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Chapter 9

Responses of Aleppo pine to ozone

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Abstract

Tropospheric ozone (O_3) has become a pollutant of major concern in southern Europe. Aleppo pine (*Pinus halepensis*) exhibits O_3-induced visible injury across the Mediterranean Basin. Therefore, two experiments were carried out in open-top chambers to assess the influence of O_3 on growth and physiology of this species and potential interactions between O_3 and the summer environment. Elevated levels of O_3 were found to alter some antioxidant enzyme activities and reduce chlorophyll content, photosynthetic activity, and stomatal conductance of Aleppo pine seedlings, although no significant effects on growth rates were detected after 3 years' exposure. These effects were observed even when O_3 exposure was restricted to the summer, i.e., the time of year with the lowest O_3 uptake. Combined exposure to both O_3 and water stress resulted in reductions in needle biomass and net photosynthesis rates, as well as disturbances in the response of plant defense systems. Our results suggest that O_3 may be a factor contributing to reductions in the vitality of Aleppo pine forests across the Mediterranean.

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1. Introduction

Tropospheric ozone (O_3) is the most widespread air pollutant in the Mediterranean area. In this region, meteorological conditions are especially favorable to its formation and persistence (Millán et al., 1996). Much work has focused on O_3-induced damage on agricultural crops in this area, and visible injury, yield losses, and reductions in fruit and grain quality have been reported (see reviews by Lorenzini, 1993; Schenone, 1993; Gimeno et al., 1994; Velissariou et al., 1996; Fumagalli et al., 2001). However, natural vegetation has received much less attention, although concerns have been expressed about the impacts of the pollutant on southern European forests (Bussotti and Ferretti, 1998; Barnes et al., 2000). In the early 1980s, Naveh et al. (1980) described O_3-induced visible injury on some pine species, highlighting the potentially important threat that photochemical pollution could represent to Mediterranean ecosystems. Aleppo pine (*Pinus halepensis* Mill.) is considered to be especially susceptible to O_3 damage because the O_3-induced visible symptoms have been reported in forests across the Mediterranean Basin (Velissariou et al., 1992; Gimeno et al., 1992; Davison et al., 1995; Barnes et al., 2000; Sanz et al., 2000). These symptoms have been replicated under experimental fumigation conditions (Wellburn and Wellburn, 1994; Anttonen et al. 1995, 1998; Manninen et al., 1999).

In recent years, concern over the health of Aleppo pine forests has increased prompted by reports of declines in tree condition in eastern Spain (Montoya, 1995; Sanz et al., 2000). It has been proposed that O_3 might be one factor contributing to the reduction in vitality of Aleppo pine forests, a phenomenon that could have significant ecological implications because of the important role this species plays in soil stabilization in the Mediterranean region.

This paper presents an overview of the results of two experiments performed within the context of a pan-European study on the interactive effects of O_3 and other environmental factors, such as water stress, on *Pinus halepensis* physiology.

2. Materials and methods

The experiments were carried out in field-based, open-top chambers located at the Ebro Delta in northeastern Spain. This facility is described in detail elsewhere (Reinert et al., 1992; Pujadas et al., 1997; Elvira et al., 1998). Two-year-old Aleppo pine seedlings were planted in 18-dm^3 pots containing 50% peat, 30% sand, 20% vermiculite and a slow–release fertilizer (Osmocote Plus; NPK 15 : 18 : 11) to prevent nutrient limitations. Seedlings were exposed to the following O_3 treatments: charcoal-filtered air (CFA), non-filtered air (NFA), and non-filtered air plus 40 ppb O_3 (NFA+40). In the latter treatment, 40 ppb O_3 was added from 8 to 18 hrs daily to the ambient levels of the pollutant. Three chambers per treatment were used and three additional chamberless plots (Ambient Air, AA) were established to evaluate chamber effects.

In the first experiment, 20 plants per plot (60 plants per treatment) were exposed to the different O_3 treatments just during summer months. In the second experiment, 24 seedlings per plot were continuously exposed to elevated O_3 over 3 years; then after 20 months, a water stress treatment was introduced. Two watering regimes were established in order to assess the interactive effects of O_3 and water stress. In the well-watered treatment (WW), water was supplied according to requirement, while water-stressed (WS) seedlings received half the water supplied to WW seedlings. After WS plants had been exposed to 10 weeks' water shortage, water was withheld altogether for 11 days. Well-watered seedlings were irrigated daily to field capacity over the same period. The water deficit resulted in a loss of 7–10% in needle water content; pre-dawn needle water potential (Scholander pressure-bomb, SKPM 1400, Skye Instruments Ltd., UK) fell to -3.8 MPa in the WS plants, while it remained in the range of -1.6 to -2.5 MPa in the WW seedlings.

