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CHRONIC OZONE EXPOSURE INCREASES THE SUSCEPTIBILITY OF HYBRID *POPULUS* TO DISEASE CAUSED BY *SEPTORIA MUSIVA*

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Abstract

Rooted cuttings of hybrid Populus *(DN34,* Populus deltoides X nigra*) were grown outdoors in pots in open-top chambers at Ithaca, NY (74·5° W, 42·5° N), during 1988 and 1989 (Experiment 1) and during 1989 and 1990 (Experiment 2). Ambient air was passed through charcoal filters to produce a 0·5 times ambient ozone treatment, and ozone generated from oxygen was added to produce one and two times ambient ozone treatments. In Experiment 1, treatments were applied for 8–12 h each day for 112 days of the 1988 growing season; then the plants were grown outdoors with ambient ozone in 1989. In Experiment 2, treatments were applied for 9 h each day for 98 days of the 1989 growing season; then the plants were grown outdoors with ambient ozone in 1990. Shallow wounds were made into the bark tissue and inoculated with either an aqueous suspension of conidia of* Mycosphaerella populorum *or sterile water on 1 and 2 September 1988 (Experiment 1) or 16 and 17 August 1989 (Experiment 2). In Experiment 1, wounds were inoculated either 0, 7, or 14 days after wounding. In Experiment 2, wounds were inoculated either 0, 3, or 6 days after wounding. Canker development was measured after harvest on 16 and 17 July 1989 (Experiment 1) and 28 May 1990 (Experiment 2).*

In both experiments, chronic exposure to ozone significantly increased the incidence of canker formation in inoculated wounds, and no cankers formed in wounds that received only sterile water. In Experiment 1, cankers formed only on plants inoculated the same day as wounding. No cankers formed on plants inoculated either 7 or 14 days after wounding. In Experiment 2, cankers formed on plants inoculated on the same day as wounding, and on a few plants inoculated 3 days after wounding. No cankers formed on plants inoculated 6 days after wounding. Additionally, in Experiment 2, exposure to increased concentrations of ozone caused a significantly higher number of plants to die during the subsequent winter. Analysis of partial correlation coefficients among plant growth and plant disease variables suggested that the observed ozone-induced increase in the susceptibility of the plants to disease was not mediated by alterations in plant growth.

Keywords: disease, ozone, plant, air pollution, canker.

INTRODUCTION

Little is known about the effects of ambient ozone on the interactions between plants and their parasites. In particular, the question of whether ozone predisposes plants to disease, thus increasing disease levels, has not been adequately examined. Much research has been conducted on this topic for many years (Heagle, 1973; Laurence, 1981) but most experiments have employed unrealistically high concentrations of ozone for short periods of time. Little is known about how diseases and ozone may interactively alter plant growth (Heagle, 1989). Furthermore, most research has focused on foliar diseases. No previous studies have investigated how chronic ozone exposure affects the susceptibility of trees to canker diseases. For these reasons, the effects of ozone exposure on the susceptibility of *Populus* to Septoria canker were examined.

Populus was selected because most species and hybrids are relatively susceptible to ozone. Decreased growth and physiological alterations occur even at ambient concentrations of ozone that do not cause foliar symptoms (Jensen, 1981; Reich & Lassoie, 1984; Woodbury *et al.*, 1994). Because ozone has been implicated as the most widespread and damaging air pollutant in the United States and elsewhere (Heagle, 1989; Heggestad & Bennett, 1984) it is important to determine whether ozone alters the susceptibility of *Populus* to disease.

The plant pathogen *Septoria musiva* Peck (= *Mycosphaerella populorum* Thompson) was selected because it is a serious problem in nursery and forest plantations of certain *Populus* species and hybrids, often causing the death of more than 50% of the trees in the plantation (Bier, 1939; Filer, 1964; McNabb *et al.*, 1982: Ostry & McNabb, 1985; Spielmann *et al.*, 1986). For these experiments, a disease-resistant clone of hybrid poplar was selected because we judged that any observed effects of ozone on disease would be of greater biological and practical significance if disease-resistant plants were used.

