

Effect of ozone and sulphur dioxide on mycorrhizae of *Pinus halepensis* Miller

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Summary – Little information exists on the effect of ozone (O₃) and sulphur dioxide (SO₂) on *Pinus halepensis* Miller. The objective of this work was to determine the effect of these gaseous pollutants alone or in combination on biomass and mycorrhizae of *P. halepensis*. Seedlings were treated for 1 year with filtered air (control), 50 ppb O₃, 40 ppb SO₂ or a mixture of 50 ppb O₃ + 40 ppb SO₂. O₃ and SO₂ treatments had no significant effect on shoot and root biomass and a slight reduction on percentage of mycorrhizal colonization was noted with SO₂. However, these parameters were significantly reduced when the pollutants acted in combination. Morphological alterations of mycorrhizae were also noted, with a reduction in the coralloid structures in favour of simple ones. Moreover, a change in species composition was observed: the ectomycorrhizae probably formed by *Suillus* species being replaced by ectendomycorrhizae in the O₃ + SO₂ treatment.

biomass / mycorrhizae / *Pinus halepensis* / ozone / sulphur dioxide

Résumé – Effet d'O₃ et SO₂ sur les mycorhizes de *Pinus halepensis* Miller. Les informations concernant l'effet d'O₃ et SO₂ sur *Pinus halepensis* Miller sont rares. L'objectif de ce travail était de déterminer l'effet de ces polluants gazeux, seuls ou en combinaison, sur la croissance et les mycorhizes de *P. halepensis*. Des semis ont été soumis pendant 1 an à de l'air purifié (témoin), 50 ppb O₃, 40 ppb SO₂ ou la mélange de 50 ppb O₃ + 40 ppb SO₂. Les traitements O₃ et SO₂ n'ont pas eu d'effet significatif sur la masse des tiges et des racines, ni sur le pourcentage de colonisation mycorhizienne. Cependant, ces paramètres ont été significativement réduits lorsque les deux polluants agissaient en combinaison. Des modifications de la morphologie des mycorhizes ont été notées, avec une réduction des structures coralloïdes en faveur des structures plus simples. De plus, un changement d'espèces fongiques a été observé avec le traitement O₃ + SO₂: les ectomycorhizes attribués à *Suillus sp* ont été remplacés par des ectendomycorhizes.

biomasse / mycorhizes / *Pinus halepensis* / ozone / sulfure dioxide

INTRODUCTION

Atmospheric pollutants may directly or indirectly affect forest productivity. In particular,

ozone and sulphur dioxide have been indicated as contributing factors to forest decline in central Europe and North America (McLaughlin, 1985). Ozone has been re-

ported to reduce root growth (Hogsett et al, 1985; Chappelka and Chevone, 1986; Temple, 1988; Schier et al, 1990), and to induce reductions in needle length, seedling height and dry weight (Schier et al, 1990). Reduced photosynthesis (Reich, 1985), changes in allocation patterns (Cooley and Mauning, 1987) and alterations in needles (Ebel et al, 1990; Sutinen et al, 1990; Evans and Leonard, 1991; McQuattie and Schier, 1993) have also been observed in plants exposed to ozone.

Soil microbiological components, such as ectomycorrhizal fungi, could also be affected by atmospheric contaminants. Because of their important role in plant nutrient uptake, tolerance in root diseases, water uptake, etc, effects on mycorrhizal associations could be related to reduced plant growth and damage of coniferous trees.

Nonmycorrhizal seedlings have been generally used to study plant response to ozone or SO₂. However, in natural conditions roots are mycorrhized. Therefore, the use of mycorrhizal plants in the experiments is more representative of what occurs in a natural plant-soil system.

