Flux-Based Ozone Risk Assessment for a Plant Injury Index (PII) in Three European Cool-Temperate Deciduous Tree Species

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Received: 30 November 2019; Accepted: 7 January 2020; Published: 9 January 2020

Abstract: This study investigated visible foliar ozone (O$_3$) injury in three deciduous tree species with different growth patterns (indeterminate, Alnus glutinosa (L.) Gaertn.; intermediate, Sorbus aucuparia L.; and determinate, Vaccinium myrtillus L.) from May to August 2018. Ozone effects on the timing of injury onset and a plant injury index (PII) were investigated using two O$_3$ indices, i.e., AOT40 (accumulative O$_3$ exposure over 40 ppb during daylight hours) and POD$_Y$ (phytotoxic O$_3$ dose above a flux threshold of $Y$ nmol m$^{-2}$ s$^{-1}$). A new parameterization for POD$_Y$ estimation was developed for each species. Measurements were carried out in an O$_3$ free-air controlled exposure (FACE) experiment with three levels of O$_3$ treatment (ambient, AA; 1.5 $\times$ AA; and 2.0 $\times$ AA). Injury onset was found in May at 2.0 $\times$ AA in all three species and the timing of the onset was determined by the amount of stomatal O$_3$ uptake. It required 4.0 mmol m$^{-2}$ POD$_7$ and 5.5 to 9.0 ppm$h$ AOT40. As a result, A. glutinosa with high stomatal conductance ($g_s$) showed the earliest emergence of O$_3$ visible injury among the three species. After the onset, O$_3$ visible injury expanded to the plant level as confirmed by increased PII values. In A. glutinosa with indeterminate growth pattern, a new leaf formation alleviated the expansion of O$_3$ visible injury at the plant level. V. myrtillus showed a dramatic increase of PII from June to July due to higher sensitivity to O$_3$ in its flowering and fruiting stage. Ozone impacts on PII were better explained by the flux-based index, POD$_Y$, as compared with the exposure-based index, AOT40. The critical levels (CLs) corresponding to PII = 5 were 8.1 mmol m$^{-2}$ POD$_7$ in A. glutinosa, 22 mmol m$^{-2}$ POD$_3$ in S. aucuparia, and 5.8 mmol m$^{-2}$ POD$_1$ in V. myrtillus. The results highlight that the CLs for PII are species-specific. Establishing species-specific O$_3$ flux-effect relationships should be key for a quantitative O$_3$ risk assessment.

Keywords: visible foliar ozone injury; European alder; mountain ash; European blueberry; stomatal ozone flux; free-air ozone exposure

1. Introduction

Tropospheric ozone (O$_3$) is one of the major concerns for forest health due to its phytotoxicity [1]. Despite the fact that peak O$_3$ concentrations have tended to decrease in the eastern part of United States and some European countries due to precursor emission controls [2], the global background O$_3$ concentration still remains high enough to cause negative impacts on tree physiology [3].
Visible foliar injury by O$_3$ (O$_3$ visible injury) is the first unequivocal visually detectable sign of O$_3$ damage and indicates an impairment of leaf physiological functions [4]. Hoshika et al., 2012a [5] reported that the percent of surface injury was negatively correlated with leaf gas exchange rate, highlighting a reduced photosynthesis and loss of stomatal control in poplar leaves with more than 5% injury. Ozone visible injury has been broadly investigated in native and exotic trees, shrubs, and herbs in Asia, Europe, and North America, and partly validated under controlled conditions [6–8].

