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Original article

Ozone and water deficit reduced growth of Aleppo pine seedlings

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Abstract

The effects of ambient and elevated ozone (O_3) levels on photosynthesis, growth, pigment, biomass and element contents of Aleppo pine (*Pinus halepensis* Mill.) were studied for two growing seasons (1997, 1998). Two-year-old seedlings were exposed to elevated O_3 in open-top chambers. The treatments were charcoal-filtered air and non-filtered air + 50 $nl\ l^{-1}$ O_3 (24 h per day, 7 days per week). In summer 1998, half of the seedlings were drought-stressed (leaf water potential down to approximately -2 MPa), while the other half were kept well-watered. At the beginning of the season (1998), current (c) and previous-year ($c + 1$) needles under O_3 stress showed an increase in stomatal conductance and net photosynthesis. During the drought period, only stomatal conductance increased in both needle age-classes, whereas the net photosynthesis decreased. At the end of the measuring period, both parameters were reduced in the O_3 treatment. Both O_3 and drought decreased chlorophyll a and b concentrations, growth and biomass. A carry-over effect of O_3 on pigments was also observed. Needle K content was increased in the O_3 treatment. Drought protected Aleppo pine against O_3 (less chlorotic mottle and less decrease of stem and branch biomass).

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Keywords: Biomass; Critical level; Drought; Ozone; Photosynthesis; Pigments; *Pinus halepensis*

1. Introduction

Among air pollutants, ozone (O_3) appears to be potentially one of the most harmful in the Mediterranean region, because the meteorological conditions present in this region are particularly favorable to its formation and persistence, and because it is the most ubiquitous. The available evidence strongly suggests that O_3 occurs at sufficient concentrations to cause injury to sensitive Mediterranean plant species, including trees [38]. *Pinus halepensis* (Aleppo pine) is a woody species, widespread in the Mediterranean area and useful for reforestation. In most studies, O_3 has been demon-

strated to be responsible for the chlorotic mottle and banding observed on the older needles of trees in the field [40] and similar symptoms have been induced on the current year (c) foliage when trees were exposed to sufficiently elevated levels of O_3 [15]. Changes such as reduced stomatal conductance, rates of photosynthesis [1,20] and pigment concentrations [1] have also been reported. However, the discrepancies in some results (such as starch contents) among the different experiments may be related to different O_3 exposure designs, as well as different developmental and environmental conditions.

For tropospheric O_3 , the critical level for vegetation is currently expressed as the accumulated exposure above a threshold concentration of 40 $nl\ l^{-1}$ (AOT40). The Level I approach of AOT40 does not consider any factor that may influence a plant's sensitivity to O_3 [14]. Water shortage is probably the most important factor limiting the growth of the Mediterranean vegetation. Therefore, the interactive effects of O_3 and water stress on the physiology of Aleppo pine may have ecological implications. Wellburn and Wellburn [40] also pointed out the detrimental effect of high levels of O_3 which affect the ability of the tree to resist water stress. Barnes et al. [4] concluded that future studies should address

Abbreviations: A, net carbon dioxide assimilation rate; AOT40, O_3 exposure over threshold of 40 $nl\ l^{-1}$; C, carbon; c , current year; $c + 1$, previous-year; CF, charcoal filtered air; CO_2 , carbon dioxide; car, carotenoid; DS, drought stressed; g_w , stomatal conductance to water vapor; K, potassium; Mn, manganese; NF + O_3 , non-filtered air + ozone; O_3 , ozone; OTC, open-top chamber; P, phosphorus; R/T , % biomass of roots (relative to total plant biomass); WW, well watered.

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O₃ exposure–response relationships in terms of the effective dose of the pollutant absorbed, paying specific attention to possibly important interactions between O₃ exposure and other stresses (e.g. drought).

The objective of this study was to investigate the effect of O₃, drought and their interaction on growth, biomass production, photosynthesis, pigment and foliar nutrient concentrations of *P. halepensis* in order to discuss the role of drought as a factor modifying the response of trees to O₃.

2. Results

2.1. Ozone concentrations

The ambient O₃ levels at the site were relatively high, particularly in August 1997 and May and August 1998 (Fig. 1). The maximum daily (24 h) mean concentrations reached 88 nl l⁻¹ in 1997 and 91 nl l⁻¹ in 1998. The highest hourly O₃ concentration was 108 nl l⁻¹ on 12 August 1997 and 106 nl l⁻¹ on 12 May 1998. The AOT40s were calculated for daylight hours (> 50 W m⁻²) between May and October. At the end of October, the AOT40 for ambient air (on the site) had accumulated to an AOT40 of 24.25/20.78 µl l⁻¹ h in 1997/1998 and in the non-filtered air + ozone (NF + O₃) open-top chambers (OTCs) to 124.04/117.79 µl l⁻¹ h, respectively.