Mycorrhizal seedlings exposed to ozone treatments have been reported to be very sensitive and they exhibit reduced percentages of mycorrhizal colonization. Reich et al (1986), McQuattie and Schier (1987, 1992), Stroo et al (1988) and Edwards and Kelly (1992) observed this fact in several *Pinus* species. In a similar way, mycorrhizal colonization was inhibited in *Quercus rubra* seedlings by high levels of sulphur dioxide (Reich et al, 1986).

P halepensis Miller (Aleppo pine) is a widely distributed plant species in Mediterranean ecosystems and is well adapted to semiarid conditions. Only few data are available about the effects of gaseous pollutants on *P halepensis*. Velissariou et al (1992) reported chlorotic mottle in Aleppo pine needles caused by ozone in Attica (Greece) and a possible interaction between ozone and low levels of SO₂ near

Athens. Similarly, Sánchez et al (1992) observed the same symptoms on *P halepensis* needles in specific areas of Spain and Greece, as a consequence of high levels of photochemical oxidant pollution. Ozone might affect the winter recovery of chlorophyll content in needles (Inclán et al, 1993), while Alonso et al (1993) reported an increase in peroxidase activity in ozone fumigated trees, suggesting a possible interaction between high levels of pollutants and Mediterranean climatic stresses. Wellburn and Wellburn (1994) also pointed out the detrimental effect of high levels of O₃, which affect the ability of the tree to resist water stress and reported extensive accumulations of starch in needles, particularly in the endodermis. Changes in concentration of fatty acids and chloroplast ultrastructure in response to ozone have also been reported in *P halepensis* (Anttonen et al, 1995).

Although the effect of ozone and sulphur dioxide on ectomycorrhizae has been studied with several coniferous plant species, to our knowledge, no previous information exists about Aleppo pine.

The objective of this study was to determine the effect of SO₂ and O₃ alone or in combination on biomass production and mycorrhizal development of *P halepensis*.

MATERIALS AND METHODS

Two-year-old *P halepensis* seedlings of similar height and diameter grown from seed in nursery were transplanted into 35 x 30 x 30 cm plastic pots containing natural soil collected from a previously cultivated, but now abandoned cereal field close to a *P halepensis* stand in Calanda, Teruel (Spain). Physical and chemical characteristics of the soil are shown in table I.

Plants were placed in chambers equipped for O₃ and SO₂ fumigation and charcoal filters, in order to avoid the possible entry of pollutants different from those of interest. Four treatments were established: filtered air (FA) without contaminants (control); FA + 50 ppb of O₃; FA + 40 ppb of SO₂; and FA + a mixture of 40 ppb of SO₂ + 50 ppb of O₃. These pollution concentrations, although realistic levels, were above the in-

mission levels measured in Mediterranean areas (Velissariou et al, 1992). They were chosen in order to accentuate the possible effects in a limited-in-time experiment.

The internal volume of the chambers was 6.9 m³ (O₃ treatment), 9.5 m³ (control) and 10.2 m³ (SO₂ and SO₂ + O₃ treatments). The walls of the chambers were made of glass and covered by a shading film which intercepted about 40% of incident sunlight. No other sources of light were provided.

The ozone was generated by two dried air feeded Triozon generators. Possible nitrogen oxides were then removed by bubbling the output of the ozonator through a water wash. The ozone and the 1% in nitrogen SO₂ from bottles were conducted to the chambers through stainless steel pipes by means of a pump.

The pollutant concentration was controlled by a system of flowmeters and electrovalves and was continuously measured by three Thermo-Electron 43A SO₂ analysers and one MonitorLab O₃ analyser.

There was a total of 125 seedlings: 32 replicates in control treatment, 35 in SO₂ and SO₂ + O₃ treatments and 25 in O₃ treatment.

The temperature (monthly average) oscillated between 10 °C in January 1993 and 27.5 °C in July 1992 during the experiment. The relative humidity varied between 46% in March 1993 and 90% in January 1993.

Plants were watered when necessary by means of a drip irrigation system.