MATERIALS AND METHODS

Cultural conditions and ozone exposure

Rooted cuttings of hybrid *Populus* (DN34, *Populus deltoides X nigra)* were grown outdoors in pots in

open-top chambers or ambient plots without chambers at Ithaca, NY, during 1988 and 1989 (Experiment 1) and during 1989 and 1990 (Experiment 2). Ozone concentrations in the ambient air and in each chamber were monitored continuously on a time-shared basis and calibrated following United States Environmental Protection Agency protocol. Ambient air was passed through charcoal filters to produce a 0·5 times ambient ozone treatment, and ozone generated from oxygen was added to produce one and two times ambient ozone treatments. Blowers were turned off from 2300 to 0500 h EST to permit natural dew formation in the chambers. In Experiment 1, treatments were applied for 8 to 12 h each day (from approximately 1 h after sunrise to 1 h before sunset) for 112 days of the 1988 growing season; then the plants were grown outdoors with ambient ozone in 1989. In Experiment 2, treatments were applied for 9 h each day (0800 to 1700 h EST) for 98 days of the 1989 growing season; then the plants were grown outdoors with ambient ozone in 1990. The average 12-h (0800 to 2000 EST) ozone concentrations for the ambient, 0·5 times ambient, one times ambient, and two times ambient treatments were, respectively, 0·044, 0·020, 0·044, and 0·079 μl litre^{-1} in 1988, and 0·040, 0·021, 0·042, and 0·067 μl litre^{-1} in 1989. Further details of experimental design, plant material, and ozone exposure and monitoring were described previously (Woodbury *et al.*, 1994).

Wounding protocol

Experiment 1
Six shallow wounds were made in the bark of each poplar stem at each of two regions of the stem with the edge of a large hypodermic needle. One-third of the plants were wounded at each of three times: 18 August, 25 August and 1 or 2 September 1988. One group of wounds was centred at the fifth node from the base of the stem; the other group was centred at the 28th node from the tip of the shoot. The wounds were approximately 2.5 cm apart along the axis of the stem and were arranged in a spiral. Each wound was approximately 5 mm long, 3 mm wide and 1 to 2 mm deep, with the long axis of the wound parallel to the long axis of the stem.

Experiment 2
One-third of the plants were wounded on each of three dates: 11, 14, and 16 August 1989. Twelve shallow wounds were made into the bark of each plant with a razor blade. The wounds were approximately 5 mm long, 5 mm wide, and 2 to 3 mm deep. The wounds were approximately 2 cm apart and were arranged spirally upward on the stem starting at the fifth node. No wounds were made on the upper stem.

Inoculation protocol

Experiment 1
On both 1 and 2 September 1988, a conidial suspension was axenically prepared by rinsing sterile distilled water across colonies of *S. musiva* growing on 2% V-8 juice agar (Ostry *et al.*, 1988). The resulting suspension was adjusted to 10^6 conidia ml^{-1} and one drop of the suspension was placed onto each wound with a sterile cotton-tipped applicator. Five wounds were inoculated with the fungus in each group of wounds; the uppermost wound in each group served as a control and received only sterile distilled water. All wounds were wrapped in paraffin film for two days following inoculation to promote spore germination. Plants wounded 18 and 25 August and 1 September were inoculated on 1 September; plants wounded on 2 September were inoculated on 2 September. Hence plants were inoculated either 0, 7, or 14 days after wounding.

Experiment 2
Ten of the wounds were inoculated with conidia of *S. musiva* as described above, one received only sterile distilled water, and one was excised and stored in fixative (5% glutaraldehyde) on the date that inoculations were made in order to permit subsequent microscopic examination. The results of this examination will not be reported here. Plants wounded on 11 and 14 August were inoculated on 17 August; plants wounded on 16 August were inoculated on the same day. Thus plants were inoculated either 0, 3, or 6 days after wounding.

Measurement of disease symptoms

Experiment 1
On 16 and 17 July 1989, stem segments containing wounds were removed and stored at 4°C for up to four weeks. Then each wound was examined for disease symptoms on the outer and inner surfaces of the bark and on the outer surface of the wood. Additionally, the horizontal and vertical extent of discoloration from the centre outward on each of the above tissues was recorded for the wound lowest on the plant in both the upper and lower groups of wounds.

