

Whole-Tree Water Use Efficiency Is Decreased by Ambient Ozone and Not Affected by O₃-Induced Stomatal Sluggishness

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Abstract

Steady-state and dynamic gas exchange responses to ozone visible injury were investigated in an ozone-sensitive poplar clone under field conditions. The results were translated into whole tree water loss and carbon assimilation by comparing trees exposed to ambient ozone and trees treated with the ozone-protectant ethylenediurea (EDU). Steady-state stomatal conductance and photosynthesis linearly decreased with increasing ozone visible injury. Dynamic responses simulated by severing of a leaf revealed that stomatal sluggishness increased until a threshold of 5% injury and was then fairly constant. Sluggishness resulted from longer time to respond to the closing signal and slower rate of closing. Changes in photosynthesis were driven by the dynamics of stomata. Whole-tree carbon assimilation and water loss were lower in trees exposed to ambient O₃ than in trees protected by EDU, both under steady-state and dynamic conditions. Although stomatal sluggishness is expected to increase water loss, lower stomatal conductance and premature leaf shedding of injured leaves aggravated O₃ effects on whole tree carbon gain, while compensating for water loss. On average, WUE of trees exposed to ambient ozone was 2–4% lower than that of EDU-protected control trees in September and 6–8% lower in October.

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Introduction

Tropospheric ozone (O₃) is an important phytotoxic air pollutant and is also recognized as a significant greenhouse gas [1]. Tropospheric O₃ level has been continuously increasing since the first direct measurements in 1874 and its atmospheric concentration is now twice or more than in the pre-industrial age in the northern hemisphere [2–4]. Phytotoxic nature of O₃ has been well known for decades [5–12]. Ozone concentrations recorded in rural areas are higher than those in the city [13] and thus O₃ is now considered as the air pollutant with the highest damage potential to forests [14].

As the penetration of O₃ through the cuticle can be considered as negligible [15], uptake through the stomata is a crucial factor for assessing the adverse effect of O₃ on plants [16–20]. However, effects of O₃ on stomatal responses are not straightforward, as both reductions and sluggish responses have been reported [21,22]. Reductions of stomatal conductance occur when measurements are carried out under steady-state conditions [23]. Sluggishness has been reported during dynamic stomatal responses to fluctuating photosynthetic photon flux density (PPFD) [22,24–27], vapor pressure deficit (VPD) [27], and severe water stress imposed by severing a leaf [26,28–30]. Sluggish stomatal control over transpiration may increase water loss. Plants live in a fluctuating environment. A fast gas exchange response to rapid changes in the environmental stimuli is the key for successful plant

adaptation and competition [31]. Because of climate change, forest ability of water control and carbon sequestration under O₃ pollution is of rising importance [14].

Scalar and conceptual uncertainties still limit the current understanding of the basic physiological mechanisms that underline responses of forests to O₃ [32]. The scalar uncertainties are due to transfer of results from seedlings in controlled environments to mature trees in the field, while the conceptual uncertainties are due to contrasting results about whole-tree water use responses to ambient O₃ [32–35]. In contrast, there is a general agreement about O₃ exposure as a factor of reduced tree carbon sequestration and biomass [36], although the results usually come from steady-state measurements of photosynthesis.

Ozone visible injury of leaves may be used as a clear and easily quantifiable proxy of O₃ foliar damage and is the only method to assess O₃ damage in the field [37]. Ozone visible injury has been investigated in many European and North American tree and herbaceous species, and partly validated under controlled conditions [38,39]. There are few reports of relationship between stomatal conductance and O₃ visible injury. After onset of O₃ visible injury, significant reductions in steady-state leaf gas exchange were recorded for tree species in chamber experiments [40–42]. Omasa et al. (1981) did not report any correlation between visible injury and stomatal O₃ uptake in a leaf [43]. Dynamic stomatal response was slower in injured leaves (20%

injury) compared to control leaves (0% injury) for manna ash (*Fraxinus ornus* L.) [28].

Our main objectives were to improve our knowledge of steady-state and dynamic stomatal response to O₃ visible injury in adult trees in the field, and to assess whole-tree water loss and carbon assimilation under ambient O₃ impacts. Measurements were carried out in an O₃-sensitive poplar clone (Oxford, *Populus maximoviczii* Henry × *berolinensis* Dippel) [44]. The amount of leaf injury per tree was experimentally manipulated by applying the O₃-protectant ethylenediurea (EDU, N-[2-(2-oxo-1-imidazolidinyl)ethyl]-N'-phenylurea). EDU *per se* does not affect gas exchange [45] and has been widely used to prevent O₃ visible injury and determine O₃ effects in many plant species [39,45–47].

