Phytochemistry, 1977, Vol 16, pp. 539–543 Pergamon Press. Printed in England

EFFECTS OF AQUEOUS SULPHUR DIOXIDE ON PINE NEEDLE GLYCOLIPIDS

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(*Received* 27 *September* 1976)

Key Word Index—*Pinus contorta*; Pinaceae; SO_2 effects; glycolipids; monogalactosyl diglyceride; digalactosyl diglyceride; sulphoquinovosyl diglyceride; fatty acids; soluble sugars.

Abstract—The major glycolipids in the fully developed and young needle tissues of lodgepole pine (*Pinus contorta* Dougl. var. *latifolia* Engelm.) were monogalactosyl diglyceride (MGDG), digalactosyl diglyceride (DGDG), and sulphoquinovosyl diglyceride (SQDG). The concentration of these glycolipids was considerably higher in the fully developed needles than in the young needles. The major fatty acid in the MGDG fraction (from both tissues) and DGDG fraction (from fully developed tissues) was linolenic acid. However, palmitic acid was the major fatty acid in the DGDG fraction from the young tissues and the SQDG fraction from both tissues. Treatment of needles with aq. SO_2 solutions produced marked changes in the concentration and composition of these glycolipid fractions. At 100 ppm, SO_2 produced a considerable drop in the linolenic acid content of all glycolipid fractions, more pronounced in the young needles than in the fully developed ones. SO_2 also had an effect on the release of soluble sugars from the needle tissues of both ages.

INTRODUCTION

The phytotoxic effects of SO_2, a major pollutant in industrial areas, have been well documented in the case of lichens and other plants [1–6]. However, the mechanisms of SO_2 phytotoxicity at the subcellular and molecular levels are still in the descriptive stages. The subcellular injuries from aqueous SO_2 on the ultrastructure and functions of pine needle chloroplasts have been reported previously [6]. It is probable that the structural alterations have been preceded by changes in a number of biochemical events.

Lipids, mostly glycerolipids, constitute about 50% by weight of chloroplast thylakoids [7]. The major portion of glycerolipids in photosynthetic membranes is present as MGDG, DGDG, and SQDG [8]. Among these lipids the galactolipids have been shown to be involved in the structural integrity of chloroplast thylakoids [9] and in fatty acid synthesis [10]. The stabilization of the chlorophyll phytol groups by their interlocking with the acyl chain of the lipids has also been suggested [11]. Furthermore, linolenic acid, a major fatty acid of galactolipids, has been linked to photosynthetic electron transport [12]. Although these lipids are involved in the structure and function of chloroplast membranes, nothing is known about the effect of SO_2 on the metabolism of these compounds. In this paper we describe the effect of SO_2 on (a) the levels and composition of these lipids in fully developed and young lodgepole pine needles and (b) the permeability of pine needle tissues as measured by the release of total soluble sugars.

RESULTS AND DISCUSSION

Glycolipid analysis of fully developed and young pine needles

The concentrations of major glycolipids, MGDG, DGDG, and SQDG in fully developed and young pine needle tissues are reported in Table 1. The amounts of chloropyll *a* and *b* in the tissues of both ages are included to define the development status of the tissues. The data show a marked increase in the levels of glycolipids and chlorophyll of pine needle tissues with aging and suggest that chloroplast development (as evidenced by a net increase in chlorophyll content) may be a result of a parallel increase in glycolipid and/or chlorophyll contents. The percentage changes in the amounts of individual glycolipids during the development of needle tissues were not very different from one another. The glycolipid composition of the needle tissues therefore remains almost unaltered.

The data in Table 2 (values of control set) show the relationship between the age of needles and the fatty acid composition of glycolipids. In the MGDG fraction the major fatty acid in the needles of both ages was linolenic acid. However, its amount in the fully developed tissues was considerably higher than that in the young tissues. Conversely, the levels of minor fatty acids (such as palmitic and linoleic acids) were higher in the young tissues than those in the fully developed tissues. The major fatty acids in the DGDG fraction were linolenic and palmitic acids but their concentrations in the two tissues were markedly different. The young needles contained more palmitic than linolenic acid, while the

Table 1. Chlorophyll and glycolipid concentration in fully developed and young pine needles. Mean values from four experiments.