After 1 year, eight randomly selected seedlings per treatment were harvested, and aerial and root biomass were determined after drying (80 °C, 16 h). Nine subsamples from the root system of each selected seedling were taken from the top, middle and bottom root portions and mixed in a bulked sample. Roots were examined for the presence of mycorrhizae under a stereomicroscope, and the mean percentage of mycorrhizal colonization was determined.

Mycorrhizal fungi were isolated from the mycorrhizae in petri dishes with modified-Melin-Norkrans medium (MMN) (Marx, 1969). Mycorrhizal tips were washed in a solution of 0.01% Tween 80 and then in sterile water for 30 min. They were surface sterilized with 30% H₂O₂ for 35 s and washed again in sterile water before being placed in the medium. Macroscopic and microscopic characteristics of the mycelia were examined.

Light microscopy: Short roots were fixed in formalin acetic alcohol (FAA, 4:1:1), dehydrated in

Table I. Physical and chemical characteristics of the soil used in the experiment.

pH (KCl)	7.58
CO ₃ (%)	23.06
CaO active (%)	8.56
C org (%)	0.30
Organic matter (%)	0.52
N (%)	0.03
C/N	8.82
P extractable (mg/kg)*	7.03
K exchangeable (meq/100 g)	0.15
Mg exchangeable (meq/100 g)	2.80
Ca exchangeable (meq/100 g)	12.30
Sand (%)	70.90
Silt (%)	19.60
Clay (%)	10.30

* Determined according to the Olsen method (1954).

a graded ethanol series and then infiltrated and embedded in Epon resin. Sections (0.5–1.0 μm) were stained with toluidine blue.

Data were subjected to analysis of variance and significant differences between mean values determined by Fischer's least significant difference test (shoot/root biomass) and Duncan's test (percentage of mycorrhizal colonization).

RESULTS

O₃ and SO₂ treatments had no significant effect on root biomass. On the other hand, a slight but not significant reduction in shoot and total biomass was observed in plants treated with O₃ and SO₂ alone. However, plant growth (shoot and root) was significantly affected by these contaminants when they acted in combination. Total biomass was reduced by 25% compared to controls (table II).

The morphology of pine root systems was also altered. Root elongation and lateral root formation were reduced in the SO₂ + O₃ treatment.

The percentage of mycorrhizal colonization also decreased significantly with the joint effect of these factors. Neither SO₂ nor O₃ alone had a strong inhibitory effect on

Table II. Effect of ozone and sulphur dioxide on shoot and root biomass production in *Pinus halepensis* seedlings.

Treatment	Shoot biomass (g)	Root biomass (g)	Total biomass (g)	Root/shoot (g)
Control	47.88 ^a ± 6.51	24.13 ^a ± 3.57	72.01 ^a ± 9.94	0.50 ^b ± 0.03
SO ₂	43.36 ^{ab} ± 6.89	23.11 ^a ± 3.77	66.47 ^{ab} ± 10.39	0.53 ^{ab} ± 0.04
O ₃	40.87 ^{ab} ± 8.63	23.21 ^a ± 3.37	64.08 ^{ab} ± 11.53	0.58 ^a ± 0.07
SO ₂ + O ₃	38.36 ^b ± 13.43	15.41 ^b ± 5.85	53.78 ^b ± 18.86	0.40 ^c ± 0.08

Data expressed as mean and standard error of eight replicates. Values followed by the same letter in a treatment are not significantly different ($P < 0.05$).

the formation and development of ectomycorrhizae (fig 1).

Two morphological types of mycorrhizae were observed. One of them occurred in the four treatments. Ectomycorrhizae were simple, dichotomous and mainly coralloid, 1.5–4 mm in length, normally stippled with a base up to 5 mm long. The surface was white to pink, with a silvery appearance due to air enclosed between the mantle hyphae (densely cottony mantle with extramatrical hyphae between dichotomies). Rhizomorphs were white to pinkish brown.