Materials and Methods

Experimental Site and Plant Material

The study was carried out in an experimental field site located in central Italy (Antella: 43°44' N, 11°16' E, 50 m a.s.l., 14.7°C as mean annual temperature and 1233 mm as total annual precipitation in 2010). Forty root cuttings of the O₃-sensitive Oxford clone were planted in two lines in 2007. Every week over the growing seasons 2008–2010, each tree was irrigated with 1 to 2 L of water (WAT, control line) or 450 ppm EDU solution (EDU, treated line), according to the successful application of EDU as soil drench to adult trees [48]. In 2010, the mean tree height was 2.9 m, and the mean stem diameter at breast height was 19 mm. Soil moisture was measured in the root layer (30 cm depth) by EC5 sensors equipped with an EM5b data logger (Decagon Devices, Pullman WA, USA). On average, soil moisture was 21.1±0.2% during the gas exchange measurements (September–October) and 24.5±0.1% during the growing season (April to October). The values were between field capacity (25.5%) and wilting point (17.5%) for this type of soil, i.e. sandy clay loam. Air temperature, relative humidity and precipitation were recorded by a 110-WS-16 modular weather station (NovaLynx corp., Auburn CA, USA). Average vapor pressure deficit during daylight hours and total precipitation were 1.02 kPa and 197 mm in September to October and 1.42 kPa and 625 mm from April to October, respectively. Ozone concentrations were continuously recorded at canopy height (2.0 m) by an O₃ monitor (Mod. 202, 2B Technologies, Boulder CO, USA). The AOT40 value (accumulated exposure above a threshold concentration of 40 ppb during daylight hours) during the growing season (April to October) was 25.8 ppm·h and the maximum hourly O₃ concentration reached 118 ppb.

Assessment of Ozone Visible Injury

Ozone visible injury occurred as dark stippling on the upper leaf surface since early September 2010. The injury was identified as O₃-like because it was missing in shaded leaves and more severe in older than in younger leaves [38]. The symptoms were similar to those caused by ambient O₃ in *Populus nigra* [42]. In September (22th to 28th) and October (23th to 28th), all 9502 leaves from five trees per treatment (WAT and EDU) were counted and assigned to 5%-step injury classes by the same two observers. Photoguides quantifying visible injury (0–100%) by image analysis processing were used [38,39]. Pest, pathogen and mechanical injury occurred in both EDU and WAT trees and was assessed to be <5% of total leaves. Leaves for measurements of gas exchange showed O₃ visible injury only and were evaluated on a 1%-step basis.

Measurement of Steady-state and Dynamic Gas Exchange

Fully expanded sun leaves (medium size) with visible injury from 0% to 50% at set positions from the terminal shoot (5th to 16th) of WAT trees were measured in clear sky days of September and October 2010 between 10:00 and 15:00 CET. Preliminary measurements did not show significant differences in gas exchange of healthy leaves, i.e. without visible ozone injury, at those set positions. Gas exchange was measured with a portable infra-red gas analyzer (CIRAS-2 PPSystems, Herts, UK), equipped with a 2.5 cm² leaf cuvette which controlled leaf temperature (24°C), leaf-to-air vapour pressure deficit (1.0 kPa), saturating light (1800 μmol m⁻² s⁻¹) and CO₂ concentration (375 ppm). Steady-state light-saturated photosynthesis (A_{max}), stomatal conductance to water vapor (g_s) and transpiration were measured in 41 leaves from WAT trees.

Dynamic measurements were carried out for 21 leaves from WAT trees. When both g_s and A_{max} reached equilibrium under constant light at 1800 μmol m⁻² s⁻¹, the leaf petiole was severed with a sharp scalpel, similar to the methodology in Paoletti (2005) [26]. The data were logged at 1 min intervals for 30 min after severing. As the absolute value of g_s and A_{max} varied among individual leaves, relative g_s and A_{max} were expressed as a percentage of the average of the last 5 points at equilibrium, i.e., just before leaf severing. The following parameters were estimated based on fittings of two linear lines to minimize the root mean square error between measured and predicted values for g_s or A (Figure 1A): range of relative g_s decrease at 30 min after severing, Δg_s; time to start g_s decrease, T_{resp}(g_s); rate of g_s decrease at 30 min from severing, Slope(g_s) = Δg_s/(30–T_{resp}(g_s)); range of relative A_{max} decrease at 30 min after severing, ΔA; time to start A_{max} decrease, T_{resp}(A); rate of A_{max} decrease at 30 min from severing, Slope(A) = ΔA/(30–T_{resp}(A)).

After measurements, the leaf area was measured by means of a leaf area meter (AM300, ADC, Herts, UK) for assessing a relationship between leaf size and the variation of g_s in single leaves. We hypothesized that the water content of a leaf may depend on leaf size and affect g_s response.

