Phytochemistry, Vol. 22, No. 6, pp. 1325–1328, 1983.
Printed in Great Britain.

0031–9422/83/061325–04$03.00/0

PROTEIN BIOSYNTHESIS IN JACK PINE AND ITS INHIBITION BY SULPHUR DIOXIDE

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(*Revised received* 27 *September* 1982)

Key Word Index—*Pinus banksiana*; Pinaceae; jack pine; sulphur dioxide effects; protein biosynthesis.

Abstract—Incorporation of [U-^{14}C]leucine in jack pine (*Pinus banksiana* Lamb.) occurred mainly in the chloroplast and soluble cytoplasmic fractions. In the chloroplasts a major portion of the label was associated with the membrane-bound proteins. Fumigation of pine seedlings with gaseous SO_2 (0.34 ppm) for 24 and 48 hr markedly inhibited *de novo* protein biosynthesis in the chloroplast and cytoplasmic fractions. The inhibition was greater for the biosynthesis of chloroplast proteins than for the cytoplasmic ones. The magnitude of inhibition was dependent on exposure time and appeared to be related to sulphur uptake in the treated tissues.

INTRODUCTION

Sulphur dioxide affects various biochemical and physiological processes [1–5] as well as the ultrastructural organization of cellular organelles [1] prior to development of visual injury symptoms in needles of jack and lodgepole pines. The sensitivity to SO_2 of various metabolic processes depends on a number of factors, such as SO_2 concentration, duration of exposure, and the ability of the plant tissues to convert toxic species (HSO_3^-, SO_3^{2-}) of the dissolved SO_2 to relatively less-toxic forms [6]. Thus, the factors that can limit the cellular concentration of SO_2, for example stomatal opening and closing, enzymic and nonenzymic reactions catalysing HSO_3^- and SO_3^{2-} oxidation, can also determine the extent of SO_2 phytotoxicity [6].

Cellular and organelle membranes appear to be the sites most sensitive to SO_2 injury [1, 2]. Since lipids and proteins are major components of biological membranes, we expected that SO_2 would have an effect on the biosynthesis of these components. The exposure of pine needles to SO_2 has been shown to markedly inhibit the biosynthesis of lipids characteristic of chloroplasts and other cellular membranes [3]. Decreased protein content has been reported in SO_2-treated plants [7–9]. Such a decrease could occur due either to an inhibition in the protein biosynthesis or to a stimulation in protein breakdown, or both. It was suggested that the increase in the amino acid content of jack pine needles after fumigation with SO_2 was due mainly to increased breakdown of tissue proteins [4]. At present, however, nothing is known about the effect of SO_2 on *de novo* biosynthesis of cellular proteins. In this study we, therefore, describe the influence of SO_2 fumigation on the *de novo* biosynthesis of chloroplast and cytoplasmic proteins of jack pine needles.

RESULTS AND DISCUSSION

Incorporation of [U-^{14}C]*leucine into needle proteins*

Most of the radioactivity (90–95%) from [U-^{14}C]leucine was incorporated into chloroplast and soluble cytoplasmic proteins. These two fractions incorporated about the same amount of radioactivity per g dry wt of tissues: $74 \pm 4 \times 10^3$ cpm for the chloroplast fraction and $72 \pm 5 \times 10^3$ cpm for the soluble cytoplasmic fraction. The particulate fractions representing mitochondria and microsomes incorporated very little radioactivity into their proteins; therefore, only the soluble cytoplasmic and chloroplast fractions were used for measuring protein biosynthesis.

Treatment of the soluble cytoplasmic fraction with TCA precipitated only 15–20% of the total radioactivity, which suggests that a major portion of the label in the soluble cytoplasmic fraction was in the form of free leucine. This was further supported by the observation that 80–85% of the label present in the cytoplasmic fraction was lost upon dialysis.

Unlike the soluble cytoplasmic fraction, all the radioactivity contained in the labelled chloroplast suspension was precipitated upon treatment with TCA, and the radioactivity was not lost upon washing the precipitated proteins with TCA and 80% acetone.

