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Biochemical Plant Responses to Ozone[1]

I. Differential Induction of Polyamine and Ethylene Biosynthesis in Tobacco

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ABSTRACT

Polyamine metabolism was examined in tobacco (*Nicotiana tabacum* L.) exposed to a single ozone treatment (5 or 7 hours) and then postcultivated in pollutant-free air. The levels of free and conjugated putrescine were rapidly increased in the ozone-tolerant cultivar Bel B and remained high for 3 days. This accumulation was preceded by a transient rise of L-arginine decarboxylase (ADC, EC 4.1.1.19) activity. The ozone-sensitive cultivar Bel W3 showed a rapid production of ethylene and high levels of 1-aminocyclopropane-1-carboxylic acid after 1 to 2 hours of exposure. Induction of putrescine levels and ADC activity was weak in this cultivar and was observed when necrotic lesions developed. Leaf injury occurred in both lines when the molar ratio of putrescine to 1-aminocyclopropane-1-carboxylic acid or ethylene fell short of a certain threshold value. Monocaffeoyl-putrescine, an effective scavenger for oxyradicals, was detected in the apoplastic fluid of the leaves of cv Bel B and increased upon exposure to ozone. This extracellular localization could allow scavenging of ozone-derived oxyradicals at the first site of their generation. Induction of either polyamine or ethylene pathways may represent a control mechanism for inhibition or promotion of lesion formation and thereby contribute to the disposition of plants for ozone tolerance.

Ozone (O_3) is an important constituent of photochemical air pollution and is considered to have serious effects on vegetation (14). Similar seasonal means of 0.04 to 0.06 μL/L, and peak episodes of 0.1 to 0.2 μL/L O_3 are found in Europe and North America (11, 12). In laboratory experiments, O_3 was shown to be phytotoxic at high concentrations (>0.2 μL/L) given for short time periods. O_3 applied at low levels ($\leq$0.15 μL/L) is also known to affect growth and development of plants when the exposure periods are in the range of weeks or months. The effects include a decline in net photosynthesis, foliar injury, altered patterns of flower formation, reduction in shoot and root growth, accelerated senescence of organs, and reduction in crop yield (11, 12, 14). O_3 tolerance is known to vary widely between species and cultivars. The families Fabaceae and Solanaceae, which include many crop plants, seem to be particularly sensitive (11, 14). Leaf damage as the most obvious pollutant effect can be observed as pigmented lesions, bleaching, chlorosis, and bifacial necrosis, depending on O_3 dose and plant species. Cigar wrapper varieties of tobacco were among the first plants for which O_3 damage ('weather flecks') was demonstrated (11). O_3-tolerant and -sensitive lines were selected in the late 1950s. A hypersensitive line (Bel W3) from these breeding programs has been used for over 30 years as a biomonitor plant for O_3 and responds to ambient concentrations with necrotic flecks that occur predominantly on the middle-aged leaves (11).

Exposure of plants to subacute levels of O_3 is now known to induce many biochemical and physiological changes (1, 13, 25). Several of these reactions form part of the plant's defense system toward oxidative stress, *e.g.* ascorbic acid and peroxidases (13), phenolic compounds (25), and polyamines (3, 13). The diamine putrescine, as well as spermidine and spermine, all commonly termed polyamines, exert several functions which counteract O_3 effects. They have been implicated in an inhibition of lipid peroxidation of membranes (27), an activation of membrane-bound ATPases (13), and a reduction of ethylene formation (6, 26). Polyamines were observed to accumulate in O_3-treated barley (24), wheat (23), and Norway spruce (4, 25). Ethylene, which appears to be antagonistic to polyamines in senescence, is induced by O_3 in many plant species (5, 11, 29). It has been suggested that polyamines protect plants from O_3 damage by decreasing the production of O_3-induced ethylene (24). On the other hand, ethylene and polyamine metabolism were not tightly coupled in O_3-treated barley (23).

We have shown in a previous communication (3) that root-applied polyamines increased the foliar levels of polyamines in the tobacco cultivar Bel W3. Concomitantly, O_3-induced foliar injury was reduced. It was further demonstrated that polyamine conjugates, but not the free polyamines, were effective scavengers for oxyradicals (3). The objective of the present study was to establish a role of polyamines in ozone tolerance *in vivo*. Therefore, the induction kinetics of free polyamines, polyamine conjugates, and ethylene were compared in an O_3-tolerant and an O_3-sensitive tobacco cultivar. The occurrence of polyamine conjugates in the apoplastic fluid of tobacco leaves is also reported.

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MATERIALS AND METHODS

Chemicals

Cinnamoylputrescines were a gift from Prof. G. Höfle, GBF Braunschweig. MACC[3] was provided by Prof. N. Amrhein, ETH Zürich, and DFMA and DFMO by Dr. E. W. H. Bohme, Merrell-Dow, Cincinnati, OH. All other chemicals were obtained commercially and were of analytical grade.

Plant Culture

Seeds of tobacco (*Nicotiana tabacum* L. cv Bel W3 and Bel B) were sown in a 2:1 (v/v) mixture of standard substrate (Frühstorfer, type T, Archut, D-Lauterbach) and Perlite. Seedlings emerged after 10 d and were transferred to 2-L pots (14 cm diameter) after 3 weeks of further culture. The plants were grown in a controlled environment cabinet (Heraeus-Vötsch, Balingen) at 25/20 ± 1°C day/night temperatures, 16 h photoperiod (6:00–22:00 h), 100 $\mu E \cdot s^{-1}$ m^{-2} (50 $W \cdot m^{-2}$) light intensity (cool-white fluorescent tubes, Sylvania GTE FR 96T12) and 70 ± 5% RH. The plants were fertilized weekly with a 10:4:7 NPK mixture (Substral, Bärnangen) and pots were watered daily to saturation with deionized water.