Tree Level Modeling

To assess effects of O₃ visible injury on leaf gas exchange at tree level, we constructed a simple model to scale up from single-leaf steady-state and dynamic gas exchange. The model was applied to the five trees per treatment (WAT and EDU) whose leaves were counted and assigned to a 5%-step visible injury class. Steady-state leaf water loss and photosynthesis at tree level, i.e. W_{loss}: mol H₂O tree⁻¹ s⁻¹, and A_{tree}: μmol CO₂ tree⁻¹ s⁻¹, were estimated as follows:

$$W_{loss} = \sum (T_{r_inj} \cdot LA \cdot N_{inj}) \quad (1)$$

$$A_{tree} = \sum (A_{max_inj} \cdot LA \cdot N_{inj}) \quad (2)$$

where T_{r_inj} and A_{max_inj} are transpiration rate (mmol m⁻² s⁻¹) and photosynthesis (μmol m⁻² s⁻¹), respectively, at 1800 μmol m⁻² s⁻¹ constant light for leaves showing O₃ visible injury. N_{inj} is the number of leaves in each 5%-step injury class. LA is the average leaf area per leaf (0.003 m² leaf⁻¹), calculated from subsamples of 30 randomly collected leaves per tree.

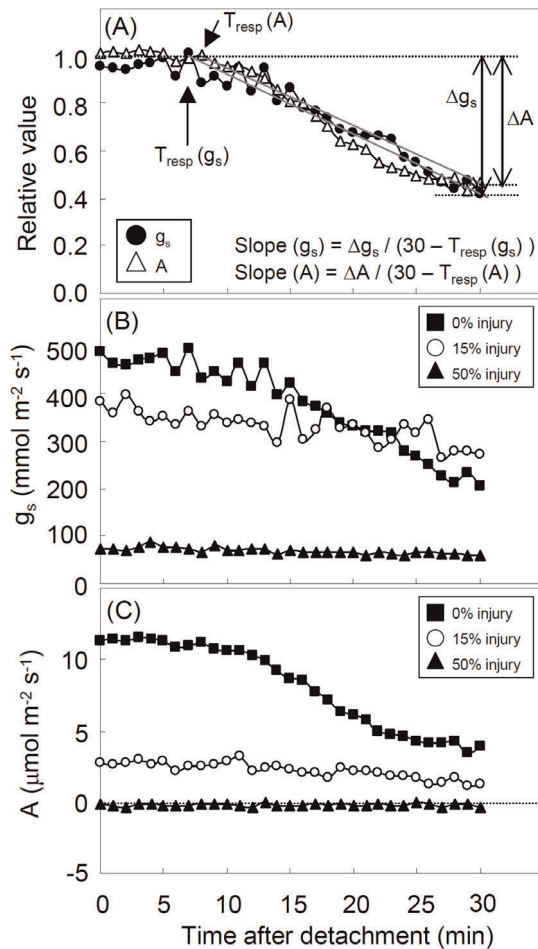


Figure 1. Examples of dynamic response of g_s and A_{max} after detachment of the leaf (A: calculation of the dynamic parameters in a leaf with 0% visible injury, B: time courses of absolute values in g_s , C: time courses of absolute values in A). Δg_s and ΔA show the range of g_s and A_{max} variation, respectively, over 30 min from the leaf severing. $T_{resp}(g_s)$ and $T_{resp}(A)$ show the time to start decrease of g_s and A_{max} , respectively. Slope(g_s) and Slope(A) show the rate of decrease for g_s and A_{max} , respectively, over 30 min. doi:10.1371/journal.pone.0039270.g001

Whole-tree leaf water loss and carbon assimilation under the severe water stress simulated by severing a leaf (W_{loss_st} : $\text{mol H}_2\text{O tree}^{-1} \text{s}^{-1}$, and A_{tree_st} : $\mu\text{mol CO}_2 \text{ tree}^{-1} \text{s}^{-1}$) were estimated by the following equations:

$$W_{loss_st} = \sum (\overline{T_{r_inj}} \cdot LA \cdot N_{inj}) \quad (3)$$

$$A_{tree_st} = \sum (\overline{A_{max_inj}} \cdot LA \cdot N_{inj}) \quad (4)$$

where $\overline{T_{r_inj}}$ is the average transpiration rate ($\text{mmol m}^{-2} \text{s}^{-1}$) and $\overline{A_{max_inj}}$ is the average photosynthesis ($\mu\text{mol m}^{-2} \text{s}^{-1}$) at $1800 \mu\text{mol m}^{-2} \text{s}^{-1}$ constant light during the 30 min after severing a leaf with O_3 visible injury.

Statistical Analysis

Effects of O_3 visible injury on steady-state leaf gas exchange and dynamic responses after severing a leaf were tested with a regression analysis. Correlation between variables of dynamic stomatal response was tested. Two-way analysis of variance (ANOVA) was used to assess the effects of measuring month and EDU treatments on number of leaves, ozone visible injury and gas exchange parameters at whole tree level. Differences among means were tested by Tukey HSD test. Percents were arcsine square root transformed prior to analysis. Data were checked for normal distribution (Kolmogorov-Smirnov D test) and homogeneity of variance (Levene's test). Results were considered significant at $p < 0.05$. All statistical analyses were performed with STATISTICA software (6.0, StatSoft Inc., Tulsa, OK, USA).