Centrifugation of the labelled chloroplasts on a discontinuous sucrose density gradient produced two distinct green bands: one at the interphases of sucrose concentrations of 30% and 40%, representing unbroken chloroplasts, and the other at the interphase between 40% and 50%, representing broken chloroplasts [10]. Analysis of the gradient fractions showed that the two chloroplast bands contained almost all of the total chlorophyll, protein and radioactivity. Since most of the radioactivity was confined to the proteins representing the two chloroplast bands, the total chloroplast fraction was used in all subsequent experiments.

Specificity of amino acid incorporation into chloroplast proteins

The incorporation of alanine (76×10^3 cpm/g dry wt) into chloroplast proteins was similar to that of leucine (75

$\times 10^3$ cpm/g dry wt), but glutamic acid (24×10^3 cpm/g dry wt) was poorly incorporated. These differences in incorporation could be attributed to carrier-mediated amino acid uptake across chloroplast membranes. It has been suggested that chloroplasts contain one specific carrier for alanine, leucine, glycine and other aliphatic amino acids and another for serine, threonine and methionine [11]. Yet another carrier located on the inner membrane of the chloroplast envelope is involved in the transport of glutamic and aspartic acids [12, 13].

Incorporation of [U-^{14}C]*leucine into membrane-bound and soluble proteins of pine chloroplasts*

Incorporation into membrane-bound and soluble proteins was, respectively, 67.6×10^3 and 3.7×10^3 cpm/g dry wt. A major portion (95%) of the radioactivity in the chloroplasts was associated with the membrane-bound protein fraction. The soluble protein fraction of the chloroplasts contained only a minor (5%) portion. Under *in vitro* conditions, isolated chloroplast suspensions have been shown to incorporate labelled amino acids into membrane-bound and soluble proteins [14–17]. The distribution of the label in the membrane-bound and soluble fractions was, however, different from the *in vivo* incorporation results of the pine needle chloroplast fractions reported here.

Effects of SO_2 *fumigation on the biosynthesis of chloroplast and cytoplasmic proteins*

Gaseous SO_2 is absorbed from the atmosphere through the stomata of plant leaves and is then dissolved within the moist tissues. It has been suggested that this dissolved gas is mainly responsible for SO_2 phytotoxicity [1, 2]. The results in Table 1 show that exposure of pine seedlings to 0.34 ppm SO_2 for 24 hr inhibited chloroplast and cytoplasmic protein biosynthesis; this inhibition became more severe after 48 hr. Fumigation for 2 hr, however, produced little or no inhibition. No visual evidence of injury on pine seedlings was observed even after 48 hr exposure. At SO_2 exposures producing visual injuries, the severity of inhibition in protein biosynthesis was greatly increased. The results in Table 1 also show an increase in sulphur content of needles with increasing fumigation time, suggesting that inhibition of protein biosynthesis was related to increasing uptake of SO_2.

The inhibitory effect of SO_2 fumigation was more severe on the biosynthesis of chloroplast proteins than on that of cytoplasmic proteins. This effect could be due to the selective accumulation of SO_2 and its products in chloroplast membranes. It has been shown that fumigation of spinach leaves with $^{35}SO_2$ results in a preferential incorporation of ^{35}S in the thylakoid membranes [18]. Among the protein fractions of the pine needle chloroplasts, the fumigation effect was considerably greater on the membrane-bound fraction than on the soluble protein fraction. A similar difference in SO_2 sensitivity has also been reported for the membrane-bound (mitochondrial) and soluble forms of glutamic–oxaloacetic transaminase in peas [19]. These results clearly demonstrated that protein biosynthesis of different origins may have different sensitivities to SO_2. It is suggested that in pine needles SO_2 inhibited protein biosynthesis by acting either as a specific inactivator of the ribosomal system or as a nonspecific modifier or both. Fumigation with SO_2 has been shown to inhibit light-dependent activities of chloroplasts [20], and such changes are in turn expected to disrupt other metabolic processes.

