheet of polyvinyl chloride 0.8 mm thick. Ozone was generated by a Welsbach Ozonator T-408 (Welsbach Corporation, Philadelphia, PA 19129). Ozone was supplied to the chamber interior through a Teflon tube, which was attached along the entire length of the upper wall of the chamber. To assure good distribution of ozone, the tubing in the chamber was perforated at 1.2-cm intervals along its entire length. Ozone flow rate (1 L/min) was maintained with a gas flow meter, and its concentration (0.5 ppm) was monitored by an Ozone Analyzer, model OA 325-2 (Meloy Laboratories, Springfield, VA 22151). Control tissues were exposed to charcoal-filtered air at the same flow rate in a similar chamber.

Callus tissues from the four lots of culture media were removed, fragmented, and distributed evenly in petri dishes. Dishes containing tissues were placed uncovered in the chamber for ozonation. Following exposure to ozone (0.5 ppm) for 2 hr or charcoal-filtered air (controls), the dishes containing tissues were covered and kept in the growth chamber for 18 hr prior to extraction. The extraction of the tissues was carried out according to a previously described procedure (5). The extracts were freeze-dried. For gel electrophoresis, LKB Ampholine PAG plates, pH range 3.5–9.5, were used. Various protein concentrations were determined (27) and used in trial runs. Finally, a 5-mg sample, which was found to contain 8 μg of protein, provided the optimum electrophoretic resolution of proteins and peroxidase. Five milligrams of freeze-dried samples were dissolved in 10 μl of water and applied to the gel. As many as eight samples were applied to a single gel plate, thus accommodating as many as four sets of treatments. Each gel was run for 4 hr at 350 V.

In order to visualize proteins, the gels were treated with fixing solution for 0.5 hr. Gels were then washed with destaining solution and finally stained for 10 min at 60 C in staining solution (0.46 gm of Coomassie Brilliant Blue R-250 in 400 ml of destaining solution). Peroxidase staining was done by using the guaiacol-hydrogen peroxide system of Ockerse et al (32).

The stained gels were examined with the aid of fluorescent light. This was achieved by placing the dish containing the gel on a milk-colored glass pane fitted on one side in a box constructed of 0.95-cm-thick plywood, 65 × 25 × 20 cm. The interior of the box was lined with glossy white paper and contained two 20-W cool-white fluorescent lamps. In a dark room, differences among the bands and profile patterns in the gels were clearly discernible. Band numbers, their intensities, and R_f values were recorded for each profile. In these experiments, comparative studies were easily made among treatments. There was, without exception, total agreement among observations of similar treatments. For future reference, all gels were photographically recorded on Kodachrome daylight film, ASA 64, and on edited photocopies. The data in this report are based upon averages from three separate experiments run under identical conditions.

RESULTS

Tissue texture, growth, and color observed in cultures from media containing 0.1 ppm EDU were nearly comparable to those in cultures on EDU-free medium. Both stem and leaf calli were light yellow to amber, firm, and significantly heavier than callus from media containing either 1.0 or 10 ppm EDU. Tissues cultured on higher concentrations of EDU were light brown. Ozone-sensitive leaf callus final wet weights were lowest overall among the tissue types and treatments in this study (Table 1).

Control leaf callus extracts from ozone-sensitive plants displayed six protein and three peroxidase bands (Table 2). Ozonation of this callus caused a loss of two bands, R_f 0.60 and 0.78, from the protein profile and a gain of one band, R_f 0.81, in the peroxidase profile.

Cultures of ozone-sensitive leaf callus on medium containing EDU showed a gain of one band, R_f 0.47, and a loss of band R_f 0.81 in its protein profile. Its peroxidase profile was a duplicate of that obtained from its ozonated counterpart grown on EDU-free medium. None of the intensities of any band of either profile from ozone-sensitive leaf callus grown on a medium, with or without EDU, was affected by ozone.

Protein profiles of ozone-insensitive leaf callus were similar among all treatments except for an increase in the intensity of band

8, R_f 0.81, following EDU and ozone treatment. Three bands were observed in the peroxidase profiles of this callus from EDU-free medium. A fourth band at R_f 0.81 was added to this profile following ozonation of the callus. The same four-band profile occurred again in this callus when grown on a medium containing EDU, all of which remained unchanged upon ozonation.

Controls from ozone-sensitive stem callus had six protein and four peroxidase bands in their respective profiles (Table 3). Ozonation of this callus effected a loss of one band, R_f 0.58, from the protein profiles and no intensity changes among the remaining bands. The peroxidase profiles from this tissue contained four bands that also were unaffected by ozone.