Fig. 1. Daily average O₃ concentrations of ambient air during the fumigation experiment, and average day profile of hourly ozone concentrations for the period 1 April–30 September for both years. (○), CF; (△), NF + O₃; (□), ambient air.

Fig. 2. The change in the pre-dawn leaf water potential during the course of drought period. Error bars indicate S.D. and $n = 16$. (○), CF WW; (●), NF + O₃ WW; (△), CF DS; (▲), NF + O₃ DS.

2.2. Pre-dawn leaf water potential

The pre-dawn leaf water potential of both the charcoal-filtered air (CF) and NF + O₃ drought-stressed (DS) seedlings had declined to about -2.0 MPa from 8 July until the end of the drought on 24 August 1998 (Fig. 2). There was no difference between the CF and NF + O₃ well-watered (WW) seedlings either.

2.3. Plant growth

The average height and diameter growth decreased slightly with increasing O₃ concentration in 1997, but the differences between the treatments were not statistically significant (Table 1). However, the diameter growth decreased due to the O₃ exposure in 1998. Drought had a marked ($P < 0.001$) negative effect on the growth. Before drought, there was no difference between WW and future DS trees within the two treatments (data not shown). There was no interaction between O₃ and drought in the case of the height and diameter growth.

The negative effect of O₃ alone was also seen as a statistically significant decrease in the wood biomass, whereas drought reduced the weight of needles and root biomass too (Table 1). Furthermore, there was an interaction between O₃ and drought since the reduction in the wood biomass was smaller in the NF + O₃ DS seedlings than in the NF + O₃ WW ones in relation to the CF DS and CF WW controls (-10 vs. -45% , respectively). Drought reduced growth and biomass more markedly than did O₃. There was no difference in the percentage of roots biomass of relative to total plant biomass between the treatments.

Table 1

The percentage (%) of relative growth rate of height and diameter at the end of 1997 and 1998 and the average needle, wood and root, total biomass (dry weight in g per plant) and % biomass of roots (relative to total plant biomass) (*R/T*) at the final harvest at the end of the experiment on 10 October 1998

Treatment	Height 1997	Diameter 1997	Height 1998	Diameter 1998	Biomass				<i>R/T</i>
					Needles	Wood	Root	Total	
CF WW	28.4 ± 9.6	38.7 ± 1.0	28.6 ± 7.5	67.3 ± 17.8	96.6 ± 21.5	68.4 ± 11.6	86.9 ± 12.5	251.9 ± 39.2	34.8 ± 4.0
NF + O ₃ WW	23.5 ± 12.5	32.2 ± 2.2	28.7 ± 7.6	55.8 ± 13.2	70.7 ± 26.2	37.6 ± 18.7	63.0 ± 24.2	171.4 ± 62.6	37.0 ± 9.0
CF DS	/	/	18.9 ± 7.8	51.1 ± 14.2	61.9 ± 20.4	48.6 ± 11.2	50.3 ± 7.3	160.9 ± 34.3	32.1 ± 6.0
NF + O ₃ DS	/	/	17.5 ± 5.6	45.4 ± 15.3	59.1 ± 12.7	43.7 ± 8.0	59.2 ± 21.2	162.1 ± 32.6	37.5 ± 6.0
O ₃	ns	ns	ns	*	ns	***	ns	***	ns
Drought	/	/	***	***	**	ns	**	*	ns
O ₃ × Drought	/	/	ns	ns	ns	**	**	ns	ns
df error	61	61	56	56	31	31	31	31	31

2.4. Gas exchange

Fig. 3 shows the net photosynthesis (*A*) and stomatal conductance for water vapor (*g_w*) of the *c* and previous-year (*c* + 1) needles before drought (May–June 1998). The *c* and *c* + 1 needles had significantly higher values of *g_w* and *A* in the NF + O₃ treatment compared to the CF treatment. The *c* + 1 needles had a higher *g_w* than the *c* ones.

During the drought period, when the diurnal variations in *g_w* and *A* of the *c* needles were monitored (Fig. 4), differences between the CF and NF + O₃ treatments were identified in the case of *g_w* (higher values in the *c* needles of the NF + O₃ WW seedlings). Drought provoked a decrease in *g_w* and *A* in both treatments, i.e. stomata were almost closed. There was no interaction between O₃ and drought stress.