Ozone, nitrogen oxides (NO_x) and sulfur dioxide (SO_2) were continuously monitored, as well as meteorological variables such as air temperature, air relative humidity, and photosynthetic active radiation (PAR). Stem height and diameter of trees in the different treatments were assessed seasonally, and aboveground biomass was determined at the end of the experiment. Net photosynthesis (A) and stomatal conductance to water vapor (g_s) were measured under the prevailing environmental conditions using a LICOR-6200 infrared gas analysis system (Licor Inc., Licoln, NB, USA). Gas exchange rates were calculated according to von Caemmerer and Farquhar (1981) and expressed on the basis of projected needle area. Both previous and current-year needles were sampled on a seasonal basis to determine their levels of photosynthetic pigments and antioxidant enzyme activities. Five replicates of needles from different trees were sampled per chamber on each sampling date to determine pigments. Regarding xanthophyll analyses, needle samples were always collected at midday and immediately frozen in liquid nitrogen. Chlorophyll and carotenoid concentrations were measured spectrophotometrically (Barnes et al., 1992) and xanthophylls were determined by HPLC according to Val et al. (1994). Antioxidant enzyme activity determination involved collecting current and previous-year needles between 09 : 00 and 10 : 00 hours (local time). Two pooled samples from six individual trees were taken per chamber on each sampling date. Cell extractions (see Alonso et al., 1999) were performed to analyze the following antioxidant enzyme activities: catalase (CAT; Aebi, 1983); glutathione reductase (GR; Castillo and Greppin, 1988), guaiacol peroxidase (POD) and superoxide dismutase (KCN-resistant SOD and CuZnSOD) (Elvira et al., 1998). Also, POD activity was assessed in extracellular fluid isolated as described elsewhere (Elvira et al., 1998).

Data were subjected to ANOVA and differences between treatments were assessed using the LSD calculated at the 5% level (STATISTICA v5.1. StatSoft Inc., USA)

3. Results

3.1. Air quality

Ozone was the most abundant air pollutant in ambient air at the field site, with SO_2 and NO_x concentrations within the range of the detection limits of the monitors used. Ozone levels showed a typical diurnal profile superimposed on a 20-ppb background level. The highest concentrations were recorded during the midday (10:00–17:00 h GMT), and the lowest concentrations were experienced around dawn (4:00–6:00 h GMT) (see Elvira et al., 1998; Alonso et al., 1999). Moreover, strong seasonal variations were also found, with the highest O_3 concentrations recorded during spring and early summer and the lowest values during winter (Table 1). Ozone concentrations were compared with the guidelines defined in the Directive 92/72/EC of the European Commission (EC) for the protection of vegetation. Thus, hourly average ambient O_3 concentrations never exceeded 100 ppb, but 24-h average concentrations were greater than 33 ppb almost every month over the experimental period. Each year, ambient levels of O_3 exceeded current United Nations (UN)—Economic Commission for Europe (ECE) critical-level guidelines for the protection of forest trees (see Table 2).

3.2. Gas exchange and ozone uptake

Values of A and g_s of Aleppo pine seedlings showed clear seasonal variations. Moreover, diurnal gas exchange patterns varied depending on the season (Fig. 1). During winter, A and g_s increased during the morning, attaining maximum values during the central part of the day, which coincided with maximum levels of solar radiation and temperature. In contrast, during summer, maximum A and g_s were recorded early in the morning (6:00–8:00 GMT) and decreased around midday, while temperature, irradiance, and vapor pressure deficit continued to increase. A partial recovery in the gas exchange values was observed during the afternoon on some summer days.

Ozone uptake rates were estimated from the relationship between g_s and time of day in the different seasons. During winter, O_3 concentrations were lower than during spring and early summer (Table 1), but the higher rates of g_s

Table 1. Monthly ozone concentrations in the different treatments during sampling dates. M10h = 10-hour average expressed as ppb calculated from 7:00 to 17:00 h GMT. M24h = 24-hour average expressed as ppb. AOT40 = Accumulated exposure over threshold of 40 ppb during daylight hours (expressed as ppb h)

		AA			NFA			NFA+40			CFA		
		M10h	M24h	AOT40	M10h	M24h	AOT40	M10h	M24h	AOT40	M10h	M24h	AOT40
1994	Feb	31	28	224	30	27	196	45	33	2410	20	18	0
	May	45	42	2714	43	41	2267	71	55	11998	4	3	0
	Jul	46	35	2457	40	34	1071	64	46	9514	7	6	0
	Sep	37	28	830	33	27	350	68	45	10534	5	4	0
1995	Feb	39	32	1188	40	33	1150	61	44	6576	19	15	0
	May	60	54	7674	56	56	6390	85	69	17139	17	14	0
	Jul	43	38	2585	43	40	2388	67	52	10799	16	13	0
	Sep	41	34	1501	40	33	1182	68	47	9755	13	11	0
1996	Mar	42	37	1755	40	36	1153	64	48	8509	13	11	0

Table 2. Cumulative ozone exposure during daylight hours for the period April to September expressed as AOT40 (Accumulated exposure over threshold of 40 ppb) in the different treatments

Year	AA	NFA+40	NFA	CFA
1994	11060	54231	7879	0
1995	23422	70042	19128	0
1996	12362	66699	9667	0

resulted in greater values of O_3 uptake (Fig. 2) than during summer months, when stomatal closure reduced O_3 uptake to a minimum. In trees exposed to the highest O_3 levels (NFA+40), O_3 uptake was usually higher in current-year than in 1-year-old needles, especially during winter. These differences were not apparent in plants exposed to non-filtered air (Fig. 2).

Maximum daily values of A and g_s also changed throughout the year, with lower values recorded during the summer compared with winter and spring (Fig. 3). These seasonal variations were detectable even when plants were not subjected to water stress. Aleppo pine seedlings exposed to elevated O_3 concentrations (NFA+40) exhibited significantly lower values of A and g_s than plants grown in charcoal-filtered air. In trees where O_3 exposure was restricted to the summer months (Fig. 3(a)), a 30% reduction in A and g_s was detected in 1-year-old needles. In contrast, trees exposed to O_3 throughout the year (Fig. 3(b)) exhibited a comparable reduction in A and g_s in current-year and 1-year-old needles.

