

# Biochemical Plant Responses to Ozone<sup>1</sup>

## II. Induction of Stilbene Biosynthesis in Scots Pine (*Pinus sylvestris* L.) Seedlings

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### ABSTRACT

Formation of the stilbenes pinosylvin and pinosylvin 3-methyl ether, as well as the activity of the biosynthetic enzyme stilbene synthase (pinosylvin-forming), were induced several hundred- to thousandfold in primary needles of 6-week-old pine (*Pinus sylvestris* L.) seedlings upon exposure to a single pulse of ozone of at least 0.15 microliters per liter. The seedlings required 4 hours of exposure as a minimum for the induction of stilbene biosynthesis when exposed to 0.2 microliters per liter ozone. Both stilbene synthase activity and stilbene accumulation increased with the duration of ozone treatment. The activity of phenylalanine ammonia-lyase and the activity of chalcone synthase, a key enzyme of the flavonoid pathway that uses the same substrates as stilbene synthase, were also stimulated about twofold by ozone. Stilbene biosynthesis appears to represent the first example of a dose-dependent biochemical response to ozone in a conifer species and may serve as a useful biomarker to study stress impacts on pine trees.

O<sub>3</sub><sup>2</sup> is a major photochemical air pollutant and is well known to be phytotoxic (4). Its average concentration in industrial countries increases 1 to 2% each year and is estimated to be 2 to 3 times higher than at the beginning of this century (12). Means of 0.04 to 0.06  $\mu\text{L/L}$  and peak episodes of 0.1 to 0.2  $\mu\text{L/L}$  O<sub>3</sub> are frequently found (4, 12). These concentrations can lead to a decline in net photosynthesis, decreases in crop yield, and visible injury in plants (4). In the last few years, O<sub>3</sub> has been discussed as a causal factor in the new type of forest decline at elevated sites in Central Europe and North America (12, 18).

Many studies of the effects of O<sub>3</sub> on the physiology and biochemistry of conifers have recently appeared (4, 21). Most of these investigations dealt with changes in photosynthesis, lipid and protein metabolism, membrane permeability, or nutrient leaching (4). Little is known, however, about the effects of O<sub>3</sub> and other stresses on secondary metabolism in conifers. More information on the subject is available in herbaceous plants (9, 14, 20), in which the biosynthesis of

phenolic compounds is one of the best studied defense reactions against infections and various abiotic stress factors, such as wounding, UV-radiation, and heavy metal ions (26). Many of these metabolites exhibit UV-absorbing, as well as radical-scavenging and antioxidative, properties (1).

Accumulation of flavonoids has been observed in the leaves of Fabaceae (9, 20) after O<sub>3</sub> exposure. Tingey *et al.* (27) reported an increase of total phenolics in O<sub>3</sub>-exposed pine seedlings, but no particular compounds have been specified. Effects of O<sub>3</sub> on the content of specific polyphenols (in particular *p*-hydroxyacetophenone, its  $\beta$ -D-glucoside picein, and kaempferol 3-O-glucoside) have been determined in spruce needles, but conflicting results were obtained (5, 10, 16).

In pine species, the stilbenes PS and PSM usually occur exclusively in the heartwood (11). Both compounds accumulate, however, in the sapwood as a response to wounding or fungal attack (11). Induction of a particulate PS-forming STS in response to UV irradiation (22) and of a soluble STS by fungal infection (3) have been demonstrated in pine seedlings. The present study describes a dose-dependent ozone-induction of stilbene biosynthesis in 6-week-old Scots pine seedlings. Some preliminary results have already been reported (21).

### MATERIALS AND METHODS

#### Chemicals

Coenzyme A esters were synthesized as described in ref. 19, modified from a method described by Stöckigt and Zenk (25). Other chemicals were obtained commercially and were of analytical grade. The radioactive chemicals were obtained from Amersham-Buchler.

#### Plant Material and Growth Conditions

Scots pine (*Pinus sylvestris* L.) seeds were purchased from the Staatliche Samenklinge Laufen (Bavaria, FRG, No. 85122, Plantage, harvest 1980). They were surface-sterilized with hydrogen peroxide (30%, v/v) for 30 min, washed with water, and sown in plastic containers (23  $\times$  7.5  $\times$  5 cm; 50 seed grains each) filled with perlite and water. The containers were covered with transparent acrylic hoods and placed into a growth chamber at 80  $\pm$  5% RH with a 14-h light period (Sylvania GTE FR 96T12, cool white; intensity: 0.15 mE  $\cdot$  m<sup>-2</sup>  $\cdot$  s<sup>-1</sup>) and 22/18  $\pm$  1°C day/night temperature. The hoods

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<sup>2</sup> Abbreviations: O<sub>3</sub>, ozone; PS, pinosylvin; PSM, pinosylvin 3-methyl ether; STS, stilbene synthase (pinosylvin-forming); PAL, phenylalanine ammonia-lyase; CHS, chalcone synthase.

were removed after 3 weeks, and a nutrient solution modified from ref. 8 was supplied (19). Every second day, seedlings were watered with demineralized water.