An exposure-based index such as AOT40 (accumulated exposure over a threshold of 40 ppb) is used to assess O$_3$ risks to European forest trees [9,10]. Previous studies have reported an AOT40-based assessment of the first symptom onset of O$_3$ visible injury in field [11,12] or open-top chambers [13,14]. Those studies suggested that an O$_3$ critical level (CL) by 5 to 10 ppm h AOT40 could protect the sensitive tree species from O$_3$ visible injury. However, it has been recognized that O$_3$ damage depends on stomatal O$_3$ uptake rather than only O$_3$ exposure [15]. To improve our quantitative assessment of O$_3$ effects on trees, a stomatal O$_3$ flux-based index such as POD$_Y$ (phytotoxic ozone dose above a flux threshold of Y nmol m$^{-2}$ s$^{-1}$) has been the focus. POD$_Y$ is estimated using the deposition of ozone and stomatal exchange model (DO3SE) [16]. Sicard et al., 2016 [17] estimated POD$_Y$ using the DO3SE model and analyzed field observation data in Southeastern France and Northwestern Italy. They proposed the stomatalflux-based standard to assess O$_3$ visible injury for two deciduous species (*Fagus sylvatica* and *Fraxinus excelsior*) and two conifer species (*Pinus cembra* and *P. halepensis*) as representative O$_3$ sensitive species (approximately 20 nmol m$^{-2}$ s$^{-1}$ of POD$_Y$ corresponded to 5% injury). Many symptomatic species have been recorded in field monitoring campaigns [8,18,19]. Paoletti et al., 2019 [8] recently found O$_3$ visible injury in 23 tree species across forest sites in France, Italy, and Romania. Nevertheless, knowledge is still limited on species-specific model parameters to calculate stomatal O$_3$ uptake for establishing the O$_3$ flux-effect relationship in most symptomatic tree species.

The species-specific tree response to O$_3$ can be affected by the growth pattern (i.e., indeterminate or determinate) [20]. In elevated O$_3$, the tree species with indeterminate pattern (e.g., poplar) can initiate new leaf formation to replace damaged older leaves [21]. This response can limit the development of O$_3$ visible injury at the plant level for those species. In this study, we selected three European cool-temperate deciduous tree species with different growth patterns, i.e., *Alnus glutinosa* (L.) Gaertn. (indeterminate) [22], *Sorbus aucuparia* L. (intermediate) [23], and *Vaccinium myrtillus* L. (determinate) [24]. These three species have often shown O$_3$ visible injury in field forest sites [8]. The aim of this study was to achieve the species-specific parameterization of the DO3SE model in these three tree species, examining both the onset date of O$_3$ visible injury and its expansion at the whole plant level using a plant injury index (PII) [4] in an O$_3$ free-air controlled exposure (O$_3$ FACE) experiment. The PII can be more closely related to the plant physiological status and especially whole plant carbon loss than to first symptom onset [14]. Three hypotheses were tested as follows: (i) Are species with higher stomatal O$_3$ uptake more sensitive to O$_3$? (ii) Does the tree growth pattern affect the expansion of O$_3$ visible injury at plant level? and (iii) Is the flux-based approach better than the exposure-based one to explain the PII?

2. Materials and Methods

2.1. Experimental Site and Plant Material

Measurements were conducted at an O$_3$ FACE facility located in Sesto Fiorentino, in central Italy (43° 48’ 59” N, 11° 12’ 01” E, 55 m a.s.l.). The details of the system are described in Paoletti et al., 2017 [25]. Five-year old saplings of *A. glutinosa* and *S. aucuparia*, and three-year old saplings of *V. myrtillus* were obtained from a nearby nursery in December 2017. Plants were transplanted into plastic pots (50 L for *A. glutinosa* and *S. aucuparia* and 25 L for *V. myrtillus*) containing a mixture of sand:peat:soil = 1:1:1 (v:v:v). In 2018, plants were exposed to the following three levels of O$_3$ concentration: Ambient air (AA), 1.5 times ambient O$_3$ concentration (1.5 × AA), and twice ambient O$_3$ concentration (2.0 × AA). Three replicated blocks (5 m × 5 m × 2 m) were set to each O$_3$ treatment
(n = 3) with three (A. glutinosa and S. aucuparia) or six (V. myrtillus) plants (total 27 plants for A. glutinosa and S. aucuparia, and 54 plants for V. myrtillus). Ozone concentrations in each treatment were recorded continuously by an O₃ monitor (Mod. 202, 2B Technologies, Boulder, CO, USA). All plants were irrigated to keep field capacity at 1- to 3-day intervals to prevent water stress. We monitored the light intensity, relative humidity, air temperature, precipitation, and wind speed above the O₃ FACE facility (2.5 m height) using a WatchDog meteorological station (Model 2000, Spectrum Technologies, Inc., Aurora, IL, USA).