Conditions of Treatment

Tobacco plants (8–10 weeks old, vegetative stage) were exposed to a single period of O_3 for 5 or 7 h in plexiglass chambers. Four exposure chambers (108 × 71 × 95 cm) were placed in a walk-in growth cabinet (10 m^2). Chamber conditions included 25/20 ± 0.5°C day/night temperatures, 16 h photoperiod (6:00–22:00 h), 100 $\mu E \cdot s^{-1}$ m^{-2} light intensity and 70 ± 5% RH and were similar to those of the precultivation period. The subchambers drew conditioned air from the large cabinet and used a vertical, single-pass through air circulation (air exchange 2.5 volumes/min). O_3-Free air was applied to all plants during pre- and postcultivation and to control plants during the experimental period. To obtain pollutant-free air, ambient air was passed through a filter system consisting of five units (particle, activated charcoal, $KMnO_4$-coated alumina granules (Purafil II), charcoal, particle). This system effectively removed O_3, SO_2, and nitrous oxides to concentrations below 0.01 μL/L. For pollutant treatments, O_3 was added to conditioned air by mass flow controllers (MKS). It was generated by electrical discharge in dry oxygen (500 M, Fischer, Köln). Air from the exposure chambers was sampled with teflon lines, and O_3 concentrations were measured at the plant level with a CSI 3100 O_3 analyzer (Messer-Griesheim, München). O_3 analyzers were calibrated in regular time intervals by the stoichiometric reaction of $NO + O_3 \rightarrow NO_2 + O_2$ and NO and NO_2 were measured by chemoluminescence (CSI 1600). A switching system was used to monitor air samples from the four subchambers (5 min each) and O_3 concentrations were automatically recorded.

[3] Abbreviations: MACC, *N*-malonyl-ACC; DFMA, difluoromethylarginine; DFMO, difluoromethylornithine; ADC, L-arginine decarboxylase; ODC, L-ornithine decarboxylase; ACC, 1-aminocyclopropane-1-carboxylic acid; DAO, diamine oxidase.

Determination of Leaf Injury and Harvests for Biochemical Analysis

Leaf injury determinations and biochemical analyses were always carried out in leaves 3 and 4 counted from the top (length of leaf 1 was >8 cm). These leaves showed a uniform distribution of lesions on the entire surface (cv Bel W3) after 48 h (*cf.* ref. 11). Injury was determined visually as percentage of leaf area, and the data were calibrated with a planimeter (Delta T). For biochemical analyses, leaf discs (10 mm diameter) were cut from the midleaf with a cork borer avoiding major veins, rapidly frozen in liquid N_2, and stored at −80°C.

Polyamine Extraction and Analysis

Leaf samples (0.2 g) were homogenized in liquid N_2 and taken up in 5 mL of 5% (v/v) perchloric acid. Soluble free polyamines were analyzed in the supernatant fraction after centrifugation at 20,000*g* for 20 min. Total soluble polyamines were analyzed after acid hydrolysis of the supernatant. Aliquots (1.5 mL) were treated with 1.5 mL of 12 N HCl and hydrolyzed for 16 to 18 h at 110°C in sealed glass ampoules. Dansylation of the polyamines was performed as previously described (3). Dansyl chloride was allowed to react overnight, and dansylated products were extracted into toluene (2 × 1 mL). Toluene from 24 samples was simultaneously removed with a Savant Speed Vac concentrator (UniEquip, Martinsried). The dansylated polyamines were redissolved in MeOH and stored at −80°C. They were separated by HPLC on a Spherisorb ODS II 5 μm column (250 × 4.6 mm; Bischoff), with a MeOH-water gradient (0–5 min: 65%, 6–9 min: 65–80%, 10–25 min: 80–95% MeOH). A Beckman HPLC system (2 M4M solvent delivery modules, 450 data system controller) was used. Polyamine contents were determined with a spectrofluorimeter (RF-530, Shimadzu) at excitation and emission wavelengths of 360 and 510 nm, respectively. Amounts of free polyamines were subtracted from total polyamines after acid hydrolysis in order to estimate the amounts of soluble conjugates. Agmatine, 1,3-diaminopropane, putrescine, spermidine, tyramine, and spermine standards were run each time polyamine levels were analyzed. 1,6-Diaminohexane was used as an internal standard. Polyamine conjugates were separated by TLC according to Negrel (21).

Enzyme Extraction and Assays

ADC and ODC activities were assayed by a procedure modified from Tiburcio *et al.* (28). Leaf tissue (1 g) was ground with quartz sand in a chilled mortar in 5 mL of 0.1 M Tris-HCl (pH 7.5) containing 10% (v/v) glycerol, 10 mM DTE, 20 mM Na ascorbate, 5 mM EDTA, and 1 mM pyridoxal phosphate. The homogenate was centrifuged at 20,000*g* for 15 min (4°C). The assay mixture contained (in a final volume of 1 mL) 0.5 mL of clear supernatant, 0.1 M Tris-HCl (pH 7.5), 3.4 kBq L-[U-^{14}C]arginine (12.7 $TBq \cdot mol^{-1}$), and unlabeled arginine to give a final concentration of 10 mM for ADC assays. L-[1-^{14}C]Ornithine (2.17 $TBq \cdot mol^{-1}$), 3.4 kBq, and unlabeled L-ornithine (25 mM final concentration) were added for the determination of ODC activity. Incubations were carried out for 60 min at 37°C in glass tubes under N_2

atmosphere and were terminated by adding 1 mL of 2 N H_2SO_4. $^{14}CO_2$ was subsequently trapped in a center vial containing 0.4 mL of 2-aminoethanol and 2-methoxyethanol (1:2, v/v) for 45 min and was quantified by liquid scintillation counting (LS 1801, Beckman). DFMA or DFMO (1 mM) were added to the reaction mixture 40 min prior to substrate. DFMA, but not DFMO, reduced $^{14}CO_2$ release in ADC assays to less than 10%.