### Ozone Treatment

Six-week-old Scots pine seedlings were subjected to O<sub>3</sub> in four acrylic cuvettes (108 × 71 × 95 cm) placed in a walk-in growth cabinet (10 m<sup>2</sup>) as described previously (14). Each subchamber was provided with a different O<sub>3</sub> concentration. The environmental conditions were the same as used during precultivation except that RH was 90 ± 5% during the photoperiod. The O<sub>3</sub> concentration was recorded automatically every 5 min. The ozone analyzers were routinely calibrated according to ref. 14. Primary needles were harvested after the O<sub>3</sub> treatment, frozen in liquid N<sub>2</sub>, and stored at -80°C.

### Preparation of Enzyme Extracts

Primary needles from three seedlings (about 0.2 g) were ground in a chilled mortar with 0.5 g sea sand (Fluka, catalog No. 84880) and 4 mL 50 mM Hepes-KOH buffer (pH 8.2) containing 5 mM EDTA, 1 mM DTE, and 2% (w/v) soluble PVP-10 (Sigma, catalog No. 6755). The buffer was boiled and cooled under an N<sub>2</sub> atmosphere before the addition of DTE in order to deaerate it. The extraction was performed under an argon atmosphere. The homogenate was centrifuged for 5 min at 1000g (4°C) and the supernatant subjected to a second centrifugation for 5 min at 13,000g (4°C). The clear supernatant was directly used for enzyme assays. In a few experiments, the resulting pellet was resuspended in 0.1 mL of extraction buffer for enzyme measurements and the supernatant passed through a PD-10 Sephadex G-25M column (Pharmacia) equilibrated with the extraction buffer.

### Enzyme Assays

PAL activity was determined by mixing 0.1 mL enzyme extract with 10 µL L-[2,6-<sup>3</sup>H]phenylalanine (final concentration: 2 mM, 7.4 kBq · mmol<sup>-1</sup>) and 90 µL sodium borate buffer (0.2 M, pH 8.8) containing 2% (w/v) PVP-10. Incubation was for 2 h at 30°C. The reaction was stopped by the addition of 50 µL 6 M hydrochloric acid and 1.5 mL toluene. The mixture was thoroughly shaken for 20 min, centrifuged for 5 min at 13,000g (4°C), and the radioactivity of the organic phase determined by liquid scintillation counting. STS was assayed by a method modified from Schoeppner and Kindl (22). In a final volume of 0.1 mL, 1.5 nmol [2-<sup>14</sup>C]malonyl CoA (1.45 GBq · mmol<sup>-1</sup>), 2 nmol cinnamoyl CoA, and 30 µL enzyme extract were incubated at 30°C for 30 min in a buffer solution containing 20 mM Hepes-KOH (pH 7.5), 5 mM EDTA, 1 mM DTE, and 2% (w/v) PVP-10. Products formed were extracted twice with 0.3 mL ethyl acetate. The combined organic extracts were concentrated in a Speedvac centrifuge (UniEquip, Martinsried), and compounds present were separated on cellulose TLC plates with a 20% (v/v) acetic acid solvent system. Both PS and PSM had R<sub>F</sub> values of 0.2 ± 0.03. The amount of labeled stilbenes was quantified by use of a TLC Linear Analyzer (Berthold, Wildbad). CHS activity was measured with the same assay system, because both STS and CHS

enzymes utilize the same substrates. The R<sub>F</sub> value of the flavanone pinocembrin, which is the isomerization product of the initially formed chalcone, was 0.6 ± 0.03. The enzyme tests were also carried out with 4-coumaroyl CoA (4 nmol) as substrate. The R<sub>F</sub> values of the products formed were 0.05 ± 0.01 for the stilbene resveratrol and 0.4 ± 0.04 for the flavanone naringenin.

### Protein Determination

Protein was determined by the method of Bradford (2) with BSA as a standard.

### Molecular Mass Determination

O<sub>3</sub>-treated seedlings were extracted with 50 mM Tris/HCl (pH 7.5), 150 mM NaCl, 5% (v/v) glycerol, 2% (w/v) PVP-10, and 0.02% (w/v) NaN<sub>3</sub> and a 30 to 90% ammonium sulfate protein fraction was prepared. The protein solution was first passed through a sterile filter (Millex-GS, 0.22 µm, Millipore) and then loaded on a Sephacryl S 200 superfine column (2.5 × 56 cm) equilibrated in the extraction buffer and calibrated three times with a mixture of six protein standards (BSA, 66 kD, [Sigma, catalog No. 7517]; ovalbumin, 45 kD, [Sigma, catalog No. 2512]; glyceraldehyde-3-phosphate dehydrogenase, 36 kD, [Sigma, catalog No. 0763]; carboanhydrase, 29 kD, [Fluka, catalog No. 21805]; β-lactoglobulin, 18.4 kD, [Sigma, catalog No. 0130]; Cyt c, 12.3 kD [Sigma, catalog No. 3131]). Elution was performed with the extraction buffer and a flow rate of 0.7 mL · min<sup>-1</sup>. Fractions of 4 mL were collected, and 90-µL aliquots were used to determine STS activity.