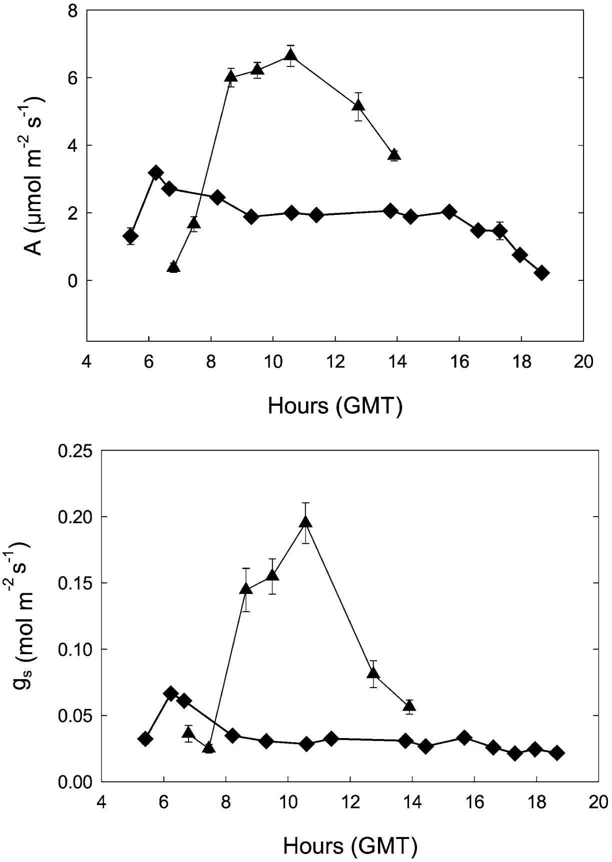

Figure 1. Daily profile of gas exchange parameters of Aleppo pine seedlings growing in charcoal-filtered air during summer (◆) and winter (▲). Means ± SE.

Soil water deficit induced a stronger depression (Fig. 3(b)) in A (up to 76%) and g_s (up to 37%) in 1-year-old needles than O_3 (around 20–23%). Water shortage, in combination with ozone, did not induce additive reductions in net photosynthesis. However, maximum stomatal conductance values for seedlings exposed to O_3 plus water stress were higher than observed in trees exposed to water stress alone.

3.3. Photosynthetic pigments

Photosynthetic pigments of Aleppo pine also exhibited marked seasonal fluctuations. During summer, the activation of the xanthophyll cycle (de-epoxidation

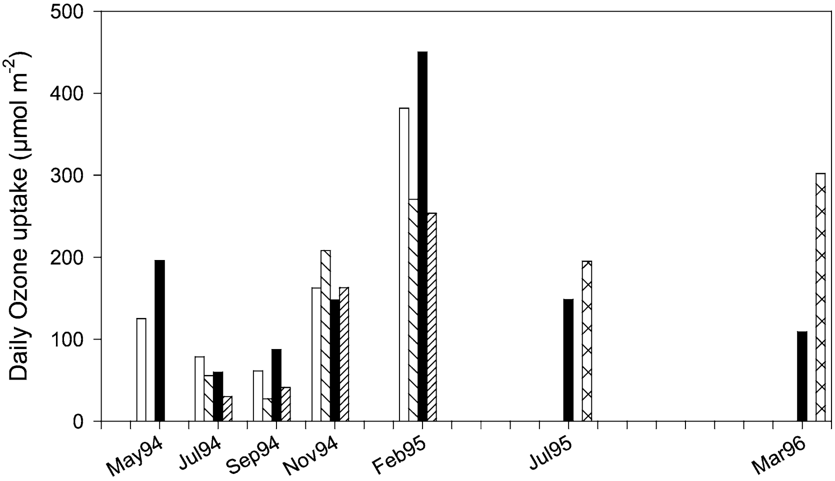

Figure 2. Daily ozone uptake on sampling dates in Aleppo pine seedlings exposed to ozone. □ 1993-needles exposed to NFA+40; \\\\\\\\ 1993-needles exposed to NFA; ■ 1994-needles exposed to NFA+40; //// 1994-needles exposed to NFA; × × × 1995-needles exposed to NFA+40.

state of xanthophyll cycle pool, DPS index) was higher ($p < 0.001$) at midday in both current and 1-year-old needles of trees exposed to elevated levels of O_3 than in seedlings raised in clean air (Fig. 4(a), (b)). In winter, an ozone-induced increment ($p < 0.005$) in DPS index was recorded in 1-year-old needles during the morning and the evening (Fig. 4(c), (d)).

Soil water deficit also caused a significant increase in the conversion state of xanthophyll cycle pool intermediates in both needle age classes, regardless of O_3 treatment (Fig. 4(a), (b)). No interactive effects of O_3 and water stress on DPS were detected at midday, but water stress induced a smaller activation ($p < 0.001$) of the xanthophyll cycle during the morning and the evening in 1-year-old needles of plants exposed to O_3. In contrast, current-year needles exposed to O_3 and water stress exhibited greater DPS during the evening than trees exposed to charcoal-filtered air.