Analysis of Phenolic Compounds in the Apoplastic Fluid

Leaves were harvested 24 h after the start of O_3 exposure, washed with Milli Q water, and infiltrated *in vacuo* (at 200 mbar, 60 s, followed by a controlled release over 30 s) with 50 mM Tris-HCl (pH 7.0), 100 mM KCl. The infiltration fluid obtained by this method contained 0.5% or less of the glucose-6-phosphate dehydrogenase activity of the leaf and no measurable Chl. After infiltration, the leaves were blotted with absorbent tissue and rolled up cylindrically into 5-mL Combitips (Eppendorf). These were placed into centrifuge tubes and centrifuged at 1000*g* for 10 min at 4°C. The infiltration fluid was collected in Eppendorf vials at the bottom of the tubes. One g of leaf tissue typically yielded 0.25 to 0.35 mL of infiltration fluid. The fluid was filtered (Millipore HV, 0.45 μm) and 20 μL samples were separated by reversed phase HPLC (25). The following conditions were used: 0.1% ammonium formate containing 2% (v/v) formic acid for 5 min, followed by a gradient from 0 to 90% (v/v) MeOH within 45 min, 1 mL·min^{-1}. Peaks were detected with a Beckman 165 UV/Vis analyzer at 280 and 340 nm, a Shimadzu spectrofluorimeter (excitation 340, emission 400 nm), or a Waters 990 diode array detector. Monocaffeoyl-putrescine had a retention time of 19.8 min, and both the *p*-coumaroyl and feruloyl conjugates eluted at 22.0 min.

Determination of Ethylene Production and ACC and MACC Levels

Ethylene production from leaf discs was determined according to Grossmann *et al.* (9). Twelve leaf discs (0.16 g) were placed adaxially on filter papers moistened with 1 mL of 50 mM Mes/NaOH (pH 5.6) containing 2% (w/v) sucrose. The filter papers were rolled cylindrically and placed into glass tubes. These were sealed with a silicone septum (Verneret, France) and incubated for 1 h at 25 ± 1°C. One-milliliter gas samples were withdrawn with a syringe, and ethylene was measured by gas chromatography on a HP-5890A equipped with a Porapak Q (80–100 mesh, 1.83 m × 2 mm) column and a flame ionization detector. Column, injector, and detector temperatures were 50, 150, and 200°C, respectively. Retention times for ethylene and ethane were 1.9 and 2.6 min, respectively. For ACC determinations, leaf samples (0.2 g) were homogenized in liquid N_2 and extracted with 1 mL 80% (v/v) ethanol. The pellet after centrifugation (10,000*g*, 5 min) was reextracted two times (0.5 mL). The supernatants were combined and concentrated as above. The residue was dissolved in 1 mL of Milli Q water and was extracted with 1 mL of $CHCl_3$. The water phase was concentrated to dryness *in vacuo* and dissolved in 1 mL of H_2O. The ACC content was assayed according to Lizada and Yang (17) and ethylene determined as above. MACC was quantified after acid hydrolysis (2 N HCl, 3 h, 120°C) of the ethanol extracts by subtracting the ACC value of the nonhydrolyzed sample from that of the hydrolyzed.

All experiments were repeated on two to four separate occasions with three to six parallels. Representative results are shown. Where indicated, the least significant difference (LSD) multiple range test was used to test for differences among treatment means.

RESULTS

Ozone-Induced Foliar Injury

A single exposure to O_3 for 7 h resulted in foliar necrosis of the middle-aged leaves of tobacco plants (Fig. 1). Lesion formation increased linearly with the O_3 dose from 10 to 80% of the leaf area in both cultivars. Threshold values were 0.15 μL/L O_3 for the O_3-sensitive cv Bel W3 and 0.35 μL/L O_3 for the O_3-tolerant cv Bel B ($P < 0.05$). This variation in O_3 tolerance was probably not due to differences in the internal O_3 dose within the leaf. The number of stomata (95 ± 6 and 103 ± 4 mm^{-2} lower leaf area) as well as stomatal conductance during short term exposure (46 ± 10 and 38 ± 9 mmol·m^{-2} s^{-1}) of the cv Bel B and Bel W3 were not significantly different. The symptoms were similar in both lines and consisted of small (1–2 mm diameter) flecks which developed between 15 and 72 h postcultivation (data not shown) and were randomly distributed on the middle-aged leaves (*cf.* ref. 11). A standard exposure of 0.15 μL/L O_3 for 5 or 7 h was chosen. This treatment resembles common peak concentrations during the summer (11, 12). It resulted in foliar lesions on 30% of the

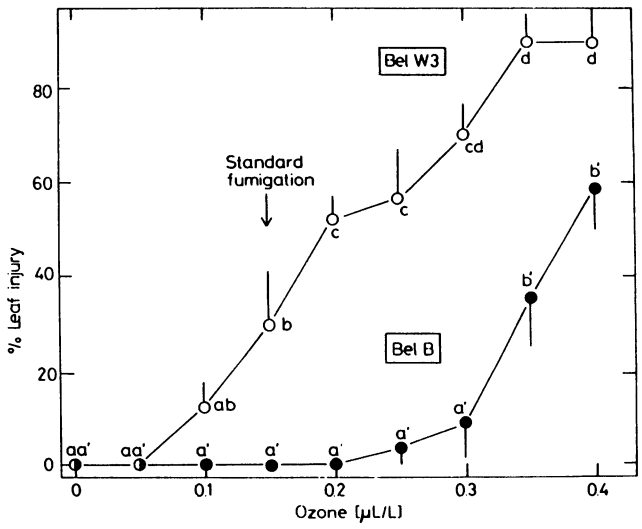

Figure 1. Leaf injury of tobacco cv Bel W3 and Bel B in response to increasing ozone concentrations. The plants were exposed to filtered air (control) or one of eight ozone levels for 7 h and postcultivated in pollutant-free air. Leaf injury in percentage of total leaf area was scored after 2 d on the middle-aged leaves 3 and 4. For the following time course studies, a standard fumigation (0.15 μL/L, 5–7 h) was used. Means (n = 3–5) followed by the same letter are not significantly different ($P < 0.05$) according to the LSD multiple range test.

