Bridging experimental and monitoring research for visible foliar injury as bio-indicator of ozone impacts on forests

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ABSTRACT

Tropospheric ozone (O\textsubscript{3}) is a phytotoxic air pollutant and the O\textsubscript{3} induced visible foliar injury (O\textsubscript{3} VFI) is a biomarker. A recently developed Free-air O\textsubscript{3} eXposure (FO\textsubscript{3}X) is a promising facility to verify field-observed O\textsubscript{3}-like VFIs and to establish a flux-based threshold for the O\textsubscript{3} VFI onset. The present study compared O\textsubscript{3}-like VFI registered in the southern European forest sites with actual O\textsubscript{3} VFI observed in a FO\textsubscript{3}X experiment. The O\textsubscript{3}-like VFIs were evaluated by eye in forests and thus was subjective. According to the imaging analysis, we firstly demonstrated that major parts of the colors were similar in the field and the FO\textsubscript{3}X. The color pallets for O\textsubscript{3} VFI was species-specific and considered a advanced tool for the O\textsubscript{3} VFI diagnosis. In addition, we calculated a flux-based threshold for the O\textsubscript{3} VFI onset at the FO\textsubscript{3}X based on a Phytotoxic Ozone Dose (POD\textsubscript{3}X), which ranged from 4.9 to 18.1 mmol m\textsuperscript{-2} POD\textsubscript{3}X. This POD\textsubscript{3}X-derived threshold partly explained but did not necessarily match with the observation for several tree species in actual forests. The multivariate analysis showed that O\textsubscript{3} VFI was decreased by the presence of various species and suggested the importance of continuous monitoring activities in the field for the further analysis.

Introduction

Tropospheric ozone (O\textsubscript{3}) is a significant greenhouse gas and phytotoxic air pollutant, which has a deleterious effect on forest health (Watanabe et al. 2021). Therefore, monitoring forest health and functionality under climate change and air pollution is crucial for estimating forest vulnerability to the ongoing changes and for establishing mitigation measures (Paoletti et al. 2022). Furthermore, the recently revised National Emission Ceilings Directive of the European Union (2016/2284/EU, hereafter NEC Directive 2016) targets a 30% reduction in air pollutant emissions by 2030, compared with 2005 levels, and it is necessary to ensure the monitoring of air pollution impacts on ecosystems in EU member states as specified in Art. 9 of NEC Directive. Various O\textsubscript{3} metrics such as AOT40 (Accumulated exposure Over a Threshold of 40 ppb; abbreviations are listed in Table S1) have been proposed to examine forest responses to O\textsubscript{3} (Lefohn et al. 2018). However, scientific evidence has pointed out that O\textsubscript{3} damage is closely related to O\textsubscript{3} uptake through stomata (CLRTAP 2017). Therefore, Phytotoxic Ozone Dose above a stomatal-flux threshold \textit{Y} (POD\textsubscript{3}), which is derived from a cumulative O\textsubscript{3} uptake over the growing season, is now included in the NEC Directive for the assessment of quantitative O\textsubscript{3} impacts on forest trees (de Marco et al. 2019).

When plants are exposed to O\textsubscript{3}, they often show specific O\textsubscript{3}-induced visible foliar injury (O\textsubscript{3} VFI), which has been used as a bio-marker to assess potential negative impacts of O\textsubscript{3} (Feng et al. 2014; Hoshika et al. 2018). Ozone-like VFIs have been found in various tree species across forest sites in Europe, indicating a possible phytotoxicity of the ambient level of O\textsubscript{3} (annual O\textsubscript{3} mean concentrations: 35 to 50 ppb) in forests (Paoletti et al. 2019; Vollenweider et al. 2019; Sicard et al. 2020).