	Fully developed needles fr. μ mol/g fr. wt	Young needles
Chlorophyll		
a	1.06	0.46
b	0.81	0.25
Glycolipids		
MGDG	5.61	2.91
DGDG	3.44	1.92
SQDG	1.84	1.13

reverse was true in the fully developed needles. Among the minor fatty acids, linoleic acid was present only in the fully developed tissues. The SQDG fraction from the needles of both ages contained mostly palmitic acid, but a substantial amount of linolenic acid was also present. Among the minor fatty acids the two tissues differed from each other in that linoleic acid was present only in the fully developed needles.

In general, during the development of needles there was a net increase in the amounts of polyunsaturated fatty acids and a decrease in the palmitic acid content of the galactolipids. The change in the levels of glycolipids and fatty acids composition is associated with the development of chloroplasts in the leaves of maize [13] and barley [14].

Our preliminary experiments with pine needle chloroplasts indicated that linolenic acid-[1-^{14}C] is incorporated selectively into MGDG and palmitic acid-[1-^{14}C] into phosphatidylcholine and very poorly into MGDG fractions. It is therefore suggested that, depending on the availability of fatty acyl moieties synthesized in the tissues, their selective acylation is determined by the lipid acceptors. The lipid acceptors control the utilization of fatty acyl moieties in other tissues [15].

Effects of SO_2 on tissue glycolipids

Aqueous solutions of SO_2 were used in determining the effects of SO_2 on needle glycolipids. The details on the choice of aqueous SO_2 concentrations are described in the Experimental.

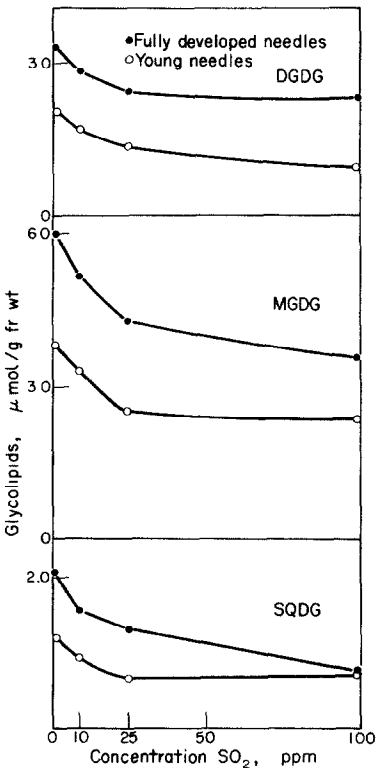

Fig. 1. The effect of aqueous SO_2 on the levels of glycolipids in fully developed and young pine needles.

The data in Fig. 1 show changes in the levels of glycolipids from fully developed and young needles as affected by various concentrations of aqueous SO_2. The concentrations of MGDG, DGDG, and SQDG declined sharply with increasing concentrations of SO_2 up to 25 ppm. Further increase in SO_2 concentration (100 ppm) resulted in only small changes in the concentration of these glycolipids. An interesting observation is that the effect of SO_2 on the level of SQDG was more pronounced in the fully developed tissues than in the young tissues. This was demonstrated by the fact that 100 ppm SO_2 resulted in a 65% decrease of SQDG in the fully developed needles compared to a 32% decrease in the

Table 2. The fatty acid composition of glycolipids in fully developed and young pine needle tissues after exposure to aqueous SO_2

