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Leaf age affects the responses of foliar injury and gas exchange to tropospheric ozone in *Prunus serotina* seedlings

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Ozone effects on symptom development and leaf gas exchange interacted with leaf age and N-content on black cherry seedlings.

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ABSTRACT

We investigated the effect of leaf age on the response of net photosynthesis (A), stomatal conductance (g_{wv}), foliar injury, and leaf nitrogen concentration (N_L) to tropospheric ozone (O_3) on *Prunus serotina* seedlings grown in open-plots (AA) and open-top chambers, supplied with either carbon-filtered or non-filtered air. We found significant variation in A, g_{wv}, foliar injury, and N_L ($P < 0.05$) among O_3 treatments. Seedlings in AA showed the highest A and g_{wv} due to relatively low vapor pressure deficit (VPD). Older leaves showed significantly lower A, g_{wv}, N_L, and higher foliar injury ($P < 0.001$) than younger leaves. Leaf age affected the response of A, g_{wv}, and foliar injury to O_3. Both VPD and N_L had a strong influence on leaf gas exchange. Foliar O_3-induced injury appeared when cumulative O_3 uptake reached 8–12 mmol m^{-2}, depending on soil water availability. The mechanistic assessment of O_3-induced injury is a valuable approach for a biologically relevant O_3 risk assessment for forest trees.

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1. Introduction

Tropospheric ozone (O_3) has been, and is predicted to remain, the most harmful to vegetation of all regionally dispersed air pollutants (Hogsett et al., 1988; Davison and Barnes, 1998; Vingarzan, 2004; Karnosky et al., 2007). Effects of commonly observed atmospheric O_3 concentrations on plant functionning have been well-documented (Winner, 1994; Sandermann, 1996; Matyssek et al., 2008). Because O_3 diffuses into leaves together with carbon dioxide, stomatal regulation has been considered as an important factor in controlling O_3 sensitivity of plants (Reich, 1987; Becker et al., 1989; Taylor and Hanson, 1992; Matyssek and Sandermann, 2003; Paoletti and Grulke, 2005). After O_3 enters the intercellular space of stomata, O_3 dissociates quickly to form oxygen and peroxides (Laisk et al., 1989; Lange et al., 1989; Larcher, 1995). It is the peroxide that oxidizes protein and lipid components of cellular membranes and thereby disrupts enzymes which are playing an important role in physiological processes (Mudd, 1982; Guderian, 1985; Paoletti, 2007). Thus, stomatal closure can be considered as an avoidance mechanism of plants in response to high O_3 concentrations (Kolb and Matyssek, 2001). On the other hand, affected plants need photosynthate to construct and maintain their defense and repair systems (Kolb and Matyssek, 2001; Andersen, 2003; Wieser and Matyssek, 2007). Stomata must be open for CO_2 to enter. Therefore, a tradeoff between an increased carbon gain and a reduced O_3 uptake becomes a critical mechanism for plants to either avoid or tolerate adverse O_3-induced effects.

Because many biotic and abiotic factors affect plant gas exchange, plant response to O_3 is likely confounded with these factors as well (Matyssek and Sandermann, 2003; Paoletti, 2007), which may include tree age, genetic make-up, pests, competition, soil moisture, humidity, light intensity, temperature, and other co-occurring greenhouse gases (Reich, 1984; Karnosky et al., 2007). For example, plants tend to close their stomata to conserve water in response to water stress (Kramer, 1983) and thereby coincidentally avoid O_3 uptake (Reich, 1987; Matyssek and Sandermann, 2003). Previous studies have demonstrated that more O_3 uptake and more severe leaf injury of forest trees were correlated with a higher availability of soil water (Showman, 1991; Hildebrand et al., 1996;

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Chappelka and Samuelson, 1998; Grulke et al., 2003; Schaub et al., 2003; Ribas et al., 2005; McLaughlin et al., 2007). Although some studies have failed to demonstrate an effect of soil water availability on foliar injury (Heagle et al., 1988; Temple and Benoit, 1988), soil water availability by itself or additional other interacting factors are likely to complicate such comparisons (Grulke et al., 2003; Schaub et al., 2005; McLaughlin et al., 2007).

Leaf nitrogen concentration (N_L) is another factor that significantly influences photosynthetic gas exchange (Field and Mooney, 1986). Its effects on plant responses to O_3 have similar tradeoffs as water does (Greitner and Winner, 1989; Pell et al., 1990; Landolt et al., 1997). Because at least 75% of total N_L is found within compounds that relate to the biochemical and photobiological processes of photosynthesis, N perhaps is the most important factor in replacement of mesophyll tissue damaged by O_3 (Grulke, 2003). Trees with higher N_L seem to have higher photosynthetic rates, accompanied with higher stomatal conductance, and subsequently higher O_3 uptake, suggesting that plants with a higher N_L should be more sensitive to O_3 (Pell et al., 1990). However, a high supply of N tends to reduce O_3 injury and premature leaf loss in birch (Paakkonen and Holopainen, 1995). Therefore, N effects on the sensitivity of trees to O_3 are inconsistent (Greitner and Winner, 1989), which might be confounded by processes of leaf senescence and nutrient retranslocation (Matyssek and Sandermann, 2003).

