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EFFECTS OF SULPHUR DIOXIDE FUMIGATION ON LIPID BIOSYNTHESIS IN PINE NEEDLES

SURJIT S. MALHOTRA and ABDUL A. KHAN
Northern Forest Research Centre, Canadian Forestry Service, Environment Canada, 5320–122 Street, Edmonton, Alberta T6H 3S5, Canada

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Abstract—Pine needle tissues were shown to incorporate acetate [1-^{14}C] into phospho-, galacto- and neutral lipids. The major incorporation of the label among these lipids was always in the phosphatidyl choline (PC) fraction. The amount of label among the other lipid fractions varied depending on the age and source of the needle tissues (lodgepole or jack pine). In general, the biosynthesis of these lipids was more efficient in the developing than in the fully developed tissues. Treatment of the needle tissues with either gaseous or aqueous SO_2 markedly inhibited their lipid biosynthesis. These effects were more pronounced in the developing than in the fully developed needles. Fumigation with gaseous SO_2 showed that SO_2 concentration and length of exposure determine the extent to which the lipid biosynthetic capacity of the tissues is affected. Lipid biosynthetic capacity was partially or completely recovered when plants were removed from the SO_2 environment. Plants exposed to moderate SO_2 concentrations (0.18–0.20 ppm) for a period of 24 hr recovered faster than those exposed to near lethal SO_2 concentrations (0.34–0.37 ppm) for only 1 hr.

INTRODUCTION

Gaseous SO_2 is discharged around many industrial areas in measurable quantities, and sometimes its concentration in the ambient air is enough to produce phytotoxic effects [1, 2]. Occasionally some of this industrial pollution affects useful forest vegetation nearby.

In an attempt to characterize the invisible effects of SO_2 on pine needle tissues it was demonstrated that aqueous SO_2 produces ultrastructural and functional changes in the chloroplasts [3]. Recently, a marked effect of aqueous SO_2 on the composition of needle glycolipids was also demonstrated [4]. Since glycolipids are involved in the structural integrity of the chloroplast membranes [5], it is concluded that ultrastructural disorganization of pine needle chloroplasts [3] is brought about by SO_2 interference with the glycolipids [4]. It is suggested that SO_2 may have affected the *de novo* synthesis of these and other important cellular lipids. The present study describes lipid biosynthesis in (a) needle tissues treated with aqueous or gaseous SO_2, and (b) fumigated needle tissues after a recovery period in an SO_2-free environment.

RESULTS AND DISCUSSION

Biosynthesis of total lipids from acetate [1-^{14}C] in pine needle tissues

The preliminary studies showed that the incorporation of acetate [1-^{14}C] into pine needle lipids was linear only up to 3 hr. Therefore, in all the studies the needles were routinely incubated for 1–3 hr. The needle tissues incorporated the label into lipids under both light and dark conditions, but the total lipid biosynthesis was 30% higher in tissues incubated under light. Maximum incorporation of acetate [1-^{14}C] into lipids was obtained when the needle tissues were incubated in distilled water (pH 4.5–7); buffer ions such as Tris or phosphate at various pH's were inhibitory. Therefore, the incubations were carried out in distilled water (pH 5.5). Saponification of the labeled lipids showed that the acetate [1-^{14}C] was incorporated exclusively into the fatty acid moieties of the lipids.

Biosynthesis of polar and neutral lipids in pine needle tissues

Analyses of the acetate [1-^{14}C] label incorporation into polar and neutral lipids of the needle tissues are shown in Table 1. A major portion of acetate [1-^{14}C] incorporation in all the needle tissues was found to be associated with the phospholipid fractions. Among the labeled phospholipids, the incorporation into phosphatidyl choline (PC) was more than either phosphatidyl ethanolamine (PE) or phosphatidyl glycerol (PG). In galactolipids, the incorporation of the label into monogalactosyl diglyceride (MGDG) was almost twice as much as in diagalactosyl diglyceride (DGDG). Among the neutral lipids, the maximum incorporation was in the triacyl glycerol (TG) fraction, followed by diacyl glycerol (DG) and free fatty acids (FFA).

In lodgepole pine, the biosynthesis of lipids appeared to be more efficient in the developing (young) than in the fully developed (mature) tissues (Table 1). The differences in the biosynthetic abilities probably reflect wide fluctuations in the levels of either the participating enzymes or other factors involved in the lipid biosynthesis. Since all of our work on jack pine was carried out on developing needles, no attempt was made to characterize lipids in the fully developed needles.