Traditionally, the O\textsubscript{3}-like VFIs have been evaluated by eye and, thus, the approach was rather subjective, although much efforts have been made to harmonize the VFI monitoring through the inter-calibration courses by international organizations such as ICP-Forests (Schaub et al. 2016). A manipulative O\textsubscript{3} exposure experiment is therefore considered as one of the key approaches for the validation of O\textsubscript{3}-like VFI to diagnose and identify foliar symptoms caused by O\textsubscript{3} (Moura et al. 2018; Vollenweider et al. 2019). Previously, O\textsubscript{3}-like VFIs have been verified in a wide variety of settings, including greenhouses and...
chambers of various sizes and designs (e.g., Bussotti et al. 2003). Although those experiments provided an insight into potential mechanisms, a difference in meteorological factors such as light, air temperature, and wind speed between chambers and natural conditions may also affect a quantitative analysis of O$_3$ VFI relative to actual field conditions (Nussbaum and Fuhrer 2000). Free-air O$_3$ eXposure (FO$_3$X) has been established in Italy since 2015, which is a unique facility in Mediterranean Europe to assess the effects of O$_3$ on vegetation within the framework of the European AnaEE (Analysis and Experimentation on Ecosystems) Research Infrastructure. As is the case with free-air CO$_2$ enrichment, experiments by free-air O$_3$ enrichment are considered as the best approach to provide realistic estimates of tree responses under real-world conditions (Paoletti et al. 2017). Symptoms of O$_3$ VFIs were reported in several tree species at the FO$_3$X (Hoshika et al. 2018, 2020). However, there is a need for validation studies to confirm whether the O$_3$-like VFIs observed in actual forests are similar enough to real O$_3$ VFIs produced in the FO$_3$X. In addition, little is known whether the FO$_3$X-derived thresholds for O$_3$ VFI can be applied to actual forest conditions, although such manipulative experiments have been used to develop standards for forest protection against O$_3$ (Büker et al. 2015). A flux-based threshold for the O$_3$ VFI onset is considered essential for the evaluation of regional O$_3$ pollution (Sicard et al. 2020). According to the forest monitoring data, Sicard et al. (2020) proposed flux-based thresholds for O$_3$-like VFI of 5 and 12 mmol m$^{-2}$ POD$_1$ in dominant conifers and broadleaved trees, respectively.

The present study compared O$_3$ VFI for forest tree species at the FO$_3$X facility with the symptoms observed at actual forest sites with the main aim of validating the field observations. For this purpose, a dataset obtained at southern European forest sites (France, Italy, and Romania) was utilized thanks to the European project MOTTLES (MOonitoring ozone injury for seTTing new critical LevelS) (Paoletti et al. 2019). A novel color pallet approach was developed to analyze the color composition of symptoms for categorizing and standardizing the O$_3$ VFI. The onset of O$_3$ VFI was also analyzed on a stomatal flux basis using POD$_1$. This study aimed to answer the following specific questions: 1) Are O$_3$-like foliar symptoms observed in the field comparable to actual O$_3$ VFIs produced at the FO$_3$X? and 2) Does the FO$_3$X-derived flux-based threshold also explain the incidence of O$_3$ VFI at the forest sites?

Materials and methods

Ozone fumigation experiments at the FO$_3$X facility

Ozone fumigation studies were carried out at the FO$_3$X facility in Italy (43° 49’N, 11° 12’E, 55 m a.s.l.). We set three levels of O$_3$ treatments (ambient O$_3$ concentration [AA], 1.2 times ambient concentration [1.2×AA], and 1.4 times ambient concentration [1.4×AA]) in 2015; AA, 1.5 times ambient concentration [1.5×AA], and twice ambient concentration [2.0×AA] in 2017, 2018, 2019, 2020. The experimental design was a split-plot with three replicated blocks (L × W × H: 5 × 5 × 2 m) for each O$_3$ treatment. The specific detail of the fumigation system can be found in Paoletti et al. (2017). Targeted species were three oaks (Quercus ilex L., Quercus pubescens Willd., Quercus robur L.) in 2015, strawberry tree (Arbutus unedo L.) in 2017, black alder (Alnus glutinosa (L.) Gaertn.), phillyrea (Phillyrea angustifolia L.), mountain ash (Sorbus aucuparia L.) and bilberry (Vaccinium myrtillus L.) in 2018, three pines (Pinus halepensis Mill., P. pinea L.) in 2019 and elm leaf blackberry (Rubus ulmifolius Schott) in 2020. Those species were common in the MOTTLES monitoring sites. All plants were transplanted into plastic pots (oaks: 10 L, phillyrea: bilberry, pines, and elmleaf blackberry: 25 L; strawberry tree: 30 L; black alder and mountain ash: 50 L) with a mixture of sand: peat: soil (1:1:1 as volume). Before the O$_3$ fumigation, the pots were irrigated to keep the well-watered conditions, i.e., volumetric soil water content was kept to field capacity (0.295 m$^3$ m$^{-3}$). Paoletti et al. (2017). Target species, exposure period, hourly mean O$_3$ concentrations, and mean meteorological parameters during the experiments are shown in Table 1.

