Mycorrhiza (1996) 6:73–75

SHORT NOTE

Z. Miszalski · B. Botton

Effect of sulphite on superoxide dismutase activity in the mycorrhizal fungus *Rhizopogon roseolus*

Accepted: 20 July 1995

Abstract Sulphite at a concentration of 1 mM did not strongly affect the growth of mycelium. Higher concentrations of 5–20 mM almost completely inhibited the growth of mycelium and superoxide dismutase (SOD) [EC.1.15.1.1.] activity. The activity of this enzyme was not detectable on polyacrylamide gels. The lack of induction of SOD and the resulting oxidative stress may in part be responsible for the growth inhibition caused by high concentrations of sulphite.

Key words Sulphur dioxide · Superoxide dismutase · Sulphite · *Rhizopogon roseolus*

Introduction

Sulphur dioxide (SO_2) is the main component of the gaseous pollutants contributing to the formation of acid rain. It dissolves in water and penetrates into cells as the sulphite ion, where it exerts a very marked effect. In plant cells, the products of its hydration and dissociation are trapped in the cytoplasm and must be removed to avoid impairment of metabolism (Ghisi et al. 1990). Oxygen radicals formed in the presence of SO_2 and its oxidation products through a chain reaction of free radicals are believed to be a major cause of the decline of forest trees (Wingsle et al. 1991). SO_2, O_3 paraquat, and various metals can induce oxidative stress via free radicals (Daza et al. 1993).

Sulphite oxidation within higher plants is carried out mostly by green tissues in connection with photosynthesis (Miszalski and Niewiadomska 1993). In green leaves in light, chloroplasts are capable of oxidizing sulphite and dealing effectively with oxygen radicals (Laisk et al. 1988; Miszalski and Ziegler 1989). Plants can also protect themselves against SO_2-induced oxygen toxicity by using scavengers of active oxygen such as superoxide dismutase (SOD) [EC.1.15.1.1], the metaloenzyme that catalyses the dismutation of $O_2^{\cdot -}$ (Tanaka and Sugahara 1980; Madamanchi and Alscher 1991; Miszalski and Ziegler 1992).

According to Høiland (1993), the effects of SO_2 on ectomycorrhizal symbiosis are mainly secondary and due to inhibition of photosynthesis. The fumigation of plants with SO_2 had no effect on the frequency of mycorrhizae formation on *Pinus strobus* or *Betula papyrifora* (Dighton and Jansen 1991). However, Shafer and Schoenberger (1991) reported a 65–85% reduction in the respiration of mycelia in pure cultures of mycorrhizal fungi after a 1-h exposure to 500 ppb SO_2. SO_2 very strongly affects spore germination in many species of fungus (Magan 1994), and for many years this gas has been used to inhibit the growth of fungi (Smilanic et al. 1990).

It has been reported that sulphite oxidase is present in fungi (Wellburn 1988), but it is not known whether SO_2 stimulates SOD, thus inducing resistance to oxidative stress. The aim of the present work was to test whether a selected strain (A_2) of the mycorrhizal fungus *Rhizopogon roseolus,* which is resistant to other environmental stress factors (Turnau et al. 1995), reacts to sulphite-induced oxidative stress by increased SOD activity.

Material and methods

Fungal culture

R. roseolus (Corda in Sturm) Th. M. Fries strain A_2 was obtained from the Institute of Botany of the Jagiellonian University in Cracow. Modified MMN liquid medium according to Kottke et al. (1987) was used for its culture. In some experiments, as indicated in the text, thiamine was omitted. Culture flasks were sealed with

Z. Miszalski (✉)
Department of Plant Physiology, Polish Academiy of Sciences,
PL-31-016 Cracow, ul. Sławkowska 17, Poland
Fax: +48-12-217901

B. Botton
Laboratoire de Physiologie Végétale et Forestière,
Université de Nancy I, B. P. 239,
F-54506 Vandoeuvre-les-Nancy Cedex, France

aluminium foil, and after 4 days of culture at room temperature (about 25 °C) in experiments with thiamine, or after 6 days in experiments without thiamine, sulphite was added to concentrations of 1, 5, 10 and 20 mM. All measurements of growth and the effect of sulphite on SOD activity were carried out after 24 h incubation in the presence of sulphite.