3.4. Antioxidant enzyme activities

Current-year needles of Aleppo pine generally exhibited higher antioxidant enzyme activities than 1-year-old needles (Table 3) plus a greater capacity to react to stress. Ozone exposure resulted in alterations in the activity of antioxidant enzymes on some sampling dates. Effects were not related to accumulated O_3 exposure or to episodic O_3 concentrations before sampling, but appeared to be influenced by needle age tree ontogeny. The most significant

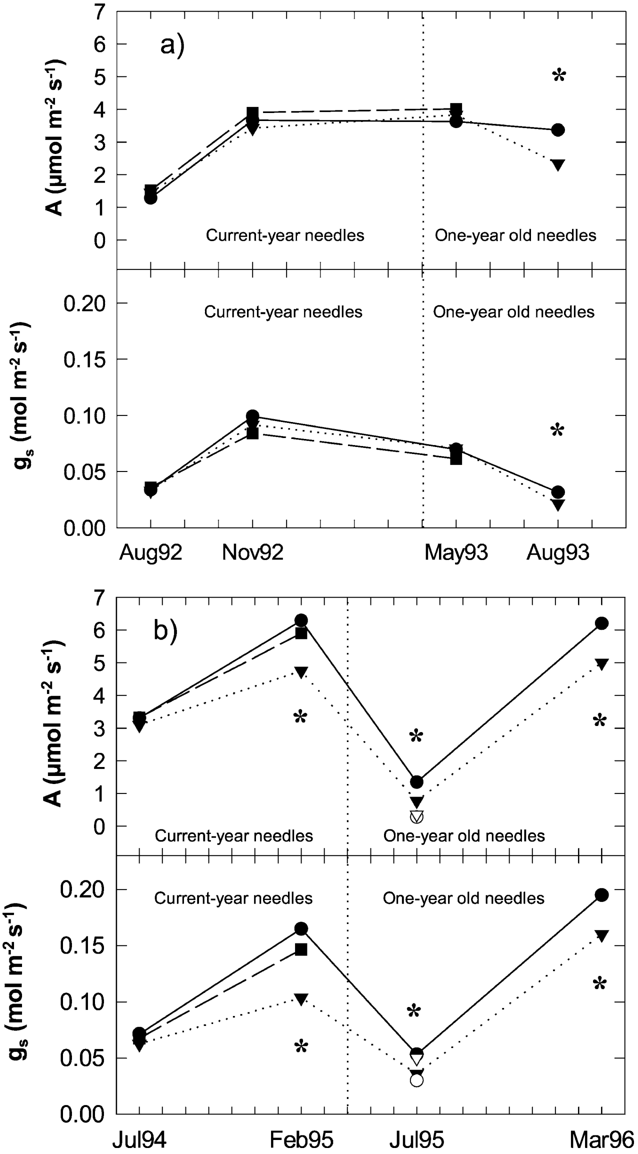

Figure 3. Maximum daily net photosynthesis (A) and stomatal conductance (g_S) measured on Aleppo pine seedlings exposed to the different ozone treatments: ● CFA WW; ○ CFA WS; ■ NFA; ▼ NFA+40 WW; ▽ NFA+40 WS. (a) 1992-needles exposed to O_3 during summer; (b) 1994-needles exposed to O_3 throughout the year. $*$ indicates significant differences between treatments.

Table 3. Antioxidant enzyme activities in Aleppo pine seedlings exposed continuously to the different ozone treatments. Means ± SE, $n = 3$–6. ex.POD = extracellular POD; cel.POD = cellular POD; KCNr.SOD = KCN-resistant SOD; POD activities expressed as $\Delta Abs_{470}\ min^{-1}\ g^{-1}$ fwt. Other activities expressed as Unit g^{-1} fwt. Different letters indicate significant differences among O_3 treatments on each sampling date

		ex.POD	cel.POD	GR	CAT	KCNr. SOD	CuZnSOD
1993 needles							
May 94	CFA	11.1 ± 2.2	178 ± 9b*	1170 ± 109	95 ± 23	22.6 ± 2.2a*	90 ± 7
	NF	10.5 ± 3.1	183 ± 17b	1269 ± 150	125 ± 18	31.9 ± 3.4b	87 ± 7
	NFA+40	10.8 ± 1.7	138 ± 4a	984 ± 89	92 ± 3	20.5 ± 2.3a	82 ± 7
Jul 94	CFA	22.7 ± 3.1b*	187 ± 9	420 ± 70	35 ± 7a†	19.7 ± 5.8	57 ± 5
	NF	11.5 ± 0.6a	157 ± 13	473 ± 15	38 ± 3a	14.3 ± 3.1	68 ± 5
	NFA+40	10.9 ± 1.2a	167 ± 8	535 ± 40	75 ± 8b	15.5 ± 3.5	60 ± 2
Sep 94	CFA	10.6 ± 3	185 ± 13b†	676 ± 106	84 ± 15	18.9 ± 2.3	86 ± 12
	NF	6.4 ± 1.1	103 ± 8a	883 ± 42	121 ± 9	19.7 ± 3.1	95 ± 10
	NFA+40	6.6 ± 1.8	152 ± 14b	744 ± 108	101 ± 8	13.2 ± 1.1	82 ± 3
Nov 94	CFA	14.1 ± 2.7b†	186 ± 11	641 ± 96	50 ± 3	28.4 ± 2.7	72 ± 5
	NF	6.4 ± 0.9a	161 ± 11	861 ± 132	78 ± 11	42.9 ± 5	81 ± 5
	NFA+40	4.8 ± 1a	155 ± 14	997 ± 100	96 ± 14	27.1 ± 3.7	64 ± 4
Feb 95	CFA	8.8 ± 1.1	198 ± 13	1060 ± 66	111 ± 10	25 ± 1.8	64 ± 6
	NF	9.3 ± 0.7	254 ± 38	1013 ± 34	106 ± 10	22.3 ± 1.1	68 ± 7
	NFA+40	11.7 ± 1	203 ± 15	1115 ± 29	130 ± 13	25.3 ± 2.5	53 ± 5
1994 needles							
Sep 94	CFA	$2 \times 10^{-4} \pm 8 \times 10^{-5}$	82 ± 7	1177 ± 61	154 ± 12	55.2 ± 4.3	144 ± 16
	NF	$3 \times 10^{-4} \pm 8 \times 10^{-5}$	90 ± 5	1093 ± 91	143 ± 16	57.7 ± 3.4	159 ± 6
	NFA+40	$4 \times 10^{-4} \pm 1 \times 10^{-4}$	91 ± 4	1208 ± 68	164 ± 9	53.5 ± 3.6	147 ± 8
Nov 94	CFA	1.2 ± 0.3	188 ± 5	2373 ± 102	280 ± 10	82.1 ± 8.6	147 ± 8
	NF	1.7 ± 0.4	174 ± 8	2089 ± 108	255 ± 11	69.6 ± 6.9	136 ± 6
	NFA+40	2.5 ± 0.7	153 ± 22	1920 ± 125	244 ± 22	79 ± 2.4	144 ± 10
Feb 95	CFA	7.1 ± 0.6a‡	209 ± 17	2043 ± 115	231 ± 12	83.3 ± 7	151 ± 13a*
	NF	16.4 ± 2.4b	242 ± 22	2438 ± 239	250 ± 15	71.7 ± 8.6	136 ± 6a
	NFA+40	36.3 ± 5.4*c*	253 ± 10	2784 ± 354	333 ± 44	76.7 ± 13.6	228 ± 33b