The symptoms were carefully checked using a ×10 hand lens for the closer examination of injuries, and all leaves were targeted for each plant. Two-well experienced observers periodically assessed O$_3$ VFI to determine the date for first-symptom onset. The observers were involved in validation activities, attended field courses and performed annual inter-comparison exercises, organized by ICP-Forests. Foliar injury was compared with the reference picture atlas provided by the validation center for central Europe (www.wsl.ch) and identified according to the previous O$_3$ VFI studies in tree species (Calatayud et al. 2010; Hoshika et al. 2013; Schaub and Calatayud 2013).

MOTTLES forest sites and field observations

The study is based on data recorded by the MOTTLES O$_3$ monitoring network from 2017 to 2021 for Italian sites, and 2017–2019 for other countries. The network includes various biogeographical regions in southern European forest sites in three countries (France, Italy, and Romania). The target forest sites cover representative species in these regions, such as Fagus sylvatica in mountainous Alpine areas and Quercus ilex in Mediterranean forest areas. As a result, the MOTTLES network considered 7 broadleaved and 4 coniferous species (Table 2).
Table 1. Exposure period, target species, and environmental conditions during the experiment at the free-air O₃ eXposure (FOX). n.A. denotes not available. Ambient O₃ concentration (AA), 1.2 times ambient concentration (1.2×AA), 1.4 times ambient concentration (1.4×AA), 1.5 times ambient concentration (1.5×AA) and twice ambient concentration (2.0×AA).

| Exposure period | Target species | 2015 | 2017 | 2018 | 2019 | 2020 |
|-----------------|----------------|------|------|------|------|------|
| 1 Jun – 15 Oct  | Quercus ilex | 12 Jun – 15 Oct | Arbutus unedo | 1 May – 31 Oct | Alnus glutinosa | 15 May – 20 Oct | Pinus halepensis | 15 May – 31 Oct | Rubus ulmifolius |
| Q. pubescens | Q. robur | | | | Phillyrea angustifolia | | P. pinaster | | |
| Q. rubra | | | | | Sorbus aucuparia | | | | |
| V. myrtillus | | | | | | | | | |

Hourly mean O₃ concentration (ppb)

| | 2015 | 2017 | 2018 | 2019 | 2020 |
|---|------|------|------|------|------|
| AA | 35.2 | 40.3 | 35.2 | 38.8 | 37.5 |
| 1.2×AA | n.a. | 57.1 | 53.1 | 56.2 | 52.3 |
| 1.4×AA | n.a. | 71.8 | 65.2 | 68.6 | 73.3 |

Meteorological parameters

| | 2015 | 2017 | 2018 | 2019 | 2020 |
|---|------|------|------|------|------|
| Daily mean air temperature (°C) | 24.4 | 24.5 | 22.8 | 23.5 | 22.1 |
| Daily mean solar radiation (W m⁻²) | 250 | 252 | 230 | 236 | 192 |
| Daily mean relative humidity (%) | 52.9 | 47.6 | 55.6 | 55.1 | 61.8 |
| Total precipitation (mm) | 592 | 195 | 136 | 161 | 313 |

Each site consisted of i) an open area (open field – OFD) to record remotely and continuously meteorological and O₃ values with active sensors, ii) a nearby Light Exposed Sampling Site – LESS (Schaub et al. 2016), located in the light-exposed forest edge closest to the OFD station (maximum radius of 500 m) LESS area, and iii) an “in the plot” (ITP) area, where soil moisture and forest-health indicators are recorded into the forest. Specific details on MOTTLES active O₃ monitoring sites are available in Paoletti et al. (2019). The field protocols for assessing O₃ VFI by MOTTLES surveyors followed the ICP-Forests manual (Schaub et al. 2016). Surveys were carried out by the same two people in each country from August to early-mid September, i.e., when O₃ VFI are more likely to be observed in the MOTTLES sites (Dalstein et al. 2005; Paoletti et al. 2010). Two campaigns of trans-country inter-calibration were carried out within MOTTLES, and the surveyors had taken part in the international cross-calibration courses organized by ICP Forests.

