WATER STATUS, PHOTOSYNTHETIC PIGMENTS, C/N RATIOS AND RESPIRATION RATES OF SITKA SPRUCE SEEDLINGS EXPOSED TO 70 PPBV OZONE FOR A SUMMER

J. PEÑUELAS,* M. RIBAS-CARBÓ,† M. GONZÁLEZ-MELER‡ and J. AZCÓN-BIETO†

*CREAF, Universitat Autonoma 08193 Bellaterra, Barcelona, Spain; †Departament de Biologia Vegetal, Universitat de Barcelona, Diagonal 645, 08028 Barcelona, Spain; ‡Smithsonian Environmental Research Center, P.O. Box 28, Edgewater, MD 21037-0028, U.S.A.

(Received 28 July 1993; accepted in revised form 14 March 1994)

Peñuelas J., Ribas-Carbó M., González-Meler M. and Azcón-Bieto J. Some observations on the water status, photosynthetic pigments, C/N ratios and respiration rates of Sitka spruce trees exposed to 70 ppbv ozone for a summer. Environmental and Experimental Botany 34, 443–449, 1994.—Three-year old seedlings of *Picea sitchensis* (Bong.) Carr. were exposed in large-scale chambers (solardomes) to 70 ppbv O₃ for an entire summer, 7 hr day⁻¹, under approximately ambient conditions. No macro- or microscopic injury, no fluorescence differences and no growth rate effects were found in the fumigated spruce. However, fumigated seedlings showed significantly lowered water potentials, chlorophyll concentrations, nitrogen and sulfur contents, and respiration rates. The old needles had higher cyanide-resistant respiration. These results were consistent with symptoms of forest decline affecting high-altitude forest and showed the presence of physiological changes even though there were no visible symptoms.

Key words: Water potential, Chlorophyll, Nitrogen, Respiration, Sitka spruce.

INTRODUCTION

Ozone is considered to be an important air pollutant in the U.S.A. and Europe and could have persistent and lasting effects on plants. In addition to the well known periodic high ozone concentrations in summer, monitoring of ozone has also shown potential phytotoxic concentrations under certain circumstances in autumn and winter.¹⁰,¹₂,¹³

The decline of conifers, hardwoods and field crops in Spain and elsewhere in Europe and the U.S.A. is believed to be caused by a combination of stressors. Although several causes have been ascribed to forest decline,²⁷ ozone formation and distribution in the atmosphere generally correlate with the spatial and temporal development of forest decline¹¹,¹²,¹³ and may enhance the action of mountain stressors such as frost.²₂,²₅ Ozone levels may be high enough to cause acute injury to sensitive plant species²⁴,²₆ but spruce are considered to be relatively resistant to the direct effects of ozone. Controlled fumigation studies have not yet reproduced visible symptoms comparable to those in declining forests.²₅

We studied a spruce of importance for commercial forestry in Europe, the Sitka spruce *Picea sitchensis* (Bong.) Carr. native to northwestern North America. The objective was to determine whether exposure of this plantation species to moderately high (70 ppb) concentrations of ozone that are usual in some areas of Catalonia and Europe during the
summer months could influence the growth and morphological and physiological characteristics of spruce seedlings. With this aim we surveyed several morphological and physiological parameters. Our hypothesis was that although no visible injuries are initially developed after exposure to a moderately high ozone concentration, physiology is altered and precedes visible damage.

MATERIAL AND METHODS

Eighty uniform 3-year old spruce seedlings raised in greenhouses were sown in pots containing a pit-grit mixture and a slow-release fertilizer. They were then transferred to ozone fumigation treatments in four solardomes (20 seedlings in each) of the Institute of Environmental and Biological Sciences, University of Lancaster. The ozone fumigation treatment lasted all summer (from 1 June to 10 September). Seedlings were watered every morning to container capacity.

Solardomes are hemispherical chambers supplied by Rosedale Engineering Ltd, U.K. (diameter 4.6 m, height 2 m, approximate volume 20 m³). They are constructed of glass mirrored in an anodised aluminium framework. Before entering the solardome, the air is passed at a flow rate of about 40 m³ min⁻¹ through large charcoal filtration units that provide a chamber environment with low spatial variability of pollutant gas concentration and rapid air circulation. This allows exposure of plants at near ambient temperature and relative humidity.²³ In two of these solardomes (70 ppbv), ozone was generated by passing compressed dry air over discharge tubes (Penwalt, Wallace and Tierman, Tonbridge, England). The other two solardomes were not ozone fumigated (5 ppbv). Control of the gas flow to each dome was achieved through individual flowmeters mounted on a stainless steel manifold system. Each dome had a monitoring tube to the monitoring instruments that measured the concentration of ozone (by means of an Ultra Violet Analyser Dasibi 1008 PC). On average, irradiance in the solardomes was reduced by about 20% with respect to that outdoors and the temperature difference between inside and outside the chamber was generally lower than 1°C (at outside temperatures lower than 25°C), making it very unlikely that spruce suffered heat stress.