2.2. Assessment of Ozone Visible Injury

The onset of O₃ visible injury was assessed every 2 to 3 days in May 2018. After we found the first symptom in any O₃ treatment, the percentage of symptomatic leaves per plant (LA) and the percentage injured area in the symptomatic leaves (AA) were scored (A. glutinosa on 31 May, 21 June, 10 and 21 July, and 8 and 20 August; S. aucuparia on 31 May, 21 June, 10 July, and 8 and 20 August; V. myrtillus on 31 May, 21 June, 4, and 11 and 19 July) with a ×10 hand lens and the help of photoguides (Innes et al. 2001 and Paoletti et al. 2009). All attached leaves were scored and counted by the same two observers. To assess the whole plant injury, a PII was calculated combining the two parameters, PII = (LA × AA)/100 [4].

2.3. Modeling of Stomatal Conductance

Leaf gas exchange was measured in fully expanded sun-exposed leaves (1 to 3 plants per replicated plot per each O₃ treatment) using a portable infrared gas analyzer (CIRAS-2 PP Systems, Herts, UK). Measurements were made on the days with clear sky in the morning (8 h to 10 h), afternoon (13 h to 15 h) and evening (16 h to 19 h) from May to October 2018. Natural illumination was used for the measurement. The CO₂ concentration in the chamber (Ca) was set to 400 ppm. The temperature and relative humidity in the chamber were adjusted manually to the ambient condition. Pooled data (210 for A. glutinosa, 217 for S. aucuparia, and 216 for V. myrtillus) were used to estimate the parameters of the DO3SE model [16], as follows:

\[ g_s = g_{\text{max}} \times f_{\text{phen}} \times f_{\text{light}} \times \left\{ f_{\text{min}} \times f_{\text{VPD}} \times f_{\text{SWC}} \right\} \]  

(1)

where \( g_{\text{max}} \) is the maximum stomatal conductance, i.e., mmol O₃ m⁻² projected leaf area (PLA) s⁻¹. The other functions are all expressed as relative terms and are scaled from 0 to 1. The model accounts for the minimum stomatal conductance (\( f_{\text{min}} \)) and the variation in \( g_s \) according to phenology (\( f_{\text{phen}} \)), photosynthetic photon flux density (PPFD) (\( f_{\text{light}} \)), temperature (\( f_{\text{temp}} \)), vapor pressure deficit (VPD) (\( f_{\text{VPD}} \)), and soil water content (\( f_{\text{SWC}} \)). The \( f_{\text{SWC}} \) was not applied in this study (\( f_{\text{SWC}} = 1 \)) because the soil moisture was equivalent to field capacity. The \( g_{\text{max}} \) and \( f_{\text{min}} \) values were set as 95th and 5th percentile values recorded in the experiment. Parameterizations of other functions were carried out using a boundary line analysis [26,27]. Further details on \( f_{\text{phen}}, f_{\text{light}}, f_{\text{temp}}, \) and \( f_{\text{VPD}} \) calculations are provided in CLRTAP (2017) [16].

2.4. Calculation of Ozone Indices

AOT40 was calculated by using hourly O₃ concentrations during daylight hours (short wave radiation > 50 W m⁻²) according to CLRTAP (2017) [16]. It is given by:

\[ \text{AOT40} = \sum_{i=1}^{n} \max\left( [O_3]_i - 40, 0 \right) \]  