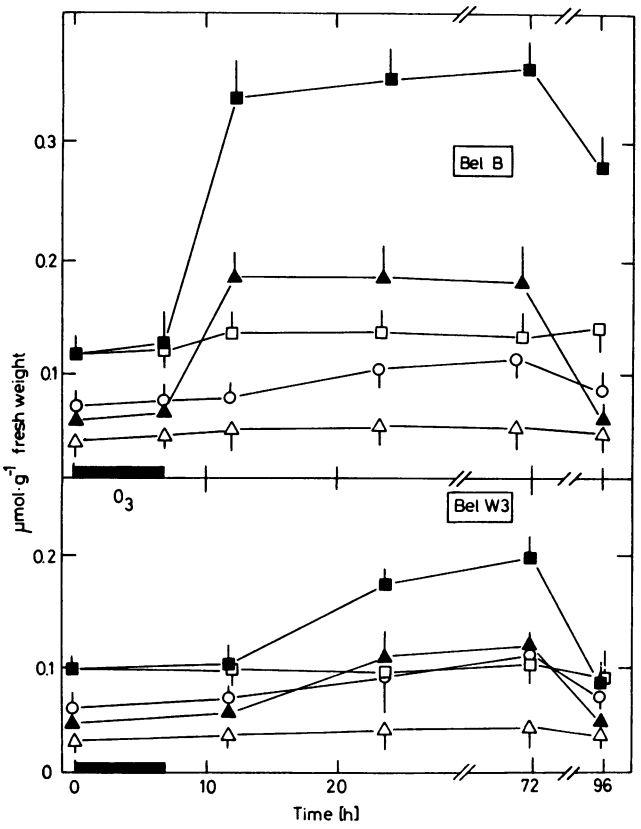

Figure 2. Time course of polyamine titers in leaves from ozone-treated and control tobacco plants. Plants were exposed to 0.15 µL/L ozone or pollutant-free air for 7 h and free and conjugated polyamines were determined at the times indicated according to "Materials and Methods." Symbols: Treated plants: conjugated putrescine (putrescine after acid hydrolysis of the supernatant minus free putrescine)(■); free putrescine (▲); total (free and conjugated) spermidine (○); total spermine (△). Control plants: conjugated putrescine (□). Bars represent ± SE (n = 3).

leaf area in cv Bel W3 and no visible injury in the O_3-tolerant cv Bel B.

Polyamine Induction in Response to Ozone

Polyamine titers were followed over 4 d after exposure to O_3 (7 h; Fig. 2). Twelve hours after the start of exposure, perchloric acid-soluble fractions of O_3-treated Bel B contained 3 to 4 times more free and soluble conjugated putrescine than control plants ($P < 0.001$). The contents remained high for 3 d of postcultivation in filtered air and then declined. In contrast, putrescine levels of the cv Bel W3 were slightly lower initially (Table I) and were not induced before 24 h (Fig. 2). The induction (twofold) of putrescine correlated in time with lesion formation in this cultivar. The levels of free and conjugated spermidine were only slightly elevated by 40 or 100%, respectively, within 24 h (Fig. 2, Table I), while no changes in spermine (Fig. 2) and cadaverine concentrations (data not shown) were found.

The activity of ADC rose sevenfold in O_3-treated tobacco Bel B with no apparent lag phase and peaked 8 h after the beginning of O_3 exposure (Fig. 3). ADC activity fell to the original levels within 24 h in this cv. In cv Bel W3, ADC activity slowly increased fourfold between 12 and 24 h. ODC (EC 4.1.1.17) had a comparable activity in both cultivars (0.2 pkat g^{-1} fresh weight) and was not significantly altered upon O_3 exposure. ADC and ODC activities remained constant in control plants cultivated in pollutant-free air.

The effects of increasing O_3 concentrations (given for 7 h) on putrescine and spermidine levels after 24 h are shown in Table I. At this time, near maximum levels were reached in both cultivars. Putrescine and spermidine levels were generally lower in the O_3-sensitive as opposed to the O_3-tolerant tobacco cultivar. Soluble free putrescine in both cultivars was increased 2- to 3-fold by the lowest O_3 concentration applied (0.1 µL/L; 0.7 µL/L·h) and declined at higher concentrations. Putrescine conjugates in cv Bel B increased markedly with the O_3 concentration applied and were highest (5.5-fold compared to control values) at 0.25 µL/L (1.75 µL/L·h) O_3 where lesion formation started to some extent in this cultivar (Fig.

Table I. *Effect of Ozone Exposure on Putrescine and Spermidine Levels of Leaves of Tobacco cv Bel W3 (O_3-sensitive) and Bel B (O_3-tolerant)*

Free and soluble conjugated polyamines were determined 24 h after exposure to O_3 (7 h) by HPLC analysis of dansylated polyamines. Means (n = 4–6) within the same column followed by the same letter are not significantly ($P < 0.05$) different according to the LSD Multiple Range Test.

Ozone	Putrescine				Spermidine			
	Free		Conjugated		Free		Conjugated	
	Bel W3	Bel B	Bel W3	Bel B	Bel W3	Bel B	Bel W3	Bel B
µL/L	nmol·g⁻¹ fresh wt							
0	51a	77a	111a	137a	21a	38a	54a	76a
0.1	131b	223c	130ab	339b	31ab	52ab	53a	69a
0.15	118b	225c	168b	367b	28a	44a	71b	72a
0.2	108b	158b	258c	ND[a]	29a	41a	93b	78a
0.25	97b	149b	337c	569c	30a	42a	68ab	157b

[a] Not determined.