The reduction in the protein content of SO_2-fumigated tissues [7–9] could, therefore, be attributed partly to inhibition in the *de novo* synthesis, as demonstrated in this investigation. We have found, however, that in addition to inhibiting *de novo* synthesis, SO_2 also caused increased breakdown of proteins. The results in Table 2 show that fumigation with SO_2 caused a decrease in the total protein content of both chloroplast and soluble cytoplasmic fractions. This decrease was more pronounced in the chloroplast proteins than in the cytoplasmic ones. This supports the suggestion that the increase found in the amino acid contents of SO_2-fumigated pine needles were due to breakdown of needle proteins [4]. In *Lemna minor*, stress has been shown to induce proteolysis and a decrease in the rate of protein synthesis causing decreases in the activities of a number of enzymes [21]. It is suggested that environmental stress, such as SO_2 fumigation, would bring about similar metabolic alterations.

The deleterious effects of SO_2 on the biosynthesis of proteins, especially of chloroplast membranes, would affect their availability as a structural component of new membranes and may also cause alteration in the existing membranes. The inhibition of polar lipid biosynthesis by SO_2 [3] would further aggravate the above process, since these lipids are the other structural components of the membrane. Alterations in the membrane lipids of the chloroplasts (galactolipids) affect membrane structure and the activity of Photosystem I [22]. In pine, treatment of needles with aqueous SO_2 produced similar changes in the membrane structure [1], lipid composition [2], and Hill reaction activity of the chloroplasts [1].

EXPERIMENTAL

Chemicals. Omnifluor and 1-[U-^{14}C]amino acids (leucine, glutamic acid and alanine) were purchased from New England

Table 1. Effects of SO_2 fumigation (0.34 ppm) on the biosynthesis of chloroplast and cytoplasmic proteins and on sulphur content of jack pine needles

Duration of SO_2 fumigation (hr)	Chloroplast proteins (% of control)			Cytoplasmic proteins	Sulphur* (mg/g dry wt)
	Total	Membrane-bound	Soluble		
2	97.0	96.7	97.0	100.0	0.9
24	72.2	71.6	85.4	79.6	1.3
48	54.9	54.0	69.5	70.4	2.4

*Sulphur content of the unfumigated needles = 0.9 ± 0.1 mg/g dry wt ($\pm$s.d.).

Table 2. Effect of SO_2 fumigation (0.34 ppm) on total protein content of chloroplast and soluble cytoplasmic fractions of pine needles

Duration of SO_2 fumigation (hr)	Chloroplasts (% of control)	Soluble cytoplasm (% of control)
24	81.7	95.4
48	74.1	89.3

Values of protein contents in the fractions isolated from control plants were: chloroplasts = 20.8 ± 1.0 mg/g dry wt; soluble cytoplasm = 24.6 ± 0.8 mg/g dry wt (± s.d.).

Nuclear Corporation. Other chemicals were of commercially available highest grade.

Plant material and growth conditions. Jack pine (*Pinus banksiana* Lamb.) seedlings were grown as described earlier [1]. Seedlings were watered as required and fertilized every week with Hoagland nutrient solution 2 diluted 1:1 with H_2O [23]. Mature needles from 4- to 5-month-old seedlings were used as experimental material.

Fumigation of seedlings with SO_2. Plant seedlings of uniform growth were carefully removed from the styroblock trays and transferred to cuvettes. Details of cuvette construction, seedling arrangement and fumigation procedure were similar to those described earlier [3] except that the cuvettes used in the present study had an airflow rate of 100–120 l/min. The flow rates of SO_2 and air were measured and controlled with rotameters and valves before being mixed and introduced into the cuvettes. The concn of SO_2 was monitored at the outport by a Phillips PW 9700 SO_2 analyser and was maintained by a feedback controller. In each expt 18–24 seedlings were equally divided into two sets and transferred to the cuvettes; one set received clean air, while the other set received 0.34 ppm SO_2. The cuvettes containing the plants were placed in a controlled environment chamber 24 hr prior to treatment with a 19.5 klx light intensity (18 hr photoperiod) and 60–65% relative humidity. Temps. in the growth chamber were 22° and 18° during the light and dark periods, respectively. The shoots were harvested after 2, 24 and 48 hr fumigation periods, and the excised fully mature needles (1 cm sections) were used for exptal purposes.