At each site, the assessment of O₃ VFI was conducted every year within the ITP and the LESS. In ITP, the evaluation of O₃ VFI were performed at each plot on five trees randomly selected. For each tree, five light-exposed branches with ≥30 needles/leaves per branch or needle age class were removed from the upper crown. For deciduous species, current year (C) leaves/needles were assessed. For evergreen species, C, one – year–old (C + 1) and two – year–old (C + 2) leaves/needles were assessed. On each leaf/needle, the extent of O₃ VFI (as a percentage of the total leaf area) was visually scored by using the actual percentage, and results were then averaged for the five branches, resulting in one value per tree; finally, the tree values were averaged per plot. In the MOTTLES network, the LESS is 50 m long and divided into 25 x 2 m² non-overlapping quadrats. In 20 of the 25 quadrants, which were randomly chosen, all plant species were listed, and the presence or absence of O₃ VFI was recorded on the same day in which the ITP survey was carried out.

For the species with validated O₃ VFI, the available literature (Innes, Skelly, and Schaub 2001) and atlas provided by the validation center for central Europe (www.wsl.ch) were used to support the observations. Here, O₃ VFI datasets at MOTTLES sites during 2017–2021 were used for the analysis.

Calculation of ozone indices

Both exposure- and flux-based O₃ metrics were already calculated at MOTTLES sites over the study period from measured parameters (Sicard et al. 2020, 2021). In addition, the metrics, i.e., AOT40 and species-specific POD₁, were calculated by using meteorological and O₃ concentration data measured at the FOX facility. Accumulated exposure over a threshold of 40 ppb (AOT40) was calculated using the hourly O₃ data during daytime (solar radiation >50 W m⁻², CLRTAP, 2017) measured at each site:

$$AOT40 = \sum \text{max}([O₃] – 40, 0) \cdot dt$$  \hspace{1cm} (1)

where \([O₃]\) is the hourly concentration of O₃ (ppb), and \(dt\) is the time step (1 h).

Phytotoxic Ozone Dose above a detoxification threshold of 1 nmol m⁻² s⁻¹ (POD₁) was calculated according to the standard methodology suggested by CLRTAP (2017).

$$POD₁ = \sum \text{max}(F_{st} - 1, 0) \cdot dt$$  \hspace{1cm} (2)

where \(F_{st}\) is the hourly stomatal uptake of O₃ (nmol m⁻² s⁻¹), which is derived from leaf surface resistance \(r_s\) and boundary layer resistance \(r_b\) (CLRTAP, 2017). \(F_{st}\) is thus given by:

$$F_{st} = \frac{[O₃]}{g_i} \cdot \frac{r_c}{r_b + r_c}$$  \hspace{1cm} (3)
Table 2. Site information of the 17 monitoring ozone injury for setting new critical Level 5 (MOTTLES) sites in France, Italy, and Romania. Daily mean air temperature (T), Daily mean solar radiation (RAD), Daily mean relative humidity (RH), Annual total precipitation (Prep.) and Hourly mean ozone concentration (O₃) are calculated from 2017 to 2021 for Italian sites and from 2017 to 2019 for France and Romania (± standard deviation).