Fig. 5 shows the diurnal variations in gas exchange parameters in the *c* + 1 needles. Again, in the case of the WW seedlings, the NF + O₃ seedlings had a higher *g_w* than the CF ones. However, this O₃-related increase in *g_w* did not give rise to higher photosynthetic rates. Drought stress had a smaller effect on the *g_w* of *c* + 1 needles compared to the *g_w* of *c* needles (Fig. 4), i.e. the stomata of *c* + 1 needles were not as closed as were the stomata of *c* needles. The NF + O₃ DS trees had the lowest *g_w* and *A*. This suggests that there was an interaction between O₃ and drought stress.

After rewatering the DS seedlings, the *c* needles of both, the NF + O₃ WW and NF + O₃ DS seedlings showed a decrease in *g_w* and *A* in September 1998 (Fig. 6), but the

Fig. 3. The net photosynthesis and stomatal conductance for the *c* and *c* + 1 needles before drought (May–June 1998). Error bars indicate S.D. and *n* = 12. (□), CF; (■), NF + O₃.

decrease was smaller (not significant) in the NF + O₃ DS than CF DS seedlings.

2.5. Pigment concentrations

O₃ fumigation caused chlorotic mottle (data not shown) and decreased pigment concentrations in summer 1998. The chlorophyll *a* (chl *a*) and *b* (chl *b*) concentrations and the chl *a* + *b*/carotenoid (*car*) ratio in the *c* and *c* + 1 needles as well as the *car* concentration in the *c* + 1 needles of the NF + O₃ seedlings were markedly lower than those in the needles of the CF seedlings in October 1998 (Table 2). On the other hand, drought markedly (*P* < 0.05) decreased the chl *b* and *a* + *b* concentrations and chl *a* + *b*/*car* ratio in the *c* needles of the CF seedlings as well as the chl *a* concentration and chl *a* + *b*/*car* ratio in the *c* + 1 needles of the CF seedlings.

In order to check the health of trees after several months with no O₃ fumigation, the pigment concentrations were measured again at the end of May 1999. The new *c* needles of the NF + O₃ seedlings only had a markedly lower chl *a* + *b*/*car* ratio compared to those of the CF seedlings, whereas in the case of the *c* + 1 needles both the chl *a* and *b* concentra-

Fig. 4. The diurnal gas exchange response of the *c* needles during drought period (July–August 1998). Error bars indicate S.D. and *n* = 8. (○), CF WW; (●), NF + O₃ WW; (△), CF DS; (▲), NF + O₃ DS.

Fig. 5. The diurnal gas exchange response of the $c + 1$ needles during drought period (July–August 1998). Error bars indicate S.D. and $n = 8$. (○), CF WW; (●), NF + O₃ WW; (△), CF DS; (▲), NF + O₃ DS.

Fig. 6. The net photosynthesis and stomatal conductance for the c needles after rewatering (September 1998). Error bars indicate S.D. and $n = 8$. (□), CF; (■), NF + O₃.

tions and the chl $a + b$ /car ratio were decreased under elevated O₃ (Table 2). The $c + 1$ needles of the NF + O₃ DS seedlings had now markedly ($P < 0.05$) higher chl a , chl $a + b$ and car concentrations than those of the NF + O₃ WW seedlings. There were no differences in the pigment concentrations and ratios between the CF WW and CF DS seedlings.

2.6. Nutrient contents

O₃ had a significant effect on C (decrease) and K (increase) levels of the c needles. Drought stress significantly affected the foliar C (increase), P (decrease), Mg (increase), Mn (increase) and K (decrease) contents (Table 3). The interaction between water stress and O₃ was significant on the C and K levels, particularly in this latter case for K

content in the NF + O₃ DS seedlings. There were also significant changes in the elemental contents of the $c + 1$ needles; there was a significant decrease in the N content and an increase in the Mn content of the $c + 1$ needles with increasing O₃ dose. The drought stress provoked a decrease in both the K and P content of the c needles of the CF seedlings.

Ca, K and P were found to be most abundant in the epidermis, mesophyll and in the guard cells (data not shown). Both needle age-classes showed increasing concentrations of Ca in the mesophyll tissue with increasing O₃ exposure. Potassium, in turn, had a tendency to decrease in the guard cells of the both needle age-classes and in the mesophyll of the $c + 1$ needles.

3. Discussion

O₃ had no effect on growth (height and diameter) in 1997, and there were no changes in gas exchange and pigments either [26]. Thus we did not expect any O₃ response in 1998, as the AOT40 was lower than in 1997 and, especially, if we ignore possible cumulative effects of the 1997 exposure. However, some significant changes in gas exchange and pigments were already observed in May 1998 [26] (but no change for growth, data not shown). There are two possible explanations for that, (a) the observed responses were due to peak O₃ concentrations (Fig. 1) and/or (b) there was a marked carry-over effect.