Metabolic expts with [U-^{14}C]*amino acids.* The excised needle tissues (2 g) were incubated in 20 ml H_2O containing 5 μCi 1-[U-^{14}C]amino acid (sp. act. adjusted to 135 mCi/mmol with unlabelled amino acid) in a shaker at 30° under 10 klx light intensity. Unless otherwise specified, [U-^{14}C]leucine was routinely used as the labelled amino acid. Under these conditions the rate of amino acid incorporation into cellular proteins was linear up to 3 hr; therefore, a 3 hr period was routinely used in all expts. After incubation, each tissue sample was removed by filtration through Miracloth, washed several times with cold H_2O and then used for isolation of chloroplast and soluble cytoplasmic fractions.

Isolation of subcellular fractions. The labelled tissue was homogenized in 30 ml 0.05 M KPi buffer (pH 7) containing 0.4 M sucrose, 1 mM dithioerythritol (DTE), 1 mM $MgCl_2$ and 1% PVP-10 for 30 sec by a Brinkman Polytron Homogenizer (Model PT-10) at low speed. Care was taken to macerate all the needle tissues. The homogenate was filtered through two layers of moist Miracloth, and the filtrate was subjected to differential centrifugation: 1500 g for 5 min for chloroplasts and 15 000 g for 20 min for mitochondria. The sedimented particulate fractions were washed once with a suspending medium containing 0.05 M KPi buffer (pH 7), 0.4 M sucrose, 1 mM DTE and 1 mM $MgCl_2$. The pellets were then suspended in a small vol. of the above medium. The supernatant obtained after removing a 15 000 g pellet was centrifuged at 120 000 g for 1 hr to obtain microsomal and soluble cytoplasmic fractions. All the above operations were carried out at 0–4°.

Density gradient centrifugation of the chloroplast fraction. Discontinuous sucrose gradients were prepared in centrifuge tubes by layering 2 ml each of 20%, 30%, 40%, 50% and 55% sucrose solns in a 0.05 M KPi buffer (pH 7) containing 1 mM DTE and 1 mM $MgCl_2$. A 0.5 ml aliquot of the chloroplast fraction was layered on the top of the gradient and then centrifuged at 0–4°, for 20 min at 8000 g in a swinging bucket rotor (Beckman SW41). Starting from the top, 1 ml fractions were collected and analysed for chlorophyll and protein contents and ^{14}C incorporation.

Membrane-bound and soluble protein fractions of chloroplasts. To determine the incorporation of [U-^{14}C]leucine in membrane-bound and soluble proteins, the chloroplast suspensions were ruptured by freezing and thawing followed by sonication for 1 min with a Biosonic-III Ultrasonicator at a medium setting. The suspension was then centrifuged at 120 000 g for 1 hr. The resulting fractions were identified as soluble (supernatant) and membrane-bound (pellet). All the above operations were carried out at 0–4°.

Protein biosynthesis. A suitable aliquot of the isolated cellular fraction was mixed with TCA (final TCA concn of 5%), and after 1–2 hr at 0°, the suspension was centrifuged. The ppt was washed once with 1% cold TCA and twice with 80% cold Me_2CO. The washed ppt was suspended in 0.5 M NaOH and was allowed to stand for 15 min at 50°. Aliquots of the clear protein solns were used for assaying the ^{14}C incorporation into proteins and the total protein content.