(*continued on next page*)

Table 3. (*Continued from previous page*)

		ex.POD	cel.POD	GR	CAT	KCNr. SOD	CuZnSOD
May95	CFA	12 ± 2.1a‡	287 ± 26	1534 ± 62a*	156 ± 23	56.1 ± 4.3	94 ± 12
	NF	21.8 ± 3.4b	257 ± 22	1810 ± 35b	234 ± 3	69.6 ± 6.6	124 ± 18
	NFA+40	14.1 ± 1a	292 ± 10	1442 ± 45a	204 ± 14	63.4 ± 3.7	94 ± 5
Jul 95	CFA	8.5 ± 0.3ab*	132 ± 10a‡	641 ± 67a*	53 ± 6a*	47.3 ± 6.2	90 ± 7
	NF	11.2 ± 1.6b	202 ± 5b	820 ± 15b	82 ± 10b	44.9 ± 2.6	70 ± 6
	NFA+40	4.9 ± 0.7a	140 ± 7a	668 ± 40a	59 ± 5a	53.9 ± 1.4	86 ± 11
Sep 95	CFA	14.7 ± 3	183 ± 15ab*	886 ± 97b‡	41 ± 8a†	37.4 ± 3.1a*	79 ± 6
	NF	12.8 ± 1	142 ± 10a	665 ± 37a	67 ± 17ab	53.8 ± 5.6b	82 ± 10
	NFA+40	9.4 ± 1.8	204 ± 12b	1092 ± 55c	86 ± 6b	53.8 ± 4.6b	70 ± 4
Mar 96	CFA	6.8 ± 1.2	170 ± 9	904 ± 63a*	73 ± 7	46.5 ± 3.7	73 ± 5
	NF	8.8 ± 1.3	167 ± 10	1007 ± 99ab	43 ± 9	45.5 ± 4.1	49 ± 3
	NFA+40	6 ± 1.1	160 ± 7	1152 ± 49b	72 ± 8	51.6 ± 3.8	62 ± 5
1995 needles							
Jul 95	CFA	–	39 ± 3	882 ± 17b*	107 ± 5b*	71.7 ± 4.9	89 ± 8
	NF	–	–	–	–	–	–
	NFA+40	–	41 ± 3	549 ± 85a	70 ± 7a	60.6 ± 6.9	62 ± 15
Sep 95	CFA	–	68 ± 4	1672 ± 90	119 ± 15	68.2 ± 3.1	72 ± 5
	NF	–	–	–	–	–	–
	NFA+40	–	72 ± 4	1478 ± 50	109 ± 8	63.8 ± 5	85 ± 7
Mar 96	CFA	13.8 ± 2.2	175 ± 8	2067 ± 81ab*	170 ± 11	87.9 ± 9ab†	103 ± 11b†
	NF	9 ± 1.7	184 ± 25	1795 ± 93a	145 ± 26	62 ± 9.7a	52 ± 6a
	NFA+40	16.6 ± 1.8	219 ± 12	2251 ± 98b	197 ± 18	89.1 ± 7.6b	125 ± 10b

* $p \leqslant 0.05$.
† $p \leqslant 0.01$.
‡ $p \leqslant 0.001$.