### Analysis of Stilbene Metabolites

About 0.1 g primary needles, 0.3 g Celite (Sigma, catalog No. 5384), and 0.3 g anhydrous sodium sulfate were homogenized at liquid N<sub>2</sub> temperature with the aid of a Microdis-membrator II (Braun-Melsungen, FRG). After extraction with 10 mL methanol and solvent evaporation, the residue was dissolved in 2.0 mL methanol and centrifuged at 13,000g for 5 min. The clear supernatant was analyzed by reverse-phase HPLC on a Hypersil-ODS column (Beckman, particle size 5 µm; 25 cm × 4.6 mm) using the Beckman HPLC-System Gold. Samples were eluted from the column with 0.1% (w/v) ammonium formate in 2% (v/v) formic acid (5 min) followed by a gradient from 0 to 90% methanol (45 min, 1 mL/min). Detection was at 280 nm with a UV/visible light analyzer (Beckman, model 165) and by fluorescence spectrometry (excitation 300 nm; emission 400 nm) with a spectrofluorimeter (Shimadzu model RF 530). Authentic samples of PS and PSM, isolated from Scots pine heartwood, were used for identification and quantification of the compounds. Representative HPLC profiles have been published earlier (21).

### Statistics

Data are means ± SE of several replicates (*n* = 3–5). *K<sub>m</sub>* and *V<sub>max</sub>* values for enzyme substrates were determined by nonlinear regression analysis using the least squares method. Where

indicated, the LSD multiple range test was used to test for differences among treatment means.

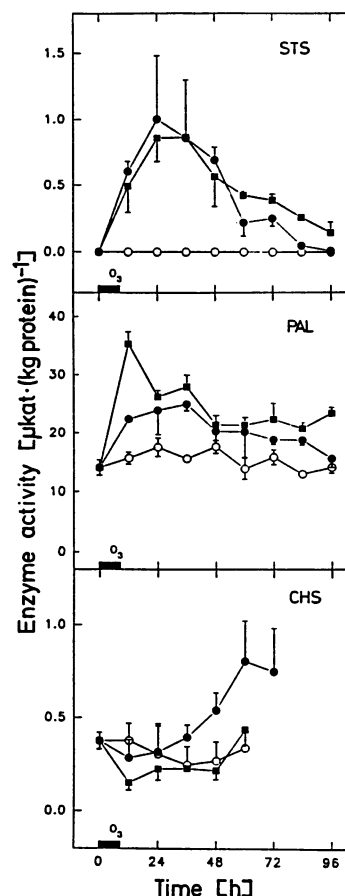
## RESULTS

### Assay of PAL, CHS, and STS

The determination of enzyme activities from conifer needle tissues is generally known to be difficult because of the presence of high amounts of phenolic and terpene metabolites. In the present study, we optimized the assay procedures for PAL, CHS, and STS, which allowed the determination of these enzymes from the same crude extract. Due to the high absorbance of the enzyme extracts, PAL was determined by a radioactive assay with [2,6-<sup>3</sup>H]phenylalanine as a substrate. The radioactive product formed was extracted with toluene and identified as cinnamic acid by TLC and HPLC. This radiometric assay was compared to the usual optical assay with light-induced PAL from parsley cell suspension cultures. Both tests gave identical enzyme activities (19). The test was linear with regard to protein (5–40  $\mu\text{g}/\text{assay}$ ) and time (at least for 3 h). A  $K_m$  value of  $1.1 \pm 0.1$  mM for L-phenylalanine was determined. The substrate concentration used in the standard assay (2 mM) led to 60 to 70% of  $V_{\max}$ . CHS and STS activities were determined in the same enzyme test. Whereas the STS of pine seedlings was previously reported to be bound to the 20,000g pellet (22), 97% of the activity was found here in the supernatant after ultracentrifugation (40,000g, 2.5 h). This is in agreement with a recent report on pine STS (3). A TLC profile of the product analysis has been published (21). The structures of the products PS and resveratrol, the stilbenes formed by STS from cinnamoyl CoA and 4-coumaroyl CoA, respectively, as well as pinocembrin and naringenin, the flavanones formed from the same substrates by CHS and a subsequent chalcone isomerase reaction, were verified by further TLC and HPLC analysis. STS activity with 4-coumaroyl CoA as substrate leading to resveratrol could not be detected in all assays. The reason for the inconsistent occurrence of this reaction has not been elucidated. STS and CHS activities showed a pH optimum near 7.8. Both reactions were linear with regard to protein between 3 and 30  $\mu\text{g}$  and with time up to 40 min. The following  $K_m$  values at 15  $\mu\text{M}$  malonyl CoA were estimated for cinnamoyl CoA:  $1.9 \pm 0.4$   $\mu\text{M}$  for STS and 7.5  $\mu\text{M}$  for CHS.  $V_{\max}$  values for STS and CHS were comparable and ranged in these experiments between 0.4 and 0.6  $\mu\text{kat} \cdot \text{kg protein}^{-1}$ . Removal of low molecular mass material from the crude extract by use of Sephadex G-25M columns did not result in a higher specific activity of the measured enzyme. STS with a specific activity of about 15  $\mu\text{kat} \cdot \text{kg protein}^{-1}$  appeared between BSA and ovalbumin after elution from a Sephacryl S200 superfine column. The molecular mass of the enzyme was estimated in two determinations as 61 and 54 kD, respectively.