Concentration of aqueous SO_2 (ppm)	Fatty acids (%) Fully developed needles 16:0	18:0	18:1	18:2	18:3	Young needles 16:0	18:0	18:1	18:2	18:3
MGDG										
0	3.5	6.7	—	2.5	86.8	12.4	7.1	—	9.2	71.1
10	7.5	8.4	—	2.8	81.2	14.9	5.8	5.8	8.3	65.1
100	10.7	9.2	—	2.7	77.8	26.6	7.2	11.0	10.7	44.4
DGDG										
0	27.6	3.5	—	6.6	62.3	54.4	4.3	—	—	41.3
10	23.7	7.7	—	6.9	61.7	48.2	7.3	—	4.1	40.4
100	33.6	4.3	—	9.2	52.9	54.1	8.7	3.1	7.3	26.9
SQDG										
0	60.7	6.6	—	10.6	22.1	65.4	4.4	—	—	30.3
10	60.8	4.6	—	9.6	24.8	81.6	3.6	—	—	14.8
100	74.4	6.6	—	4.0	15.0	87.9	—	—	—	12.1

young tissues. On the other hand, the same SO_2 concentration (100 ppm) caused a 50% decrease in the DGDG fraction in the young tissues and 33% in the fully developed tissues. These differences clearly indicate that the biosensitivity of various cellular metabolites to SO_2 depends on the age of the needles.

The decrease or loss of tissue glycolipids due to SO_2 may be brought about by either a reduced synthesis of these lipids, an increased lipase activity in the needles, peroxidation of the unsaturated fatty acids of the diglycerides, or a combination of the above.

The results from acetate-[1-^{14}C] incorporation studies (Khan and Malhotra, Unpublished results) showed that SO_2 produced a net decrease in the synthesis of glycolipids as well as free fatty acid content. This suggests that inhibition of glycolipid synthesis may not be due to the effect of SO_2 on lipase. On the other hand, it is possible that fatty acids liberated as a result of lipase activity may have been utilized elsewhere.

Effects on fatty acid composition

Besides influencing the levels of the glycolipids, SO_2 produced a strong effect on the fatty acid composition in tissues of both ages (Table 2). These effects were dependent on SO_2 concentration. At 10 ppm the effect was minimal; however, treatment of the needles with 100 ppm SO_2 produced marked changes in the fatty acid composition of glycolipids from the tissues of both ages. In general, the glycolipids isolated from 100 ppm SO_2-treated tissues showed a decrease in their linolenic acid contents. However, this decrease was more pronounced in the young tissues than that in fully developed ones. As to other major fatty acids, there was a general increase in the palmitic acid levels in all glycolipids except for the DGDG fraction from the young needles, which remained almost unchanged (Table 2).

The mechanism(s) by which SO_2 could bring about the loss of linolenic acid (of glycolipids) from the tissues is not clear. However, preliminary experiments have shown that SO_2 causes an increase in the release of malonyl dialdehyde from the pine needle tissue, indicating that the unsaturated fatty acyl chains of the lipids are undergoing oxidation. The above observation is further supported by the fact that linolenic acid, when incubated with increasing concentrations of SO_2, produces a parallel increase in the production of malonyl dialdehyde. Presently we do not have any concrete evidence that SO_2 would produce a similar effect on the polyunsaturated component of the biological membranes. However, production of malonyl dialdehyde can affect other cellular processes [16, 17].

The localization of the glycolipids in the chloroplast membranes [18, 19] suggests their involvement with either the structures and/or functions of the chloroplast membranes. Recently Shaw *et al.* [9] have shown that removal of galactolipids from spinach chloroplasts by a galactolipid lipase resulted in the enlargement and unstalking of the thyalkoids. In pine needles, aqueous solutions of SO_2 have been shown to produce swelling of thylakoid discs and cause disintegration of intrachloroplast membranes [6]. It is possible that the changes in the ultrastructures of pine needle chloroplasts could have resulted from the loss of these glycolipids upon SO_2 treatment. In addition to being involved in the chloroplast structural framework, the lipids such as galactolipids [9] and triacyl glycerols and α-tocopherols [20] are also required for photosystem–I–dependent oxidation of TMPD (*N,N,N',N'*-tetramethylphenylenediamine) in lipase-treated and heptane-extracted spin ach chloroplasts. It is also known that cellular permeability towards water-soluble nonelectrolytes across the membranes depends on the degree of unsaturation and the length of fatty acids in the membranes [21]. It is therefore probable that the transport of such nonelectrolytes across the cellular membranes could be influenced by SO_2.