Effects of SO_2 on lipid biosynthesis

It has been reported that after penetration into plant

Table 1. Biosynthesis of phospho-, galacto- and neutral lipids from acetate [1-^{14}C] in pine needle tissues

Tissue	PC	PE	PG	DGDG	MGDG	DG	FFA	TG
	(cpm × 10^3/hr/g fr. wt)							
Lodgepole pine								
Developing needles	327 1	167.8	52 8	28.1	58.5	29.5	29.3	188 7
Fully developed needles	114.3	40.2	39.5	24.9	55.7	18.5	8 7	33 8
Jack pine								
Developing needles	87.0	67.0	48.0	14 8	23.3	6.0	5 3	14.3

tissues, gaseous SO_2 becomes hydrated, and it is the hydrated species that affects various cellular processes [6]. Therefore, in several studies aqueous SO_2 solutions have been employed to study SO_2 effects on vegetation [3, 4, 7, 8]. In the present study, however, we investigated the effect of both aqueous and gaseous SO_2 on lipid biosynthesis of pine needle tissues.

Treatment with aqueous SO_2

At a SO_2 concentration of 25 ppm, biosyntheses of the phospho- and galactolipids were equally inhibited in developing and fully developed lodgepole pine needle tissues (Table 2). At 100 ppm, biosyntheses of the phospholipids and MGDG were inhibited more in the developing than in the fully developed needle tissues. The biosynthesis of the DGDG fraction, however, was inhibited more in the fully developed tissues than in the developing tissues. At both concentrations, SO_2 considerably reduced the biosynthesis of neutral lipids, DG, and FFA. This effect appeared to be more pronounced in the fully developed tissues than in the developing tissues. However, SO_2 affected the biosynthesis of the TG fraction more in the developing tissues than in the fully developed ones. These results confirm our earlier observations that the sensitivity of various metabolic processes to SO_2 is a function of age of the needles [4].

Fumigation with gaseous SO_2

Effects of gaseous SO_2 on the biosynthesis of jack pine lipids were similar to those of aqueous SO_2 on lodgepole pine seedlings. Exposure of jack pine needles for 24 hr to a concentration of 0.18–0.20 ppm SO_2 markedly inhibited the biosynthesis of various lipids (Table 3, expt. 1, 2). The inhibitory effect among the phospholipids was always slightly higher in PG and PE than in PC. Among the galactolipids, DGDG was generally inhibited more than MGDG. Fumigation of seedlings for 24 hr with a high SO_2 concentration (0 35 ppm) drastically inhibited all lipid biosynthesis (Table 1, expt. 1) and caused needle tip burn. It is therefore suggested that invisible and visible effects of prolonged SO_2 exposure are directly related to impaired cellular metabolism.

Recovery of fumigated tissues

Under field conditions, plants are generally subjected either to low SO_2 concentrations for long periods or to high concentrations for short and intermittent periods. Such conditions provide the vegetation an opportunity to recover from or withstand SO_2 for extended periods of time without showing any visual injury. In order to simulate the field conditions (varying concentrations of SO_2) as closely as possible, the fumigations were carried out in the controlled environment cuvettes and followed by different lengths of recovery time in an SO_2-free environment. The experimental conditions chosen and the results obtained are given in Table 3 (expt. 2, 3). It was clearly shown that a high SO_2 concentration (0.37 ppm) for a short exposure time (1 hr) resulted in much more inhibition of lipid biosynthesis than a low SO_2 concentration (0.2 ppm) for a longer period of time (24 hr) (Table 3, expt. 2, 3). The inhibitory effects of either fumigation were partially or completely reversed upon removal of plants from the SO_2 environment. However, the recovery in the biosynthetic ability of plants exposed to a high SO_2 concentration for the short duration was slower than that of the plants exposed to low SO_2 for the longer duration. It is also interesting to note that at the high SO_2 concentration much of the damage to lipid biosynthesis of the tissue occurred during the initial period of exposure (Table 3, expt. 1 vs expt. 3).