In the present experiment, with high daily O₃ concentration (Fig. 1), we found a significant increase in the stomatal conductance of *Pinus halepensis* before and during the water stress period and a decrease at the end of experiment. In other O₃ studies on *Pinus halepensis*, the authors have found a decrease in the stomatal conductance, except in the case of Fontaine et al. [13], who found the highest values in the one-year old needles (no significant difference). Several hypotheses have been presented to explain such an increase in g_w . In general, the increase in stomatal conductance may depend on the fumigation history or on the relative humidity [21] as well as on different leaf types [35] or leaf anatomy and water status of the plant [27]. Eamus et al. [11], for example, concluded that this result may be attributed to a decrease in the intercellular CO₂ concentration due to enhanced photosynthesis, or it may reflect a long-lasting impairment of stomatal regulation by O₃ in Norway spruce. The increase in stomatal conductance may depend on the fumigation history or on the relative humidity [21] as well as on different leaf types [35] or leaf anatomy and water status of the plant [27]. The work of Hassan et al. [17] provides another explanation: a direct effect of O₃ on the epidermal complex leading to stomatal opening. Furthermore, whole plants may compensate for small losses of leaf tissue or photosynthetic capacity with increased photosynthesis in younger undamaged leaves. For example, Beyers et al. [6] found that the c needles of O₃-exposed ponderosa pine had

Table 2

Pigment concentrations and ratios of the *c* and *c* + 1 needles in October 1998 (*n* = 16) and May 1999 (*n* = 6–8). *F* values are given for the effect of O₃, water supply and interaction between them

	Pigment concentrations (mg g ⁻¹ dw)					
	Chl a	Chl b	Chl a + b	Car	Chl a/b	Chl a + b/car
Oct. 1998, <i>c</i> needles						
CF WW	5.02 ± 0.91	1.83 ± 0.41	6.85 ± 0.73	1.04 ± 0.13	2.87 ± 0.63	6.64 ± 0.41
CF DS	4.63 ± 0.55	1.54 ± 0.23	6.17 ± 0.76	1.01 ± 0.09	3.02 ± 0.16	6.01 ± 0.29
NF+O ₃ WW	3.97 ± 1.10	1.52 ± 0.40	5.49 ± 1.02	0.94 ± 0.17	2.77 ± 0.71	5.89 ± 0.50
NF+O ₃ DS	4.38 ± 0.72	1.48 ± 0.26	5.86 ± 0.97	1.00 ± 0.12	2.98 ± 0.12	5.84 ± 0.36
O ₃	9.362**	5.034*	14.351***	3.128 ns	0.345 ns	24.418***
Drought	0.001 ns	3.824 ns	0.505 ns	0.413 ns	2.077 ns	10.100**
O ₃ × Drought	3.646 ns	2.160 ns	5.715*	1.741 ns	0.069 ns	6.873*
df error	60	60	60	60	60	60
Oct. 1998, <i>c</i> + 1 needles						
CF WW	5.39 ± 0.66	1.80 ± 0.24	7.19 ± 0.89	1.15 ± 0.12	3.01 ± 0.16	6.22 ± 0.29
CF DS	4.93 ± 0.55	1.68 ± 0.22	6.61 ± 0.75	1.13 ± 0.10	2.95 ± 0.14	5.83 ± 0.30
NF+O ₃ WW	3.63 ± 0.88	1.28 ± 0.34	4.90 ± 0.21	0.91 ± 0.13	2.88 ± 0.33	5.31 ± 0.68
NF+O ₃ DS	3.97 ± 0.69	1.40 ± 0.36	5.37 ± 1.00	0.99 ± 0.12	2.91 ± 0.35	5.44 ± 0.63
O ₃	58.826***	28.577***	51.284***	42.554***	1.659 ns	25.593***
Drought	0.094 ns	0.001 ns	0.053 ns	0.727 ns	0.014 ns	1.052 ns
O ₃ × Drought	5.094*	2.580 ns	4.483*	2.503 ns	0.427 ns	4.061*
df error	59	59	59	59	59	59
May 1999, <i>c</i> needles						
CF WW	4.37 ± 0.23	1.50 ± 0.15	5.87 ± 0.37	0.83 ± 0.04	2.92 ± 0.17	7.04 ± 0.34
CF DS	4.63 ± 0.24	1.53 ± 0.10	6.16 ± 0.33	0.86 ± 0.04	3.02 ± 0.08	7.17 ± 0.27
NF+O ₃ WW	4.52 ± 0.55	1.49 ± 0.32	6.00 ± 0.86	0.91 ± 0.09	3.09 ± 0.32	6.58 ± 0.49
NF+O ₃ DS	4.21 ± 0.35	1.35 ± 0.21	5.56 ± 0.53	0.84 ± 0.07	3.17 ± 0.31	6.65 ± 0.35
O ₃	0.896 ns	1.554 ns	1.172 ns	1.086 ns	3.019 ns	12.076**
Drought	0.018 ns	0.474 ns	0.119 ns	0.954 ns	0.979 ns	0.546 ns
O ₃ × Drought	4.029 ns	1.114 ns	2.888 ns	4.071 ns	0.022 ns	0.040 ns
df error	25	25	25	25	25	25
May 1999, <i>c</i> + 1 needles						
CF WW	5.82 ± 0.70	2.31 ± 0.34	8.13 ± 1.03	1.19 ± 0.11	2.53 ± 0.10	6.81 ± 0.32
CF DS	5.81 ± 0.41	2.41 ± 0.36	8.22 ± 0.74	1.19 ± 0.07	2.44 ± 0.21	6.88 ± 0.28
NF+O ₃ WW	4.56 ± 0.97	1.76 ± 0.51	6.31 ± 1.46	1.01 ± 0.16	2.64 ± 0.25	6.19 ± 0.70
NF+O ₃ DS	5.76 ± 0.45	2.17 ± 0.42	7.94 ± 0.64	1.23 ± 0.16	2.78 ± 0.83	6.53 ± 0.80
O ₃	6.692*	6.459*	7.494*	2.027 ns	1.672 ns	4.632*
Drought	5.632*	2.752 ns	5.012*	4.825*	0.018 ns	0.831 ns
O ₃ × Drought	5.683*	1.037 ns	3.945 ns	4.561*	0.419 ns	0.359 ns
df error	25	25	25	25	25	25