Results

Number of Leaves and Ozone Visible Injury

In September, EDU trees had 83% more leaves per tree than WAT trees (Figure 2A). In October, leaf abscission had progressed faster in WAT trees (-36% of leaves relative to September) than in EDU trees (-15%), resulting in EDU trees showing significantly more leaves ($+144\%$) than WAT trees. The percentage of injured leaves ($>5\%$ of visible injury) was significantly higher in WAT trees than in EDU trees in both September and October (Figure 2B). In October, the percentage of injured leaves was 3.13 and 7 times higher than in September in WAT and EDU trees, respectively.

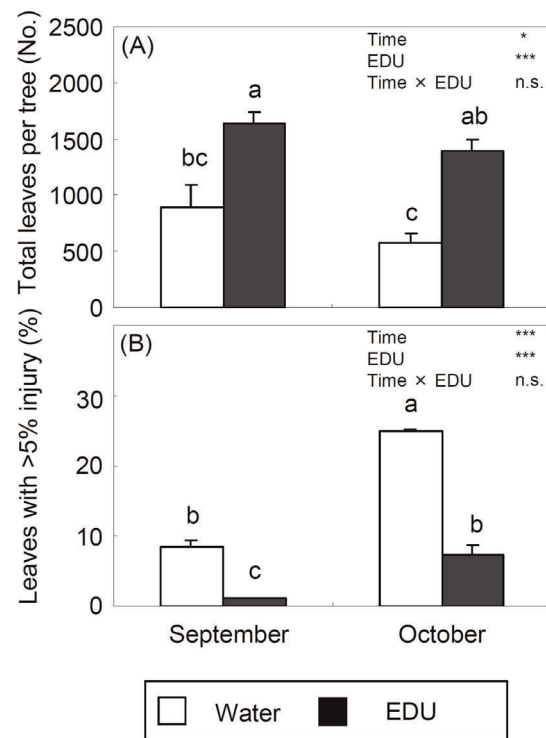


Figure 2. Total number of leaves (A) and percentage of ozone injured leaves (more than 5% of injured surface) (B) per tree (+SE) (WAT: water treated plants; EDU: EDU treated plants). * and *** denote significance at the 5% and 0.1% level, respectively; n.s. indicates no significance. Different letters above the bars indicate significant differences among bars (Tukey HSD test, $P < 0.05$, $n = 5$ trees). doi:10.1371/journal.pone.0039270.g002

Steady-state g_s and A_{\max}

Steady-state leaf gas exchange for both g_s and A_{\max} decreased with increasing O_3 visible injury (Figure 1, 3). In healthy leaves (0% injury), g_s was 400 to 800 $\text{mmol m}^{-2} \text{s}^{-1}$ whereas it was less than 100 $\text{mmol m}^{-2} \text{s}^{-1}$ in leaves with 50% injury (Figure 3A). Leaves with higher injury (>50% injury) were tested but did not show a measurable g_s . In control leaves, A_{\max} was 5 to 15 $\mu\text{mol m}^{-2} \text{s}^{-1}$, and dropped to around 0 $\mu\text{mol m}^{-2} \text{s}^{-1}$ in leaves with more than 35% injury (Figure 3B).

Variation of g_s and A_{\max} after Detachment of a Leaf

After detachment of a leaf, two phases of gas exchange response were observed (Figure 1A): no response until T_{resp} and then a linear decrease. The magnitude of change in g_s at 30 min after severing a leaf (Δg_s) did not depend on leaf size (data not shown; $R^2 = 0.02$, $p = 0.537$) and thus on the total water content of a leaf.

Figure 4 shows the relationships between O_3 visible injury and dynamic response of g_s and A_{\max} . Δg_s showed a non-linear response to O_3 visible injury (Figure 4A). It sharply decreased from 45–60% in healthy leaves (0% injury) to 15–30% in leaves with >5% visible injury. $\text{Slope}(g_s)$ sharply decreased from 2.5–3.2% min^{-1} in healthy leaves (0% injury) to 0.8–1.8% min^{-1} in leaves with >5% visible injury, and did not vary in leaves with 5–50% of injury (Figure 4B). The response time to start stomatal closing ($T_{\text{resp}}(g_s)$) was linearly correlated to O_3 visible injury (Figure 4C). $T_{\text{resp}}(g_s)$ increased from about 10 min in healthy leaves to >13 min in leaves with >20% injury. The magnitude of decrease in photosynthetic rate (ΔA) sharply decreased from about 55% in healthy leaves to about 25% in leaves with >5% visible injury (Figure 4D). $\text{Slope}(A)$ sharply decreased from about 3.3% min^{-1} in healthy leaves to about 1.6% min^{-1} in leaves with >5% visible injury (Figure 4E). There was a linear relationship between the response time to start decrease of photosynthesis ($T_{\text{resp}}(A)$) and O_3 visible injury (Figure 4F). $T_{\text{resp}}(A)$ increased from 5–13 min in