| Code  | Country | Lat. ¹ N | Lon. ¹ E | Elevation a.s.l. | Dominant species | Slope aspect | Soil type          | T (°C)   | RAD (W m⁻²) | RH (%) | Prep. (mm) | O₃ (ppb) |
|-------|---------|----------|----------|-----------------|------------------|--------------|-------------------|----------|-------------|--------|------------|--------|
| LCAS  | France  | 44.99703 | 6.48082  | 1755            | Larix decidua    | 30° E        | Orthic Luvisols   | 7.95 ± 6.4 | 402.06 ± 176.7 | 63.43 ± 7.3 | 415.52 ± 248.0 | 48.18 ± 6.6 |
| MNTR  | France  | 45.80500 | 2.06200  | 810             | Pinus sylvestris | -            | Dystric Cambisols | 10.57 ± 6.3 | 293.31 ± 173.7 | 77.17 ± 1.8  | 745.64 ± 492.2 | 38.46 ± 5.6  |
| MORV  | France  | 47.27491 | 4.09921  | 620             | Abies alba      | 6.5° SE       | Chromic Cambisols | 9.51 ± 6.1  | 327.16 ± 191.5  | 82.48 ± 7.6  | 186.64 ± 116.2 | 35.02 ± 5.3  |
| REV   | France  | 49.90776 | 4.62972  | 390             | Pinus abies     | 2°            | Calcaric Leptosol | 10.83 ± 6.1  | 284.52 ± 176.6  | 80.72 ± 10.6 | 635.10 ± 387.9  | 32.30 ± 7.2  |
| ABR1  | Italy   | 41.86604 | 13.57482 | 1500            | Fagus sylvatica | 5-10° S       | Humic Acrisols   | 8.02 ± 7.1  | 369.71 ± 186.0  | 78.33 ± 7.4  | 663.83 ± 522.4 | 54.87 ± 9.9  |
| CPZ1  | Italy   | 41.70423 | 12.35719 | 0               | Quercus ilex    | Plain         | Fluvisol         | 16.71 ± 6.2 | 424.28 ± 224.9  | 78.95 ± 3.5  | 455.40 ± 342.6 | 33.05 ± 6.3  |
| CPZ2  | Italy   | 41.70429 | 12.35722 | 0               | Phylleia latifolia | Plain       | Fluvisol         | 16.71 ± 6.2 | 424.28 ± 224.9  | 78.95 ± 3.5  | 455.40 ± 342.6 | 33.05 ± 6.3  |
| EMI1  | Italy   | 44.71998 | 10.20345 | 200             | Quercus petraea | Plain         | Flavivisols      | 11.60 ± 7.6  | 321.76 ± 209.4  | 70.62 ± 12.3 | 484.47 ± 311.9 | 39.68 ± 14.7 |
| LAZ1  | Italy   | 42.82746 | 11.89817 | 690             | Quercus cerris  | 5° NWW        | Dystric Cambisols | 13.75 ± 7.3 | 388.65 ± 181.8  | 73.16 ± 11.2 | 1027.06 ± 1060.3 | 46.99 ± 7.3  |
| PIE1  | Italy   | 45.68374 | 8.06994  | 1150            | Fagus sylvatica | 30° NWW       | Dystric Podzols  | 7.42 ± 6.2  | 309.64 ± 140.4  | 71.77 ± 8.7  | 1138.12 ± 1151.0 | 47.72 ± 8.7  |
| TREP1 | Italy   | 46.35425 | 11.49405 | 1800            | Pinus abies     | 10° NWW       | Fluvic Podzols   | 5.76 ± 7.2  | 337.89 ± 173.3  | 71.54 ± 8.2  | 674.76 ± 403.7 | 48.24 ± 8.7  |
| VEN1  | Italy   | 46.06335 | 12.38810 | 1100            | Fagus sylvatica | 5° E          | Fluvic Luvisols   | 8.25 ± 7.2  | 329.94 ± 179.8  | 86.54 ± 5.2  | 1137.28 ± 705.3 | 35.46 ± 8.0  |
| FAG   | Romania | 45.43305 | 25.26972 | 1300            | Fagus sylvatica | 20° W         | Rendzin          | 7.21 ± 7.1  | 370.26 ± 155.0  | 78.25 ± 7.8  | 584.36 ± 363.0 | 41.28 ± 6.6  |
| GORUN | Romania | 45.02874 | 24.99576 | 500             | Quercus petraea | 10° W         | Luvisol          | 11.02 ± 7.7 | 444.63 ± 201.3  | 78.84 ± 6.4  | 539.94 ± 243.7 | 20.17 ± 6.6  |
| MOUD  | Romania | 45.50694 | 25.58916 | 1185            | Pinus abies     | 20° E         | Eutricambisols    | 7.44 ± 7.2  | 271.76 ± 148.4  | 80.46 ± 6.1  | 438.97 ± 274.0 | 26.23 ± 5.9  |
| STEAR | Romania | 44.50926 | 26.17320 | 86              | Quercus robur   | Plain         | Pluvivisols      | 12.47 ± 7.9 | 434.19 ± 191.3  | 75.98 ± 8.2  | 541.67 ± 429.0 | 21.22 ± 7.2  |
where $g_s$ is the stomatal conductance (mmol O$_3$ m$^{-2}$ projected leaf area [PLA] s$^{-1}$), $r_s$ is calculated by wind speed and leaf dimension, and $r_o$ is defined as 1/(g$_s$+g$_{ext}$) (s m$^{-1}$). $g_{ext}$ is the cuticular conductance (0.0004 s m$^{-1}$). For the details of the calculation of $r_s$ and $r_o$, see CLRTAP (2017).