SOD activity

Approximately 0.5 g of fresh material was ground at 4 °C with sand in 1.5 ml of 0.1 M phosphate buffer at pH 7.8 containing 1 mM EDTA in a precooled mortar and then centrifuged for 20 min at 12000 g. SOD activity was assayed in 50- to 200-µl aliquots of the supernatant at room temperature (Elstner and Heupel 1976). Dismutated oxygen radicals were quantified using buffer containing 0.01 mM xanthine, 0.1 U ml^{-1} xanthine oxidase, and 0.5 mM hydroxylamine. The reaction was started by addition of xanthine and stopped after 30 min in darkness with 30 mM sulphanilamide in 5% HCl. N-1-naphthylethylene-diamine was added to a final concentration of 0.25 mM and SOD activity determined by measuring nitrite formation at 540 nm. In some experiments, the crude extract was precipitated with 90% ammonium sulphate or 20% polyethylene glycol 4000 at 4 °C for 30 min, stirred for 5 min and then centrifuged at 40000 g for 20 min. The precipitate was dissolved in the homogenization buffer and aliquots assayed for SOD. Protein content of the extracts was measured according to Bradford (1976).

Polyacrylamide gel electrophoresis

Electrophoresis was performed in a vertical slab apparatus on 7% polyacrylamide gels. Approximately 10 µg of protein in 50 µl of extract was loaded in each lane. Electrophoresis was carried out at 4 °C with a constant voltage of 180 V in 0.1 M Tris-glycine buffer at pH 8.3. The SOD isoenzymes were visualized on gels by the negatively stained photochemical procedure in natural light according to Beauchamp and Fridovich (1971), using 30 min preincubation in darkness with 0.1 M phosphate buffer at pH 7.8, 2.45 mM nitro blue tetrazolium, 1 mM EDTA, 0.028 mM riboflavin, and 28 mM N,N,N,N′-tetramethyl-ethylene-diamine.

Results and discussion

The addition of thiamine (100 µg l^{-1}) to the medium stimulated the growth of *R. roseolus,* but its presence was not essential (Figs. 1, 2). The addition of sulphite at a concentration of 5, 10 or 20 mM strongly inhibited the growth of mycelium, but at 1 mM it slightly stimulated growth if added in the presence of thiamine (Fig. 1).

The activity of crude extracts from *R. roseolus* in removing $O_2^{\cdot -}$ decreased after the addition of 5 mM sulphite to the medium (Fig.3), and the activity was reduced to about 33% of the control by 10 mM sulphite. Sulphite at 1 mM was slightly inhibitory. The activity detectable in crude extracts cannot be attributed solely to SOD, since other low molecular weight substances, such as vitamin C, glutathione, and tocopherol, are also active in removing $O_2^{\cdot -}$.

In experiments using proteins purified after 90% ammonium sulphate precipitation, the data indicate that SOD activity was also inhibited by sulphite (Fig. 4). However, SOD activity was always very low in both the ammonium sulphate and the PEG precipitates (data not shown).

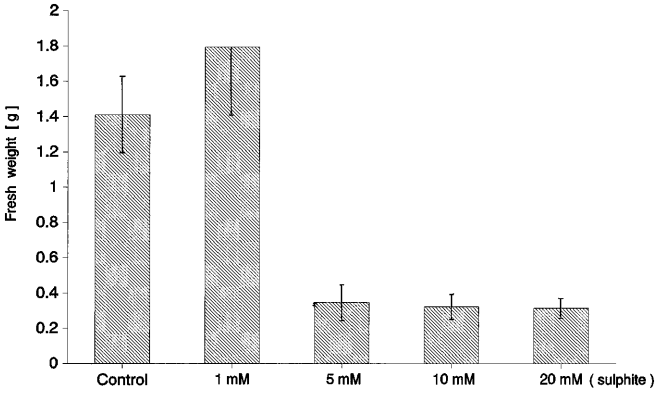

Fig. 1 Fresh weight of *Rhizopogon roseolus* mycelium on the 5th day after inoculation in 30 ml medium with thiamine. Sulphite was added on the 4th day, 24 h before weighing (control without sulphite). The values are the means of five series of experiments. *Bars* show standard deviations