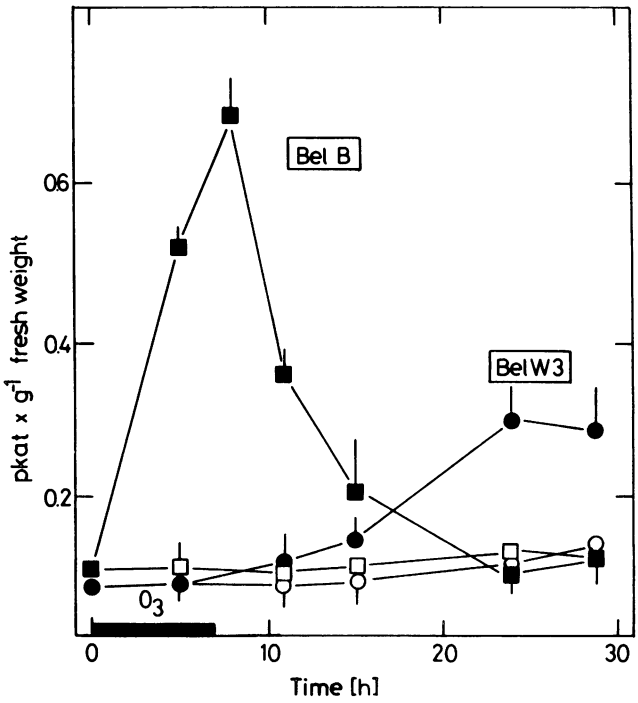

Figure 3. Time course of arginine decarboxylase activity. Tobacco plants were treated with 0.15 μL/L ozone for 7 h or received pollutant-free air. The assay and preparation of plant material were carried out as described in "Materials and Methods." Symbols: activity of extracts from fumigated plants of cv Bel B (■); activity from fumigated plants of cv Bel W3 (●); activity from control plants, cv Bel B (□); activity from control plants, cv Bel W3 (○). Bars represent ± SE (n = 4–6).

1). Bel W3 responded significantly ($P < 0.05$) with elevated levels of putrescine conjugates at 0.15 μL/L O_3 and above (Fig. 2, Table I). The level of free spermidine increased to some extent at ≥0.1 μL/L O_3, while soluble conjugated spermidine was induced at 0.15 or 0.25 μL/L O_3 in cv Bel W3 and Bel B, respectively.

Extracellular Localization of Putrescine Conjugates

Monocaffeoyl-putrescine was the main putrescine conjugate in both tobacco cultivars and occurred together with feruloyl- and *p*-coumaroyl-putrescine (3). To study the occurrence of polyamine conjugates in the apoplastic space, leaves of the tobacco cultivar Bel B were vacuum-infiltrated with buffer. The infiltration fluid was subsequently collected by centrifugation of the leaves and was subjected to HPLC analysis. Several UV-absorbing compounds were detected (Fig. 4). Three of them showed elevated levels (two- to fourfold) upon O_3 exposure. The component No. 1 eluting at 19.8 min (Fig. 4) was increased fourfold in O_3-treated Bel B plants. It was identified as monocaffeoyl-putrescine, by cochromatography with reference material in TLC, and HPLC combined with UV/VIS diode-array analysis (λ_{max} = 293 and 319 nm). The monocaffeoyl-putrescine content of the infiltration fluid was 5 to 10% of the total conjugated putrescine of the leaf (20–140 nmol/mL of infiltration fluid from control and treated plants, respectively). Assuming that the cell wall comprises 50% of the fresh weight of the cell and that the cell wall is 60% water, a caffeoyl putrescine concentration of 2×10^{-5} M (controls) up to 4×10^{-4} M in the apoplastic fluid of O_3-treated plants was calculated.

Ethylene Induction in Response to Ozone

O_3 treatment (0.15 μL/L, 5 h) had significant effects on ethylene biosynthesis in the O_3-sensitive cultivar. Leaf discs taken from O_3-treated Bel W3 showed a rapid (maximum after 1–2 h) and massive (7-fold) surge of ethylene production (Fig. 5). Standard errors for O_3-induced ethylene formation were high (up to 20%), but consistent effects (induction 5- to 12-fold) were found in five independent experiments in all plants which developed necrotic lesions at a later stage of cultivation. A 2-fold increase (not significant at $P < 0.05$) was found for cv Bel B. In both cultivars, ethylene returned to the original levels by the end of the 5 h exposure period. The amount of ethylene production and ACC levels was influenced by both the O_3 dose and the plant cultivar (Table II). Ethylene formation was significantly ($P < 0.05$) increased at 0.2 (Bel W3) and at 0.3 μL/L O_3 (Bel B). ACC concentrations after 1 h of exposure were elevated up to 5-fold (Bel B) or 65-fold (Bel W3) at 0.4 μL/L O_3. MACC levels in O_3-treated Bel W3 plants were slightly higher as opposed to those of cv Bel B but were not significantly altered by the pollutant treatment.

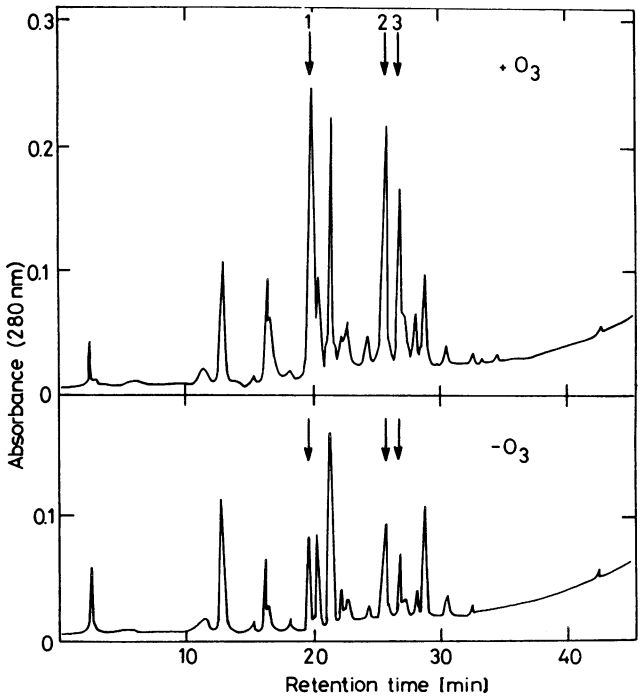

Figure 4. HPLC analysis of phenolic compounds in the infiltration fluid of O_3-treated (+ O_3) and control (− O_3) tobacco plants of cv Bel B. Leaves were harvested 24 h after the beginning of ozone exposure (0.15 μL/L, 5 h) and were vacuum infiltrated with buffer. The infiltration fluid was collected by centrifugation of infiltrated leaves and was subjected to reversed phase HPLC as described in "Materials and Methods." The elution positions of ozone-induced compounds are marked by arrows.