the highest photosynthetic rates, whereas there was no change in stomatal conductance.

3.1. The role of stomata

The needles of Aleppo pine seedlings have sunken stomata and the epistomatal cavity is filled with epicuticular waxes; it is suggested that this morphology represents a water conservation mechanism, creating a greater barrier to water loss than to CO₂ entry into the needle interior [2]. Long-term O₃ exposure has been reported to degrade and erode the waxes [3], and presumably this structural alteration reduces the effectiveness of the wax as a water barrier. This may lead to an apparent increase in the water loss. However, we did not observe any O₃-related, enhanced degradation of epicuticular waxes around the stomata or in the substomatal

cavity (data not shown). The conflicting evidences suggest that further research will have to be done to explain increased stomatal conductance. Fact is that, in our study, the stomata were first open, but were closed later, and finally the photosynthesis decreased due to more and more severe damage with the increasing absorbed O₃ dose [17].

3.2. The contribution of drought

In this drought experiment, the *c* needles of both the CF DS and NF + O₃ DS seedlings showed significant reductions in *g_w* and *A* (stomata were quite closed). The *g_w* of *c* + 1 needles also decreased, but the stomata seemed to remain more open than in the *c* needles. The O₃ exposure led to a reduction in the *g_w* of *c* + 1 needles in the NF + O₃ DS seedlings. This suggests that drought protected the seedlings

Table 3

The total element content (% dry weight) of the *c* and *c* + 1 needles sampled on 9 October 1998 (df error = 12)

Element Treatment	C	N	C/N	P	K	Ca	Mg	Mn
<i>C</i>								
CF WW	51.75 ± 0.22	3.42 ± 0.07	15.11 ± 0.33	0.31 ± 0.02	1.29 ± 0.06	0.18 ± 0.06	0.098 ± 0.007	0.016 ± 0.003
NF + O ₃ WW	51.25 ± 0.07	3.30 ± 0.23	15.56 ± 1.32	0.33 ± 0.03	1.48 ± 0.04	0.20 ± 0.08	0.093 ± 0.005	0.015 ± 0.004
CF DS	51.90 ± 0.14	3.26 ± 0.07	15.88 ± 0.18	0.25 ± 0.02	1.29 ± 0.06	0.20 ± 0.05	0.111 ± 0.010	0.021 ± 0.002
NF + O ₃ DS	51.85 ± 0.07	3.25 ± 0.12	15.97 ± 0.61	0.26 ± 0.02	1.25 ± 0.06	0.24 ± 0.03	0.104 ± 0.005	0.022 ± 0.004
O ₃	**	ns	ns	ns	*	ns	ns	ns
Drought	**	ns	ns	***	**	ns	*	**
O ₃ × Drought	*	ns	ns	ns	**	ns	ns	ns
<i>c</i> + 1								
CF WW	52.65 ± 0.13	3.29 ± 0.10	16.01 ± 0.45	0.20 ± 0.06	0.69 ± 0.23	0.28 ± 0.11	0.096 ± 0.009	0.018 ± 0.005
NF + O ₃ WW	52.40 ± 0.16	3.05 ± 0.18	17.22 ± 1.01	0.26 ± 0.02	0.84 ± 0.02	0.33 ± 0.03	0.090 ± 0.007	0.023 ± 0.004
CF DS	52.09 ± 0.16	3.13 ± 0.07	16.60 ± 0.32	0.23 ± 0.03	1.02 ± 0.10	0.32 ± 0.10	0.093 ± 0.013	0.021 ± 0.003
NF + O ₃ DS	52.54 ± 0.19	3.00 ± 0.15	17.54 ± 0.88	0.21 ± 0.02	0.85 ± 0.07	0.30 ± 0.03	0.091 ± 0.009	0.022 ± 0.002
O ₃	ns	*	ns	ns	ns	ns	ns	*
Drought	*	ns	ns	ns	*	ns	ns	ns
O ₃ × Drought	**	ns	ns	ns	*	ns	ns	ns