Experiment 2
The protocol was as described above except that measurements were made only on the outer bark, and on intact plants rather than excised segments. Measurements were made on 28 May 1990. Canker length and width measurements were made only on plants inoculated on the same day as wounding.

Experimental design and analysis
A split-plot design was used with four ozone treatments (whole plot treatments) arranged in a completely randomized design. The four ozone treatments were ambient (in chamberless plots), and 0·5 times ambient, 1 times ambient, 2 times ambient (in open-top chambers). The sub-plot treatments were three wounding treatments (different numbers of days between wounding and inoculation) applied to individual trees in a completely randomized design within each chamber.

In both experiments, there were eight trees wounded on each of three dates prior to inoculation in each

open-top chamber or ambient plot. In Experiment 1, there were two replicate open-top chambers for each ozone treatment; in Experiment 2 there were three open-top chambers for each ozone treatment. The experimental unit was the open-top chamber, while the sampling unit was the individual tree.

Analyses of variance were performed separately for each wounding treatment to test for effects of ozone on several measures of disease symptoms on all plants in the three treatments in open-top chambers. The 12-h seasonal mean ozone concentrations were used for these analyses. Linear and quadratic contrasts were calculated using orthogonalized coefficients to test for dose-response effects of ozone on disease symptoms. An additional analysis of variance was performed to test for effects of the open-top chambers by comparing the ambient (chamberless) and 1 times ambient treatments.

For Experiment 1, data are limited to plants wounded and inoculated on the same day because cankers were induced only on these plants. For Experiment 2, all data are presented because cankers were induced on some plants inoculated three days after wounding.

The effect of ozone on measures of growth such as height, basal diameter, root and shoot dry mass, number of leaves produced, and leaf senescence was reported previously (Woodbury *et al.*, 1994). To assess if effects of ozone on disease might be mediated by effects on plant growth, partial correlation coefficients were calculated to test for correlation between each measure of disease and each measure of plant growth. These coefficients were calculated after adjusting for the effects of the ozone and wounding treatments on these variables. Statistical analyses were performed using the GLM procedure in the SAS system (SAS, 1985).

RESULTS

Effect of ozone on Septoria canker induction

Experiment 1

Wounds inoculated the same day that they were inflicted developed cankers, whereas those inoculated 7 or 14 days after wounding did not. No cankers resulted from the wounds inoculated with sterile water. Cankers increased in size very slowly in all ozone treatments. Nine months after inoculation, the discolored areas on the bark surface extended from the centre less than 10 mm vertically and less than 6 mm horizontally on average for any ozone treatment (Tables 1 and 2).

Exposure to ozone caused a linear increase in the number of inoculated wounds that developed cankers. In the group of wounds at the base of the stem, ozone increased the number of wounds with symptoms on both the outer bark and the wood ($p = 0{\cdot}05$ and $p = 0{\cdot}04$, respectively, Table 1). A similar trend was noted for symptoms on the inner bark, but it was not statistically significant ($p = 0{\cdot}16$, Table 1). In the group of wounds higher on the stem, ozone increased the number of wounds with symptoms on the wood ($p = 0{\cdot}04$, Table 2). Similar trends were noted for symptoms on the inner bark and bark surface, but these trends were not statistically significant ($p = 0{\cdot}15$ and $p = 0{\cdot}88$, respectively, Table 2).

There was some evidence that ozone also increased the extent of discoloration of the wood both horizontally ($p = 0{\cdot}023$) and vertically ($p = 0{\cdot}095$) at the stem base, but not on the upper stem (Tables 1 and 2). No

Table 1. Effect of ozone on Septoria canker on the lower stems of hybrid *Populus*, Experiment 1