### Ozone Induction of PAL, STS, and CHS Activities

The induction kinetics of PAL, STS, and CHS activities in 6-week-old pine seedlings exposed to 0.15 as well as 0.3  $\mu\text{L}/\text{L}$   $\text{O}_3$  were followed over a period of 96 h. A single  $\text{O}_3$  pulse of 8 h resulted in a strong (about 100-fold) induction of STS activity (Fig. 1). Maximum activity of about 1  $\mu\text{kat} \cdot \text{kg pro-}$

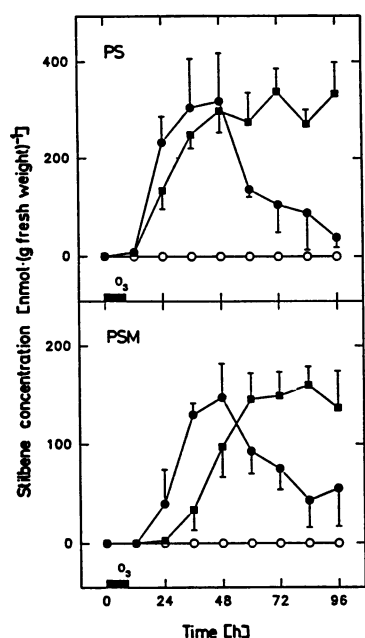


**Figure 1.** Time course of STS, PAL, and CHS activity with cinnamoyl CoA as substrate in needles of 6-week-old pine seedlings upon exposure to ozone. The seedlings were treated with 0.15 and 0.3  $\mu\text{L}/\text{L}$  ozone or pollutant-free air for 8 h, and the enzyme activities were determined according to "Materials and Methods." Symbols: enzyme activities of extracts from seedlings treated with 0.15  $\mu\text{L}/\text{L}$  ozone (●); 0.3  $\mu\text{L}/\text{L}$  ozone (■); or pollutant-free air (○). Bars represent  $\pm$  SE ( $n = 3-5$ ).

tein<sup>-1</sup> was reached between 24 and 36 h after onset of  $\text{O}_3$  and was followed by a linear decrease. The time course and degree of STS activity were nearly the same at both  $\text{O}_3$  concentrations. STS activity was not detectable in needles of control seedlings. In the same experiment, PAL activity was increased 1.5-fold with 0.15  $\mu\text{L}/\text{L}$   $\text{O}_3$  and 2.5-fold with 0.3  $\mu\text{L}/\text{L}$   $\text{O}_3$  from a control level of about 15  $\mu\text{kat} \cdot \text{kg protein}^{-1}$  (Fig. 1). In a similar experiment, CHS activity was increased in  $\text{O}_3$ -treated seedlings in addition to STS and PAL (Fig. 1). The CHS activity increased constantly after 24 h and reached levels of about twofold over controls (0.8  $\mu\text{kat} \cdot \text{kg protein}^{-1}$ ) when exposed to 0.12  $\mu\text{L}/\text{L}$   $\text{O}_3$ . Exposures to 0.3  $\mu\text{L}/\text{L}$   $\text{O}_3$  did not cause an increase of CHS activity. In contrast to PAL and STS, the activity of CHS was low and near the detection limit in some experiments.

### Ozone Induction of Stilbene Metabolites

Corresponding to the induction of the biosynthetic enzyme activities, a pronounced accumulation of the stilbenes PS and



**Figure 2.** Time course of PS and PSM accumulation in needles of 6-week-old pine seedlings upon exposure to ozone. The seedlings were treated with 0.15 and 0.3  $\mu\text{L/L}$  ozone or pollutant-free air for 8 h, and the stilbene metabolites were determined according to "Materials and Methods." Symbols: pinosylvin/pinosylvin methyl ether of extracts from seedlings treated with 0.15  $\mu\text{L/L}$  ozone (●); 0.3  $\mu\text{L/L}$  ozone (■); or pollutant-free air (○). Bars represent  $\pm$  SE ( $n = 5$ ).

PSM was observed in the primary needles of  $\text{O}_3$ -treated seedlings. Stilbenes were not detectable in control seedlings (detection limit, 5 pmol/test). The time course of PS and PSM accumulation after exposure to 0.15 as well as 0.3  $\mu\text{L/L}$   $\text{O}_3$  is shown in Figure 2. PS appeared 12 to 24 h after the onset of  $\text{O}_3$  exposure and reached a maximum of about 350 nmol·g fresh weight<sup>-1</sup> after about 48 h (Fig. 2). The increase was several thousandfold in relation to the detection limit. The amount of PS remained at the high level in seedlings exposed to 0.3  $\mu\text{L/L}$   $\text{O}_3$ . However, a steep decrease of PS to nearly

control levels within another 48 h occurred in seedlings exposed to 0.15  $\mu\text{L/L}$   $\text{O}_3$ .