The protein profiles of non-ozonated sensitive stem callus grown on medium with EDU differed from those of their counterparts cultured on EDU-free medium. Callus from medium containing EDU showed a loss of band R_f 0.78 while gaining bands R_f 0.54 and 0.60. Following ozonation in intensity of protein band R_f 0.71 increased and that of peroxidase bands R_f 0.60 and 0.75 increased. Peroxidase band R_f 0.71 was diminished by this treatment.

TABLE 1. Final wet weights (gm) of callus from two cultivars of *Medicago sativa* after 6 wk of growth on media containing EDU[a]

	Final wet wt (g)			
	Williamsburg (ozone-sensitive)		MSB (ozone-insensitive)	
Treatment	Stem callus	Leaf callus	Stem callus	Leaf callus
Control	3.48[b]	1.82	4.21	4.34
EDU				
0.1 mg/l	2.94	1.75	2.69	3.62
1.0 mg/l	0.22	0.28	0.18	0.12
10.0 mg/l	0.00	0.00	0.00	0.00

[a]EDU is *N*-[2-(2-oxo-1-imidazolidinyl)-ethyl-*N'*-phenylurea, and ozone injury retardant.
[b]All values represent the mean of three replications. Initial inoculum weight average, 0.065 g.

TABLE 2. Total protein and peroxidase banding patterns of extracts of leaf callus from two cultivars of *Medicago sativa*

	Total protein				Peroxidase			
	−EDU		+EDU[a]		−EDU		+EDU	
Cultivar and band no.	$-O_3$	$+O_3$[b]	$-O_3$	$+O_3$	$-O_3$	$+O_3$	$-O_3$	$+O_3$
Williamsburg (ozone-sensitive)								
1			0.47[c]	++[d]	0.47[c]	++[d]	0.47[c]	++[d]
2	0.60[c]	−−[d]	0.60	++	0.60	++	0.60	++
3					0.63	++	0.63	++
4								
5	0.69	++	0.69	++				
6	0.71	++	0.71	++				
7	0.76	++	0.76	++				
8	0.78	−−	0.78	++				
9	0.81	++			0.81		0.81[b]	++
MSB (ozone-insensitive)								
1					0.45	++	0.45	++
2	0.46	++	0.46	++				
3	0.60	++	0.60	++	0.60	++	0.60	++
4	0.64	++	0.64	++	0.64	++	0.64	++
5	0.70	++	0.70	++				
6	0.74	++	0.74	++				
7	0.78	++	0.78	++				
8	0.81	++	0.81	+++		0.81	0.81	++

[a]EDU, 0.1 ppm. EDU is *N*-[2-(2-oxo-1-imidazolidinyl)-ethyl]-*N'*-phenylurea.
[b]Ozone, 0.5 ppm for 2 hr.
[c]R_f values for bands.
[d]Status of bands in extracts from ozonated calli: band absent (−−); band intensity decreased (+); band intensity unchanged (++); band intensity increased (+++).

Protein profiles of ozone-insensitive stem callus were similar among all treatments. Their peroxidase profiles, whether cultured with or without EDU, contained four bands in repeating patterns. Upon ozonation of this callus from EDU-free medium, the intensity of peroxidase band R_f 0.72 was diminished. On the other hand, insensitive stem callus from medium containing EDU had lost peroxidase bands R_f 0.50 and 0.75 and had gained a new band at R_f 0.53 following ozonation.

DISCUSSION

Callus tissue from stems and leaves of two plant cultivars with different ozone susceptibilities from a single alfalfa species can be successfully initiated and maintained in vitro on a modified Blaydes medium. Growth responses of stem and leaf callus tissues of the ozone-insensitive strain were similar and also greater than their respective counterparts from the ozone-sensitive strain. Furthermore, within the latter strain, leaf callus growth was about half that of stem callus. Irrespective of the growth responses of these cultures, their textures and colors were similar.

This is the first report on the incorporation of EDU into a callus culture medium and there is no prior information on how it may affect plant growth. In this study, plant callus growth in vitro was suppressed most noticeably by EDU at 1.0 and 10 mg/L. The question as to how EDU may influence growth and development of plant cells and tissues should be given consideration in future research. Data provided would be important indeed if EDU becomes widely used as a protectant against ozone injury in plants.