Effects of SO_2 on the release of soluble sugars from the tissues

The influence of SO_2 on cellular metabolites such as soluble sugars was determined to ascertain whether the effects of SO_2 discussed above were confined to the glycolipids or whether other cellular metabolites were also influenced. The effects of SO_2 on the release of sugars would indicate the changes in cellular permeability. Soluble sugars released from the young needles was 7.3, 7.5, 8.8 and 15.5 μmol/g fr. wt for 0, 10, 25 and 100 ppm SO_2 resp.; for the older needles 8.1, 8.4, 8.8 and 10.5 μmol/g fr. wt were released at these concentrations of SO_2. The incubation of tissues from both fully developed and young needles resulted in a release of soluble sugars from the tissues, even in the absence of SO_2. However, the presence of SO_2 in the incubation solution caused a dramatic increase in the amount of sugars released from both the tissues. The amount of sugars released was markedly higher from the younger needles than that from the fully developed ones. The release of soluble sugars from tissues of both ages might have occurred because of a decrease in glycolipid concentration in the SO_2-treated tissues. However, the amount of sugars released from the young tissues was considerably higher than the glycolipid losses, suggesting that SO_2 caused a release of some other bound form(s) of sugars.

Choice for SO_2 treatment

It has been reported that SO_2 is effective on vegetation only in aqueous solutions [22]. The changes in the hydration state of lichen thalli were shown to determine to a great extent the resistance of the tissues to SO_2 [22]. In equating the effects of gaseous and aqueous SO_2 on the biological activity of the spores of the fungus *Diplocarpon rosae*, Saunders [23] observed that a $\times 1000$ higher concentration of SO_2 had to be employed in the aqueous phase than in the gaseous phase. This observation provided a basis for studying the effects of SO_2 injury on vegetation by using aqueous solutions of SO_2 [6, 24, 25]. The above relationship between the aqueous and gaseous SO_2 concentrations, however, may not be linear at all concentrations [26, 27] and is temperature-dependent [27].

Recently it has been suggested [27] that the principal species of SO_2 in solution is HSO_3^- (to the extent of at least 99% of the total SO_2); other species such as H_2SO_3, SO_3^{2-}, and $S_2O_5^{2-}$ were present in negligible amounts, and also present as a minor component was dissolved unreacted SO_2. On the assumption that nonionic species would permeate more rapidly across cellular membranes than ionic ones, Hocking and Hocking [27] inferred that given the moist conditions,

the dissolved unreacted SO_2 would be the most likely biologically active species for plant injury. The concentration of dissolved unreacted SO_2 at 25° for 1, 10 and 100 ppm total SO_2 in water was calculated to be 0.0012, 0.011 and 0.092 ppm respectively, or about 1/1000 of the total SO_2 in water [27]. The Hocking and Hocking report [27] provided a rationale for working with aqueous SO_2 concentrations and relating them to the concentrations of biologically active species or to the biological activity of SO_2 that might be observed in natural systems.

GENERAL CONCLUSIONS

The difference in biochemical responses to aqueous SO_2 between the fully developed and young pine needle tissues indicates that the phytotoxicity of SO_2 is at least partially dependent upon the stage of plant tissue development. In view of the importance of galactolipids [9] for the structural integrity of the chloroplast thylakoids, any changes in these lipids caused by SO_2 would result in structural and functional injuries to the biomembranes. We feel that the process of hidden SO_2 injuries such as swelling of thylakoid discs and disintegration of intrachloroplast membranes [6] may be initiated as a result of alterations in glycolipid components. Since very low concentrations of aqueous SO_2 (10–25 ppm) usually do not produce any visual symptoms [6] but cause marked changes in the glycolipid content and composition, the analysis of such lipids may provide an effective tool for determining the extent of hidden SO_2 injury to vegetation.