The objective of this study was to examine the inter-relationships among leaf age, N_L, leaf gas exchange, and O_3-induced injury on black cherry (*Prunus serotina* Ehrh.) seedlings grown within open-top chambers. We tested the hypothesis that net photosynthesis, stomatal conductance, and N_L are strongly interrelated and collectively influence the incidence and severity of O_3-induced foliar injury. Using these data, we tried to determine whether we can find a mechanistic way to assess O_3 risk for forest seedlings. We used black cherry because: (1) it is one of the tree species native to the northeastern USA that is most sensitive to O_3 (Davis and Skelly, 1992; Simini et al., 1992); (2) it has a relatively high gas exchange rate (Fredericksen et al., 1995, 1996a, 1996b); (3) it has been extensively studied both *in situ* and in bio-indicator gardens (Simini et al., 1992; Kouterick et al., 2000; Ferdinand et al., 1999; Lee et al., 1999, 2002; Schaub et al., 2003, 2005); and (4) it is a very valuable commercial timber species (Marquis, 1990).

2. Materials and methods

2.1. Study site and experimental design

The study site was located at the Pennsylvania's Bureau of Forestry's Penn Nursery, (40° 40′ N latitude, 77° 37′ W longitude, 490 m a.s.l.) in Centre County, Pennsylvania, USA. Soils are Laidig series in the taxonomic subgroup Typic Fragidults, 0.6–1.8 m deep over colluvial material from sandstone and siltstone (USDA, 1981). The climate is a composite of relatively dry Midwestern continental climate. Maximum and minimum air temperatures and precipitation during study in 1998 are shown in Fig. 1. The experimental design consisted of three blocks with each block split into two water regimes with two open-top chambers (for controlling ozone exposure) and one open-plot (AA: ambient air) randomly arranged within each water regime (Schaub et al., 2003). One of each pair of chambers was supplied with carbon-filtered air containing approximately 50% of ambient O_3 concentrations (CF: ca. 0.5 × AA) and the other one was supplied with non-filtered air (NF: ca. 0.96 × AA). The entire experiment consisted of a total of 18 plots (6 AA + 6 CF + 6 NF), all of which received natural rainfall and nine of which were supplementary irrigated every 2–5 days to any drought effects. Each plot included four black cherry seedlings from each of four different, open-pollinated families (2 sensitive and 2 tolerant to O_3; see Lee et al., 1999), 10 white ash (*Fraxinus americana* L.), and 10 red maple (*Acer rubrum* L.) seedlings that were planted within the well-tilled nursery soil during late September 1997. The open-top chambers were operated throughout the growing seasons of 1998 and 1999. Seasonal and annual assessments for seedling phenology, growth, physiology, and typical ozone-induced symptoms were conducted during both growing seasons (Schaub et al., 2003).

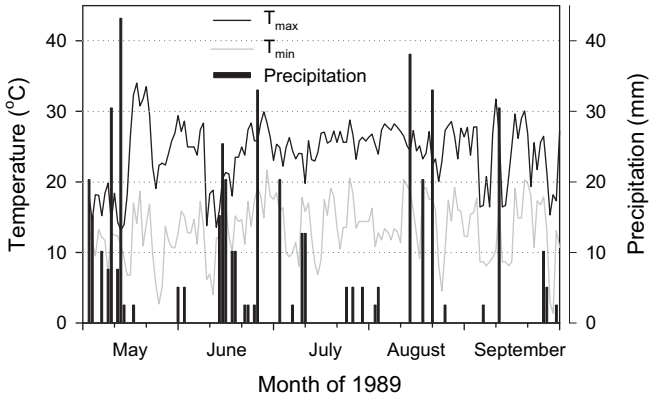

Fig. 1. Daily maximum and minimum air temperature and precipitation at the Penn Nursery site from May to September, 1998.

2.2. Physiological measurements

In this study and in respect to the hypothesis as outlined above, we used a sampling procedure that differed from the one as applied by Schaub et al. (2003). We used black cherry seedlings to study the effect of N_L and leaf age on the leaf gas exchange to O_3. We randomly chose one seedling per family within each plot during each of seven sampling dates for the physiological study from July 30 to August 7, 1998. During each sampling day, we measured a complete block of six plots and rotated within three blocks. Four leaves of one seedling were randomly selected within each of four age-classes from the youngest (1st age-class) to the oldest leaf (4th age-class). When we started to monitor O_3 on 16 May 1998, we identified and tagged 48 unfold leaves as our oldest leaves. The other leaf age-classes were determined based on leaf position and shoot growth rate between the oldest leaf position and the current shoot tip. On each sampling date, the leaves of the oldest 4th age-class were 11 weeks old, 3rd age-class ca. 7 weeks old, 2nd age-class ca. 4 weeks old, and the youngest, the 1st age-class, ca. 2 weeks old (and not yet fully expanded).

Net photosynthesis (A) and stomatal conductance to water vapor (g_{wv}) were measured under constant light conditions (PAR = 1200 μmol m^{-2} s^{-1}) with a Li-Cor 6400 portable photosynthesis system with a red/blue LED light source and CO_2 injector (Li-Cor Inc. Lincoln, Nebraska, USA). Measurements took place from 0900 to 1200 h and from 1300 to 1600 h on sampling days. The temperature within the cuvette was maintained near ambient air temperature, which varied between 24 and 35 °C depending on time of day and the day when measurements were taken. A constant CO_2 concentration at 355 μmol mol^{-1} was supplied with a fixed air flow of 500 μmol s^{-1}. Pertinent environmental variables were closely monitored including air temperature, relative humidity, and vapor pressure deficit.