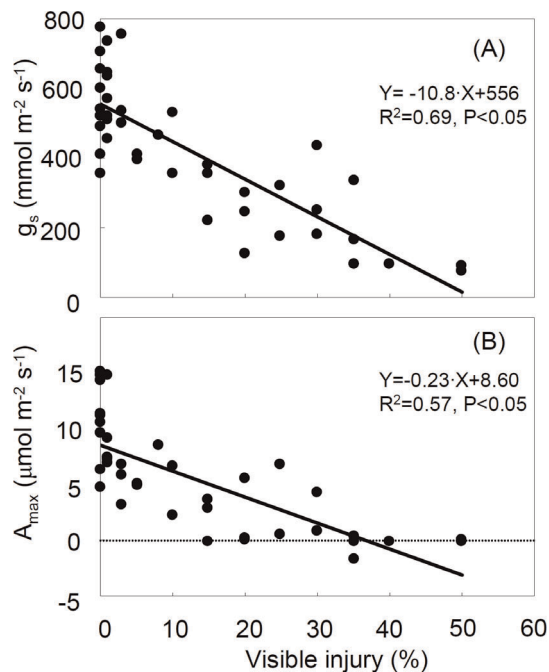


Figure 3. Relationships between steady-state leaf gas exchange (A: stomatal conductance (g_s), B: light-saturated photosynthesis (A_{\max})) and visible ozone foliar injury.
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healthy leaves to 25 min in a leaf with 50% injury. Table 1 shows correlation between the A_{\max} and g_s variables obtained from dynamic response after severing of a leaf. The magnitude of change in A_{\max} (ΔA) increased with increasing Δg_s . The rate of reduction in A_{\max} , i.e. $\text{Slope}(A)$, was positively correlated with $\text{Slope}(g_s)$. The response times to start decrease of A_{\max} and g_s , i.e., T_{resp} , were not significantly correlated, although they showed a statistical tendency to a positive correlation ($p < 0.1$).

Carbon Assimilation and Water Loss at Tree Level

In September, A_{tree} and W_{loss} were significantly lower in WAT trees, being half of the values in EDU trees (Figure 5A–B). In October, the difference between WAT and EDU trees became even larger. Whole-tree water use efficiency ($A_{\text{tree}}/W_{\text{loss}}$) at steady-state was significantly higher in EDU trees than in WAT trees both in September and October (Figure 5C). WUE decreased over time, but the decrease was larger in WAT (–6%) than in EDU trees (–2%), resulting in a significant Time x EDU interaction. Both in September and October, both $A_{\text{tree-st}}$ and $W_{\text{loss-st}}$, i.e. whole-tree carbon assimilation and water loss under the simulated severe water stress, were significantly lower in WAT trees (Figure 6A–B), similarly to the results from steady-state measurements (Figure 5A–B). Whole-tree instantaneous water use efficiency, expressed as $A_{\text{tree-st}}/W_{\text{loss-st}}$, was significantly higher in EDU trees than in WAT trees both in September and October (Figure 6C). Again, the decrease of WUE_{st} over time was larger in WAT (–8%) than in EDU trees (–3%).

Discussion

According to previous reports in different species [28,40–42], the steady-state measurements indicated that g_s and A_{\max} linearly decreased with increasing leaf visible injury in the O_3 -sensitive Oxford clone (Figure 3). A_{\max} dropped to around 0 $\mu\text{mol m}^{-2} \text{s}^{-1}$ in leaves with more than 35% injury and g_s was not measurable in leaves with more than 50% injury. In a previous field study, leaves of manna ash with 20% visible injury showed a 33% reduction in g_s and A_{\max} relative to healthy leaves (measurement in September) [28]. The result of the present study showed a larger reduction in g_s (about 39%) and A_{\max} (about 54%) of 20% injured leaves, suggesting effects of O_3 visible injury on gas exchange are species-specific. Paoletti et al. (2009a) suggested that the modifications of stomatal conductance in O_3 injured leaves were driven by the structural alterations found in the mesophyll rather than by structural changes in stomata or other epidermal cells [28]. Omasa et al. (1981) suggested that stomatal opening in leaves with O_3 visible injury varied with changes in the pressure balance between guard cells and epidermal cells caused by the water-soaking of epidermal cells [43]. The most likely changes, however, are due to photosynthetic impairment [21,49].