In addition to PS, the consecutive product PSM was also formed after  $\text{O}_3$  treatment and showed a similar time course of accumulation. A delay of about 12 h relating to PS (Fig. 2) was observed. The accumulation of PSM in needles exposed to 0.15  $\mu\text{L/L}$   $\text{O}_3$  reached its maximum more than 12 h earlier than at 0.3  $\mu\text{L/L}$   $\text{O}_3$ . Both stilbenes were below the detection limit in the hypocotyls or in the roots of treated and untreated seedlings. The levels of the flavonoids pinocembrin and catechin ranged between 1 and 2  $\mu\text{mol}\cdot\text{g}$  fresh weight<sup>-1</sup> and were independent on the treatment.

### Dependence of Stilbene Biosynthesis on $\text{O}_3$ Concentration

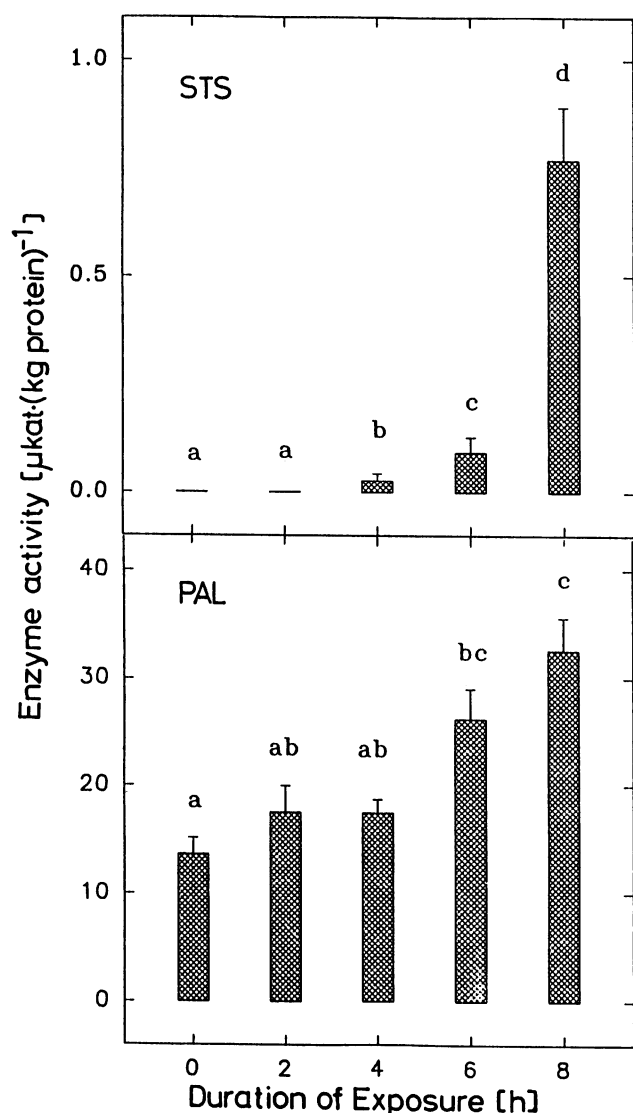
The effects of 8-h pulses of increasing  $\text{O}_3$  concentrations on enzyme activities, the stilbene concentrations, and the appearance of visible injuries are summarized in Table I. Near maximum levels of enzyme activities (at 12 or 24 h after onset of  $\text{O}_3$ ) and of stilbene contents (at 36 or 48 h after onset of  $\text{O}_3$ ) are shown. A considerable induction of STS activity (up to 100-fold) was found when a concentration of at least 0.15  $\mu\text{L/L}$   $\text{O}_3$  was applied to the seedlings. The STS activity did not change markedly at increasing  $\text{O}_3$  concentrations. Only a moderate increase in STS activity occurred at 0.12  $\mu\text{L/L}$   $\text{O}_3$ . PAL activity was slightly increased at 0.15  $\mu\text{L/L}$   $\text{O}_3$  (1.3-fold compared with the control) and rose to not more than two times higher levels at increasing  $\text{O}_3$  concentrations. A strong stilbene formation (>1000-fold) was detected in seedlings treated with more than 0.15  $\mu\text{L/L}$   $\text{O}_3$  in accordance with the induction of the biosynthetic enzymes. The amount of stilbene metabolites was at about the same level in all treatments, which was also in accordance with the induction of STS activity. Visible injuries of the needles could be detected when seedlings were exposed to at least 0.2  $\mu\text{L/L}$   $\text{O}_3$ . Lower  $\text{O}_3$  concentrations did not result in visible symptom development. About one-third of the needles lost their turgor, became first olive green, and finally necrotic between 24 and 30 h after the onset of treatment with 0.2  $\mu\text{L/L}$   $\text{O}_3$ . Needle tips became necrotic within 12 to 24 h, and injury comprised