A simplified formula of the multiplicative stomatal conductance model was applied because all plants were grown under well-watered conditions at the FO$_3$X (Hoshika et al. 2020). It is given by:

$$g_s = g_{max} \times f_{light} \times max\{f_{min}, (f_{temp} + f_{VPD})\}$$

(4)

where $g_{max}$ is the maximum stomatal conductance to O$_3$ expressed on a total leaf surface area (mmol O$_3$ m$^{-2}$ PLA s$^{-1}$), $f_{min}$ is the minimum conductance, $f_{light}$, $f_{temp}$, and $f_{VPD}$ indicate the stomatal response functions to photosynthetic photon flux density (PPFD), air temperature (T) and vapor pressure deficit (VPD), respectively. For several species (A. glutinosa, Q. ilex, Q. pubescens, Q. robus, S. aucuparia, V. myrtillus), species-specific stomatal conductance parameters were already reported in our previous studies (Hoshika et al. 2018, 2020). Stomatal conductance for the other species was parameterized according to the measurements with various meteorological conditions using a portable leaf gas exchange measurement system (CIRAS-2 PP Systems, Herts, UK). Measurements were carried out at least once a month (7 to 19 days in total for each species) throughout the experimental period (Table 1). Pooled data (A. unedo: 128 data, P. angustifolia: 374 data, P. halepensis: 174 data, P. pinaster: 303 data, P. pinea: 164 data, R. ulmifolius: 249 data) were used for the species-specific parameterization according to the boundary line technique (Elvira et al. 2004; Bükner et al. 2015; Hoshika, Paolletti, and Omasa 2012). To draw the boundary line (i.e., upper limits of point $g_s$ data in a scatter diagram) for each environmental variable, the data were divided into the following stepwise classes: PPFD: 200 µmol m$^{-2}$ s$^{-1}$ steps (when PPFD <200 µmol m$^{-2}$ s$^{-1}$, 50 µmol m$^{-2}$ s$^{-1}$ steps were applied), T: 2°C steps, VPD: 0.2 kPa steps. Each model function was then fitted according to 95th percentile values per each stepwise class of environmental factors. $g_{max}$ and $f_{min}$ were estimated as the 95th and 5th percentile, respectively (Bičárová et al. 2019; Hoshika et al. 2020). For the detailed formula of $f_{light}$, $f_{temp}$, and $f_{VPD}$ functions, see CLRTAP (2017).

**Data analysis**

For three species, Rubus ulmifolius, Vaccinium myrtillus, and Sorbus aucuparia, an O$_3$ VFI image analysis was developed to compare the symptoms observed in the field with those developed at the FO$_3$X. Three pictures with minimum 180 dpi (RGB – red, green, blue color mode) of leaves presenting O$_3$ VFI of each species were selected from the field and FO$_3$X conditions. First, the mid-part of each leaf was cut off from the original picture with the same size for each species. Next, each cut of the sample picture was transformed in Indexed Color mode, and a Local (Perceptual) and an 8-color pallet was generated. Next, the color pallets were merged, forming a general 8-color pallet for the O$_3$ VFI pictures obtained from MOTTLES or FO$_3$ X conditions. The final 8-color pallets, which characterize the color composition of O$_3$ VFI, were then compared between field and FO$_3$X conditions, considering the percentage of the same colors appearing in both conditions. We surveyed the significant O$_3$ VFI colors of the final color pallet (50% of the total colors range) as the ones better describing the O$_3$ VFI (O$_3$ VFI/Color), while the other colors were related to leaf typical chlorophyll pigments (LTCP). All analyses were conducted using Adobe Photoshop CC 2017, and the color names were verified in the Hex dictionary (https://www.hexdictionary.com/). All pictures analyzed with its, respectively, 8-color pallet are available as supplementary material (Fig. S1).