(2)

where \([O_3]_i\) is the ith measured hourly O₃ concentration (ppb) with \( i \) equal to 1 . . . \( n \) in the integral and \( n \) is the number of hours included in the calculation period.
Stomatal O₃ uptake ($F_{st}$, nmol m⁻² s⁻¹) was calculated as:

$$F_{st} = \left[ O_3 \right] \times g_s \times \frac{r_c}{r_b + r_c}$$

(3)

where $r_c$ is the leaf surface resistance ($= 1/(g_s + g_{ext})$, s m⁻¹), $g_{ext}$ is the external leaf or cuticular conductance (s m⁻¹) [16], and $r_b$ is the leaf boundary layer resistance, given as:

$$r_b = 1.3 \times 150 \times (L_d / u)^{0.5}$$

(4)

where $u$ is wind speed (m s⁻¹) and $L_d$ is the species-specific leaf dimension (A. glutinosa 0.07 m, S. aucuparia 0.04 m, and V. myrtillus 0.04 m obtained as averaged value of 3 to 5 leaves of two plants in each block in each O₃ treatment) [16].

$POD_Y$ (nmol m⁻²) was estimated from hourly data as:

$$POD_Y = \sum_{i=1}^{n} \max \{ F_{st,i} - Y, 0 \}$$

(5)

where $F_{st,i}$ is the $i$th hourly stomatal O₃ uptake (nmol m⁻² s⁻¹) and $n$ is the number of hours included in the calculation period. $Y$ is a species-specific threshold of stomatal O₃ uptake (nmol m⁻² s⁻¹).

Exposure- or flux-based dose-response functions were determined from a linear regression between PII and AOT40 or $POD_Y$ over a threshold of $Y$ ($Y$ from 0 to 10, with an increment of 0.5 nmol m⁻² s⁻¹). Two criteria were applied to select the best dose-response function which included: (1) the confidence interval (C.I.) must include $Y$-intercept $= 0$, and (2) among the functions meeting criterion 1, the equation with the highest $R^2$ value was chosen. CLs were calculated as the level when PII reaches 5. In fact, a significant decline of physiological performance was found in leaves with more than 5 of PII [14].

2.5. Data Analysis

Statistical analyses were performed using SPSS (20.0, SPSS, Chicago, IL, USA). To assess the effects of O₃ on the number of attached leaves, a two-way analysis of variance (ANOVA) was applied. Data were checked for normal distribution and homogeneity of variance (Levene’s test). Since the PII data were not normally distributed, the Kruskal–Wallis analysis of variance was applied to examine the effect of O₃. The relationships between PII and O₃ indices were fitted using a simple linear regression. Results were considered significant at $p < 0.05$.

3. Results

3.1. Ozone Concentration and Meteorological Factors

Daily mean PPFD, air temperature, wind speed, and relative humidity (mean ± S.E.) during the exposure period (May to October 2018) were 527 ± 13 µmol m⁻² s⁻¹, 22.8 ± 0.3 °C, 0.3 ± 0.0 m s⁻¹, and 55.6% ± 0.8%, respectively (Figure 1). Total rainfall was 136 mm. Daily mean O₃ concentration (mean ± S.E.) in AA, 1.5 × AA, and 2.0 × AA was 35.2 ± 0.7 ppb, 53.1 ± 1.1 ppb, and 65.2 ± 1.4 ppb, respectively.
A. glutinosa Y-

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S. aucuparia not found in

The number of attached leaves in

× 2.0

Ozone-induced increases of PII were also found in

S. aucuparia

A. glutinosa

increased number of symptomatic leaves and injured leaf area (data not shown). Ozone significantly

POD

leaves. The AOT40 corresponding to the onset of O

dark reddish stippling on the upper leaf surface (Figure 2) and was more severe in older than in younger