When analyzing dynamic g_s response to severing of a leaf, stomata of injured leaves were shown to be slower than those of healthy leaves in responding to the closing signal ($T_{\text{resp}}(g_s)$) and in the rate of closing ($\text{Slope}(g_s)$) (Figure 4B–C). These combined effects translated in a lower ability of injured leaves to close stomata, i.e. in a lower Δg_s than healthy leaves, resulting in a sluggish stomatal control over water loss. In a previous study, Paoletti et al. (2009a) also reported a slower response of stomata to severing in leaves of manna ash with O_3 visible injury [28], even though only leaves with 0% and 20% injury were compared. Here, we compared leaves with a range of O_3 visible injury, i.e. from no injury (control) until a measurable g_s was recorded (~50% injury) and showed that Δg_s decreased sharply above 5% injury and did not change any more (Figure 4A).

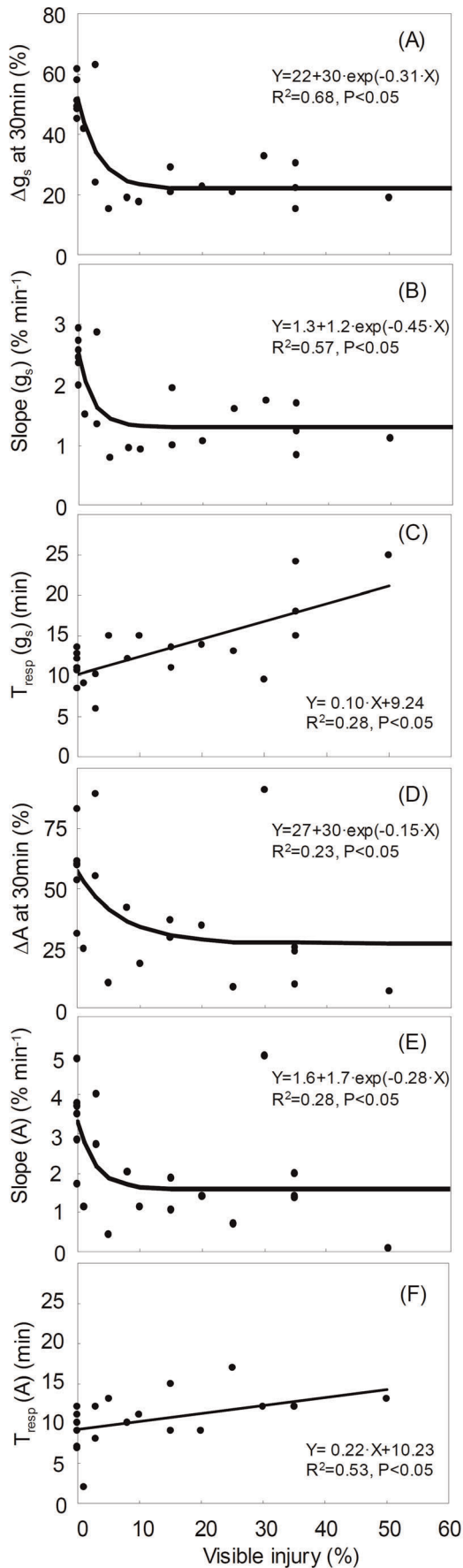


Figure 4. Relationships between visible ozone foliar injury and dynamic response of stomatal conductance (g_s) and photosynthesis (A_{max}) over 30 min after leaf severing (A: Δg_s at 30 min; B: Slope(g_s); C: $T_{resp}(g_s)$; D: ΔA ; E: Slope(A); F: $T_{resp}(A)$). doi:10.1371/journal.pone.0039270.g004

Literature results highlight several mechanisms by which O_3 may induce sluggishness. Omasa (1990) reported a slight increase in permeability of epidermal cell membranes and alteration of the osmotic pressure after O_3 exposure, that may modulate a balance in turgor between guard and subsidiary cells [50]. Vahisalu et al. (2010) found that Ca^{2+} -dependent signaling and O_3 -induced stomatal movements were independent, and highlighted a temporary desensitization of the guard cells due to blocking of the K^+ channels [51]. Another cause of sluggishness may be O_3 -induced lower rates of transpiration in which leaves take longer to perceive the same change in water status following petiole excision [26,28–30] or light variation [22,26]. All the above mechanisms, however, cannot explain the non-linear response of Δg_s to visible injury observed in the present study. Ozone may also delay stomatal responses by stimulating ethylene production and reducing stomatal sensitivity to ABA [52]. Ethylene production is known to increase with increasing O_3 visible injury [53,54]. In tomato plants, concentration of ACC (1-aminocyclopropane-1-carboxylic acid), a precursor of ethylene, increased when visible injury reached 5% and remained constant until the maximum injury recorded in the experiment, i.e. 35% [55]. A sharp rise of ethylene emission as soon as visible injury reaches 5% and a constant emission over this threshold would explain why Δg_s decreased sharply above 5% injury and did not change any more when injury was >5% (Figure 4A). Tuomainen et al. (1997) also showed that ethylene emission from detached leaves was enhanced fourfold in ozone-treated plants, while no changes were observed in control leaves that were similarly cut at the petiole [55].