against the O₃ impact. After rewatering (Fig. 6), we also observed that the NF + O₃ DS seedlings had higher values of g_w and A than the NF + O₃ WW seedlings. Tingey and Hogsett [37] clearly indicated that water stress can protect plants from O₃ injury mainly through its influence on stomatal conductance, rather than through biochemical mechanisms. The chlorophyll results provide further evidence for the effect of water stress that may be seen as beneficial in this context (see below).

The changes in the gas exchange parameters caused by drought explain the decrease in the height and diameter growth and also in the biomass of needles and woody parts. The reductions in the biomass of different plant parts were proportional to each other, and therefore, the R/T ratio did not change. O₃ also provoked a decrease in the above-ground and below-ground biomass of the WW seedlings; these results are in contrast with those of Wellburn and Wellburn [40]. The decrease in the photosynthetic rate at the end of the experiment could not alone explain the significant decrease in the biomass. Possible factors further decreasing biomass accumulation could be enhancing respiration as suggested by Dizengremel and Gérant [10] or increase root exudation. Also, photosynthetic measurements were taken in the morning between 08:00 and 10:00 h; i.e. during the period of highest daily net CO₂ uptake (Figs. 4 and 5). This photosynthetic behavior is typical of arid-growth conifers, such as ponderosa pine in the mountains of southern California, USA or as showed for Aleppo pine by Inclán et al. [20]. Both *c* and *c* + 1 needles have equal photosynthetic rates; however, while A in the *c* needles of the CF and NF + O₃ seedlings decline similarly during the day, A in the *c* + 1 needles of the NF + O₃ seedlings is lower than in the CF seedlings during the majority of the day (Fig. 5). If the daily pattern of A in the *c* + 1 needles of the CF and NF + O₃ seedlings continued throughout the growing season, then this could explain a decline in biomass.

Foliar chlorophyll content has been found to have a high correlation with the amount of O₃-induced chlorotic mottle [30]. In the present study, the pigments were chosen as indicators of possible O₃-induced needle damage as for occurrence of chlorotic mottle, only the sensitive individuals in a population (of a detector bioindicator) will respond to O₃ and (only when they experience) appropriate edaphic and tropospheric conditions coupled with O₃ exposures and concentrations sufficient to cause foliar injury. The results show that both treatments, O₃ and drought, decreased the needle pigment concentrations and the chl *a* + *b*/car ratio. The higher pigment concentrations in the DS than WW NF + O₃ seedlings suggest that drought stress decreased O₃ flux into the needles [20]. In mature Aleppo pines under field conditions, longer needles (i.e. needles developed during years of abundant rain fall) have been found to be associated with higher occurrence of chlorotic mottle [33].

3.3. Cumulative and carry-over O₃ effects on the pigments

Robinson and Wellburn [31] report a decrease in the chl *a*/*b* ratio of Norway spruce needles under O₃ stress. Such a change was not observed in the present study, as chl *b* concentrations were also reduced by O₃. Indeed, marked changes in the chl *b* concentration may be expected especially in the needles showing visible O₃ damage, as chl *b* is broken down in senescence [36] and O₃ injury seems to be among the factors affecting needle retention in mature Aleppo pines in the field [33]. The differences in the chl *a*, chl *a* + *b* and car concentrations in the *c* + 1 needles between the WW and DS NF + O₃ seedlings in May 1999 also suggest that the WW NF + O₃ seedlings had not (yet) been able to compensate for the O₃-induced chlorophyll loss [31]. The lower chl *a* + *b*/car ratio in the *c* needles of the NF + O₃ seedlings compared to that in the *c* needles of the CF seedlings points to a carry-over effect [23].