S. aucuparia

3.2. Ozone Visible Injury

The first O3 visible injuries were observed in 2.0 × AA on 18 May for A. glutinosa, on 21 May for S. aucuparia, and on 26 May for V. myrtillus (Table 1). Ozone visible injury occurred as dark or reddish stippling on the upper leaf surface (Figure 2) and was more severe in older than in younger leaves. The AOT40 corresponding to the onset of O3 visible injury was 5.6 to 8.9 ppm·h, and the POD0 corresponding to the occurrence of injury was approximately 4.0 mmol m\(^{-2}\) regardless of tree species. After the onset of O3 visible injury, the PII value increased in all species (Figure 3) due to an increased number of symptomatic leaves and injured leaf area (data not shown). Ozone significantly increased PII values in A. glutinosa on 21 June (1.5 × AA, +224% and 2.0 × AA, +656%), 8 August (1.5 × AA, +400% and 2.0 × AA, +1163%), and 20 August (1.5 × AA, +440% and 2.0 × AA, +2169%). Ozone-induced increases of PII were also found in S. aucuparia on 20 August (1.5 × AA, +101% and 2.0 × AA, +182%) and V. myrtillus on 19 July (1.5 × AA, +65% and 2.0 × AA, +172%). Ozone stimulated the number of attached leaves in A. glutinosa on 20 August (Figure 4); however, such an increase was not found in S. aucuparia and V. myrtillus.

Table 1. The date of first symptom onset of O3 visible foliar injury and corresponding ozone indices (POD0, phytotoxic ozone dose above a flux threshold of 0 nmol m\(^{-2}\) s\(^{-1}\) and AOT40, accumulated exposure over a threshold of 40 ppb) for Alnus glutinosa, Sorbus aucuparia, and Vaccinium myrtillus in 2.0 × AA (twice ambient O3 concentration).

| Species        | A. glutinosa | S. aucuparia | V. myrtillus |
|----------------|--------------|--------------|--------------|
| Onset date     | 18 May       | 21 May       | 26 May       |
| POD0 (mmol m\(^{-2}\)) | 3.2          | 4.3          | 3.5          |
| AOT40 (ppm·h)  | 5.6          | 6.3          | 8.9          |
The response of the parameter $f_{\text{phen}}$ to PPFD ($f_{\text{light}}$) indicated that $V. \text{myrtillus}$ had a higher $a$ value (0.0104) relative to $A. \text{glutinosa}$ (0.0024) and $S. \text{aucuparia}$ (0.0043). The optimal temperature for stomatal opening was 20 to 30 °C in all species. A VPD higher than around 1 kPa induced stomatal closure regardless of the species. The $f_{\text{phen}}$ values peaked from June to August in all three species. Estimated $g_s$ values were in good agreement with the measured values as confirmed by the coefficient of determination ($R^2 = 0.46$ to 0.61) and root mean square error (RMSE = 31 to 57 mmol O$_3$ m$^{-2}$ s$^{-1}$) (Figure S1).
Table 2. Summary of parameters in the DOSE stomatal conductance model for *Alnus glutinosa*, *Sorbus aucuparia*, and *Vaccinium myrtillus*.

| Parameter | Unit | *A. glutinosa* | *S. aucuparia* | *V. myrtillus* |
|-----------|------|----------------|----------------|----------------|
| $g_{\text{max}}$ | (mmol O$_3$ m$^{-2}$ PLA s$^{-1}$) | 300 | 240 | 140 |
| $f_{\text{min}}$ | (fraction) | 0.13 | 0.17 | 0.17 |
| $A_{\text{start}}$ | (day of year) | 121 | 121 | 121 |
| $A_{\text{end}}$ | (day of year) | 304 | 304 | 304 |
| $f_{\text{phen}}$ | (days) | 50 | 50 | 50 |
| $f_{\text{phen},a}$ | (fraction) | 0.3 | 0.3 | 0.3 |
| $f_{\text{phen},b}$ | (days) | 50 | 50 | 50 |
| $f_{\text{phen},c}$ | (fraction) | 0.3 | 0.3 | 0.3 |
| $f_{\text{phen},d}$ | (fraction) | 0.3 | 0.3 | 0.3 |
| $f_{\text{light}}$ | (constant) | 0.0024 | 0.0043 | 0.0104 |
| $f_{\text{temp}}$ | $T_{\text{opt}}$ | 29 | 23 | 20 |
| | $T_{\text{min}}$ | 5 | 0 | 5 |
| | $T_{\text{max}}$ | 40 | 40 | 40 |
| $f_{\text{VPD}}$ | VPD$_{\text{max}}$ | 1.8 | 1.2 | 1.2 |
| | VPD$_{\text{min}}$ | 5.7 | 7.0 | 4.7 |