Sluggish A_{max} responses with increasing O_3 visible injury were also found in the measurements of dynamic leaf gas exchange (Figure 4D–F). The response of A_{max} was similar to that of g_s after severing a leaf (Figure 1), i.e. no response until T_{resp} and then a linear decrease during stomatal closure. Although the response time to start reduction of A_{max} was not significantly correlated with the response time to closing stomata, the magnitude and rate of reduction in A_{max} were linearly correlated to those of stomatal closure (Table 1). Heber et al. (1986) showed that photosynthetic rate decreased following stomatal closure after severing of a leaf [56]. Slightly shorter $T_{resp}(g_s)$ than $T_{resp}(A)$ confirmed that the reduction of A_{max} was mediated by the response of g_s . The slower reduction of A_{max} in injured leaves than in healthy leaves would increase carbon assimilation under water stress conditions and may be interpreted as a feedback mechanism to maximize

Table 1. Correlation between A_{max} vs. g_s variables obtained during the dynamic response to severing of a leaf (Δ : magnitude of change in A_{max} and g_s over 30 min from the leaf severing; T_{resp} : time to start decrease in A_{max} and g_s after severing a leaf; Slope: rate of A_{max} and g_s decrease).

Parameter	Pearson coefficient	Level of significance
Δ	0.626	0.002**
Slope	0.622	0.003**
T_{resp}	0.371	0.098 n.s.

**denotes the significance at 1% level; n.s. indicates no significant correlation. doi:10.1371/journal.pone.0039270.t001

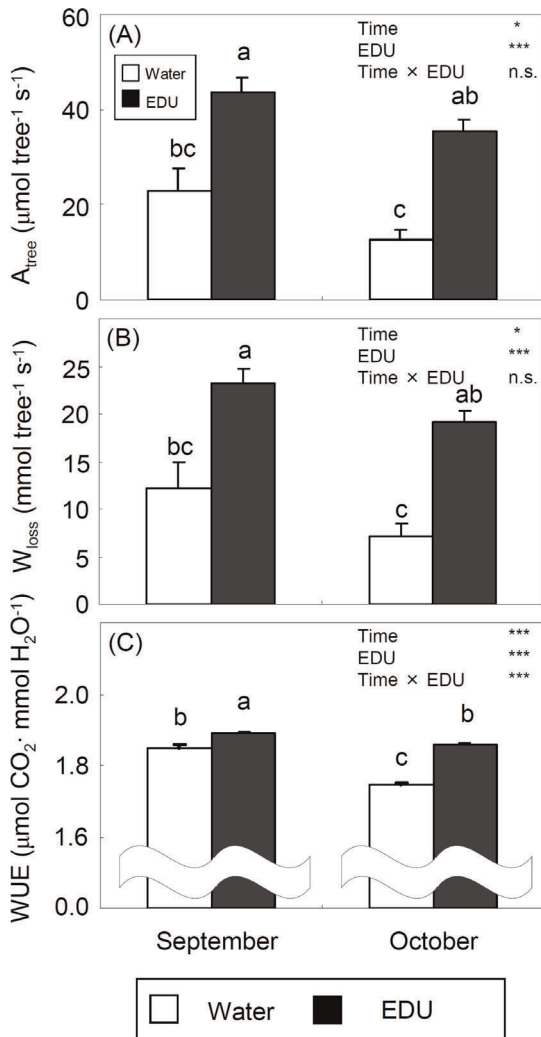


Figure 5. Estimated steady-state carbon assimilation (A: A_{tree}), water loss (B: W_{loss}) and instantaneous water use efficiency expressed as A_{tree}/W_{loss} (C: WUE) at tree level (+SE) (WAT: water treated plants; EDU: EDU treated plants). * and * denote significance at the 5% and 0.1% level, respectively; n.s. indicates no significance. Different letters above the bars indicate significant differences among bars (Tukey HSD test, $P < 0.05$, $n = 5$ trees). doi:10.1371/journal.pone.0039270.g005**

photosynthesis under stress. However, severe O_3 visible injury (>35%) shifted carbon sink to source because A_{max} was $< 0 \mu\text{mol m}^{-2} \text{s}^{-1}$ (Figure 3B).