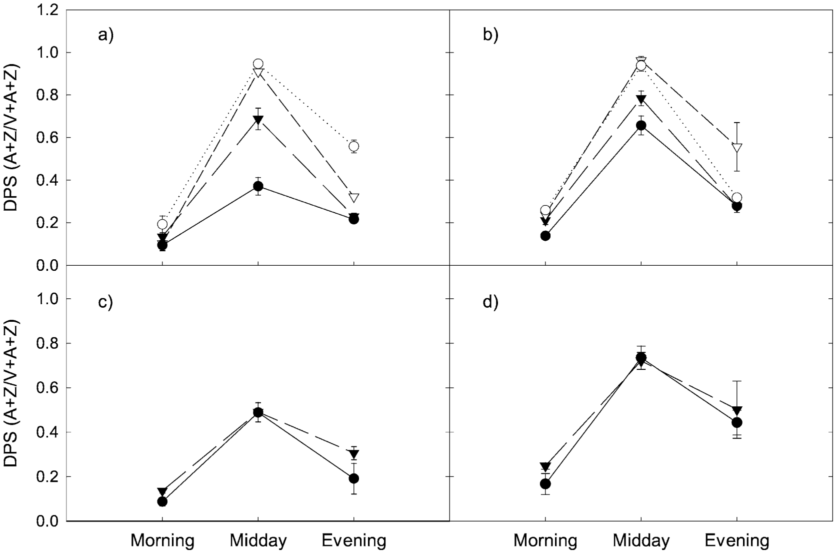

Figure 4. De-epoxidation state of the xanthophyll cycle pool $(A + Z)/(V + A + Z)$ in 1-year-old needles (a), (c) and current-year needles (b), (d) during summer (a), (b) and winter (c), (d) in trees exposed to ozone and/or soil water deficit: ● CFA WW; ○ CFA WS; ▼ NFA+40 WW; ▽ NFA+40 WS. Means ± SD, $n = 4$–12.

effect of O_3 on antioxidant systems was recorded during winter in 10-month-old needles (1994 needles measured in February 1995, Table 3). At this stage, seedlings previously exposed to the highest O_3 levels exhibited significantly higher extracellular-POD and CuZn SOD activity and a trend, though not statistically significant $(p < 0.1)$, toward an increase in CAT, GR, and cellular-POD activity compared with plants grown in clean air. Similar responses were observed when antioxidant enzyme activities were expressed on a chlorophyll basis.

Extracellular-POD activity showed the greatest increase in response to O_3 exposure, up to fivefold in the NFA+40 treatment and twice in the NFA treatment during the winter (February 1995). The increase in extracellular-POD activity was due to an increase in the activity of acid (1.5 times higher activity in NFA+40 compared with CFA), but especially basic (3.3 times higher) isoperoxidases (Fig. 5). Although acid extracellular-POD activity was similar throughout the year, the activity of basic isozymes increased during spring and summer in control trees. In contrast, the basic POD activity of the trees exposed to O_3 increased sharply during wintertime before the activation of these activities was detected in CFA plants.

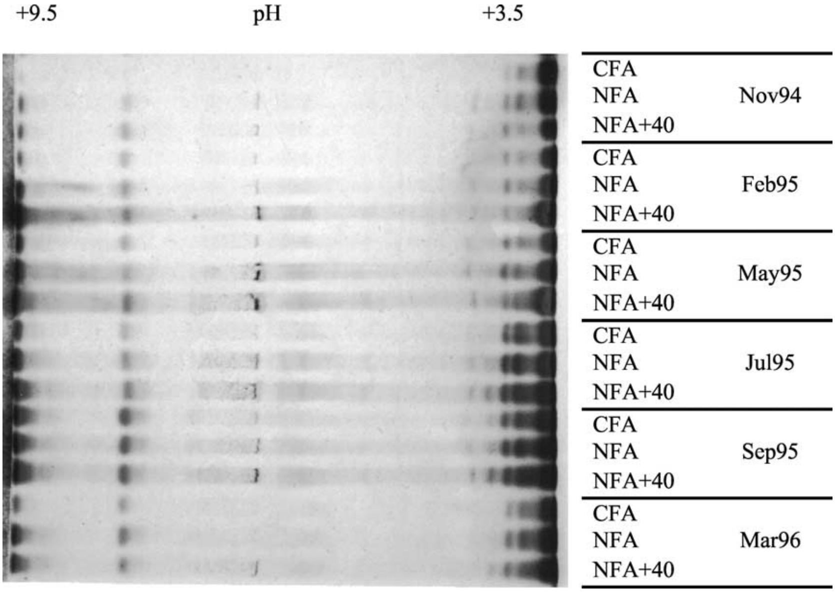

Figure 5. Extracellular isoperoxidase pattern of 1994-needles exposed to the different ozone treatments. Isoperoxidases were separated by isoelectricfocusing (IEF) on a 3.5–9.5 pH gradient following Elvira et al. (1998).

When Aleppo pine seedlings were subjected to water stress, 1-year-old needles exhibited a decrease in cellular-POD and KCN-resistant SOD activities, while current-year needles exhibited a significant increase in GR and CuZn SOD (Data presented in Alonso et al., 2001). Ozone exposure in combination with water stress resulted in significant decreases in POD, GR, CAT and KCN-resistant SOD activities in current-year needles.

3.5. Growth

Aleppo pine seedlings exhibited active growth throughout the year with the highest rates recorded during springtime (Fig. 6). No significant effects of O_3 were observed on height and stem diameter growth after 3 years of continuous exposure to the respective O_3 treatments. In contrast, water stress significantly decreased stem and diameter growth up to 16%. The combination of O_3 and water stress resulted in no additional decreases in relative growth rate.