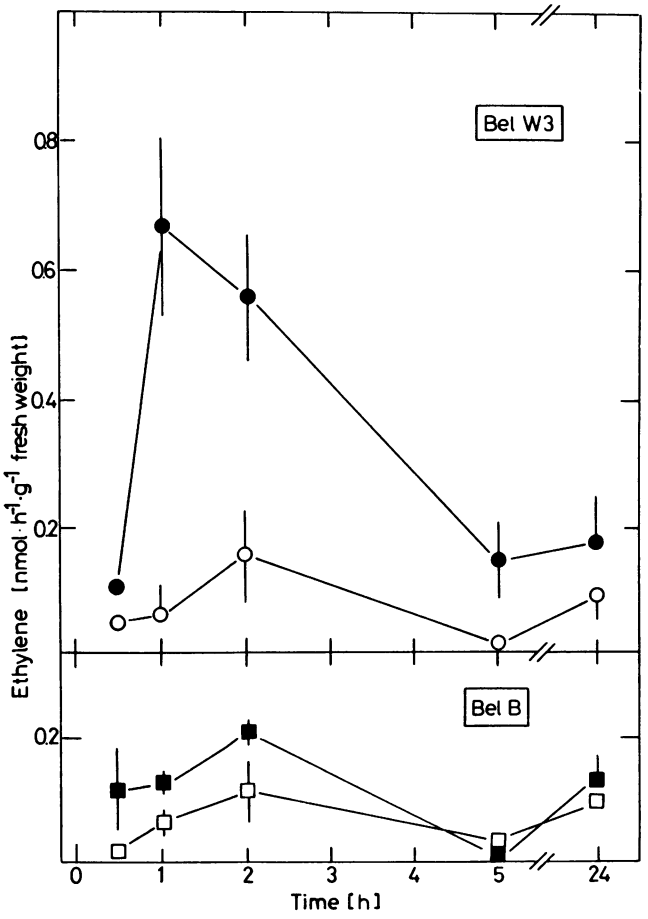

Figure 5. Time course of ethylene production from tobacco leaf discs. Plants of the cultivars Bel W3 and Bel B were treated with 0.15 μL/L ozone (0.5–5 h, ●, ■) or received ozone-free air (○, □). Leaf discs were taken at the indicated times and were incubated for 1 h in glass tubes. Ethylene formation by leaf discs was determined by gas chromatography. Bars represent ± SE (n = 4–6). Symbols as in Figure 3.

DISCUSSION

Feeding of exogenous polyamines to tomato (22) and tobacco plants (3) resulted in a significant suppression of O_3-induced leaf injury. In the O_3-sensitive tobacco cultivar Bel W3, this protective effect correlated with increased foliar levels of free, soluble conjugated, and wall-bound polyamines (3). The results presented in this paper show that endogenous polyamine metabolism is also responsive to O_3 exposure *in vivo* and that either polyamines or ethylene are predominantly induced in tolerant *versus* sensitive tobacco cultivars.

Polyamine metabolism in barley, wheat, and Norway spruce responded to O_3 exposure for several days (4, 23, 24). ADC activity and spermidine levels rose in parallel in O_3-treated barley. A pretreatment of the plants with DFMA, a specific inhibitor of ADC, resulted in an increase of the O_3-induced foliar injury. It was postulated that ADC modulates the plant's sensitivity to the pollutant (24). The results obtained here with tobacco strengthen the role of polyamines in an O_3 response. O_3 caused induction of both free and conjugated endogenous polyamines, with marked effects for putrescine and less prominent for spermidine. Kinetic studies revealed two types of polyamine induction in cultivars of differential O_3 sensitivity. (a) In the O_3-tolerant cv Bel B, ADC activity was increased and putrescine was accumulated very rapidly (maxima at 8 and 12 h, respectively) and at O_3 doses which did not cause any visible injury. The endogenous putrescine levels of the leaves were similar to those found to protect cv Bel W3 from O_3 damage after exogenous supply (3). Putrescine induction in cv Bel B was one of the earliest O_3-induced biochemical responses observed in tobacco and preceded that of phenylalanine ammonia-lyase and lytic enzymes (15, 25). These results suggest that the response of polyamines was rapid and massive enough to play a role in the inhibition of foliar injury. (b) In the O_3-sensitive cv Bel W3, as well as in visually injured cv Bel B, a second type of induction occurred. This reaction was slower and correlated with lesion development. *De novo* formation of polyamine conjugates is also known to occur during the hypersensitive response following infection of tobacco plants with TMV and

Table II. *Effect of Ozone Exposure on Ethylene Production and on ACC and MACC Levels in Leaves of Tobacco cv Bel W3 (O_3-sensitive) and Bel B (O_3-tolerant)*

Plants were exposed to ozone for 1 h. Leaf discs from treated and control plants were incubated in sealed glass tubes and the ethylene formed during 1 h incubation was determined by GC. ACC and MACC contents of the leaves after 1 h of ozone exposure were assayed according to "Materials and Methods." The molar ratio of the total putrescine (after 24 h) to ACC content (after 1 h) of the leaves was calculated for all ozone treatments (putrescine and ACC values for control plants of cv Bel B = 100%). Standard errors (n = 4–6) did not exceed 5% (ACC and MACC) or 10% (ethylene), respectively. Statistical analysis was carried out as in Table I.