3.4. Changes in the nutrient contents

The role of nutrition in the O₃ sensitivity of woody plants is still under debate [29]. Response of nutrient uptake and distribution within different tissues to O₃ exposure has been found to be variable depending on species and environmental conditions. In Norway spruce, Keller and Matyssek [22] reported increased concentrations of Ca, Mg and N. Le Thiec et al. [24] found increased concentrations of K and Ca in the same species, but a decrease in the K content of the beech leaves after O₃ fumigation. On the other hand, Samuelson et al. [32] found that O₃ exposure reduced foliar N concentrations and Fangmeier et al. [12] did not detect any O₃ effect on the nutrient content in spring wheat.

In our experiment, O₃ reduced the N content, especially of the *c* + 1 needles, which may have contributed to the decrease of pigment concentrations and, consequently, to photosynthesis, growth and biomass production. The needle K content also changed significantly and this change could result from a perturbation of the mineral nutrition at the root level. According to Barnes and Pfirrmann [5], the increase in the K levels could be explained by an increasing ion uptake at root level, but our microanalyses revealed a decrease in the K levels (at the end of experiment), especially in the guard cells. We explain this phenomenon by the fact that O₃ exposure may lead to changes in the cell membrane permeability [8] which could cause perturbation in the nutrient compartmentalization [18]. The increase in the Ca content of the mesophyll tissue may also be due to this leakage of membrane [9] or due to an involvement of Ca in detoxification [28]. Thus it may be linked to early events under O₃ attack. Drought tended to decrease the P concentrations. This suggests that drought has, by reducing the water supply, also reduced the nutrient supply to the plant, or changed the availability of the ions from the soil solution [16].

The cumulative effects of high O₃ concentrations on Aleppo pine seedlings were seen as lower net photosynthesis in the *c* needles. This may be due to chloroplast damage, which was observed as decreased chl *a* + *b* concentration in both the *c* and *c* + 1 needles. Consequently, growth and biomass were reduced (total biomass was reduced by 32% for an AOT40 of about 120 µl l⁻¹ h, consequently we could have -10% for a value of 37.5 µl l⁻¹ h). Our study also suggests that drought stress reduced the O₃ flux into the needles by reducing stomatal conductance. At any rate, drought reduced the growth and biomass more markedly than did O₃ (except for wood).

4. Methods

4.1. Experimental site, plant material and treatments

The experiment was performed in an OTC facility at Col du Donon (48°29'N, 7°05'E) in France; the site is at 727 m altitude. A total of four chambers were used, each with a

diameter of 3 m and a height of 2.8 m. On 22 April 1997, 2-year-old seedlings of *P. halepensis* Mill. (provenance Languedoc) were potted in 22 l pots filled with 50% peat, 30% sand and 20% perlite, i.e. 16 plants per chamber. A slow-release fertilizer (Nutricot 100, 13/13/13 N/P/K) was mixed with the substrate upon planting. The nutrient additions were the same for each pot and for both years in April. From 25 April 1997, seedlings were exposed to two treatments: CF and non-filtered air + 50 nl l⁻¹ (ppb) O₃ (NF + O₃) (24 h per day, 7 days per week) until 17 October 1997. Treatments were replicated in two OTCs each. The fumigations were continued from 6 May 1998, when the NF + O₃ seedlings were exposed to ambient air + 50 nl l⁻¹ O₃ until 9 October 1998. An electric discharge generator (Trailgaz Labo 76, France) supplied with pure oxygen produced O₃. O₃ was distributed to the chambers through Teflon tubes under constant flow conditions. Using an UV analyzer (Environment S.A., 1003RS, Poissy, France), air from each chamber and ambient air was continuously pulled through a manifold system and recorded for two 5 min periods every hour. Photosynthetically active radiation (PAR), air temperature and relative air humidity (RH) were also recorded. All data were stored by an automated data acquisition system. Trees were irrigated to field capacity at two days intervals by weighing individual pots. Seedlings were grown in a greenhouse during the winter and brought back to the experimental field at the end of April. Half of the seedlings were exposed randomly to drought stress (no watering) from 8 July to 24 August 1998. On 24 July and 12 August, DS trees were irrigated with an amount of water equivalent to 50% of their evapotranspiration (pots were weighted in order to determine water loss). At the end of drought period, all the DS trees were irrigated as the WW trees. On 10 October 1998, half of the seedlings were harvested and the rest were moved into a greenhouse for winter in order to avoid strong frost because the experimental site was not a natural habitat for Aleppo pine.

4.2. Pre-dawn leaf water potential

At regular periods throughout the drought treatment, the pre-dawn leaf water potential was measured using a Skye pressure chamber [34]. Measurements were made between 04:00 and 06:00 hours local time. The pre-dawn water potential was measured on the stem of *c* shoots.