After 10, 20 and 30 days of ozone fumigation, four samples of each solardome (8 for each treatment and measurement) were taken to measure water potentials, needle dry weight/fresh weight ratios, and fluorescence stress indexes. Visible injury and relative growth were measured in all the seedlings.

Growth measurements

Spruces seedlings were measured for height to the top of the terminal bud from a black ink mark approximately 1 cm above the soil level. Stem diameter was measured at the black mark. Relative growth rates (RGR) were calculated every 15 days throughout the summer using this non-destructive technique, in which measurements of stem height and diameter were used to estimate above-ground biomass as reported by Lucas et al.²⁴

Fresh and dry weights

Needles were weighed to the nearest 0.1 mg (fresh weight) and then dried at 60°C to constant dry weight (Mettler AM50 electronic balance).

Water potential measurements

Shoots (six for each treatment and age) were excised at midday and placed in a Soilmoisture 3500 pressure chamber with some xylem tissue extending through a seal to the outside of the chamber.²⁹,³⁹ Pressure was applied until free liquid appeared in the xylem. The gas source was nitrogen to avoid possible membrane oxidation. A strong light source and a low power microscope were used to observe the xylem exudation during pressuration. For prevention of water loss, the shoots were wrapped with clingfilm as suggested by Leach et al.¹⁷ To avoid exudates other than that of the xylem, the terminal part of each shoot was peeled away.

Chlorophyll fluorescence index of stress

The rate of rise of induced chlorophyll fluorescence was measured to provide an early indication of possible ozone injury because it depends on the efficiency with which light energy is transformed within the photosynthetic apparatus. Fluorescence rises quickly to a maximum peak \( F_{m} \) and then decreases slowly to a steady state \( F_{s} \) value. We calculated \( F_{s}/F_{m} \) as a fluorescence coefficient of stress. Lower values of this ratio indicate higher photosynthetic efficiency.

Samples of previous and current year's needles
(eight for each age and treatment) were collected. Shoots were placed adaxial surface uppermost on moist filter paper and held in place by an overlaying black plastic sheet\(^2\) for 20 min (dark adaptation) prior to fluorescence emission measurements. We previously checked that the coefficient of fluorescence reached constant values after this period. Light intensity was higher than 300 \(\mu\text{E m}^{-2} \text{s}^{-1}\) to obtain saturation responses of the coefficient. Then chl fluorescence was monitored \textit{in vivo} using a Hansatech fluorometer and the output recorded in a Servogor 120 BBC chart recorder.

Finally, after the 90 days (all summer) of ozone fumigation, the above parameters and the following were studied.

\textbf{Scanning electron microscope observations}

In these observations, a scale was used to quantify the rates of damage. The scale was similar to that of Crossley and Fowler\(^8\) in which 0 represented a perfect, newly formed crystalline epicuticular wax structure and 4 represented a surface on which no crystalline structure was visible.

Needles (eight from the current year and eight from the previous year, randomised for each treatment) were dried at 60°C to constant weight, removed and checked for handling damage before excising central 5 mm length pieces. These pieces were then secured by silver dag abaxial and adaxial surfaces onto different aluminium stubs (four needles/stub). The prepared stubs were then coated with 40 nm of gold in a Polaron E5000 sputter coater; examinations were made using a Stereoscan S-120 Scanning Electron Microscope (Cambridge Instruments Ltd.). Representative areas of each needle were photographed at different magnifications using Kodak Tri-X Pan film over 40 s at an accelerating voltage of 15 kV.

\textbf{Photosynthetic pigments}

Chlorophyll concentration was measured after maintaining needles (six for each age and treatment) in dimethylformamide for 4 hr at 60°C. Calculations were carried out according to Inskeep and Bloom.\(^15\) All procedures of extraction were carried out at low light levels.

\textbf{C, N and S needle contents}

Carbon, nitrogen and sulfur contents of needles (six for each age and treatment) were analysed with a Carlo Erba NA 1500 Analyzer by using a standard configuration for those determinations. The samples were previously dried at 60°C to constant weight. They were weighed by using a Mettler UM3 microbalance in tin containers.