Variable	Ozone treatment								Ozone effects			Chamber effect p value[d]
	Ambient		0·5 × Amb.		1 × Amb.		2 × Amb.		Main p value[b]	Linear p value[c]	Quadratic p value[c]	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE				
Bark surface												
Number of cankers[e]	4·67	0·19	4·06	0·15	4·59	0·13	4·95	0·14	0·052	0·024	0·399	0·785
Vert. extent[f] (mm)	8·27	1·25	7·20	1·25	9·95	1·12	9·17	1·23	0·378	0·399	0·268	0·435
Horiz. extent[f] (mm)	4·69	0·33	4·76	0·31	5·64	0·27	4·98	0·30	0·233	0·710	0·114	0·179
Inner bark												
Number of cankers	3·01	0·30	2·80	0·26	3·45	0·25	3·77	0·27	0·162	0·088	0·458	0·394
Vert. extent (mm)	6·12	1·38	4·90	1·10	8·77	1·08	7·85	1·18	0·172	0·206	0·142	0·298
Horiz. extent (mm)	1·52	0·02	2·75	0·52	2·66	0·50	1·87	0·55	0·522	0·310	0·709	0·001
Wood												
Number of cankers	2·96	0·14	2·57	0·16	3·13	0·14	3·66	0·15	0·040	0·018	0·550	0·461
Vert. extent (mm)	4·66	0·87	3·94	0·75	7·39	0·68	5·76	0·74	0·095	0·095	0·053	0·153
Horiz. extent (mm)	1·12	0·14	1·06	0·11	1·91	0·11	1·34	0·12	0·023	0·378	0·012	0·059

[a] Mean and standard error as determined by analysis of variance; see text for details.
[b] Overall p value for ANOVA of the effect of ozone on all plants grown in chambers.
[c] p value for the linear or quadratic contrast.
[d] Overall p value for ANOVA of the effect of open-top chambers on plants grown in chambers and those grown outside chambers at the ambient ozone concentration.
[e] Average number of cankers present on each stem (eight stems/wounding date/chamber, two chambers/treatment, five inoculated wounds/stem).
[f] Vertical and horizontal extent of discoloration measured from the centre of the lowest canker on each stem.

Table 2. Effect of ozone on Septoria canker on the upper stems of hybrid *Populus*, Experiment 1

Variable	Ozone treatment								Ozone effects			Chamber effect *p* value
	Ambient		0·5 × Amb.		1 × Amb.		2 × Amb.		Main *p* value	Linear *p* value	Quadratic *p* value	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE				
Bark surface												
Number of cankers	3·90	0·28	4·20	0·41	4·45	0·38	4·46	0·37	0·881	0·693	0·783	0·300
Vert. extent (mm)	6·87	0·82	6·33	1·13	8·91	1·05	8·36	1·11	0·356	0·337	0·279	0·227
Horiz. extent (mm)	4·02	0·40	4·37	0·65	5·00	0·61	5·10	0·64	0·720	0·508	0·694	0·228
Inner bark												
Number of cankers	3·24	0·13	3·16	0·29	4·18	0·28	4·10	0·30	0·148	0·132	0·116	0·039
Vert. extent (mm)	4·71	0·93	3·76	1·22	6·23	1·21	6·19	1·30	0·389	0·295	0·396	0·008
Horiz. extent (mm)	1·34	0·11	1·45	0·20	1·93	0·20	1·97	0·21	0·280	0·196	0·357	0·069
Wood												
Number of cankers	3·12	0·02	2·95	0·19	4·00	0·18	4·07	0·20	0·044	0·032	0·082	0·001
Vert. extent (mm)	3·17	0·44	3·15	0·94	5·55	0·88	4·98	0·92	0·300	0·305	0·231	0·065
Horiz. extent (mm)	0·87	0·07	0·93	0·22	1·32	0·21	1·35	0·22	0·440	0·301	0·471	0·051

For explanations see notes to Table 1.

such trends were observed for discoloration of the inner or outer bark (Tables 1 and 2).

Finally, more symptoms were produced on the inner bark and wood of the upper stem and the extent of discoloration in the inner bark and wood of both the upper and lower stem inoculations was greater on plants in chambers than those in ambient plots (Tables 1 and 2).

Experiment 2

Cankers were induced on nearly all plants that were inoculated and wounded on the same day and on a few plants inoculated three days after wounding. No cankers were induced on plants inoculated six days after wounding. No cankers formed in the wounds inoculated with sterile water. Cankers increased in size very slowly in all ozone treatments.