$g_{\text{max}}$ is the maximum stomatal conductance; $f_{\text{min}}$ is a fraction of minimum stomatal conductance to $g_{\text{max}}$; $f_{\text{phen}}$ is the variation of stomatal conductance with season; $f_{\text{light}}, f_{\text{temp}},$ and $f_{\text{VPD}}$ depend on photosynthetically relevant photon flux density at the leaf surface (PPFD, μmol m$^{-2}$ s$^{-1}$), temperature ($T$, °C), and vapor pressure deficit (VPD, kPa), respectively; $A_{\text{start}}$ and $A_{\text{end}}$ are the year days for the start and end of the experiment; $f_{\text{phen},a}$ and $f_{\text{phen},b}$ represent the number of days of $f_{\text{phen}}$ to reach its maximum and the number of days during the decline of $f_{\text{phen}}$ to the minimum value, respectively; $f_{\text{phen},c}$ and $f_{\text{phen},d}$ represent maximum fraction of $f_{\text{phen}}$ at $A_{\text{start}}$ and $A_{\text{end}}$, respectively; $a$ is the parameter determining an exponential curve of stomatal response to light; $T_{\text{opt}}, T_{\text{min}},$ and $T_{\text{max}}$ denote optimal, minimum, and maximum temperature for stomatal opening, respectively; and VPD$_{\text{min}}$ and VPD$_{\text{max}}$ denote the threshold of VPD for attaining minimum and full stomatal opening, respectively.

Figure 5. Parameterization of $f_{\text{light}}, f_{\text{temp}}, f_{\text{VPD}},$ and $f_{\text{phen}}$ in stomatal conductance model for *Alnus glutinosa*, *Sorbus aucuparia*, and *Vaccinium myrtillus* exposed to three levels of O$_3$ concentration (AA, ambient O$_3$ concentration 1.5 × AA, and 2.0 × AA). Red lines show model functions and black points show measured stomatal conductance.

3.4. Dose-Response Relationship for Plant Injury Index

In *A. glutinosa*, the first criterion (Y-intercept = 0 included in C.I.) was reached in the regressions between PII and AOT40 or POD$_{3-7}$. Among these indices, POD$_7$ had the highest $R^2$ (0.54) (Table S1). On the one hand, the CL corresponding to PII = 5 based on POD$_7$ was 8.1 mmol m$^{-2}$ (Figure 6) and, on the other hand, the AOT40-based CL was 33 ppm·h. In *S. aucuparia*, the first criterion was achieved for AOT40 and POD$_{1-5}$·. The highest $R^2$ value was found in POD$_2$ (0.87). The CLs in this species were found to be 22 mmol m$^{-2}$ POD$_2$ and 30 ppm·h AOT40. In *V. myrtillus*, the first criterion was
achieved for AOT40 and POD1−4. POD1 had the highest R² value (0.82), while the exposure-based index AOT40 performed equally well (R² = 0.81). The CLs was 5.8 mmol m⁻² POD1 and 20 ppm·h AOT40 in this species.

4. Discussion

4.1. New DO3SE Parameterization in Three Deciduous Tree Species

An accurate parameterization of the gₐ model is essential to develop a flux-based approach for O₃ risk assessment [26,28]. The model performance with new parameterization was comparable to that in previous studies [26,29]. A comparison of the three target species showed that the gₘₐₓ value was relatively high in A. glutinosa (300 mmol m⁻² s⁻¹) as compared with the other species (S. aucuparia, 240 mmol m⁻² s⁻¹ and V. myrtillus, 140 mmol m⁻² s⁻¹). This value was within the range for a previous field observation of this species (170 to 380 mmol m⁻² s⁻¹) [30–32]. A high gₐ enhanced stomatal O₃ uptake, thus, leading to higher O₃ damage [15]. The level of gₘₐₓ in A. glutinosa was comparable to that of the other O₃-sensitive species such as Oxford poplar clone (340 to 520 mmol m⁻² s⁻¹) [27,33] and Fagus crenata (315 mmol m⁻² s⁻¹) [34,35].