In the present study, a different protein and peroxidase banding pattern was found for each extract from the callus tissue clones. Dissimilar patterns were also found for leaf and stem callus tissues from within each strain, and the patterns for leaf and stem callus tissues were strain-specific. Although differences in banding patterns have been demonstrated between extracts from different

TABLE 3. Effect of an ozone injury retardant (EDU) on total protein and peroxidase banding patterns for extracts of stem callus from two cultivars of *Medicago sativa*

	Total protein				Peroxidase			
	−EDU		+EDU[a]		−EDU		+EDU	
Cultivar and band no.	$-O_3$	$+O_3$[b]	$-O_3$	$+O_3$	$-O_3$	$+O_3$	$-O_3$	$+O_3$
Williamsburg (ozone-sensitive)								
1					0.52[c]	++[d]	0.52[c]	++[d]
2			0.54[c]	++[d]				
3	0.58[c]	−−[d]	0.58	++	0.58	++	0.58	++
4			0.60	++				
5	0.63	++	0.63	++				
6					0.65	++	0.65	++
7	0.68	++	0.68	++	0.68	++	0.68	+++
8	0.71	++	0.71	+++			0.71	+
9	0.75	++	0.75	++			0.75	+++
10	0.78	++						
MSB (ozone-insensitive)								
1					0.50	++	0.50	−−
2								0.53
3	0.55	++	0.55	++				
4	0.58	++	0.58	++				
5	0.62	++	0.62	++				
6	0.65	++	0.65	++	0.65	++	0.65	++
7	0.68	++	0.68	++				
8	0.70	++	0.70	++				
9					0.72	+	0.72	++
10	0.75	++	0.75	++	0.75	++	0.75	−−
11	0.79	++	0.79	++				

[a]EDU, 0.1 ppm. EDU is *N*-[2-(2-oxo-1-imidazolidinyl)-ethyl]-*N'*-phenylurea.
[b]Ozone, 0.5 ppm for 2 hr.
[c]R_f values for bands.
[d]Status of peroxidase bands in extracts from ozonated calli: band absent (−−); band intensity decreased (+); band intensity unchanged (++); band intensity increased (+++).

plant and tissue types (eg, leaf extracts from ozone-sensitive and ozone-insensitive soybean [12] and pinto bean [17] plants) similar findings for callus tissues are unprecedented. It is suggested that, at least in alfalfa, callus tissues from two of its cultivars may maintain separate and distinct identities that can be distinguished electrophoretically.

Profiles of extracts from ozonated alfalfa callus tissues differed from those of the controls. Two bands were lost from the profiles of ozone-sensitive leaf cells, one band was lost from the ozone-sensitive stem cells, one band was induced in both peroxidase profiles of leaf callus, and the intensity of one band was reduced in the peroxidase profile of ozone-insensitive stem callus.

Contrasting isozymic patterns have been reported in gels of extracts from healthy and ozone-injured leaves of ozone-sensitive and ozone-insensitive plants (12). Possible correlations between isozyme patterns derived from field-grown strains of alfalfa that exhibit various levels of ozone susceptibility and their corresponding callus tissues in vitro are not known. Furthermore, any judgment as to the significance of ozone-induced modifications in the isozymic patterns of plant tissues in vitro must await further research.

It has been proposed that EDU negates ozone injury in plant tissue through the induction of free-radical-scavenging enzymes that protect the cells against ozone-induced toxins (19). Data in the present study suggest that EDU can induce new isozymes, protect ozone-sensitive isozymes against alterations by ozone, and possibly modify isozyme quantities. The results presented in this report, however, raise a major unanswered question regarding EDU activity in plant tissue. Why were there dissimilar isozymic responses between callus tissue from the two alfalfa strains when grown on media with EDU added? The isozymic composition of ozone-sensitive stem callus was especially responsive to the presence of EDU.

Some isozymic bands that appeared in the profiles stained for total proteins were seen again in the corresponding peroxidase profiles. In both strains of leaf callus, there were groups of three or four fast-moving bands among the proteins that failed to appear in either of the corresponding peroxidase profiles. This was not the situation, however, for stem callus of either strain. Instead, bands in their peroxidase profiles were distributed generally among the ranges of R_f values that were determined for bands in analogous profiles for total proteins.

All profiles of leaf callus contained band R_f 0.60; however, it was found elsewhere only once which was in the total protein profile of ozone-sensitive stem callus from media containing EDU. Furthermore, it was affected by ozone only when present in ozone-sensitive leaf callus from EDU-free medium.

Results have been presented in this study which can form a basis for reexamining certain previously held ideas concerning the predisposal of certain plants to ozone injury. Certainly, in a callus tissue system, many of the structural and physiological encumbrances associated with studying the mechanism of ozone injury in whole plants are eliminated. Furthermore, callus tissues from plant strains with demonstrated differences in ozone susceptibility will produce isozymic profiles that are distinctly different. These profiles can be modified through ozonation of the callus or by the addition of substances in its growth medium.

Further work is needed to characterize the isozymes of callus from a variety of ozone-sensitive and ozone-insensitive plants. Furthermore, the composition of the culture media, particularly the content of EDU, should be studied for possible influence on gene expression in ozone-sensitive germplasm.

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