Radioactivity. Incorporation of ^{14}C in the proteins was assayed by mixing 0.1–0.5 ml protein aliquot with 7 ml scintillation fluid (0.4 g Omnifluor dissolved in a mixture of 30 ml EtOH and 70 ml toluene).

Other estimations. Protein was measured according to ref. [24] and chlorophyll according to ref. [25]. The tissue dry wt was measured by oven-drying the fresh needles at 80° for 24 hr. For S analysis, the dried sample of needle tissues was ground to a fine powder and a portion was used for oxygen flask combustion [26]. S in the combusted sample was determined according to ref. [27].

Acknowledgements—We thank M. W. Ali and J. Shuya for technical assistance and P. A. Hurdle for maintaining SO_2 fumigation facilities. This work was funded in part by a grant from the Alberta Oil Sands Environmental Research Program.

REFERENCES

1. Malhotra, S. S. (1976) *New Phytol.* **76**, 239.
2. Khan, A. A. and Malhotra, S. S. (1977) *Phytochemistry* **16**, 539.
3. Malhotra, S. S. and Khan, A. A. (1978) *Phytochemistry* **17**, 241.
4. Malhotra, S. S. and Sarkar, S. K. (1979) *Physiol. Plant.* **47**, 223.
5. Malhotra, S. S. and Khan, A. A. (1980) *Biochem. Physiol. Pflanz.* **175**, 228.
6. Malhotra, S. S. and Khan, A. A. (1982) in *Air Pollution and Plant Life* (Treshow, M., ed.). John Wiley, London (in press).
7. Fisher, K. (1971) *Mitt. Forstl. Bundes-Versuchsanst. Wien* **92**, 209.
8. Godzik, S. and Linskens, H. F. (1974) *Environ. Pollut.* **7**, 25.
9. Constantinidou, H. A. and Kozlowski, T. T. (1979) *Can. J. Botany* **57**, 176.

10. Wagner, G. J. and Siegelman, H. W. (1975) *Science* **190**, 1298.
11. Nobel, P. S. and Cheung, Y. N. S. (1972) *Nature New Biol.* **237**, 207.
12. Heldt, H. W. and Saver, F. (1971) *Biochim. Biophys. Acta* **234**, 83.
13. Heldt, H. W. and Repley, L. (1971) in *Proceedings of the 2nd International Congress on Photosynthesis Research* (Forti, G., Avron, M. and Melandri, A., eds.) p. 1345. Stressa, Junk, Hague.
14. Boardman, N. K., Franki, R. I. B. and Wildman, S. G. (1966) *J. Mol. Biol.* **14**, 470.
15. Chen, J. L. and Wildman, S. G. (1970) *Biochim. Biophys. Acta* **209**, 207.
16. Margulies, M. M. and Parenti, F. (1968) *Plant Physiol.* **43**, 504.
17. Harris, E. H., Preston, J. F. and Eisenstadt, J. M. (1973) *Biochemistry* **12**, 1227.
18. Ziegler, I. (1977) *Planta* **135**, 25.
19. Pahlich, E. (1975) *Atmos. Environ.* **9**, 261.
20. Shimazaki, K. and Sugahara, K. (1980) *Res. Rep. Natl. Inst. Environ. Stud. Jpn.* **11**, 79.
21. Cooke, R. J., Oliver, J. and Davies, D. D. (1979) *Plant Physiol.* **64**, 1109.
22. Shaw, A. B., Anderson, M. M. and McCarty, R. E. (1976) *Plant Physiol.* **57**, 724.
23. Hoagland, D. R. and Arnon, D. I. (1950) *Calif. Agric. Exp. Stn. Circ.* 347.
24. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265.
25. Arnon, D. I. (1949) *Plant Physiol.* **24**, 1.
26. Chan, C. C. Y. (1975) *Analyt. Letters* **8**, 655.
27. Carson, J. A., Crepin, J. A. and Nemunis Siugzdines, P. (1972) *Can. J. Soil Sci.* **52**, 278.