Interestingly, the parameter a, in the fₗₐₜₜₜ function, was relatively high in V. myrtillus among the three species, suggesting a lower light saturating point of gₐ [27]. Karlsson, 1987 [36] and Gerdol et al., 2000 [37] reported a relatively low light saturating point of photosynthesis (200 to 300 μmol m⁻² s⁻¹ of PPFD) in this species. V. myrtillus is known as a shade tolerant species [38] while the other two species are light demanding [39,40]. In fact, a high a value in fₗₐₜₜₜ function was found in other shade tolerant species such as F. crenata (a = 0.0086) [34], while a lower a was obtained in a light-demanding poplar clone (Populus maximowiczii Henry x berolinensis Dippel, a = 0.0020) [27].

In the afternoon, a high VPD often closes stomata together with high air temperature [41]. This was supported by the parameters in fᵥᵥᵥᵥᵥ and fₜₜₜₜ for the three species. In fact, gₐ was decreased by 29%, 29%, and 49%, in A. glutinosa, S. aucuparia, and V. myrtillus, respectively, when VPD reached 3 kPa. In addition, gₐ was decreased by 13%, 38%, and 68%, in A. glutinosa, S. aucuparia, and V. myrtillus, respectively, when air temperature reached 35 °C. Since ambient O₃ concentrations were elevated in the afternoon, those functions were fundamental for the stomatal O₃ flux calculation [28].
4.2. Flux-Based Assessment of Ozone Visible Injury

The foliar symptoms of *A. glutinosa*, *S. aucuparia*, and *V. myrtillus* in this experiment were similar to those observed in the field at ambient O₃ levels [8]. The first foliar symptoms were observed in May in all species exposed to 2.0 × O₃. The onset occurred at 5 to 9 ppm-h AOT40. This is supported by the findings in previous studies where 5 to 10 ppm-h AOT40 caused an emergence of O₃ visible injury for sensitive tree species such as *F. sylvatica* [11]. In addition, the present study found that approximately 4 mmol m⁻² POD₀ was enough to cause the onset of O₃ visible injury regardless of tree species. *A. glutinosa* with a high $g_{\text{max}}$ quickly reached this critical point of POD₀ and showed the first symptom earlier than the other two species. In species with a high $g_{\text{s}}$, the O₃ dose can easily exceed the metabolic capacity for detoxification, and therefore can quickly cause O₃ visible injury [42].

After the onset, the O₃ visible injury expanded to the plant level in all three species as confirmed by the increase in PII values. The increases of PII were well correlated with flux-based indices (POD_{Y}) in each species. The POD_{Y} showed a higher $R^2$ than AOT40, suggesting that POD_{Y} was better than AOT40 to assess PII. This is supported by the fact that O₃ impacts are more closely related to O₃ uptake than to external O₃ exposure [15]. Previous studies suggested a threshold Y as an assumed threshold below which stomatal O₃ flux by the plant may be detoxified [16]. The result shows that Y was relatively higher in *A. glutinosa* (Y = 7) as compared with other two species (Y = 0 to 1). This suggests that *A. glutinosa* can have a higher capacity for O₃ detoxification than the other two species, although this species had a high stomatal O₃ uptake.

Sicard et al., 2016 [17] indicated that 22 mmol m⁻² of POD₀ corresponded to 5% visible injury in O₃-sensitive deciduous *F. sylvatica* according to field measurements. Our results in *A. glutinosa* and *S. aucuparia* support their findings because the CLs corresponding to PII = 5 on the basis of POD₀ were 22 and 29 mmol m⁻² in *A. glutinosa* and *S. aucuparia*, respectively. However, the CL in *V. myrtillus* was much lower than that of the other two species. On the basis of PII, *V. myrtillus* was more sensitive to O₃ than *A. glutinosa* and *S. aucuparia*. This is because *V. myrtillus* had a dramatic increase in PII from June to July. Although it had a relatively low $g_{s}$ and thus low stomatal O₃ uptake, this species was highly susceptible to O₃ in these months. *V. myrtillus* had a vegetative stage in May and then flowering and fruiting stages from June to July [43]. In fact, previous studies found that the capacity to detoxify O₃ was lower when the plants were flowering or producing fruits [44–46].