At whole-tree level, the total carbon assimilation (A_{tree}) and water loss (W_{loss}) assessed under steady-state conditions were significantly lower in WAT trees exposed to ambient ozone than in EDU-protected trees in both September and October (Figure 5A–B). Such O_3 -induced reduction of photosynthesis and water loss was in agreement with meta-analysis results [36]. Dynamic and steady-state whole-tree WUEs showed a similar seasonal trend. WUE was significantly higher in EDU trees than in WAT trees, both in September and October and both when assessed under steady-state and dynamic conditions (Figure 5C and 6C). On average, WUE of trees exposed to ambient ozone was 2–4% lower than that of EDU-protected control trees in September and 6–8% lower in October. The decrease of tree-level WUE over time, in fact, was larger in WAT than in EDU trees, confirming the

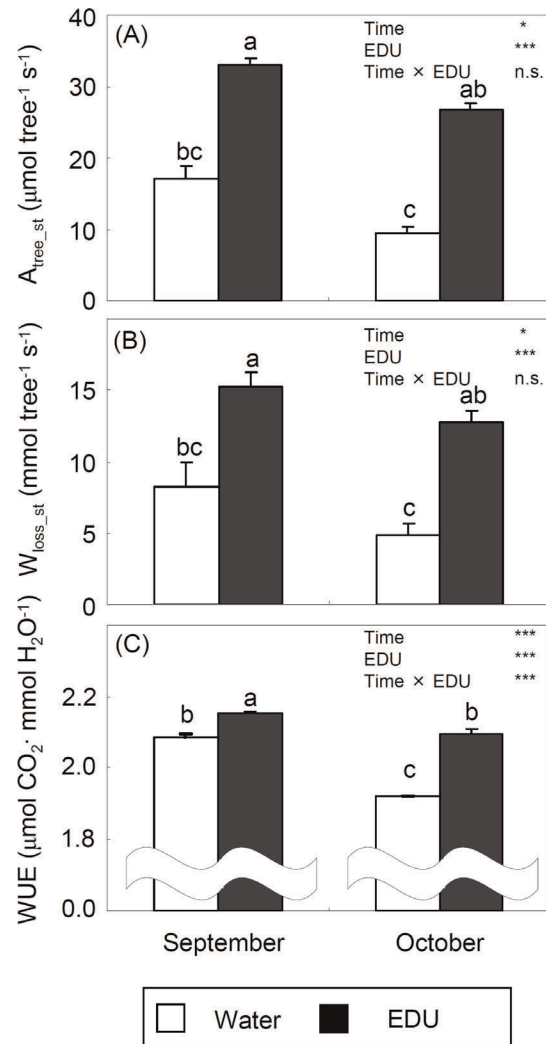


Figure 6. Estimated carbon assimilation (A: A_{tree_st}), water loss (B: W_{loss_st}) and instantaneous water use efficiency expressed as A_{tree_st}/W_{loss_st} (C: WUE_{st}) at tree level under severe water stress imposed by severing a leaf (+SE) (WAT: water treated plants; EDU: EDU treated plants). * and * denote significance at the 5% and 0.1% level, respectively; n.s. indicates no significance. Different letters above the bars indicate significant differences among bars (Tukey HSD test, $P < 0.05$, $n = 5$ trees). doi:10.1371/journal.pone.0039270.g006**

frequently reported decrease in leaf-level WUE in O_3 -exposed plants [33] and O_3 -injured leaves [41]. Also whole-tree dynamic carbon assimilation (A_{tree_st}) and water loss (W_{loss_st}) were significantly lower in WAT trees than in EDU-protected trees (Figure 6A–B). In contrast, ozone-induced stomatal sluggishness would be expected to increase whole-tree water loss. This response, however, was balanced by lower gas exchange (Figure 3) and premature shedding of injured leaves. After the onset of O_3 visible injury in early September, ozone visible injury increased quickly (Figure 2B). In parallel, leaf abscission also progressed (Figure 2A), so that both whole-tree water loss and carbon assimilation were reduced. However, McLaughlin et al. (2007) reported that ambient O_3 spikes significantly increased water loss of trees, as assessed from sap-flow measurements, suggesting that ozone-induced aberrations in the stomatal dynamics may differ depending on the species and the environmental conditions [32].

Conclusions

One of the topical subjects in the assessment of O₃ risk to forests is scaling up from leaf level to the stand and landscape level [4]. Further improvement of our understanding about stomatal responses to ambient O₃ can be regarded as an essential factor in modelling and predicting forest responses to both O₃ and climate [21]. Occurrence of O₃ visible injury resulted in loss of stomatal control for water loss, but was compensated by lower stomatal conductance and premature leaf shedding. The resulting decline in whole tree ability of transpiring and sequestering atmospheric carbon is a significant effect of ambient ozone pollution.

Stomata play a crucial role in regulating plant gas exchange with the atmosphere, including O₃ uptake [16–20]. Surface O₃ concentrations are continuously increasing [4]. The climate

change brings about the risk of drought and flooding [1]. The results of this study contribute new knowledge about water control and carbon sequestration of trees under ambient O₃ exposure and suggest that the effects of O₃-induced stomatal sluggishness on the whole-tree carbon and water balance are negligible.

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Author Contributions

Conceived and designed the experiments: EP KO. Performed the experiments: YH EP. Analyzed the data: YH EP. Contributed reagents/materials/analysis tools: EP. Wrote the paper: YH EP.

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