Ozone	Ethylene		ACC		MACC		Put/ACC Ratio	
	Bel W3	Bel B	Bel W3	Bel B	Bel W3	Bel B	Bel W3	Bel B
μL/L	nmol·g⁻¹ fresh wt·h⁻¹		nmol·g⁻¹ fresh wt					
0	0.14a	0.13a	0.17a	0.13a	3.33ab	3.35a	0.56	1.00
0.1	0.16a	0.09a	0.17a	0.17a	4.53a	3.32a	0.63	1.93
0.2	0.48b	0.16a	0.25a	0.20a	3.60ab	1.77b	0.44	1.93
0.3	0.56b	0.39b	4.52b	0.28a	2.95a	2.58ab	0.028	1.58
0.4	1.56c	0.47b	11.12c	0.75b	3.05ab	2.20ab	0.011	0.61

fungal pathogens (8). The conjugates accumulate in living cells surrounding the necrotic spots. A similar induction of putrescine and spermidine conjugates may occur under O_3 doses which lead to acute damage.

Early accumulation of polyamines could exert a protective function toward O_3 exposure in several ways that include plasmalemma and cell wall microenvironments. O_3 is known to increase membrane permeability and efflux of cations in plants (13). An increased cell permeability for organic compounds and concomitant loss of K^+, Ca^{2+}, and Mg^{2+} from the leaf interior was shown in O_3-treated European beech. The efflux of cations correlated with an increase in their extracellular fractions (16). Polyamine levels may therefore respond to Ca^{2+}/K^+ imbalances or pH changes in the cell wall and act through stabilizing membranes and reestablishing ionic balances across the plasmalemma (13, 26).

O_3 exposure leads to an increase of oxyradicals and peroxides in the liquid layer surrounding the mesophyll cells, which is the first barrier for O_3 once it enters the leaf (1, 13). Due to the short lifetime of oxyradicals, effective scavengers for O_3-derived radicals should be located at this site of generation. O_3-induced changes in apoplastic ascorbic acid have been reported for several plant species (13). It has been shown here that hydroxycinnamic acid amide conjugates of putrescine, which are scavengers for oxyradicals (3), accumulated upon O_3 exposure. Caffeoyl-putrescine occurred extracellularly (5–10% of the molar content in the leaf) and represented a major phenolic component of the apoplastic fluid. It was induced fourfold in O_3-treated plants. Extracellular polyamine conjugates and other yet unidentified phenolic compounds of the apoplast may thus protect cells by being scavengers for extracellular oxyradicals.

Several other reports point to an extracellular localization of amines. Free and wall-bound polyamines were found in bean (10). Tyramine and tyramine conjugates were incorporated into cell walls of tobacco (8). This pathway was induced after TMV infection (20). Catabolic enzymes for polyamines, DAO (EC 1.4.3.6) and PAO (EC 1.4.3.4) are present in the cell walls of the *Fabaceae* and *Poaceae*, respectively, and are involved in the generation of H_2O_2 in the apoplast (7, 13). Extracellular H_2O_2 may be used for peroxide-dependent reactions such as lignin or suberin biosynthesis or IAA catabolism. A close correlation between DAO activity and diamine levels on one side and lignification on the other was found in pea epicotyls (7). Other cell wall components which may undergo oxidative coupling by H_2O_2 include wall-bound phenolics and extensin subunits. DAO was also recently found in tobacco root cultures (30). In addition to their radical scavenging properties, polyamine conjugates are good substrates for peroxidases *in vitro* (18). Peroxidase isoenzymes were found extracellularly and were induced upon O_3 exposure (13). Peroxidases which utilize ascorbic acid or phenolic compounds, *e.g.* putrescine conjugates, may remove excess H_2O_2 in the apoplast. Polyamines are thus involved in both the generation and the decay of peroxides and oxyradicals. We therefore assume that extracellular polyamines have a functional role in the regulation of activated oxygen species in the apoplast. O_3 exposure may raise the existing levels of H_2O_2 and oxyradicals above a certain threshold level. A burst of superoxide and H_2O_2 was recently found as a very rapid response of plants after microbial infection (2). The common trigger in both O_3 and biotic stresses may thus be a sudden, high extracellular level of oxidants which, in turn, induces defensive pathways such as polyamine formation.

The polyamines, spermidine and spermine, and the phytohormone, ethylene, which arise from a common precursor, show opposite physiological effects by delaying and promoting senescence processes, respectively, and mutually inhibit each other's synthesis in several plant systems (6, 26). Tingey *et al.* (29) have shown for more than 20 plant species that O_3 induced the production of stress ethylene. The amount of ethylene formed correlated well with the degree of foliar injury that developed hours or days later. O_3 rapidly induced ethylene formation in wheat, but polyamine metabolism was altered only after exposure for several days. It was assumed that the two pathways were not tightly coupled (23). Inhibitor studies of either ethylene or polyamine biosynthesis indicated the involvement of both pathways in the determination of O_3 tolerance in pea and barley, respectively (19, 24). In the tobacco cultivars investigated here, either ethylene or polyamines were predominantly induced. Ethylene production and ACC levels closely correlated with foliar injury but peaked as soon as 1 to 2 h of O_3 treatment. The time course of ethylene formation and polyamine induction suggest that putrescine formation in cv Bel W3 was not started before ethylene returned to the original levels. The induction for both polyamines and ethylene was rapid enough to inhibit or promote lesion development. When the molar ratio of putrescine to ACC contents of the leaves (normalized to the levels of control plants of cv Bel B = 100%) was calculated for both cultivars, the values were below 1 for all O_3 concentrations in cv Bel W3 (Table II) and were drastically reduced at 0.3 and 0.4 μL/L O_3. Bel B showed a ratio of 1.9 at 0.1 and 0.2 μL/L O_3 which was due to the strongly induced putrescine levels. Putrescine/ACC (Table II) and putrescine/ethylene ratios (data not shown) fell below 1 when these plants were treated with higher O_3 doses which caused visible injury.

It is concluded that plant cultivars differ in their disposition for O_3 damage (25). This may be based on differential responses in antioxidant or hormone levels or in stomatal conductance as found for other plant species. It could be shown here that polyamine and ethylene pathways reacted very early and sensitively to O_3 exposure and were differentially induced in O_3-tolerant and sensitive tobacco cultivars. Once initiated, polyamines and ethylene may affect other biochemical and physiological reactions (*e.g.* hydrolytic enzyme activities) which then promote or inhibit lesion formation. Induction of either pathway may thus represent an important physiological switch to amplify O_3 effects in plants.