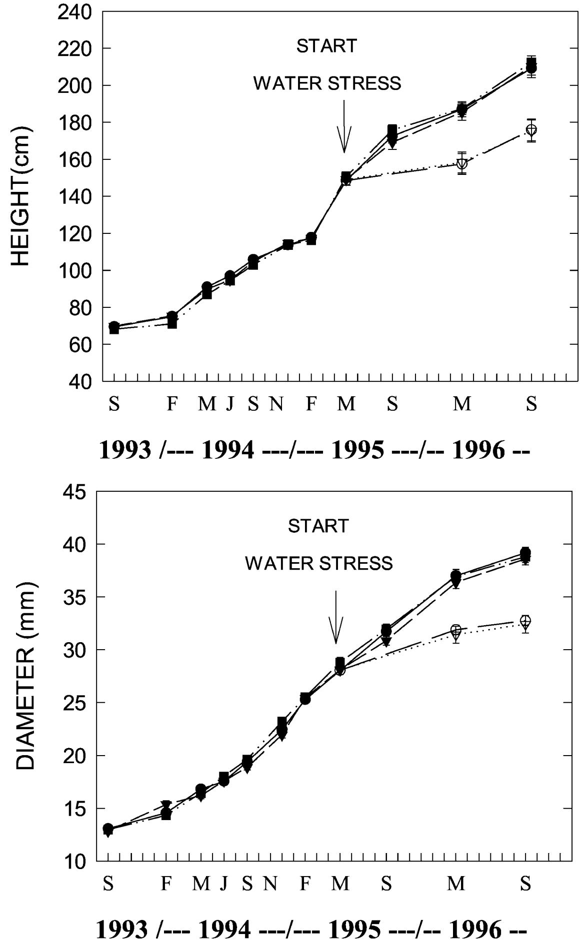

Figure 6. Incremental stem and height growth of Aleppo pine seedlings grown in the different ozone treatments: ● CFA WW; ○ CFA WS; ▼ NFA+40 WW; ▽ NFA+40 WS; ■ NFA WW. Means ± SD. $n = 25$–35 for WW treatments and $n = 12$ for WS treatments.

4. Discussion

The European Commission (EC) Directive 92/72/EC established two guidelines to protect vegetation from O_3 injury: 100 ppb as an hourly average and 33 ppb as a 24-h average. Ambient O_3 concentrations at the Ebro Delta over the experimental period were almost continuously above the 33 ppb 24-h average value. Ambient O_3 levels each year exceeded the UN-ECE critical level for the protection of forest trees (10 000 ppbh in 6 months, April to September).

The experiments involved realistic O_3 exposures, as concentrations in the same range of those reported in NFA+40 treatment have been recorded at different sites on the Spanish Mediterranean coast (Millán et al., 1996; Gimeno et al., 1996; Sanz and Millán, 1998).

The mild temperatures at the field site, along with the fact that trees were irrigated for experimental purposes, enabled Aleppo pine seedlings to keep growing throughout the year. A similar year-round pattern of growth is also observed in natural forests, where trees frequently show several flushes during the same year, depending on weather conditions (Gil et al., 1996). Despite the fact that trees showed continuous growth throughout the year, most of the physiological parameters analyzed showed clear seasonal variations. Chlorophyll content, net photosynthesis, and stomatal conductance decreased during the summer even when plants were not subjected to water stress. For instance, chlorophyll levels exhibited a 40–50% reduction during the summer, followed by recovery during autumn and winter regardless of O_3 treatment (data presented in Elvira and Gimeno, 1996). A similar pattern was also observed for changes in carotenoid content. Summer chlorosis has also been observed in natural forests of Aleppo pine (Davison et al., 1995) and in other Mediterranean species (Kyparissis et al., 1995). The recovery in chlorophyll levels observed during the winter suggests that the reduction of photosynthetic pigments during summer might afford a protection mechanism to avoid the absorption of potentially damaging excitation energy (Kyparissis et al., 1995; Elvira et al., 1998).

Reductions in net photosynthesis and stomatal conductance during the summer have frequently been reported for other Mediterranean species in their natural environment, usually associated with water stress (Epron and Dreyer, 1993; Damesin and Rambal, 1995; Faria et al., 1998). In our experiment, Aleppo pine seedlings exhibited seasonal variations in gas exchange independent of water status. Factors other than soil water deficit, such as vapor pressure deficit, high temperatures, or high levels of irradiance, presumably contributed to the decline in photosynthesis (Faria et al., 1998).

Changes in physiological parameters induced by seasonal weather variations and/or water stress, were more important than changes induced by O_3. Nevertheless, O_3 decreased the rate of net photosynthesis, stomatal conductance, and the levels of photosynthetic pigments (data presented in Elvira and Gimeno, 1996) and triggered alterations in detoxification systems. These effects were detectable even when exposure to the pollutant was restricted to the summer months (i.e., the season with the lowest O_3 uptake because of the low stomatal conductance values).

Ozone-induced declines in A and g_s have been reported previously for Aleppo pine (Anttonen et al., 1998), as in other tree species (Chappelka and Samuelson, 1998). Despite these results, long-term effects of O_3 exposure on photosynthesis are not very conclusive and increases, decreases, or no effects could be found. Although some data suggest that photosynthesis can be lowered without a concomitant fall in stomatal conductance (Heath and Taylor, 1997), Aleppo pine seedlings exhibited reductions in g_s in the same range as net photosynthesis. However, the higher maximum stomatal conductance exhibited by plants subjected to combined O_3 and water stress compared with those grown in charcoal-filtered air might be indicative of poorer stomatal control. Such "sluggish" behavior of stomata in O_3-exposed foliage has been reported previously (Tjoelker et al., 1995; Grulke, 1999). This loss of stomatal regulation might be particularly dangerous for Mediterranean species that have to cope with harsh environmental conditions during the summer.