Light-response curves were measured by setting photosynthetically active radiation (PAR) from 1400 to 1200, 1000, 800, 600, 400, 200, 100, 50, and 0 μmol m^{-2} s^{-1}. We let the leaves acclimate to the preset light conditions within the cuvette for 3 min before each measurement. We measured light-response curves on four healthy leaves from the 2nd age-class and on four leaves with O_3-induced symptoms from 3rd to 4th age classes within NF chambers either within irrigated or in non-irrigated regimes. We fit these data with the non-rectangular hyperbolic model developed by Hanson et al. (1987). We then estimated light-saturated maximum assimilation rates (A_{max}), light compensation points (Γ), dark respiration (R_d), and quantum yield (Φ).

2.3. Leaf nitrogen concentration

After leaf gas exchange was measured, we collected four leaves from each age-class per family within each of the 18 plots. These leaves were ground and N_L (%) was determined by combustion with Fisons Elemental Analyzer (Fisons, Italy) within the Agricultural Analytical Services Laboratory, Pennsylvania State University.

2.4. O_3-Induced injury evaluation

We visually rated the percentage adaxial stipple on individual leaves that we used for gas exchange measurements. The Horsfall-Barratt rating system (Horsfall and Barratt, 1945) developed for the Forest Health expert system (Nash et al., 1992) was used to determine an average percent leaf area being affected (%) by visible O_3-induced injury. Ratings were recorded as 0, 3, 6, 12, 25, 50, 75, 88, 94, 97, and 100% leaf area affected. Other than in Schaub et al. (2003), these leaves were individually selected and rated and represent their respective age-class as selected for this study only.

2.5. Ozone monitoring and uptake

Ambient O_3 concentrations were recorded from May to September, 1998 for each open-top chamber and within one open-plot. Ozone concentrations were continuously measured at 5-min intervals with a TECO Model-49 O_3 analyzer (Thermo Environmental Instrumentals, Inc., Franklin, MA, USA) with data stored via Odessa DSM 3260 data logger (Odessa Engineering, Austin, TX, USA). A six point calibration (0, 50, 100, 150, 200, and 300 ppb) was performed on the monitors at each sampling point every 10–14 days using a Thermo Electron Corporation Model 49 PS calibrator and a tanked source of compressed air that had all hydrocarbons and external pollutants removed. The calibrator was checked against a National Standard on an annual basis at the USEPA, Region II Office in Edison, NJ. Because day-length was about 12 h at the study site during the summer months (Campbell and Norman, 1998), we calculated 12-h (07:00 am–07:00 pm) O_3 exposures for hourly O_3 concentrations equaling or exceeding 0 ppb (SUM0) and 60 ppb (SUM60) for the oldest leaves within the respective O_3 plots.

Cumulative O_3 uptake (COU) was calculated for leaves by the surrogate water vapor techniques (Laisk et al., 1989; Matyssek et al., 2008) with g_{wv} and O_3 concentrations for the period between May 16 (when O_3 monitoring began) and until the time we measured gas exchange. The values were then corrected for conductance of ozone molecules by dividing g_{wv} by 1.68, the ratio of diffusivity between O_3 and water vapor. O_3 concentrations in the intercellular space were assumed to be zero (Laisk et al., 1989). We used average g_{wv} in the younger age-class leaves to calculate O_3 uptake not only for these leaves, but also for older age-class leaves during their corresponding developmental stage. Our assumption was that gas exchange should not differ significantly for the same age-class leaves growing in the early and late season understanding that indeterminant shoot growth is typical for seedlings of this species. Because leaf lifespan was not recorded precisely except for the oldest leaves, COU for the younger (1st–3rd) age-classes must be used with caution.

2.6. Statistical analysis

Analysis of variance ($\alpha = 0.05$) was used for N_L, gas exchange, and individual leaf injury based on a split split-plot design with SAS GLM procedure (SAS Institute Inc., Cary, North Carolina, 1995). Water regime comprised the main plot, O_3 treatment was the subplot, and leaf age-class formed the sub subplot. Because we did not measure the exact same leaves or even the same seedlings within a block for gas exchange and leaf injury, no repeated measure analysis of variance was conducted. Instead, we treated the day of measurement as the block effect because we measured a single block each day. Family effect was not considered due to a lack of replication within each family and family effect was not significant from each age class across sampling dates. Injury data were transformed by square root of (injury + 0.5) before analysis. The relationships between any two variables were evaluated by correlation analysis with SAS.

3. Results

3.1. Gas exchange

Significant differences in A and g_{wv} were detected among O_3 treatments ($P < 0.01$) and among leaf-age classes ($P < 0.001$) (Table 1). Although the water-regime effect was not significant for A and g_{wv}, most two-term interactions associated with water, O_3 and age-class were significant.

There was a clear trend in A and g_{wv} among age-classes with significantly higher leaf gas exchange in the younger than older leaves (Fig. 2). The 1st age-class leaves were not fully developed and therefore showed lower A and g_{wv} compared to the 2nd age-class leaves. The oldest leaves within the 4th age-class showed the lowest gas exchange rates. Plants in open-plots (AA) generally had the highest rates of gas exchange (A and g_{wv}) when compared to plants grown within chambers under the non-irrigated water regime (Fig. 2). Under irrigation, plants in CF plots showed similar A and g_{wv} as plants in AA plots (Fig. 2). Because older leaves showed more injury in AA and NF plots than leaves in CF plots did, plants in CF plots showed the highest A on the oldest leaves at both water regimes, but not for g_{wv}. There was no difference in g_{wv} between CF and NF grown within the non-irrigated regime. But g_{wv} was significantly higher in CF than that in NF chambers for all age-classes within the irrigated regime.