The combination of O₃ and SO₂ also affected the morphology and development of mycorrhizae. Control, O₃ and SO₂ usually exhibited dichotomous and coralloid structures, with a well-developed mantle. In contrast, roots treated with SO₂ + O₃ showed a reduction of coralloid structures with a predominance of simple, unramified ectomycorrhizal tips. The percentage of coralloid structures decreased significantly in this treatment (fig 1).

Several isolates were obtained after the culture of this morphotype. Their comparison with a fungal isolate collection made their identification possible in some cases. The morphological features were as follows:

i) Mycelium pink brown, superficial, regular margin, brown reverse. Hyaline hyphae, simple ramification, 3–5 µm wide, thin walls. Similar to *Suillus* sp.

ii) Mycelium pinkish-brown. Aerial mycelium white, cottony, densely distributed on the colony. Regular margin, sometimes dark brown. Brown-yellowish reverse. With exudates of yellow pigments. Hyaline hyphae, simple ramification, 3–5 µm wide, thin walls. Similar to *Suillus* sp.

iii) Mycelium initially white, then cream. Aerial mycelium white, cottony, densely dis-

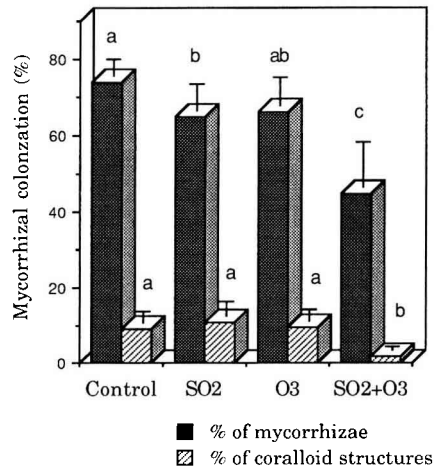


Fig 1. Effect of ozone (O₃) and sulphur dioxide (SO₂) on total percentage of mycorrhizal colonization and percentage of coralloid structures on *Pinus halepensis* seedlings. Data expressed as mean and standard error of eight replicates. Values with the same letter in a parameter are not significantly different ($P < 0.05$).