Ozone-induced reductions in gas exchange were observed before changes in chlorophyll content. Similar findings have been reported for Aleppo pine (Manninen et al., 1999) and other conifers (Takemoto et al., 1997). Thus, chlorosis does not seem to be a primary effect of O_3 exposure, but rather a secondary effect due to impaired photosynthetic capacity (Heath and Taylor, 1997). Moreover, when CO_2 fixation is reduced without reducing light capture, there is the potential for photoinhibition (Demmig-Adams and Adams III, 1992). On the one hand, this may explain the decline in chlorophyll levels, while on the other hand, photoinhibition also affects defense mechanisms. In this sense, O_3 altered some of the photoprotective capabilities of Aleppo pine. The activation of the xanthophyll cycle pool at midday was higher in seedlings exposed to O_3 than in trees raised in charcoal-filtered air. This could be linked with the reduction in CO_2 assimilation rates and was also observed in plants exposed to O_3 only during summer months (Elvira et al., 1998). Other tree species have also exhibited an activation of the xanthophyll cycle induced by O_3 (Mikkelsen et al., 1995; Reichenauer and Bolhar-Nordenkampf, 1999). These findings suggest the involvement of photooxidative stress in the development of O_3-induced injury (see Heath and Taylor, 1997; Tausz et al., 1999). When plants were exposed to a combination of ozone and water stress, a further increase of DPS index was recorded in current-year needles while 1-year-old needles showed lower activation of xanthophyll cycle components than plants raised in charcoal-filtered air. This behavior of 1-year-old needles was observed even though chlorophyll and net photosynthetic rates were similar in water-stressed plants regardless of the O_3 treatment (data presented in Inclán et al., 1998a and Alonso et al., 2001). Thus, effects observed on xanthoplyll cycle intermediates indicate a greater capacity to respond to stress in current-year needles than in 1-year-old needles.

Ozone exposure also caused alterations in the antioxidant systems of Aleppo pine needles. The most significant increase in the activity of the antioxidant-related enzymes coincided with the period of highest O_3 uptake: during winter. At this time of year, O_3 concentrations were lower than in spring and early summer but high stomatal conductance resulted in a higher rate of uptake of the pollutant. During the summer months, stomatal closure reduced O_3 uptake to a minimum. However, a trend toward an increase in some antioxidant activities in plants exposed to elevated O_3 was also detected. Ozone effects on antioxidant enzymes also depended on needle age, as 1-year-old needles did not show the same increase in antioxidant-related enzyme activities as was observed in current-year needles.

The greatest change induced by O_3 in Aleppo pine was found to be the activity of extracellular POD. Similarly, when plants were exposed to O_3 only during summer months, extracellular-POD activation was the first effect recorded (Elvira et al., 1998). Extracellular-POD activity thus appeared to be more O_3 responsive than cellular POD. Similar findings have been reported for other species (Castillo et al., 1984, 1987). These results suggest that the principal effects of O_3 are located in the apoplast with the plasma membrane as the chief target (Kangasjärvi et al., 1994; Alscher et al., 1997).

Ozone exposure did not induce new extracellular-POD isozymes, although the response of basic isoperoxidases was more pronounced than that in acid isozymes. The function of isoperoxidases in conifer physiology is not clear. Acid isoperoxidases seem more related to lignification processes, while basic forms are related to ethylene and indolacetic acid metabolism (Gaspar et al., 1991). Also, basic extracellular isoperoxidases have been linked to a detoxification role under O_3 exposure, using ascorbate as an electron donor (Gaspar et al., 1985; Castillo and Greppin, 1986).

When Aleppo pine seedlings were exposed to both O_3 and water stress, some antioxidant activities (POD, GR, CAT and KCN-resistant SOD) decreased in current-year needles compared with plants grown in charcoal-filtered air, which differed in the response induced by water stress alone. This reduction of antioxidant activities could mean that the protection capacity of these needles was overwhelmed when plants were exposed to both stresses.

No reduction in tree relative growth rates was detectable in either experiment, although small decreases in net photosynthesis compounded over many years might produce significant growth reductions (Teskey, 1995). In fact, Barnes et al. (2000) reported growth decreases in this species after 4 years of repeated O_3 exposure. Although O_3 did not reduce tree growth, seedlings exposed to the highest levels (NFA+40) throughout the year exhibited significantly lower (up to 10%) needle biomass and total aboveground biomass accumulation compared with plants grown in charcoal-filtered air (data in Inclán et al., 1998b). Similarly, water stress resulted in significant decreases in both needle weight (23%) and total aboveground biomass (27%). Ozone and water stress were found to affect biomass additively. Future studies need to address the combined action of O_3 and other environmental stresses on Mediterranean tree species.

5. Conclusions

Ambient O_3 concentrations on the eastern coast of Spain regularly exceed international guidelines for the protection of forest trees. Aleppo pine physiology was affected by O_3 exposure, but the response was strongly modified by environmental conditions. Ozone exposure caused alterations in the protective systems and decreased CO_2 assimilation rates, stomatal conductance, photosynthetic pigments, and biomass accumulation. These effects were also observed when O_3 exposure was restricted to the summer months, i.e., the period when low stomatal conductance resulted in minimal rates of pollutant uptake. Based on the high values of O_3 uptake observed during winter and the fact that the tree exhibited continuous growth, international guidelines to protect Mediterranean trees should consider O_3 concentrations throughout the year. The interactive effects of O_3 and water stress did not induce a further decrease in gas exchange or growth rates, but their combined effects produced detrimental effects in some protective systems of Aleppo pine, such as xanthophyll cycle and antioxidant enzymes, and decreased needle biomass accumulation. These responses suggest that O_3 exposure may impair the ability of this species to withstand water stress in its natural environment, as suggested by previous authors (Wellburn and Wellburn, 1994; Gerant et al., 1996; Barnes et al., 2000). The reduction in net photosynthesis induced by O_3 during the winter and spring (the most active period), along with a weaker capacity for photoprotection during the summer months (the most stressful period), could be considered important threats to the vitality of Aleppo pine forests, and are worthy of detailed consideration to predict the future of Mediterranean vegetation under changing climate conditions.

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