**Table I.** Effect of Different Ozone Concentrations (Given as an 8-h Pulse) on PAL and STS Activities, on the Formation of Stilbene Metabolites, as Well as on the Appearance of Visible Injury of Primary Needles of 6-Week-Old Pine Seedlings

Near maximum levels of enzyme activities and of amounts of PS and PSM are given (means  $\pm$  SE,  $n = 3-5$ ).

| $\text{O}_3$<br>Concentration | STS                                        | PAL            | PS                                           | PSM                | Visible Injury |
|-------------------------------|--------------------------------------------|----------------|----------------------------------------------|--------------------|----------------|
| $\mu\text{L/L}$               | $\mu\text{kat}\cdot\text{kg protein}^{-1}$ |                | $\text{nmol}\cdot\text{g fresh weight}^{-1}$ |                    |                |
| 0                             | <0.01 <sup>a</sup>                         | 17.5 $\pm$ 1.6 | <0.05 <sup>a</sup>                           | <0.05 <sup>a</sup> | No             |
| 0.1                           | <0.01 <sup>a</sup>                         | 18.0 $\pm$ 2.1 | <0.05 <sup>a</sup>                           | <0.05 <sup>a</sup> | No             |
| 0.12                          | 0.06 $\pm$ 0.03                            | 18.3 $\pm$ 1.6 | <0.05 <sup>a</sup>                           | <0.05 <sup>a</sup> | No             |
| 0.15                          | 1.0 $\pm$ 0.48                             | 23.9 $\pm$ 4.2 | 317.9 $\pm$ 97.8                             | 147.8 $\pm$ 34.3   | No             |
| 0.2                           | 1.40 $\pm$ 0.28                            | 30.5 $\pm$ 1.5 | 342.0 $\pm$ 21.0                             | 149.7 $\pm$ 26.7   | Yes            |
| 0.3                           | 0.86 $\pm$ 0.18                            | 35.3 $\pm$ 2.1 | 298.1 $\pm$ 44.7                             | 146.0 $\pm$ 26.2   | Yes            |

<sup>a</sup> Detection limit.



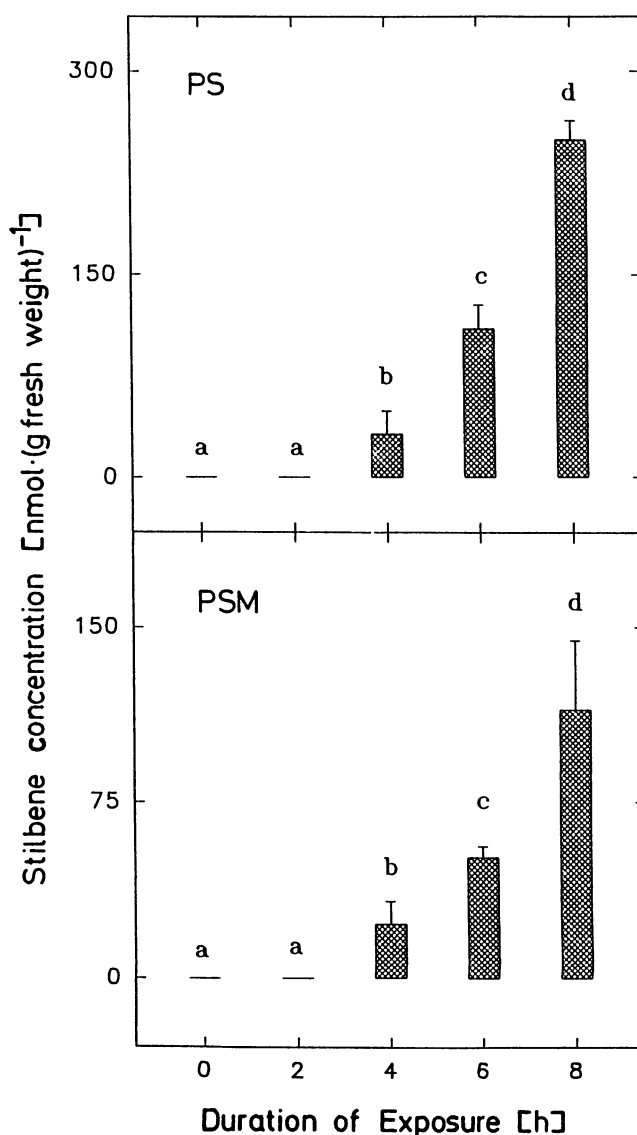
**Figure 3.** Activity of STS and PAL in needles of 6-week-old pine seedlings upon exposure to 0.2  $\mu\text{L/L}$  ozone of different durations (0, 2, 4, 6, and 8 h). The enzyme activities were determined at 36 h after onset of ozone treatment according to "Materials and Methods." Bars represent  $\pm$  SE ( $n = 4$ ). Means followed by the same letter are not significantly different according to the LSD multiple range test (STS,  $P < 0.0001$ ; PAL,  $P < 0.0005$ ).

more than two-thirds of the needle, when 0.3  $\mu\text{L/L}$   $\text{O}_3$  was applied.