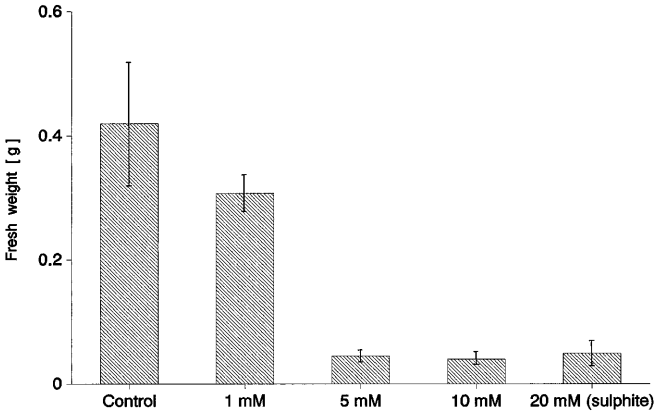

Fig. 2 Fresh weight of *R. roseolus* mycelium on the 6th day after inoculation in 30 ml medium without thiamine. Sulphite was added on the 4th day, 48 h before weighing (control without sulphite). The values are the means of five series of experiments. *Bars* show standard deviations

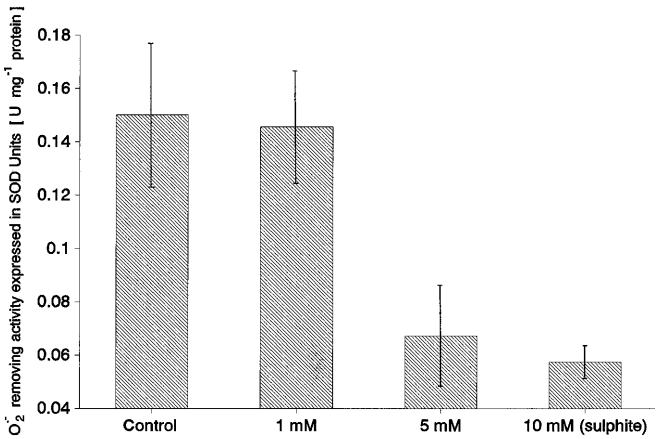

Fig. 3 The ability of crude extracts of *R. roseolus* to remove $O_2^{\cdot -}$. The mycelium was cultured for 5 days with thiamine. Sulphite was added on the 4th day, 24 h before weighing (control without sulphite). The values are the means of five series of experiments. *Bars* show standard deviations

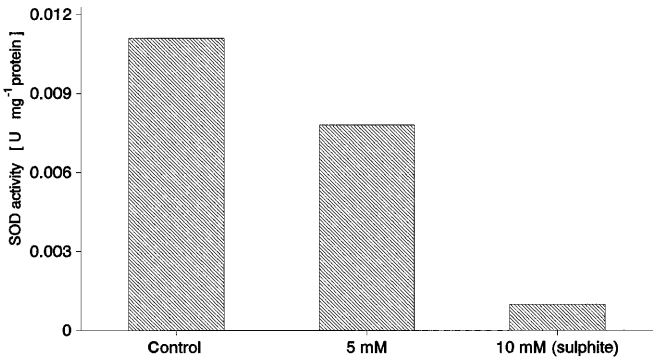

Fig. 4 The ability of the 90% ammonium sulphate precipitate of crude extracts of *R. roseolus* to remove $O_2^{\cdot -}$. The mycelium was cultured at different sulphite concentrations (control without sulphite). The values are the means of at least five independent experiments

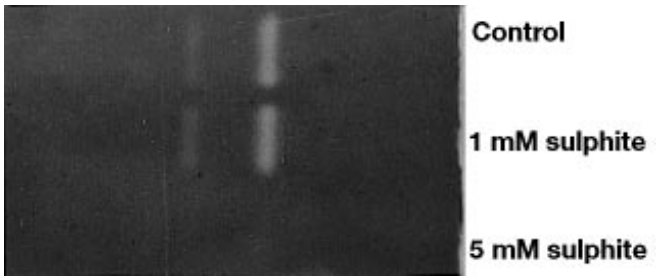

Fig. 5 Uniform (7%) polyacrylamide gel electrophoresis of superoxide dismutase extracted from *R. roseolus* treated with 1 or 5 mM sulphite (control without sulphite) . Each well was loaded with approximately 10 μg of protein (crude extract)

After gel electrophoresis (Fig. 5), SOD activity was identified in the control (without sulphite) and after exposure of mycelium to 1 mM sulphite, but after treatment with 5 mM sulphite no activity was detectable on the gels. Thus, most of the activity removing free radicals in crude extracts after treatment with 5 or 10 mM sulphite was probably due to the presence of low molecular weight compounds.