Since SO_2 treatments resulted in a general inhibition of all the lipid fractions, it is quite possible that some of the enzymes involved in the primary synthesis of fatty acids (such as fatty acid synthetase, acetyl-COA carboxylase) were affected more than those responsible for

Table 2. Effects of aqueous SO_2 on the lipid biosynthesis in needle tissues of lodgepole pine seedlings

Needle tissues	SO_2 conc ppm	PC	PE	PG	DGDG	MGDG	DG	FFA	TG
		(% of control)							
Developing needles	25	87.3	73.0	75.1	86.1	84.2	92 2	70 2	64.7
	100	71.7	62 2	40.4	82.0	75 7	81 4	57 4	55 2
Fully developed needles	25	85.5	75.1	73.0	80 2	90.4	63.2	55 7	90 6
	100	82 1	66.7	55.8	65.4	89.0	48.7	46 9	63.8

Table 3. Effects of gaseous SO_2 on lipid biosynthesis in developing needles from jack pine seedlings and their ability to recover in an SO_2-free environment

Fumigation conditions Conc (ppm)	Duration (hr)	Recovery (hr)	PC	PE	PG	DGDG	MGDG	DG	FFA	TG
						(% of control)				
Expt. 1										
0.18	24	0	78.2	74.0	68.6	57.4	81.1	75.6	75.0	80.4
0.35	24	0	30.0	44.0	41.6	29.8	26.4	26.7	15.6	39.0
Expt. 2										
0.20	24	0	81.8	60.5	61.5	73.1	81.1	80.6	76.5	88.9
0.20	24	48	82.2	70.3	67.4	100.0	100.0	100.0	100.0	100.0
Expt. 3										
0.37	1	0	43.6	37.7	33.5	42.3	44.8	40.7	42.2	39.3
0.37	1	24	63.4	65.4	69.4	66.0	59.2	63.6	115.0	52.4

various acylation reactions. This conclusion was further supported by the observation that decreased synthesis of various lipids did not result in an increase in FFA. In fact, after SO_2 treatments the amount of label in the FFA fraction dropped more dramatically than in most other lipids, suggesting a major effect on fatty acid synthesis. The primary enzymes of fatty acid biosynthesis require functional —SH groups for their activity; SO_2 may have inhibited these enzymes by affecting —SH groups. Pine needle sections incubated with —SH binding reagents (*p*-chloromercuribenzoate and *N*-ethylmaleimide) showed a severe loss in fatty acid biosynthesis (preliminary experiments). It is difficult to suggest how SO_2 affects the thiol or other functional groups of the enzymes. However, bisulphite ions at low pH are very effective as an oxidizing agent [7], which may bring about oxidation of thiol or other functional groups. In preliminary experiments, incubation of dithioerytheritol (DTE) with HSO_3^- caused oxidation of the thiol groups of DTE. It is therefore suggested that the HSO_3^- ions produced from dissolved SO_2 in the tissues can influence the status of enzymes containing —SH groups.

A number of glycerolipids discussed in this report have been shown to be important constituents of chloroplasts, mitochondria, and other subcellular organelles [9, 10]. Since actate [1-^{14}C] was incorporated into fatty acids of the lipids, it is suggested that SO_2 must affect site(s) where fatty acids are either synthesized or acylated into various lipids. The differential effects of SO_2 on glycerolipid biosynthesis (Tables 2 and 3) suggest that cellular biosynthesis of fatty acids occur at more than one site and that SO_2 affects various sites differently. However, if it is assumed that all the fatty acids are synthesized at the same site, than the different effect on glycerolipid biosynthesis by SO_2 may be due to a difference in accessibility of individual fatty acids to various sites of acylation.

The site specificity for fatty acid synthesis in the excised pine needle sections was determined by incubating the tissues with cycloheximide, a known inhibitor of cytoplasmic proteins. Fatty acids synthesis in cycloheximide-treated pine tissues 1 mg/ml incubation medium) was strongly inhibited. This is an indication that fatty acid synthesis in the needle tissues probably takes place only in the cytoplasm, and the acylation of the fatty acids by various organelles may be dependent upon their cytoplasmic synthesis. This was further supported by the observation that the pine needle chloroplast preparations were unable to carry out fatty acid biosynthesis but were capable of acylating palmitoyl-CoA. It is therefore suggested that SO_2 primarily affects the cytoplasmic fatty acid synthesis, which in turn affects acylation reaction at various sites in the cells.

EXPERIMENTAL

Growth conditions. Jack pine (*Pinus banksiana* Lamb.) and lodgepole pine (*Pinus contorta* Dougl. var. *latifolia* Engelm.) seedlings were grown under greenhouse conditions as described previously [3]. Needles from 4- to 5-month old plants were used. The needles were divided into fully developed and developing tissues as described before [4].