#### Dependence of Stilbene Biosynthesis on the Duration of $\text{O}_3$ Exposure

Pine seedlings were exposed to 0.2  $\mu\text{L/L}$   $\text{O}_3$  for 2, 4, 6, and 8 h. Samples were harvested 36 h after the onset of treatment. Induction of STS activity occurred when the seedlings were exposed to  $\text{O}_3$  for at least 4 h and increased considerably when the pulse was prolonged to 8 h (28-fold) (Fig. 3). Exposure of seedlings for 2 h did not lead to STS induction. PAL activity also increased with advanced duration of  $\text{O}_3$

treatment but was significantly ( $P < 0.0005$ ) higher than the control only in treated seedlings after 6 h of exposure (Fig. 3). The results of PS and PSM determinations performed in parallel samples are shown in Figure 4. A strong increase of stilbene content after  $\text{O}_3$  treatment could be detected with prolonged exposure time. As for STS activity, a 4-h pulse was necessary to stimulate stilbene formation. Visible injury occurred when the seedlings were treated with  $\text{O}_3$  for at least 6 h. When seedlings were exposed to 0.1  $\mu\text{L/L}$   $\text{O}_3$  for 12 h, no STS activity and no increase of PAL activity were detected (data not shown).



**Figure 4.** Amount of PS and PSM in needles of 6-week-old pine seedlings upon exposure to 0.2  $\mu\text{L/L}$  ozone of different durations (0, 2, 4, 6, and 8 h). The amount of stilbenes was determined at 36 h after onset of ozone treatment according to "Materials and Methods." Bars represent  $\pm$  SE ( $n = 4$ ). Means followed by the same letter are not significantly different according to the LSD multiple range test ( $P < 0.0001$ ).

## DISCUSSION

O<sub>3</sub> treatment may induce the formation of phenolic compounds, which are known to be phytoalexins in herbaceous plants (20, 26). However, little is known of the influence of O<sub>3</sub> on phenylpropanoid metabolism in conifers. In this paper, we demonstrate that the stilbene metabolites PS and PSM accumulate and the biosynthetic enzyme activities STS and PAL are induced in primary needles of Scots pine seedlings as a response to O<sub>3</sub> exposure. An O<sub>3</sub> pulse (8 h) of 0.15  $\mu\text{L/L}$ , which did not result in visible injury of pine seedlings, was sufficient for the induction of stilbene biosynthesis.

Enzyme assays for PAL, STS, and CHS were optimized in such a way that the determination of enzyme activities was possible from the same crude extracts. Removal of low molecular mass material did not result in higher enzyme activities. In contrast, additional purification steps were required for the reliable determination of STS activity by other authors (3). The  $K_m$  value of cinnamoyl CoA for STS (about 2  $\mu\text{M}$ ) was in the range of the  $K_m$  values of 4-coumaroyl CoA for the resveratrol-forming STS in peanut (23) and grapevine (15).  $K_m$  values for phenylalanine with PAL and cinnamoyl CoA with CHS were also comparable to those reported for various herbaceous plants. The molecular mass of STS was estimated as between 54 and 61 kD. These values are lower than the value of 95 kD recently described for STS from pine seedlings (3).

Exposure of pine seedlings to a minimum O<sub>3</sub> concentration of 0.15  $\mu\text{L/L}$  given as an 8-h pulse resulted in a strong induction of STS from levels below the detection limit of 0.01 to 1  $\mu\text{kat}\cdot\text{kg protein}^{-1}$ . This was followed by a massive accumulation of stilbene metabolites up to 300 nmol·g fresh weight<sup>-1</sup>. The calculated O<sub>3</sub> dose (concentration  $\times$  time) applied was equal to 1.2  $\mu\text{L/L} \times \text{h}$ . A concentration of 0.1  $\mu\text{L/L}$  O<sub>3</sub> never resulted in the induction of stilbene biosynthesis, even when the exposure time was prolonged to 12 h, *i.e.* the same total dose. This indicates that the induction only occurred when a threshold concentration of somewhat below 0.15  $\mu\text{L/L}$  was exceeded.

A strong dependence on the duration of the O<sub>3</sub> treatment was observed for both stilbene accumulation and STS activity. A treatment of at least 4 h was necessary to stimulate stilbene biosynthesis when pine seedlings were exposed to 0.2  $\mu\text{L/L}$  O<sub>3</sub>. With longer exposure times, the amount of stilbenes and the level of STS activity increased markedly at 36 h after the onset of O<sub>3</sub>. This indicates that pine seedlings efficiently detoxify or exclude O<sub>3</sub> or its reaction products at concentrations up to 0.15  $\mu\text{L/L}$  O<sub>3</sub>. Beyond this concentration, the defense mechanisms may be overcome and stilbene biosynthesis occurs. A similar assumption has been proposed by Peters *et al.* (17) from the results of O<sub>3</sub>-exposure experiments with extracellular enzyme activities in beans. These observations are in accordance with a typical "threshold reaction."