Ranieri et al. (1992) demonstrated an increase in SOD isoforms due to SO_2 action in different cultivars of wheat, and in experiments with white and green parts of *Chlorophytum* leaves, a much higher SOD activity was measured after exposure to SO_2 (Niewiadomska et al. 1995). Absence of the induction of either new isoforms or a higher SOD activity in the present experiments with *R. roseolus* may explain, at least in part, why this fungus is sensitive to oxidative stress.

Acknowledgements This work was supported by a EUROSILVA (Ardon) Grant, and a Polish Research Grant (KBN Nr. 6P205 037 05).

References

Beauchamp Ch, Fridovich I (1971) Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. Anal Biochem 44:276–287

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Ann Biochem 72:248–255

Daza CM, Sandalio LM, Quijano-Rico M, del Río LA (1993) Isoenzyme pattern of superoxide dismutase in coffee leaves from cultivars susceptible and resistant to the rust *Hemileia vastatrix*. J Plant Physiol 141:521–526

Dighton J, Jansen AE (1991) Atmospheric pollutants and ectomycorrhizae: more questions than answers? Environ Pollut 73:179–204

Elstner EF, Heupel A (1976) Inhibition of nitrate formation from hydrolammonium chloride. A simple assay for superoxide dismutase. Ann Biochem 70:616–620

Ghisi R, Dittrich APM, Heber U (1990) Oxidation versus reductive detoxification of SO_2 by chloroplasts. Plant Physiol 92:846–849

Høiland K (1993) Pollution, a great disaster to mycorrhiza? Agarica 12:65–88

Kottke I, Guttenberger M, Hampp R, Oberwinkler F (1987) An in vitro method for establishing mycorrhizae on coniferous tree seedlings. Trees 1:191–194

Laisk A, Pfanz H, Heber U (1988) Sulfur dioxide fluxes into different cellular compartments of leaves photosynthesizing in a polluted atmosphere. Planta 173:241–252

Madamanchi NR, Alscher RG (1991) Metabolic basis for differences in sensitivity of two poa cultivars to sulfur dioxide. Plant Physiol 97:88–93

Magan N (1994) Tolerance of fungi to sulfur dioxide. In: Jennings DH (ed) Stress tolerance of fungi. Dekker, New York, pp 173–187

Miszalski Z, Niewiadomska E (1993) SO_2 oxidation during greening of *Avena sativa* seedlings. Photosynthetica 28:577–581

Miszalski Z, Ziegler H (1989) Uptake and efflux of ^{35}S sulfite by protoplasts and their chloroplasts of oat (*Avena sativa* L.). Z Naturforsch 44c:509–513

Miszalski Z, Ziegler H (1992) Superoxide dismutase and sulfite oxidation. Z Naturforsch 47c:360–364

Niewiadomska E, Miszalski Z, Morańda J (1995) Non-uniform sensitivity to SO_2 within one variegated leaf of *Chlorophytum comosum*. Phyton Austria 35:55–61

Ranieri A, Durante M, Volterrani A, Lorenzini G, Soldatini GF (1992) Effects of low SO_2 levels on superoxide dismutase and peroxidase isoenzymes in two different wheat cultivars. Biochem Physiol Pflanz 188:67–71

Shafer SR, Schoenberger MM (1991) Mycorrhizal mediation of plant response to atmospheric change: air quality concepts and research considerations. Environ Pollut 73:163–177

Smilanic JL, Harvey JM, Hartsell PL, Henson DJ, Harris CM, Fouse DC, Assemi M (1990) Influence of sulfur dioxide fumigant dose on residues and control of postharvest decay of grapes. Plant Dis 74:418–421

Tanaka K, Sugahara K (1980) Role of superoxide dismutase in defense against SO_2 toxicity and an increase in superoxide dismutase activity with SO_2 fumigation. Plant Cell Physiol 21:601–611

Turnau K, Kottke I, Dexheimer J (1995) Toxic element filtering in *Rhizopogon roseolus/Pinus sylvestris* mycorrhizas collected from calamine dumps. Mycol Res

Wellburn A (1988) Air pollution and acid rain: the biological impact. Longman Scientific & Technical

Wingsle G, Gardeström P, Hällgren J-E, Karpiński S (1991) Isolation, purification, and subcellular localization of superoxide dismutase from Scots pine (*Pinus silvestris L.*) needles. Plant Physiol 95:21–28