Fumigation conditions. Pine seedlings were exposed to different SO_2 concns in fumigation cuvettes using a continuous flow-through system. These rectangular cuvettes, measuring 61 × 61 × 30.5 cm, were constructed from clear acrylic sheets. The cuvette was then placed on another rectangular tank containing water. Holes (2.38 cm) were drilled in the cuvette floor at equal distances in order to insert seedling root plugs into the water container. An airtight seal at the point of insertion was made by passing the lower stem region of each seedling gently through a longitudinally slitted rubber stopper. The stoppers were then gently inserted through the holes in the cuvette floor until the slit closed firmly around the stem and sealed the holes. Water was added to the bottom container until the tips of all the root plugs were well immersed. The cuvette inlet and outlet for the air were diagonally opposite to each other on the side walls. The air flow was kept at 10 l./min and SO_2 flow was adjusted to give the desired concentration at the output of the cuvette. SO_2 concns were monitored by a Phillips PW-9700 SO_2 analyzer. The air inside the cuvette was mixed by an internally mounted fan near the input port. Two identical cuvette assemblies were placed in an environmental growth chamber, one for control and the other for SO_2 treatment. The growth chamber was maintained at 24° and 20000 lx light intensity with a relative humidity inside the cuvettes around 60%. On top of each cuvette a jacket of fresh water filtered out the heat radiation. 13–18 seedlings of the same age were placed in each cuvette. After fumigation the needles were divided into fully developed and developing needles [4] and cut into 1 cm sections for experimental purposes.

Metabolic experiments with acetate [1-^{14}C]. The excised tissue (0.5 g) was transferred to flasks containing 10 ml H_2O and 10 μCi acetate [1-^{14}C]. In experiments where the needles were treated with aq. SO_2, the needle tissues were incubated with 10 ml SO_2 solns (pH adjusted to 5.5 with dil. NaOH) containing 10 μCi acetate [1-^{14}C]. Except as otherwise specified, the incubations were carried out for 1–3 hr at 30° and under 10500 lx light intensity. In experiments with cycloheximide the tissues were first incubated in the antibiotic soln for 30 min in cold. After incubation, each tissue sample was removed,

washed and homogenized in 50 ml $CHCl_3$–MeOH (2:1). The homogenate was then filtered through a glass-wool-packed Buchner funnel, and the residue was washed repeatedly with $CHCl_3$–MeOH (2:1) as in ref. [4]. The $CHCl_3$ extracts containing lipids were washed with acidic H_2O and evapd to dryness under red. press. [11] and the labeled products were analyzed.

Chromatographic analysis of labeled products. The total lipids were identified and analyzed by TLC on Si gel by adding authentic standards to the plant samples. The standards were also run individually on the same chromatogram. Radioactive regions were matched with the standards. The Si gel from these regions was removed and assayed for ^{14}C either directly or after elution from the Si gel. The chromatograms were run in lined tanks. For phospholipid analysis the chromatograms were developed in $CHCl_3$–MeOH–HOAc–H_2O (100:25:10:4). The R_f values of PC, PG and PE in this solvent system were 0.24, 0.35 and 0.47 respectively. The galactolipids were also separated in the same solvent system, and the R_f values for DGDG and MGDG were 0.40 and 0.87 respectively. The galactolipids, however, were routinely separated from the phospholipids in Me_2CO–C_6H_6–H_2O (91:30:8) [12]. The various products in these chromatograms were identified by exposure to I_2 vapour. The neutral lipids were separated in *n*-C_6H_{14}–Et_2O–HCO_2H (40:10:1) [13], and the products were visualized under UV after spraying with 0.1% soln 2′,7′-dichlorofluoresceine in EtOH. The radioactivity in the total fatty acids of the lipids was determined by alkaline hydrolysis of the total lipids with 10% KOH in EtOH under N_2. After acidification with HCl, fatty acids were extracted with $CHCl_3$. Alternatively, the fatty acids in the total lipids were transesterified by refluxing with 14% BF_3 in MeOH for 2 hr. After cooling, the reaction was stopped with excess H_2O and methyl esters of the fatty acids were extracted with $CHCl_3$. Labeled products (FA or methyl esters of FA) isolated after either treatment were analyzed by TLC on Si gel in *n*-C_6H_{14}–Et_2O–HCO_2H (40:10:1). The radioactive FA or these methyl esters regions were matched, removed, and assayed for the label in the product.

Determination of the radioactivity. Radioactive samples (liquid or scraped from thin layer plates) were added to omniflour (New England Nuclear) scintillation fluid [0.4% omniflour dissolved with 30% EtOH in toluene] and counted in a liquid scintillation spectrometer. The radioactivity on the TLC was measured by a radioisotope scanner attached to a thin layer conveyer.

Other estimations. In experiments with DTE and HSO_3^-, thiol groups were measured according to ref. [14]. Background absorption of HSO_3^- was always subtracted from the absorption values of samples containing both DTE and HSO_3^-.

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