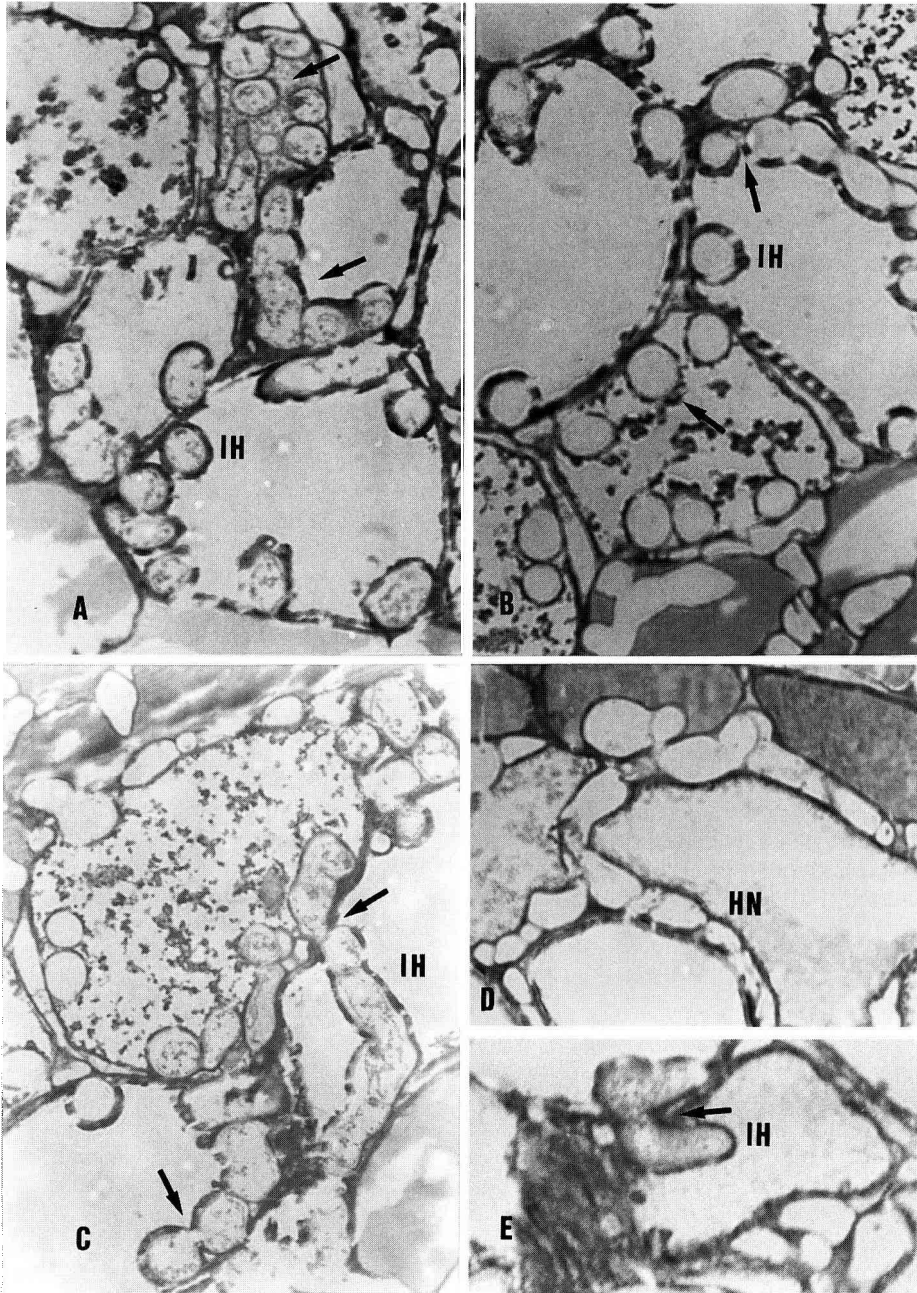


Fig 2. Semi-thin sections of ectendomycorrhizae in the SO₂ + O₃ treatment showing intracellular hyphae (IH) and the Hartig net (HN). **A–D:** x 1 000; **E:** x 1 500.

tributed on the colony. Regular margin. Reverse brown in the middle, yellowish-white in the margin. With exudates of light yellow pigments. Hyaline hyphae, simple ramification, 2.5–4.5 μm wide, thin walls.

iv) Mycelium initially white, then brown. Aerial mycelium white, cottony, irregularly distributed on the colony, with radial folds. Dark brown reverse. Hyaline hyphae, simple ramification, 3–5 μm wide, thin walls. Similar to *Suillus collinitus*.

The other morphotype was of the ectendomycorrhizal type. It appeared only in the $\text{SO}_2 + \text{O}_3$ treatment (50% of the studied seedlings) and some seedlings of the O_3 treatment (28% of seedlings). Ectendomycorrhizae were simple, 1–2.5 mm in length and 0.5–1.5 mm in width and brown to dark brown in older roots, except at the apices which were lighter. The mantle was poorly developed to lacking, with a smooth surface and sometimes emanating hyphae. They were differentiated from nonmycorrhizal root tips by the absence of root hairs, a swollen appearance and the examination of hand-cross sections for the presence of Hartig net. The presence of intracellular hyphae in stained sections, as well as a thin mantle and a well-developed Hartig net, proved that they were ectendomycorrhizae (fig 2). Attempts to isolate the mycosymbiont in MMN medium failed.