3.2. Foliar injury

All treatment effects and their two-way interactions were significant for foliar injury ($P < 0.05$) among O_3 treatments, water regimes, and age classes (Table 1). As expected, no difference in foliar injury was found between O_3 treatments for 1st and 2nd age-class; but leaves showed more severe injury within NF and AA plots compared to those leaves grown within CF chambers for the 3rd and 4th age-classes (Fig. 2). In addition, plants grown under irrigated conditions showed similar foliar injury classes between AA and NF, but the oldest leaves of non-irrigated plants were more severely injured in the NF compared to the AA plots (Fig. 2). These data must be interpreted with caution because foliar injury was only evaluated as the percentage of affected leaf area on the selected leaves for the gas exchange measurement and therefore may not be representative for O_3-induced injury for the entire seedling.

3.3. Leaf nitrogen concentration

Leaf nitrogen concentration differed significantly among O_3 treatments ($P < 0.05$), among age-classes ($P < 0.001$), and among interactions between water regime and age-class ($P < 0.05$) (Table 1). Regardless of age-classes, N_L was significantly higher for seedlings grown within AA than for seedlings grown in either CF or NF chambers within the irrigated regime (Fig. 2). In the non-irrigated regime, N_L was similar in seedlings in AA plots and CF chambers and young leaves within AA and CF contained higher N concentrations than young leaves in NF plots.

Table 1
Degree of freedom (df), mean squares (MS), and F values (F) for net photosynthesis (A), stomatal conductance to water vapor (g_{wv}), foliar injury, and leaf nitrogen concentrations for *Prunus serotina* seedlings grown in open-top chambers and in open-plots under irrigated and non-irrigated water regimes at the Penn Nursery site in central Pennsylvania, USA.

Source of variation	df	A (μmol m^{-2} s^{-1})		g_{wv} (mol m^{-2} s^{-1})		Foliar injury		df	Leaf N	
		MS	F	MS	F	MS	F		MS	F
Block	6	228.96		0.4154		2.36		2	0.0825	
Water regime	1	0.00	0.00	0.1335	3.95	11.90	6.26*	1	0.0793	0.08
Block*Water	6	24.61		0.0338		1.90		2	0.9879	
O_3 treatment	2	92.25	9.12**	0.1895	18.52***	153.32	83.56***	2	1.6911	4.46*
Water*O_3	2	41.61	4.11*	0.0573	5.60*	2.41	1.31	2	0.2401	0.63
Block*Water*O_3	24	10.11		0.0102		1.83		8	0.3790	
Age	3	1739.16	313.25***	0.3332	102.11***	467.02	401.52***	3	35.4142	879.73***
Water*Age	3	17.44	3.14*	0.0060	1.83	5.89	5.06**	9	0.1778	4.42**
O_3*Age	6	29.60	5.33***	0.0086	2.63*	79.11	68.02***	3	0.0814	2.02
Water*O_3*Age	6	5.58	1.01	0.0016	0.50	1.89	1.62	6	0.0291	0.72
Error	612	5.55		0.0033		1.16		252	0.0403	