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LITERATURE CITED

1. **Alscher RG, Amthor JS** (1988) The physiology of free-radical scavenging: maintainance and repair processes. *In* S Schulte-Hostede, NM Darrall, LW Blank, AR Wellburn, eds, Air Pollution and Plant Metabolism. Elsevier, London, pp 94–115
2. **Apostol I, Heinstein PF, Low PS** (1989) Rapid stimulation of an oxidative burst during elicitation of cultured plant cells. Plant Physiol **90:** 109–116
3. **Bors W, Langebartels C, Michel C, Sandermann H** (1989) Polyamines as radical scavengers and protectants against ozone damage. Phytochemistry **28:** 1589–1595
4. **Dohmen GP, Koppers A, Langebartels C** (1990) Biochemical response of Norway spruce *Picea abies* (L.) Karst., towards 14-month exposure to ozone and acid mist: effects on amino acid, glutathione and polyamine titers. Environ Pollut **64:** 375–383
5. **Elstner EF, Osswald W, Youngman RJ** (1985) Basic mechanisms of pigment bleaching and loss of structural resistance in spruce (*Picea abies*) needles: advances in phytomedical diagnostics. Experientia **41:** 591–597
6. **Evans PT, Malmberg RL** (1989) Do polyamines have roles in plant development? Annu Rev Plant Physiol Plant Mol Biol **40:** 235–269
7. **Federico R, Angelini R** (1986) Occurrence of diamine oxidase in the apoplast of pea epicotyls. Planta **167:** 300–302
8. **Flores HE, Protacio CM, Signs MW** (1989) Primary and secondary metabolism of polyamines in plants. Rec Adv Phytochem **23:** 329–393
9. **Grossmann K, Häuser C, Sauerbrey E, Fritsch H, Schmidt O, Jung J** (1989) Plant growth retardants as inhibitors of ethylene production. J Plant Physiol **134:** 538–543
10. **Goldberg R, Perdrizet E** (1984) Ratio of free to bound polyamines during maturation in mung-bean hypocotyl cells. Planta **161:** 531–535
11. **Guderian R,** ed (1985) Air Pollution by Photochemical Oxidants. Springer, Berlin
12. **Heagle AS** (1989) Ozone and crop yield. Annu Rev Phytopathol **27:** 397–423
13. **Heath RL** (1988) Biochemical mechanisms of pollutant stress. *In* WW Heck, OC Taylor, DT Tingey, eds, Assessment of Crop Loss From Air Pollutants. Elsevier, London, pp 259–286
14. **Heck WW, Taylor OC, Tingey DT,** eds (1988) Assessment of Crop Loss from Air Pollutants. Elsevier, London
15. **Langebartels C, Heller W, Kerner K, Leonardi S, Rosemann D, Schraudner M, Trost M, Sandermann H** (1990) Ozone-induced defense reactions in plants. *In* HD Payer, T Pfirrmann, P Mathy, eds, Environmental Research with Plants in Closed Chambers. Air Pollution Research Reports of the EC **26:** 358–368
16. **Leonardi S, Langebartels C** (1991) Fall exposure of beech saplings (*Fagus sylvatica L.*) to ozone and simulated acidic mist: effects on gas exchange and leachability. Water Air Soil Pollut (in press)
17. **Lizada MCC, Yang SF** (1979) A simple and sensitive assay for 1-aminocyclopropane-1-carboxylic acid. Anal Biochem **100:** 140–145
18. **Martin-Tanguy J** (1985) The occurrence and possible function of hydroxycinnamoyl acid amides in plants. Plant Growth Regul **3:** 381–399
19. **Mehlhorn H, Wellburn AR** (1987) Stress ethylene formation determines plant sensitivity to ozone. Nature **327:** 417–418
20. **Negrel J, Lherminier J** (1987) Peroxidase-mediated integration of tyramine into xylem cell walls of tobacco leaves. Planta **172:** 494–501
21. **Negrel J** (1989) The biosynthesis of cinnamoylputrescines in callus tissue cultures of *Nicotiana tabacum.* Phytochemistry **28:** 477–481
22. **Ormrod DP, Beckerson DW** (1986) Polyamines as antiozonants for tomato. HortScience **21:** 1070–1071
23. **Raab MM, Weinstein LH** (1990) Polyamine and ethylene metabolism in *Triticum aestivum* (var. vona). *In* HE Flores, RN Arteca, JC Shannon, eds, Polyamines and Ethylene: Biochemistry, Physiology, and Interactions. American Society of Plant Physiologists, Rockville, MD pp 408–410
24. **Rowland-Bamford AJ, Borland AM, Lea PJ, Mansfield TA** (1989) The role of arginine decarboxylase in modulating the sensitivity of barley to ozone. Environ Pollut **61:** 95–106
25. **Sandermann H, Schmitt R, Heller W, Rosemann D, Langebartels C** (1989) Ozone-induced early biochemical reactions in conifers. *In* JWS Longhurst, ed, Acid Deposition. Sources, Effects and Controls. British Library, London, pp 243–254
26. **Smith TA** (1985) Polyamines. Annu Rev Plant Physiol **36:** 117–143
27. **Tadolini B** (1988) Polyamine inhibition of lipoperoxidation. Biochem J **249:** 33–36
28. **Tiburcio AF, Masdéu MA, Dumortier FM, Galston AW** (1986) Polyamine metabolism and osmotic stress. Plant Physiol **82:** 369–374
29. **Tingey DT, Standley C, Field RW** (1976) Stress ethylene evolution: a measure of ozone effects on plants. Atmos Environ **10:** 969–974
30. **Walton NJ, McLauchlan WR** (1990) Diamine oxidation and alkaloid production in transformed root cultures of *Nicotiana tabacum.* Phytochemistry **29:** 1455–1457