As in Experiment 1, ozone increased the number of inoculated wounds that developed cankers in plants inoculated and wounded on the same day ($p = 0{\cdot}01$), and this increase was a linear response to ozone dose (Table 3). A similar trend was observed for plants inoculated three days after wounding, but this trend was not statistically significant ($p = 0{\cdot}07$, Table 3). For plants inoculated three days after wounding, ozone increased the number of plants with cankers ($p = 0{\cdot}05$), and this was a linear response to ozone dose ($p = 0{\cdot}03$, Table 3). Canker length and width were not affected by ozone treatment (Table 3).

For the plants inoculated three days after wounding, ozone increased the number of plants that died during the winter ($p = 0{\cdot}001$). This was not a linear effect of ozone dose (Table 3). Similarly, for plants inoculated either 0 or six days after wounding, more plants ex-

Table 3. Effect of ozone on Septoria canker on the lower stems of hybrid *Populus*, Experiment 2

Days after wounding	Variable	Ozone treatment						Ozone effects		
		0·5 × Amb.		1 × Amb.		2 × Amb.		Main *p* value[b]	Linear *p* value[c]	Quadratic *p* value[c]
		Mean[a]	SE	Mean	SE	Mean	SE			
0	Plants with cankers[d]	1·000	0·03	0·958	0·03	0·963	0·03	0·644	0·471	0·561
	Cankers per plant[e]	6·928	0·43	8·708	0·43	9·590	0·42	0·012	0·005	0·313
	Vert. extent[f] (mm)	8·206	3·25	10·681	3·18	18·746	3·95	0·189	0·082	0·597
	Horiz. extent[f] (mm)	7·246	0·77	8·875	0·75	9·833	0·92	0·161	0·776	0·643
	Dead plants[d]	0·137	0·10	0·048	0·10	0·394	0·94	0·095	0·092	0·136
3	Plant with cankers	0·000	0·02	0·000	0·02	0·083	0·02	0·053	0·029	0·187
	Cankers per plant	0·000	0·05	0·000	0·05	0·208	0·06	0·071	0·038	0·218
	Dead plants	0·041	0·05	0·000	0·05	0·500	0·05	0·001	0·000	0·006
6	Dead plants	0·125	0·10	0·125	0·00	0·215	0·10	0·762	0·525	0·744

[a] Least-squares mean and standard error as determined by analysis of variance; see text for details.
[b] Overall *p* value for ANOVA of the effect of ozone on all plants grown in chambers.
[c] *p* value for the linear or quadratic contrast.
[d] Proportion of plants in each chamber.
[e] Average number of cankers present on each stem (eight stems/wounding date/chamber, three chambers/treatment, 10 inoculated wounds/stem).
[f] Vertical and horizontal extent of discoloration measured from the centre of the lowest canker on each stem.

posed to twice-ambient ozone died during the winter, but these effects were not statistically significant (p = 0·095, p = 0·762, Table 3).

Effect of plant growth on Septoria canker induction

For both experiments, significant partial correlations occurred between many measures of plant growth. For example, basal diameter and shoot height were correlated: the correlation coefficient was 0·707 and p was less than 0·001. There were also correlations among many measures of disease susceptibility. For example, the width and length of cankers on the bark surface were correlated: the correlation coefficient was 0·878 and p was less than 0·001.

In Experiment 1 there were no significant correlations between any measure of growth and any measure of disease susceptibility. For example, shoot height and the length of cankers on the bark surface were not correlated (r = 0·077, p = 0·350). In Experiment 2 there was a weak correlation between canker width and stem height as measured on 1 August 1989 (r = 0·186, p = 0·021), but not at the end of the experiment. Similarly there was a weak correlation between canker width and internode length (r = 0·179, p = 0·027). These two measures of plant growth (stem height and internode length) were strongly correlated (r = 0·756, p = 0·000). Canker width was not affected by ozone in this experiment, and inasmuch as no other significant partial correlations were found, there was no evidence that the effect of ozone on susceptibility to Septoria canker was due to any effects of ozone on plant growth.

DISCUSSION

Ozone could potentially alter the interaction between pathogens and their host plants in two ways. First, ozone could directly affect the pathogen, altering its virulence or survival. Second, ozone could indirectly affect the pathogen by means of physiological or biochemical alterations in the host that alter susceptibility. Few studies have examined how ambient concentrations of ozone affect micro-organisms, but the results of these studies suggest that micro-organisms are not affected by ambient ozone concentrations (Laurence, 1981).