$^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

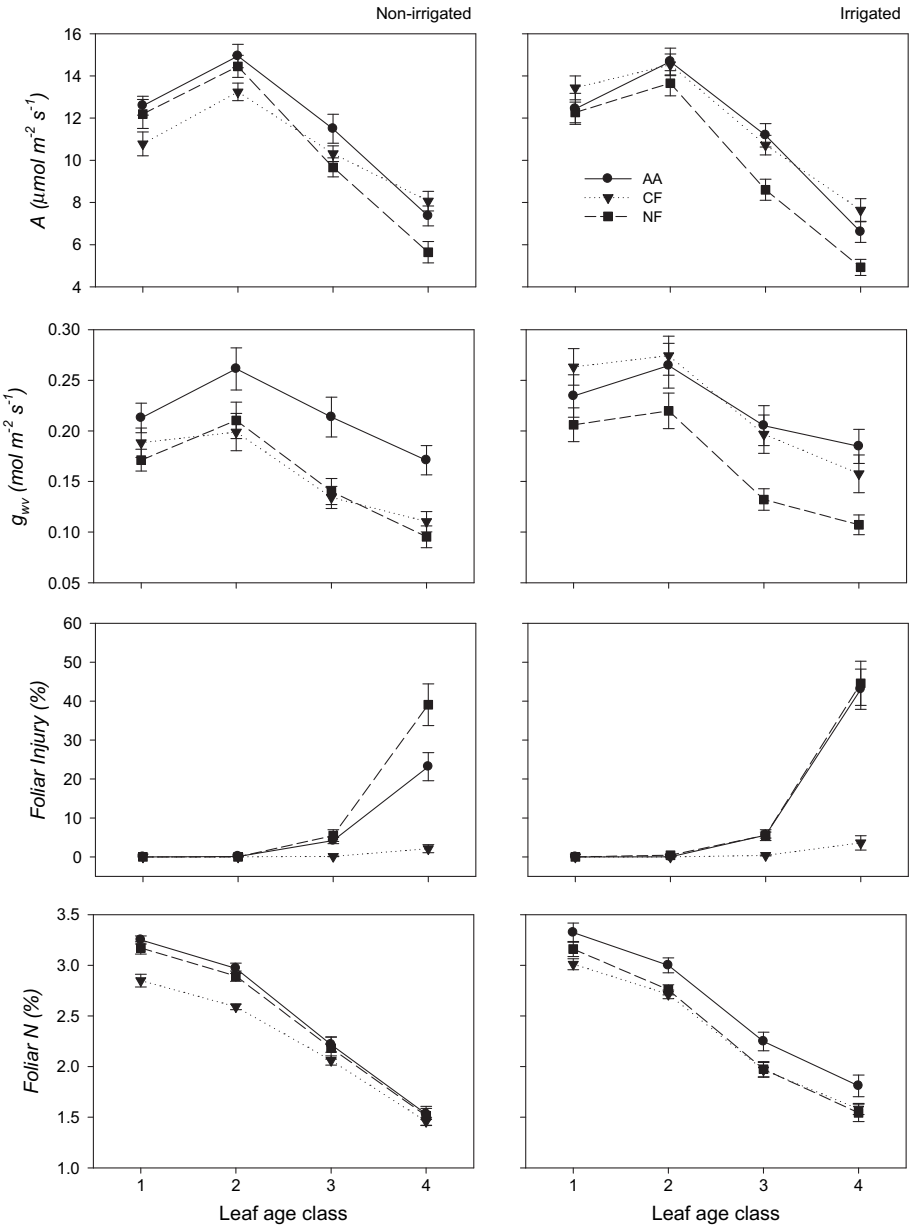

Fig. 2. Means (±se, $n = 28$) of net photosynthesis (A), stomatal conductance to water vapor (g_{wv}), foliar injury (%), and foliar nitrogen concentration measured on four age-classes of leaves from *Prunus serotina* seedlings grown in ambient plots (AA), carbon-filtered (CF), and non-filtered (NF) open-top chambers under non-irrigated and irrigated regimes at the Penn Nursery site in central Pennsylvania, USA.

3.4. Ecophysiological parameters

Maximum assimilation rate (A_{max}) differed substantially between healthy leaves and injured leaves (Fig. 3). A_{max} was 17.8 μmol m^{-2} s^{-1} for young healthy leaves and 4.59 μmol m^{-2} s^{-1} for older leaves with O_3–induced injury symptoms, respectively. Light compensation point (Γ) and dark respiration (R_d) were 58 μmol m^{-2} s^{-1} and −2.73 μmol m^{-2} s^{-1}, respectively, for the younger leaves and 48 μmol m^{-2} s^{-1} and −1.94 μmol m^{-2} s^{-1} for the older leaves, respectively. Leaf age-classes showed the same quantum yield (Φ) with 0.05 mol CO_2 (mol incident photon)$^{-1}$.

3.5. Relationships between g_{wv} and VPD, A, and N_L

To further explore the treatment effects on gas exchange, we plotted g_{wv} against vapor pressure deficit (VPD) (Fig. 4), a driving force of transpiration. We found a significant negative relationship between VPD and g_{wv} ($r^2 = 0.76$, $P < 0.001$). In addition, lower VPD was apparent for seedlings within AA plots compared to those grown within the chambers (insert of Fig. 4). As expected, N_L and A were significantly correlated ($r^2 = 0.62$, $P < 0.001$). Omitting the data from non-fully developed leaves would result in an even stronger correlation ($r^2 = 0.77$, $P < 0.001$) (Fig. 5).

3.6. O_3 concentrations and uptake

Ambient O_3 exposures were moderately high throughout most of the growing season. By the time when we measured gas exchange in these seedlings, SUM0 values for the oldest age-class leaves were ca. 72.6 ppm h within AA plots, and 36.8 ppm h and 66.3 ppm h in CF and NF chambers, respectively. SUM60 values were ca. 19.0, 1.2, and 14.1 ppm h in AA, CF, and NF, respectively

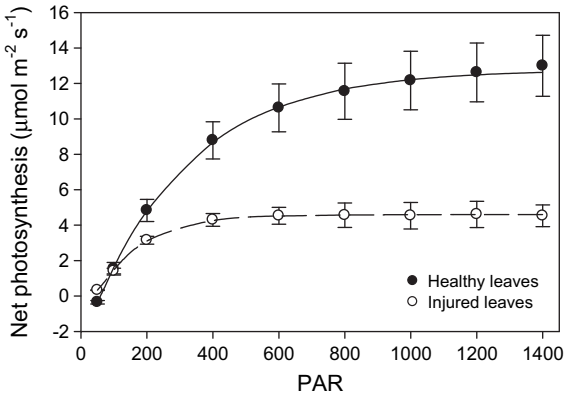

Fig. 3. Net photosynthesis (A) (μ ± 1se, $n = 4$) at different photosynthetically active radiation (PAR) for four leaves without O_3-induced injury symptoms (healthy) and four leaves with 12–25% leaf area affected with O_3-induced symptoms. Lines are modeled with Hanson et al. (1987)'s equation (both $r^2 \approx 1.00$, $P < 0.0001$).

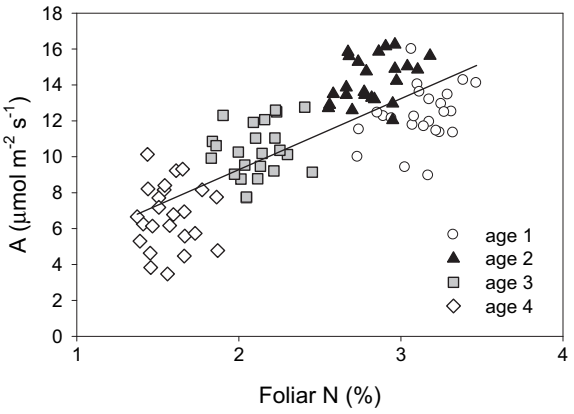

Fig. 5. Relationship ($r^2 = 0.62$, $P < 0.0001$) between net photosynthesis (A) and foliar N concentration for *Prunus serotina* seedlings sorted by water regimes and O_3 treatments at the Penn Nursery site in central Pennsylvania, USA.