\textbf{Respiration rates and cyanide resistance}

An oxygen electrode (Rank Brothers, Cambridge, England) was used to measure needle (six for each age and treatment) respiration rate, and the inhibitor KCN was added to estimate the cyanide resistant respiration.\(^16\) Oxygen uptake rates were measured at 25°C in an air saturated solution (the initial concentration of \(\text{O}_2\) was considered to be 240 \(\mu\text{M}\)) containing 10 mM HEPES and 0.2 mM \(\text{CaCl}_2\). Cut needles were introduced in the measurement cuvette. Depletion of the oxygen concentration in the strongly stirred solution of the closed cuvette was linear with time. Measurements were made in the dark between 240 and 120 \(\mu\text{M}\) \(\text{O}_2\). A nylon net separated the needles from the stirrer bar and the electrode. Cyanide was prepared and used as described earlier.\(^26\) The used concentration of KCN (2 mM) was rather high to avoid diffusion problems of the tissues. At the end of every \(\text{O}_2\) uptake measurement, the leaves were oven-dried at 60–70°C to constant weight. The fresh weight of these samples was also measured at the beginning of each experiment.

\textbf{Statistical procedures}

All statistical analyses (ANOVA, Student \(t\)-tests, and regression) were done with Systat v 5.1 for the Macintosh (Systat, Inc. Evanston, Illinois, U.S.A.).

\textbf{RESULTS}

Sitka spruce seedlings fumigated with 70 ppbv ozone 7 hr day\(^{-1}\) for a summer in solardomes did not show visible injury symptoms, differences in fluorescence index, or ultrastructural anomalies. Growth that had largely been completed by the end of August for all seedlings did not show any significant difference between control and exposed spruces.

However, after the all-summer fumigation, water potential was significantly lower in ozone-fumigated plants (Table 1). Ozone-fumigated spruces also showed a clear decrease in chlorophyll concentration and in nitrogen and sulfur leaf contents
Table 1. Effect of all-summer fumigation with 70 ppbv ozone on several physiological and morphological characteristics of Sitka spruce seedlings. Mean values and standard errors in previous and current year’s needles at the end of the summer fumigation period in the solaromes. * = P < 0.10, ** = P < 0.05 and *** = P < 0.01 level of significance of the difference between control and fumigated seedlings (Student’s test); n = 4–12 measurements for each treatment. The same statistically significant differences were maintained in an ANOVA where samples taken within chambers were considered as subsamples, and treatment effects were tested against the experimental error instead of the subsampling error.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control Previous</th>
<th>Control Current</th>
<th>Ozone fumigated Previous</th>
<th>Ozone fumigated Current</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water potential (MPa)</td>
<td>-1.10 ± 0.08</td>
<td>-0.95 ± 0.04</td>
<td>-1.35 ± 0.06</td>
<td>-1.41 ± 0.08</td>
</tr>
<tr>
<td>Chl a (μg g⁻¹FW)</td>
<td>1.12 ± 0.03</td>
<td>1.10 ± 0.02</td>
<td>0.83 ± 0.03</td>
<td>0.83 ± 0.02</td>
</tr>
<tr>
<td>Nitrogen content (%)</td>
<td>1.37 ± 0.03</td>
<td>1.36 ± 0.02</td>
<td>1.09 ± 0.02</td>
<td>1.00 ± 0.03</td>
</tr>
<tr>
<td>Sulfur content (%)</td>
<td>0.19 ± 0.01</td>
<td>0.18 ± 0.01</td>
<td>0.15 ± 0.01</td>
<td>0.11 ± 0.01</td>
</tr>
<tr>
<td>Oxygen uptake (μmol O₂ gDW⁻¹ hr⁻¹)</td>
<td>7.2 ± 0.2</td>
<td>13.5 ± 0.3</td>
<td>7.2 ± 0.1</td>
<td>8.8 ± 0.4</td>
</tr>
<tr>
<td>Cyanide-resistant oxygen uptake</td>
<td>3.2 ± 0.1</td>
<td>5.2 ± 0.2</td>
<td>5.0 ± 0.1</td>
<td>3.8 ± 0.1</td>
</tr>
<tr>
<td>(μmol O₂ gDW⁻¹ hr⁻¹)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resistance to KCN (%)</td>
<td>47 ± 3</td>
<td>41 ± 3</td>
<td>68 ± 2</td>
<td>44 ± 3</td>
</tr>
<tr>
<td>Visible and scanning microscope injury. Growth and fluorescence differences</td>
<td>NO</td>
<td>NO</td>
<td>NO</td>
<td>NO</td>
</tr>
</tbody>
</table>

in current and previous year’s needles (Table 1). Current year’s needles of ozone fumigated seedlings showed lower respiration rates than those of the control seedlings, but no difference was found in last year’s needles which already had low rates (Table 1). The rate of dark oxygen uptake in the presence of 2 mM KCN was always lower than in the absence of this inhibitor of cytochrome-c- oxidase. Current year needles of fumigated seedlings had lower cyanide resistant rates compared to controls, showing an opposite trend to that of old needles (Table 1). The percentage of resistant respiration increased in fumigated old needles compared to non-fumigated ones (Table 1).