In order to detect main predictors for symptoms in the natural environment, we correlated symptom variables with site variables. We applied two linear models by using the linear model (lm) function of R software (Team R Development Core, 2018): in the first model we considered the percentage of symptomatic species within the LESS as response variable; in the second model we considered the O$_3$ VFI (merging ITP and LESS data) as response variable. The following site variables were included in the model as predictors: year, country, slope aspect, dominant species, and biogeographical region (as categorical), elevation (as continuous), and the total number of species within the LESS (as count variable); all interactions among site variables were also considered in the model. Selection of the optimal model, among those generated by all possible combinations, was based on the Akaike Information Criterion (AIC) values to assess the quality of the model (Barton et al. 2012). For the best model selected, we also calculated R$^2$ and parameter-specific p-values for each predictor level (e.g., value).

**Results**

**Ozone visible injury at MOTTLES forest sites**

The O$_3$ VFI occurring in the MOTTLES sites were characterized by the following categories: Homogeneously distributed interveinal reddish (Rd), Reddish interveinal stippling (RdSt), Homogeneously distributed interveinal brownish (Brw), Dark brownish interveinal stippling (BrwSt), Bronzing (Br), and chlorosis (Cl) (Table 3). The 23 symptomatic species presented, in most of the cases, a combination of these symptoms. The most common O$_3$ VFI were Cl and BrwSt,
occurring in 43% of the species, followed by RdSt, detected in 35% of the species. Rd, Br, and Brw occurred in 26%, 13%, and 9% of the species, respectively. Some pictures were selected to show each of the categorical O3 VFI occurring in symptomatic species at MOTTLES sites (Figure 1.).

In MOTTLES forest sites, merging ITP and LESS data, O3 VFI was observed on 23 species, consisting of 14 trees, 8 shrubs and 1 liana (Clematis vitalba) (Table 3). Fagus sylvatica was the species found to be most frequently symptomatic (21 times across all sites and years), followed by the shrubs Rubus ulmifolius and Corylus avellana, recorded 12 and 11 times, respectively. Most of the symptomatic species were deciduous angiosperms, while only two conifers, i.e., Pinus cembra and Picea abies, were found to be symptomatic 2 times and once, respectively. Regarding the forest LESS, linear models showed that the percentage of symptomatic species, despite a significant influence of the country (Table 4; Figure 2.), decreased with the increment in the total number of tree and shrub species within the LESS (Figure 1.). The same trend was observed for O3 VFI (Figure 3); however, in this case, species richness in the LESS was the unique driver among the selected site characteristics.

Ozone visible injury at the FO3X

Pictures of the O3 VFI for the symptomatic species at FO3X are shown in Figure 1. Ozone exposure caused O3 VFI in all target deciduous species, while only two of six evergreen species were symptomatic, i.e., a Mediterranean shrub, A. unedo, and a Mediterranean pine, P. halepensis (Table 5). Among the symptomatic species, A. glutinosa, S. aucuparia, and V. myrtillus showed first symptoms even under real-world O3 conditions (18 May to 21 June), while O3 VFI was found only later for A. unedo and Q. pubescens (3 to 25 September).

Following the same classification used to identify O3 VFI in the MOTTLES sites, a similar combination of symptoms was observed in the eight symptomatic species in the FO3X facility (Table 4). The most common O3 VFI observed was Brw/St occurring in 50% of the species, followed by Brw/St and RdSt, detected in 38% of the species. Rd occurred in 25%, while Cl was found in 13% of the species. So far, Br has not been observed in the FO3X. The Cl mottling appeared on needles of P. halepensis, while the other evergreen species, A. unedo, presented RdSt occurring between the veins on the upper leaf surface. A similar symptom was found in V. myrtillus in which Rd was also observed. The deciduous

Table 3. Species showing ozone-induced visible foliar injury (O3 VFI) at the MOTTLES (Monitoring Ozone injury for seTTtting new critical Level(s)) sites (ITP – in the plot and LESS - Light Exposed Sampling Site) in France (FR), Italy (IT) and Romania (RO) or from the Free-air O3 eXposure (FO3X). O3-induced visible foliar injury (O3 VFI) classifies as homogeneously distributed interveinal reddish (Rd), Reddish interveinal stippling (RdSt), Homogeneously distributed interveinal brownish (Brw), Dark brownish interveinal stippling (Brw/St), chlorosis (Cl) and Bronzing (Br). Leaf habit (evergreen [Ev]/deciduous [De], conifer [C]/broadleaf [Br]).