The amount of stilbenes in seedlings was not significantly different up to 36 h after the onset of O<sub>3</sub> treatment at 0.15 and 0.3  $\mu\text{L/L}$  O<sub>3</sub>. However, the concentration of stilbenes remained constant during the time of investigation (96 h) in seedlings exposed to 0.3  $\mu\text{L/L}$  O<sub>3</sub>, whereas it decreased at 0.15  $\mu\text{L/L}$  O<sub>3</sub> after having reached a maximum. At 0.15  $\mu\text{L/L}$  O<sub>3</sub>, no visible injury was observed, in contrast to treatment with

0.2 and 0.3  $\mu\text{L/L}$  O<sub>3</sub>. The decrease in stilbene concentrations may be the result of translocation or of metabolic turnover. Because stilbenes were not detectable in hypocotyls or roots of these seedlings, we suppose that the decrease in the stilbenes was due to metabolism such as glycosylation or oxidation. Oxidation of the stilbene resveratrol by peroxidases and oligomerization to  $\epsilon$ -viniferin have been observed in cell cultures of grapevine; and, in grapevine leaves, stilbenes accumulated in the cell wall (7). Primary needles of pine showed damage symptoms at 0.3 but not at 0.15  $\mu\text{L/L}$  O<sub>3</sub>. Peroxidases that are involved in stilbene metabolism might be inhibited in the injured tissue.

The time course of stilbene accumulation in the needles of pine seedlings corresponded well with the increase of PAL and STS activities. Thus, the stilbenes were apparently synthesized *de novo* in the needle. This has been confirmed by pulse-labeling experiments with [U-<sup>14</sup>C]phenylalanine and [2-<sup>14</sup>C]acetate, which are metabolic precursors of phenylpropanoid metabolites. When both substances were fed to seedlings, incorporation of radioactivity into two compounds present only in O<sub>3</sub>-treated seedlings, and cochromatographing with PS and PSM in TLC, was observed (our unpublished data). The time course and the extent of STS induction remained similar at increasing O<sub>3</sub> concentrations. The effect of O<sub>3</sub> on PAL activity, which was present at high levels in control seedlings, was not as pronounced as on STS activity. Moreover, PAL activity depended on the O<sub>3</sub> concentration applied and showed increasing levels with increasing O<sub>3</sub> concentration. The activity of CHS, an enzyme that catalyzes the first committed step in the biosynthesis of flavonoids, was also stimulated in O<sub>3</sub>-exposed pine seedlings. The time course of this enzyme depended on the applied concentration, as was observed for PAL, but the activity was higher at 0.15 than at 0.3  $\mu\text{L/L}$  O<sub>3</sub>. Thus, O<sub>3</sub> had quantitatively different effects on the activities of PAL, CHS, and STS depending on the concentration applied. Other components of the phenylpropanoid pathway were also affected by O<sub>3</sub> treatment. For example, coniferylalcohol dehydrogenase, an enzyme involved in lignin biosynthesis, was induced by O<sub>3</sub> (in addition to stilbene biosynthesis) in both seedlings and 4-year-old pine trees (13). Moreover, higher amounts of the flavonol catechin compared with the control have been found in the O<sub>3</sub>-treated trees (13).

In conclusion, the induction of STS, PAL, and CHS, as well as stilbenes, establishes for the first time a dose-dependent biochemical O<sub>3</sub> response of a conifer species at the level of both enzymes and products. Although O<sub>3</sub> concentrations of at least 0.15  $\mu\text{L/L}$  resulted in a high induction of total stilbenes (up to 0.5 mmol/g fresh weight) and STS activity (up to 1  $\mu\text{kat}\cdot\text{kg protein}^{-1}$ ), no stilbene biosynthesis was observed in control seedlings. This induction, however, is not specific for O<sub>3</sub>, because stilbene biosynthesis in pine was also induced by UV irradiation (22) and by fungal infection (3). Thus, the results on O<sub>3</sub> induction of stilbene biosynthesis in Scots pine may be compared with similar reactions in grapevine and peanut, in which accumulation of resveratrol and stilbene oligomers (viniferins) has been observed upon fungal infection and UV irradiation (11, 15). Stilbene metabolites have been implicated as phytoalexins due to their fungistatic properties (11, 26). The formation of such stress metabolites is supposed to change the disposition of a tree to other stress factors (21).

This would, in turn, change the susceptibility of trees to forest decline processes (24). Monitoring stilbene biosynthesis in needles of pine, therefore, may provide a useful method to examine stress impacts in this tree species. This is of special significance if the induction of stilbene biosynthesis also occurs under outdoor conditions and in the absence of visible injury. In an outdoor experiment at Liphook (UK) started by the Central Electricity Research Laboratories, Leatherhead, current year's needles from 5-year-old pine trees showed high levels of stilbenes under SO<sub>2</sub> and O<sub>3</sub> treatments (alone and in combination), whereas in control plants only low levels of PS and no PSM were present (6). These results support the view that stilbene biosynthesis may be applicable as an early biomarker for the action of stressors on needles of pine trees.

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