**DISCUSSION**

These results show that even though there are no visible ozone effects on Sitka spruce seedlings after a summer-long exposure (7 hr day⁻¹) to 70 ppbv ozone, some physiological changes can be detected. Changes in water potential suggest that the leaves of Sitka spruce exposed to ozone are submitted to a stronger water-stress. This water-stress might be due not to a general deterioration but rather to an impairment of specific permeability sites. Ozone is known to react with unsaturated fatty acids, which are common constituents of the spruce epicuticular wax. As a pollutant that impedes or alters development of the cuticle or increases weathering of epicuticular waxes, ozone may increase water loss. However, as no visible cuticular damage was observed in the scanning electron microscope, it would seem reasonable to conclude that water loss took place through the stomata because of ozone action, which agrees with results of Lucas and Peñuelas and is in accord with the results of other studies which showed that ozone, except at extremely high concentrations, does not increase the permeability of the cuticle to either water vapour or other gases.

Ozone did not show any effect on needle flu-
orescence, but, in contrast, showed a clear effect on another photosynthetic parameter, the chlorophyll concentration, as has also been found in Norway spruce and loblolly pines exposed to similar ozone concentrations. Thus, ozone might contribute to chlorosis shown by declining forest trees. A clear decrease in nitrogen and sulfur contents was also found in fumigated trees. Decreases in total protein content due to exposure to pollution have also been reported. Such changes could either be the result of a decrease in the rate of the protein synthesis and/or an increase in the rate of protein degradation.

Although it is well known that photosynthesis is reduced in plants fumigated with high concentrations of pollutants, less research has dealt with respiration, even though this process largely participates in the production of energy in the plant cell. The treatment of plants with high levels of ozone generally causes injuries and a rise in the respiratory rate. However, in the present study we found decreases in respiration rates of current year's needles of fumigated seedlings. The older needles had relatively low respiration rates. The present results agree with those found in experiments under lower, more usual, ozone levels where no visible injuries and no respiration changes were found. McDowall’s experiments showed a sequence of damage following ozone fumigation. Respiration was first inhibited prior to the appearance of visible symptoms and was stimulated thereafter when visible leaf damage occurred. Lehnerr et al. also reported decreased respiration rates with increasing average ozone concentrations. When symptoms of ozone injury develop, the swelling of mitochondria occurs, and is probably associated with a direct effect on the structure and permeability of the mitochondrial membranes. A decrease in the efficiency of oxidative phosphorylation is observed, accompanied by an enhancement of electron transport. This could be due to an uncoupling mechanism or to the functioning of the non-phosphorylating cyanide-resistant pathway; a postulate supported by our results because there was a significant increase in the percentage of cyanide resistance of old needles (Table 1).

The symptoms of damage to the older needles were consistent with the symptoms of crown thinning and premature shedding of older needles observed in damaged forests at high altitude. The present investigation indicates that ozone may affect Sitka spruce without accompanying visible injury, and that it could predispose them to later-appearing stressors, such as drought, winter desiccation, freezing injury or parasites. Interaction between these factors may contribute to the decline of sub-alpine stands of spruce in mountains of Spain and throughout Europe.

Acknowledgements—We thank Professor T.A. Mansfield for fruitful discussion and allowing the experimentation in the Institute of Environmental Sciences of the University of Lancaster, where the research was supported by the U.K. Department of the Environment. The technical assistance of Peter Lucas, Maureen Harrison, Andrew Bullough and the Servei de Microscòpia Electrònica de la Universitat de Barcelona is also greatly appreciated. We also thank financial support from the CICYT (Spain) grants AGR90-458 and AMB94-0199, and the fellowships from FPI to M. Ribas-Carbó and M. González- Mateo and from CIIRIT to J. Peñuelas.

REFERENCES


37. Swanson E. S., Thomson W. W. and Mudd J. B.
*Can. J. Bot.* **51**, 1213