The seasonal dynamics of PII differed among the species. In *S. aucuparia* and *V. myrtillus*, the PII values showed a monotonic increase, while *A. glutinosa* had a rather constant PII during June to August. Novak et al., 2003 [13] reported that several species (*Populus nigra*, *Prunus avium*, and *Salix alba*) similarly had a leveling or even decreasing trend of total injured leaf area during the season. In general, O₃ visible injury usually appears on older leaves [4]. However, new leaf formation in *A. glutinosa* was significantly increased by elevated O₃, while damaged old leaves were shed. This new leaf growth can alleviate the expansion of O₃ visible injury at the plant level in *A. glutinosa*. An accelerated leaf turnover can be considered as a compensation response to stress in plants with indeterminate growth pattern [20]. However, PII in 2.0 × AA was still significantly higher than that in AA in *A. glutinosa*, suggesting that such leaf growth did not fully compensate for the O₃ damage.

5. Conclusions

The present O₃ FACE experiment successfully confirmed O₃ visible injury in three cool-temperate deciduous tree species, *A. glutinosa*, *S. aucuparia*, and *V. myrtillus*. The onset of O₃ visible injury in these species required 4.0 mmol m⁻² POD₀ and 5.5 to 9.0 ppm-h AOT40. The timing of the first symptom onset among the species was determined by the amount of stomatal O₃ uptake. The early emergence of O₃ visible injury in *A. glutinosa* was related to high $g_{\text{s}}$; however, PII was affected not only by stomatal O₃ uptake but also by other species-specific ecophysiological traits. The dynamics of PII suggest that an increased fructification (flowering, fruiting) can weaken the state of the *V. myrtillus* tree, then, finally the trees can be more sensitive to O₃ [47]. In addition, PII values in *A. glutinosa* were affected by its indeterminate growth pattern, and a new leaf formation alleviated the expansion of O₃ visible injury at
the plant level in this species. Nevertheless, O₃ impacts on PII were better explained by the flux-based index, POD₂, than by the exposure-based index, AOT40, especially in *A. glutinosa*, although it changed in a complex manner. The CLs corresponding to PII = 5 were 8.1 mmol m⁻² POD₂ in *A. glutinosa*, 23 mmol m⁻² POD₂ in *S. aucuparia*, and 5.8 mmol m⁻² POD₂ in *V. myrtillus*.

Forest trees also suffer from other climate change factors such as elevated CO₂, nitrogen deposition, warming, the risk of flooding, drought, and forest fire [42]. The interactions between O₃ and other climate change factors are crucial to establish the species-specific O₃ flux-effect relationship for the O₃ risk assessment.

**Supplementary Materials:** The following are available online at http://www.mdpi.com/1999-4907/11/1/82/s1, Figure S1: Comparison between measured and estimated gs in *Alnus glutinosa*, *Sorbus aucuparia* and *Vaccinium myrtillus*. Table S1: Summary for R² and the information of confidential interval (C.I.) in exposure or flux-based dose-response functions for plant injury index (PII).

**Author Contributions:** Y.H., P.S., A.D.M., and E.P. conceived the study; Y.H. and E.C. carried out the experiment and collected the data; Y.H., B.M. and S.M. undertook the statistical analyses. All authors were involved in writing the paper, although Y.H. took a lead role. All authors have read and agreed to the published version of the manuscript.

**Funding:** We are grateful for the financial support to the MITIMPACT project (INTERREG V A—Italy—France ALCOTRA), Fondazione Cassa di Risparmio di Firenze (2013/2014/2015), and the LIFE project MOTTLES (LIFE15 ENV/IT/000183) of the European Commission.

**Acknowledgments:** We thank Alessandro Materassi, Gianni Fasano, and Francesco Sabatini for maintenance of the ozone FACE; Moreno Lazzara and Iacopo Manzini for support during field work.

**Conflicts of Interest:** The authors declare no conflict of interest.

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