In interpreting the effects of ozone on plant disease, it is useful to separate pathogens based on their method of resource exploitation. A broad distinction can be made between obligate parasites that require living host tissue (biotrophic) for sustenance and survival, and facultative parasites that can survive and grow on dead tissue (necrotrophic). Such necrotrophic pathogens may actually kill the tissue before colonizing it, or may enter living tissue and continue to grow after tissue death. Ozone often restricts the incidence and severity of obligate fungal parasites, while having little effect on facultative fungal parasites (Laurence, 1981). This pattern was recently observed in cottonwood (*Populus deltoides*): exposure to ozone increased the resistance of cottonwood leaves to disease caused by an obligate parasite (*Melampsora medusae*), while having no effect on a disease caused by a facultative parasite (*Marssonina brunnea*) (Coleman *et al.*, 1987, 1988). However, ozone has increased the incidence and severity of facultative fungal parasites of some woody species. Ozone increased the infection and colonization of Ponderosa pine roots by *Heterobasidion annosum* (James *et al.*, 1980). Ozone also increased the severity of root rot of azalea caused by *Phytophthora cinnamomi* in one of numerous cultivars tested (Moore *et al.*, 1984). Overall, the indirect effects of ambient ozone on susceptibility to disease are not well understood, particularly for tree species.

The rate of canker enlargement observed in this study was much slower than has been previously reported (Bier, 1939; Filer *et al.*, 1971; Long *et al.*, 1986; Spielmann *et al.*, 1986). The growth rate of the pathogen in leaf disc inoculations under controlled conditions has also been shown to be much slower on DN34 than other clones of hybrid *Populus* (Ostry *et al.*, 1988). The slow growth rate of the pathogen is presumably due to the high level of disease resistance of this clone (Ostry & McNabb, 1985).

At the highest concentration of ozone, virtually all inoculations made on the same day as wounding resulted in cankers, but at lower concentrations fewer cankers were induced. Because the pathogen was protected from the direct effects of ozone exposure first by paraffin film and soon after by growing within the host tissue, it seems unlikely that there was a direct effect of ozone on the pathogen. Rather, it appears that the effect of ozone was to increase the susceptibility of the plant.

Ozone did not affect canker induction or canker size in the upper stem as much as in the lower stem, although there was a significant effect of ozone on symptom production on the wood of the upper stem tissues. There are two possible explanations for this difference. First, the tissues on the upper stem were younger, and hence were exposed to ozone for less time and may not have been as strongly affected as were the older tissues at the stem base. Second, younger stem tissue is known to be more susceptible to Septoria canker (Long *et al.*, 1986). If the upper stems were less disease-resistant, the effect of ozone might not be as readily detected.

The significant effects of the chambers themselves on symptom production and canker expansion may be due to differences in plant growth. In all cases where significant differences between plants grown in chambers and those grown outside occurred, the incidence or size of cankers was greater on plants grown in the chambers than those grown outside. Open-top chambers, while providing one of the best means of examining the effects of ozone on plant growth and disease susceptibility, have been shown to alter the microclimate around the plants (Heagle *et al.*, 1973; Olszyk *et al.*, 1980; Weinstock *et al.*, 1982). Alterations in temperature, light, and relative humidity all might have direct effects on the pathogen, or might affect the pathogen indirectly by means of changes in plant development or response to ozone.

There was no relationship between plant growth and measurements of disease susceptibility that were affected by ozone. Hence the ozone-induced increase in the susceptibility of *Populus* to Septoria canker is apparently not mediated by effects of ozone on plant growth.

Our results suggest that ambient concentrations of ozone may predispose hybrid *Populus* to Septoria canker. Further research will be required to understand how ozone affects the epidemiology of Septoria canker. Because leaf infections provide both primary and secondary inoculum, an understanding of the effect of ozone on leaf infections is required in order to assess the impact of ozone on Septoria canker disease.

In these experiments ozone not only increased susceptibility to disease, but there was also some evidence that ozone increased the susceptibility of trees to death during the winter. In both cases, ozone increased the susceptibility of plants to another stress. Inasmuch as trees are exposed to many such biotic and abiotic stresses, our results suggest that it is important to investigate further the role of ozone in predisposing trees to other stresses.

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