4.3. Plant growth

The height and the basal diameter of all the seedlings were recorded weekly during both years. This measurement was made on two axes at 90° to each other (circular) to reduce errors resulting from the trunk cross sections not being exactly round. The mean diameter was then calculated. At the end of fumigation the percentage of relative growth rate was calculated for height and diameter and the seedlings were separated into roots (fine roots with coarse roots), stem and

branch wood and needles. The biomass was determined after drying the material for 96 h at 65 °C.

4.4. Leaf gas exchange

The net CO₂ assimilation rate, A ($\mu\text{mol m}^{-2} \text{s}^{-1}$) and leaf conductance to water vapor, g_w ($\text{mol m}^{-2} \text{s}^{-1}$) were measured using the LiCor model 6200 (USA) portable photosynthesis chamber (4 l). During the period of shoot extension (but needles had finished to elongate), when measurements were made on the c needles, they were harvested. After this period of shoot extension, one shoot was monitored for the rest of the season, as no subsequent surface area changes occurred. Measurements were carried out between 08:00 and 10:00 GMT inside the OTC and the results were expressed per foliage total surface area. As Aleppo pine needles cannot be approximated to two-dimensional forms, a equation describing their surface area has been developed [26]. There were no significant differences in the PAR, vapor pressure deficit, RH and temperature among the treatments during the measurements, these parameters were almost constant. Measurements were made on 12 trees, every time in the same order, per treatment on c and $c + 1$ needles. They were carried out on eight separate days from May to June 1998 before the drought stress period. On two occasions the measurements were continued at hourly intervals to follow diurnal variations of the measured parameters during drought. After rewatering, measurements were made on the c needles on two occasions in the morning in September 1998 on all trees. We calculated the average for each tree across the different measurement periods and presented the means of trees.

4.5. Determination of pigments

Current-year (c) and $c + 1$ juvenile needles were sampled for pigment analyses in mid October 1998 and at the end of May 1999. The needles were frozen with liquid nitrogen and put into a freezer (– 70 °C) prior to analysis. The pigments were extracted with dimethyl sulfoxide (DMSO) at 65 °C for 6 h according to Hiscox and Israelstam [19]. Absorbances were measured at 470, 646 and 663 nm with a Beckman DU®-64 spectrophotometer. The concentrations of chl a , chl b and total car were calculated according to Wellburn [39].

4.6. Nutrient contents

The needles for microanalysis were sampled between 10:00 and 11:00 GMT on 9 October 1998. Five c and five $c + 1$ needles were sampled from each seedling, and each needle was analyzed five times. The material to be analyzed was prepared according to the method used in Le Thiec et al. [25]. Sections of plant material were examined under a scanning electron microscope (Stereoscan 90B, Cambridge) at 15 kV, equipped with a energy dispersive microanalysis system (EDX, diode Si–Li; Analyzer AN10000 10/25). It was confirmed that the beam of primary electrons did not penetrate

other cells by analyzing one layer of cells on a sheet of aluminium. All measurements were made using an X-ray take-off angle of 45°, a measuring time of 100 s, a magnification of 6000 for all examined tissues (stomata, epidermis, mesophyll). In order to convert the microanalysis data into real concentrations (% dry mass), powdered spruce needles were used as standards (CRM 100 given by Community Bureau of Reference of the Commission of the European Communities). For the chemical analyses, Aleppo pine needles were sampled on 9 October 1998. The samples were dried at 65 °C. Total P and the cations (Mg, K, Ca, Mn) were mineralized (H₂O₂ and HClO₄) and measured by ICP (Jobin Yvon JY438 Plus). The total N content was determined using the Kjeldahl method and a Technicon II Autoanalyser, total C by the Anstett method [7].

4.7. Statistical analysis

The statistical analysis of the data was performed by the analysis of variance (ANOVA) with the GLM procedure of Statistical Analysis System. Test of equality of averages using Bonferroni (t -test) was applied equally (the same letters indicate that averages are not significantly different, and the alphabetic order corresponds to decreasing values). Significance was as follows: ns, not significant; *, significant ($P < 0.05$); **, highly significant ($P < 0.01$); ***, extremely significant ($P < 0.001$). Since there were only two OTCs (replicates) per treatment, subreplicates (trees) were used rather than real replicates (chambers), in the statistical analysis. All trees from two chambers belonging to the same treatment were pooled, giving four subreplicates. For diurnal gas exchange data, a pair-wise comparison was used.

The effects of time (day of measurement), treatment, OTC were tested using a three-way ANOVA. No significant effects were found due to time or OTC within treatments. This test demonstrated that the experimental conditions were homogeneous between different OTC across the time.

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