(Fig. 6). Total COU for the oldest age-class leaves was 20.8, 13.3, and 8.2 mmol m^{-2} in AA, NF, and CF, respectively, within non-irrigated regime and 21.5, 14.2, and 11.6 mmol m^{-2} in AA, NF, and CF, respectively, within irrigated regime (Fig. 7). Unfortunately, we did not record the exact lifespan for the other age-class leaves and therefore, we are not able to precisely obtain COU for the other age classes. However, because some oldest leaves in CF chambers showed O_3-induced visible injury already at the point when we started measuring leaf gas exchange, we may consider the estimated COU from these leaves as the minimum COU for O_3-induced symptoms, i.e. 8.2 mmol m^{-2} within the non-irrigated regime and 11.6 mmol m^{-2} within the irrigated regime (Fig. 7). Based on our estimates of leaf lifespan, we found the maximum COU for 1st and 2nd age class were never above the minimum COU within their respective water treatments. The 3rd age-class leaves showed variation in COU among treatments (Fig. 7); leaf COU reached the minimum COU in AA plots (14.0 mmol m^{-2}) and NF chambers (8.4 mmol m^{-2}) within the non-irrigated treatment and only in AA plots (14.3 mmol m^{-2}) within the irrigated treatment. However, leaf COU in CF was only 4.5 mmol m^{-2} within the non-irrigated treatment and 8.7 mmol m^{-2} and 6.5 mmol m^{-2} in NF and CF, respectively, within the irrigated treatment.

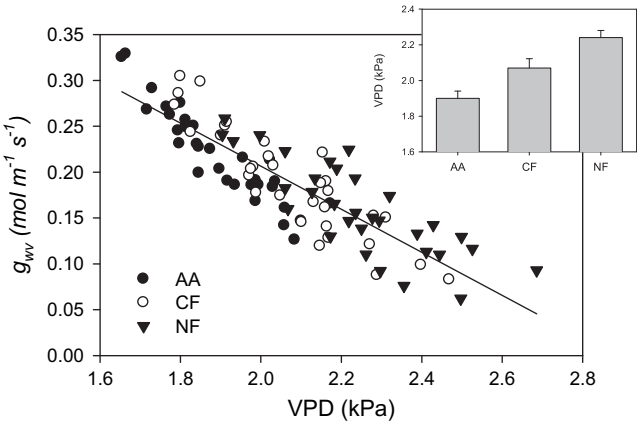

Fig. 4. Relationship ($r^2 = 0.76$, $P < 0.0001$) between stomatal conductance to water vapor (g_{wv}) and vapor pressure deficit (VPD) for *Prunus serotina* seedlings sorted by water regimes and age classes at the Penn Nursery site in central Pennsylvania, USA. Inset figure shows mean (+1 SE) of VPD in O_3 treatments for those days when gas exchange was measured.

4. Discussion

The results showed that leaf gas exchange and N_L did not differ significantly between the two water regimes, but foliar injury did ($P = 0.046$) (Table 1). The lack of difference was clearly related to high precipitation events during the early season of 1998 (Fig. 1). Seedlings in the non-irrigated plots were never under severe water stress, which is also supported by similar soil water potential values between the two regimes from May to August in 1998 (Schaub et al., 2003). Furthermore, this was also supported by the lack of

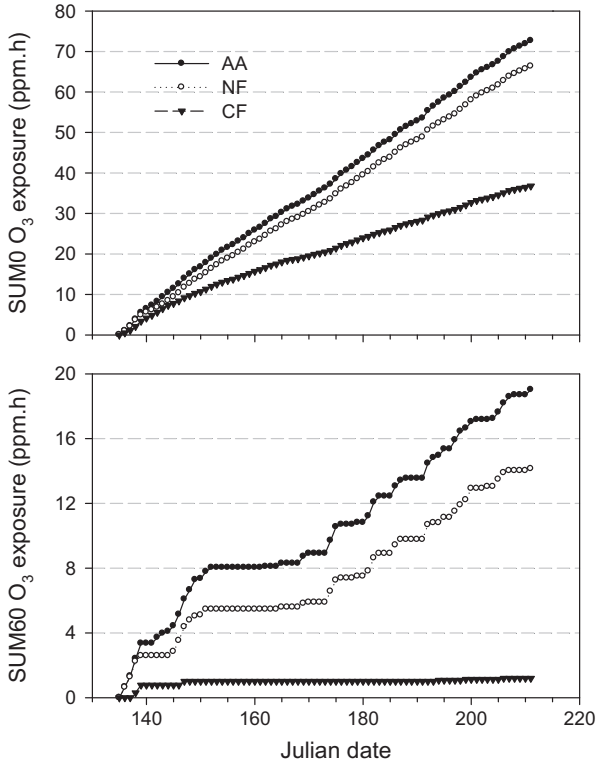

Fig. 6. Cumulative O_3 exposures of SUM0 and SUM60 during daylight hours (07:00–19:00 EST) for the oldest age-class leaves grown in ambient plots (AA), non-filtered (NF), and carbon-filtered (CF) chambers at Penn Nursery site in central Pennsylvania, USA.