DISCUSSION

The main conclusion that can be drawn under the experimental conditions is that the combination of SO_2 and O_3 had a greater negative effect on biomass and mycorrhizal colonization of *P. halepensis* than either substance alone, suggesting a synergic interaction of these gaseous pollutants. The effects of SO_2 or O_3 on biomass production and mycorrhizal associations are related to alterations of photosynthesis and metabolism of plants, mediated by changes in stomatal aperture, carbon-fixing enzymes, pH buffering ca-

capacity and disruption of the integrity of membranes (Guderian, 1985; Kozłowski et al, 1991). Whilst the mechanisms of action of SO_2 and O_3 separately are quite well known, those of the mixture remain unclear. It is generally accepted that the mixture of both SO_2 and O_3 seems to lower the threshold doses of damage for the single components (Darrall, 1989). The formation of free radicals, ie, molecules with unpaired electrons, and radical-chain mechanisms seem to be involved in this process (Weigel et al, 1989; Elstner and Osswald, 1991).

O_3 and SO_2 do not penetrate the soil surface, so direct effects of these pollutants on mycorrhizae are not likely. Indirect effects are mediated by the plant, due to the reduced photosynthesis and therefore the decreased allocation of carbohydrates to the root (Reich et al, 1985; Dighton, 1988; Dighton and Jansen, 1991). It is widely accepted that the availability of carbohydrates is a limiting factor for the development of mycorrhizal infection. On the other hand, proton excretion by the roots and sulphate translocation from leaves to roots have been reported in SO_2 fumigated plants (Kaiser et al, 1993). This fact could also explain the detrimental effect of SO_2 in mycorrhizal colonization.

Reductions in plant biomass and percentage of colonization seem to be a general fact. However, it is also remarkable that O_3 and SO_2 not only affected mycorrhizal infection but produced qualitative changes in the mycorrhizae.

Firstly, morphological alterations were noted, with a significant reduction in the coralloid structures in favour of simple or dichotomous ones. McQuattie and Schier (1987, 1992) also observed reduction of the coralloid form of a *Pisolithus tinctorius* ectomycorrhiza on *Pinus rigida* due to fumigation with O_3 and the presence of Al in the nutrient solution, as well as other anatomical alterations such as deterioration of fungal mantle and reduction of Hartig net.

Dighton and Skeffington (1987) reported the suppression of a coralloid ectomycorrhiza of *P sylvestris* due to acid rain.

On the other hand, mycorrhizal symbiosis did not disappear completely, but a change in species composition occurred. The presence of ectendomycorrhizae in the SO₂ + O₃ and O₃ treatments revealed that the pollutants may selectively inhibit certain species and favour other more resistant ones. It is generally accepted that diversity stabilizes the plant–soil system during stress. Different species differ in their tolerances, physiological requirements, etc. The altered environment results in a replacement of one mycorrhizal fungal species by another, but the plant retains the mycorrhizal component (Perry et al, 1989). These changes in species composition under stress caused by acidification or SO₂ and O₃ deposition have been reported under natural and experimental conditions (Dighton and Skeffington, 1987; Markkola and Ohtonen, 1988; Meier et al, 1989; Dighton and Jansen, 1991).

In our case, ectomycorrhizae were formed by several *Suillus* species or strains. The soil used for the experiment probably contained the mycorrhizal propagules from the surrounding pine stand. This genus is very common in *P halepensis* forests and it has been found to form ectomycorrhizae with this pine (Torres et al, 1991; Torres and Honrubia, 1994). These *Suillus* fungal species were replaced by ectendomycorrhizal associations formed by E-strain complex fungi in SO₂ + O₃ treatment, suggesting their tolerance to stress conditions. E-strain fungi are often mycorrhizal symbionts of *P halepensis* in nurseries (unpublished data) and they have recently been found to form ectendomycorrhiza with *P halepensis* in burned forests (Torres, personal communication). These observations may lead to the conclusion that they are pioneer species, resistant to stress conditions and able to form mycorrhizal associations when other fungi cannot.

Further research on the differences in susceptibility to atmospheric pollutants among fungal species and their implications in plant nutrition must be undertaken.

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