EXPERIMENTAL

Growth conditions. Lodgepole pine (*Pinus contorta* Dougl. var. *latifolia* Engelm.) seeds were grown in styroblock trays as described previously [6]. After 4 weeks of germination seedlings received nutrient soln [28] once a week and were watered as required. Plants were grown at 22–24° under *ca* 10 500 lx light-source. Needles were harvested from 5 to 6 month old seedlings. For experimental material, the needles on the plant were divided into two groups (a) fully developed (thick, dark green) and (b) young (tender, pale green top cluster) needles. Samples from both tissues were washed with H_2O and cut into 1 cm sections.

SO_2 treatment. 1.5 g excised tissue was transferred to flasks containing various concns of SO_2 in H_2O. pH of these solns was adjusted to 6 with dil. NaOH prior to the transfer of needle tissues. Flasks were sealed with glass cover slips and incubated for 22 hr on a wrist-action shaker under 10 500 lx light-source at 23–25°.

Sugar analysis. At the end of the incubation time, the soln was removed and tissues were washed 2–3 times with H_2O. The incubation soln and the washings from each sample were combined for soluble sugar analysis. HCl was added to an aliquot of the combined soln (final HCl concn 3%) and centrifuged to remove any residue. Soluble sugars in the supernatant layer were determined according to the pHOH–H_2SO_4 method [29].

Lipid analysis. Washed tissues from each treated sample were homogenized with 50 ml of $CHCl_3$–MeOH (2:1) and filtered through a glass-wool packed Buchner funnel. The residue was washed with $CHCl_3$–MeOH (2:1) until free of chlorophyll and other pigments. Total lipids were extracted from the filtrate and evaporated to dryness [30]. These lipids were dissolved in a known vol. of $CHCl_3$ and an aliquot was quantitatively analyzed for galacto- and sulpholipids by TLC on Si gel with authentic standards. The chromatograms were developed in lined tanks using $CHCl_3$–MeOH–HOAc–H_2O (100:20:10:3). The galacto- and sulpholipid fractions were identified on the chromatograms by a brief exposure to I_2 vapor and were estimated in the presence of adsorbent [31], after the removal of I_2. The isolation and purification of galacto- and sulpholipid fractions were accomplished by a combination of DEAE–cellulose column chromatography [31] and TLC on Si gel in the above solvent system. For the analysis of their fatty acid composition, the purified lipid fractions were refluxed with 14% BF_3 in MeOH for 3–4 hr. The Me esters of the fatty acids were isolated and purified by TLC on Si gel. The chromatograms were developed in lined tanks containing *n*-C_6H_{14}–Et_2O–HCO_2H (40:10:1) [15]. The purified Me esters of the fatty acids were isolated and subjected to GLC. The chain lengths of these Me esters were analyzed on a stainless steel column (2 m × 4 mm id) packed with 10% DEGS. An FID chromatograph was used in the analysis. The column temp. was programmed to hold at 160° for the initial 2 min, followed by an increase at the rate of 5°/min up to 190°. At this point, further separation was carried out isothermally. The flow rate of N_2 was 90 ml/min. Synthetic Me esters of authentic standards were used for identification. Integration and quantitation on the component peaks were achieved by a GLC computer terminal.

Other estimations. The sugar moiety in the galactolipids was identified by liberating the bound sugar [32] and analyzing it by TLC on Si gel. Chromatograms were developed in EtOAc–MeOH–HOAc–H_2O (12:3:3:2), dried, sprayed with conc H_2SO_4, and charred to detect the sugar(s). Galactose was found to be the only sugar in these fractions. Malonyl dialdehyde was estimated [33] in the aq. phase after its extraction with 2 vol. of $CHCl_3$ and the chlorophyll was estimated in 80% Me_2CO [34].

Acknowledgements—The authors thank Dr. A. P. Tulloch, Prairie Regional Laboratory, Saskatoon, Saskatchewan, for providing initial samples of MGDG, DGDG, and SQDG. We are also thankful to the National Research Council of Canada and the Alberta Oil Sands Environmental Research Program for financial assistance.

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