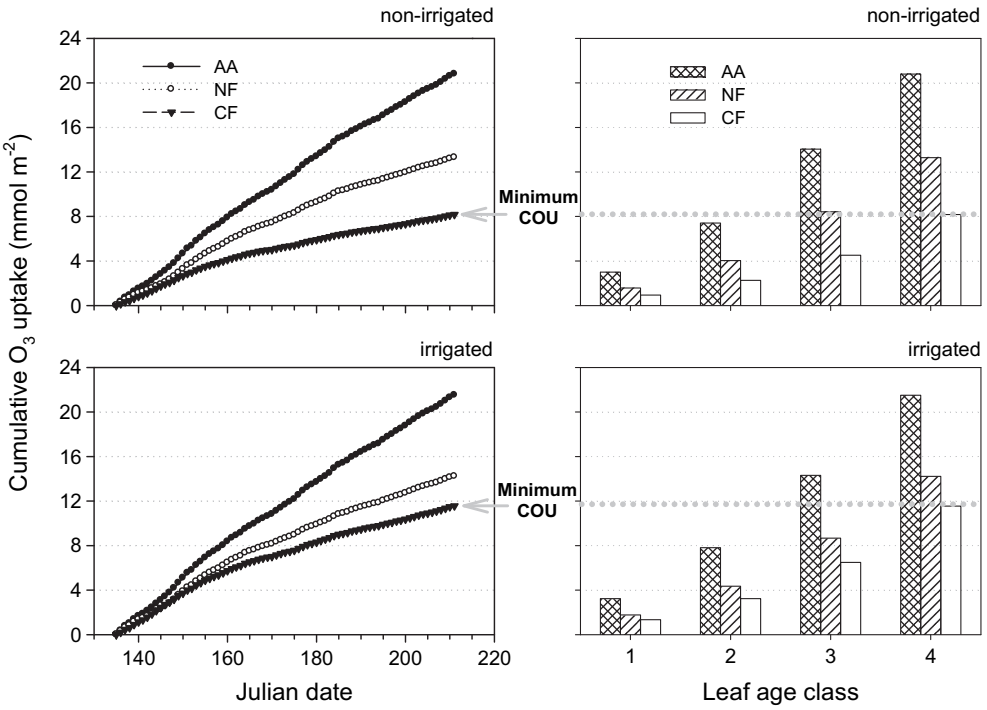

Fig. 7. Cumulative O_3 uptake based on SUM0 during daylight hours (07:00–19:00 EST) and stomatal conductance during the development of the oldest age-class leaves (left panel) and COU for all age-class leaves (right panel) in ambient plots (AA), non-filtered (NF), and carbon-filtered (CF) open-top chambers at two water regimes at the Penn Nursery site in central Pennsylvania, USA. The arrows and thicker dotted lines refer to the minimum COU for visible O_3-induced symptoms.

differences in COU based on stomatal conductance and O_3 exposure (Fig. 7). Therefore, response of black cherry seedlings to O_3 interacted with soil water availability (Hildebrand et al., 1996; Chappelka and Samuelson, 1998; Grulke et al., 2003; Schaub et al., 2005; Ribas et al., 2005; McLaughlin et al., 2007).

Although the water regime effect was not significant, we found that O_3 as well as interactions between water regime and O_3 in A and g_{wv} were significant at $\alpha = 0.05$. This is because seedlings in CF chambers varied significantly in A and g_{wv} among interactions between age and water regime relative to seedlings in AA plots and NF chambers (Fig. 2). Regardless of leaf age-classes, seedlings in AA plots showed higher A and g_{wv} compared to seedlings in NF chambers, which may be due to the differences in VPD between AA plots and open-top chambers (Fig. 4). Apparently, the microclimate was altered inside the open-top chambers by increased daytime air temperature, reduced photon flux density, and continuous air movement (Fuhrer, 1994; Heagle et al., 1979; Nussbaum and Fuhrer, 2000; Paoletti, 2007). This chamber effect must be considered if plant growth and metabolic processes are compared between chambers and ambient plots in future studies (Manning, 2005; Karnosky et al., 2007).

As expected, leaf age had a significant effect on A (Larcher, 1995). A and g_{wv} increased up to full leaf expansion for the 2nd age-class and then decreased with increasing leaf age (Fig. 2). The 1st age-class leaves were most likely undergoing cell division and elongation. Therefore, they might lack fully developed photosynthetic enzymes (Reich, 1984; Greitner et al., 1994) and the associated maturation of the light-harvesting apparatus (Hanson et al., 1988). When leaves become older (3rd and 4th age-classes), gas exchange rates in general significantly decrease (Mebrahtu and Hanover, 1991; Whitehead and Singh, 1995; Pearson and Brooks, 1995). However, high O_3 exposures tended to accelerate this declining process by damaging leaf cells, especially for photosynthetic rate (Fig. 2). This finding is consistent with previous studies (Greitner et al., 1994; Haikio et al., 2007; Zhang et al., 2001). The results are also supported by the ecophysiological parameters derived from light-response curves; the young and healthy leaves had much higher A_{max}, light compensation point, and dark respiration than the older, symptomatic leaves (Fig. 3). Yet, quantum yield, which measures light use efficiency, was relatively consistent not only between the young and old leaves but also between sensitive and non-sensitive genotypes, as found by Yun (2007).

The ability of O_3 to accelerate leaf aging has already been documented for various tree species (Baker and Allen, 1996; Pell et al., 1999; Matyssek and Sandermann, 2003). Nitrogen resorption from senescing leaves is presumed to be a more efficient nutrient-use because the resorption prior to the onset of senescence is important for N recycling (Chapin, 1980; Vitousek, 1982). In the current study, there was significantly lower N_L found in older leaves compared to younger leaves (Fig. 2), suggesting that N resorption occurred. However, because of the lack of interaction between leaf age and O_3 treatment in foliar nitrogen concentration (Table 1), it appears that lower N_L in the older leaves was probably due to retranslocation exclusively caused by natural senescence processes. In their review article, Matyssek and Sandermann (2003) argued that O_3-induced senescence differed from natural senescence in controlling leaf degradation. Nitrogen concentrations may stay rather stable in O_3-injured leaves before premature abscission sets in. Udding et al. (2005) found that ozone injury impaired the autumnal resorption of N_L in *Betula pendula* Roth.

The higher gas exchange rate in the 3rd and 4th age-class leaves in CF compared to the leaves grown in NF is due to very mild foliar O_3-induced injury occurring on seedlings within the CF chambers (Fig. 2). Yet, it was unclear why new leaves differed in A and g_{wv} in CF when compared to NF chambers, considering the higher nitrogen content in new leaves grown under NF conditions compared to leaves in CF chambers (Fig. 2). There is a possibility that the response of leaf gas exchange to O_3 may occur before or without the onset of visible symptoms (Reich and Amundson, 1985; Reich et al., 1986) so that seedlings within the NF chambers closed

their stomata avoiding O_3 uptake. Nevertheless, seedlings in AA plots showed very high A and g_{wv} (Fig. 2), confirming the above hypothesis. Another possibility may be a higher VPD for seedlings grown within NF compared to those within CF chambers, which was higher than in AA plots (Fuhrer, 1994). Therefore, the strong correlation between VPD and g_{wv} (Fig. 4) might explain the differing gas exchange rates for the three O_3 treatments.

A possible lack of stomatal response to O_3 (Paoletti and Grulke, 2005) and tradeoffs between growth and defense (Matyssek and Sandermann, 2003) make it difficult to explain foliar O_3-induced injury based on stomatal conductance and/or photosynthetic rate. For example, seedlings in AA plots showed a higher g_{wv}, and presumably higher O_3-induced injury (Fig. 2) compared to seedlings grown in the chambers (Fig. 2). However, O_3-induced injury on the oldest leaves was lower in AA compared to NF for the non-irrigated regimes. In addition, seedlings in AA generally showed higher A compared to seedlings grown under NF or CF conditions (Fig. 2). Similar issues became apparent for seedlings in NF, which showed higher O_3-induced injury on two older-age-class leaves, but lower A and g_{wv} than seedlings in grown the other plots (Fig. 2). Apparently, gas exchange may not be sufficient to explain onset and development of O_3-induced injury without considering cumulative O_3 uptake.

To relate O_3 uptake (Fig. 7) and foliar injury (Fig. 2), we found a minimum COU for visible O_3-induced symptoms at 8.2 mmol m^{-2} under lower soil water availability and 11.6 mmol m^{-2} under higher soil water availability (Fig. 7), similar to results of 11 mmol m^{-2} when this species was grown under high ozone exposures in the sub-alpine region of the Swiss Alps (Zhang et al., 2001). At both water regimes, COU of the 1st and 2nd age-class leaves never reached either 8.2 mmol m^{-2} or 11.6 mmol m^{-2} (Fig. 7) and did not show symptoms (Fig. 2). The COU of the 3rd age-class leaves was higher than 11.6 mmol m^{-2} within irrigated AA plots and 8.2 mmol m^{-2} within non-irrigated AA plots as well as NF chambers (Fig. 7) leading to visible symptom development (Fig. 2). No symptoms were apparent on seedlings from the 3rd age-class leaves grown in CF chambers because the minimum COU was not reached. However, we don't have an explanation for the 3rd age-class leaves grown under irrigated and NF conditions showing mild O_3-induced injury (Fig. 2) with a COU of only 8.7 mmol m^{-2}. Fourth age-class leaves reached their respective minimum COUs (Fig. 7) no matter they grew in AA plots or NF and CF, under irrigated or non-irrigated conditions. As a result, O_3-induced injury developed on the all leaves (Fig. 2). This analysis seems to explain the differences in O_3-induced injury development among the treatments (Fig. 2) except for the higher injury classes in NF compared to AA on the oldest leaves at the non-irrigated regime, which could be due to the lack of photosynthate for producing defense compounds (Andersen, 2003; Wieser and Matyssek, 2007). Thus, stomatal conductance and O_3 concentrations can be used to predict the development of O_3-induced injury if we have a better understanding of the inter-relationships between these variables and the phenology of O_3 sensitive plant species.

5. Conclusions

Tropospheric ozone continues to cause foliar visible injury on black cherry seedlings and thus may continue to be a threat to forest health. Whether soil water availability has an influence on the plant response to O_3 depends on the level of water stress. Although under same ozone exposures, a close negative correlation between increasing N_L and reduced foliar O_3-induced injury could be demonstrated, cause-effect mechanisms cannot be completely established without considering interfering intercorrelation among leaf age, gas exchange, senescence, N_L, and N resorption. These findings demonstrate that mechanistic O_3 uptake, although complex and correlated with phenological factors such as leaf age and N_L, can be used for effectively assessing a biologically relevant O_3 risk for forest trees.

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