| Species | FO3X Experiment | MOTTLES sites | Site name | Habitat | Figure |
|---------|-----------------|---------------|-----------|---------|--------|
| Acer pseudoplatanus | | | FR (LCAS, REV), RO (MOLID) | De Br | - |
| Abies glauca | | | FR (REV) | De Br | - |
| Carpinus betulus | | | FR (MORV), IT (EMI) | De Br | 1A |
| Clematis vitalba | | | IT (LAZ) | De Br | 1H |
| Cornus sanguinea | | | IT (ABR) | De Br | 1C |
| Corylus avellana | | | FR (MORV, REV), IT (PIE) | De Br | 1D |
| Fagus sylvatica | | | FR (MNTFR), IT (ABR, PIE, VEN) RO (FAG, GORUN, MOLID) | De Br | 1D |
| Fraxinus excelsior | | | FR (LCAS), IT (EMI) | De Br | - |
| Picea abies | | | FR (MNTFR) | Ev C | 1E |
| Pinus cembra | | | IT (TRE) | Ev C | 1E |
| Prunus avium | | | FR (LCAS) | De Br | - |
| Prunus spinosa | | | FR (LCAS, IT (LAZ) | De Br | 1F |
| Quercus robur | | | FR (MNTFR) | De Br | - |
| Ribes sp. | | | FR (LCAS, MORV) | De Br | - |
| Rosa canina | | | FR (LCAS, IT (LAZ, ABR) | De Br | 1G |
| Rubus fruticosus | | | FR (LCAS, MNTFR) | De Br | - |
| Rubus ulmifolius | | | IT (CPZ, EMI, PIE, ABR, LAZ) | De Br | 4A |
| Salix cinerea | | | FR (MNTFR) | De Br | - |
| Sorbus aria | | | FR (LCAS) | De Br | - |
| Sorbus aucuparia | | | IT (ABR), RO (FAG) | De Br | 4B |
| Ulmus minor | | | RO (STUAR) | De Br | - |
| Vaccinium myrtillus | | | IT (TRE) | De Br | 4C |
| Vitis vinifera | | | FR (MORV) | De Br | - |

* asymptomatic species
broadleaf *S. aucuparia* presented RdSt, and also Brw and BrwSt. In addition, interveinal Rd appeared on the upper leaf surface in *R. ulmifolius*. Finally, Brw and BrwSt similarly occurred between the primary leaves veins in the two deciduous oak species.

Comparing ozone visible injury at the MOTTLES and FO$_3$X sites: A color analysis

For *R. ulmifolius*, 62.5% of colors selected in the final 8-color pallets were common colors presented in both MOTTLES and FO$_3$X samples (Figure 4A–D). Considering only the O$_3$ VFI/Color, 75% of the colors were common between the MOTTLES and FO$_3$X samples (Coral Reef – 512E10, Peat – C7BAA1, and Pesto – 6C6133). One specific O$_3$ VFI/Color was not identified at FO$_3$X samples and was recorded only at the MOTTLES site (Bracken – 512E10). Furthermore, 50% of the LTCP colors were common between the MOTTLES and FO$_3$X samples.

For *S. aucuparia*, 50% of colors selected in the final 8-color pallets were present in both MOTTLES and FO$_3$X samples (Figure 4B–C). Considering only the O$_3$ VFI/
Color, 25% of the colors were common between the MOTTLES and FO$_3$X sample (Yellow Metal – 6C6133). One O$_3$ VFI/Color was recorded only at the MOTTLES site (Saddle – 472D22), whereas two O$_3$ VFI/Color was recorded at the FO$_3$X (Sepia Skin – B5A42, and Tan – D7B690). Furthermore, 75% of the LTCP colors were common between the MOTTLES and FO$_3$X samples.

For *V. myrtillus*, however, not all the considered O$_3$ VFI in the MOTTLES sites were similar to the FO$_3$X O$_3$ VFI pictures (Fig. S1), 75% of colors selected in the final 8-color pallets were present in both samples (Figure 4CD). Considering only the O$_3$ VFI/Color, 75% of the colors were common between the MOTTLES and FO$_3$X samples (Leather – 8B674F, West Coast – 695328 and Mongoose – A3926A). One specific O$_3$ VFI/Color, was not identified at FO$_3$X samples and was recorded only at the MOTTLES site (Beaver – A97E6D). Furthermore, 75% of the LTCP colors were common between the MOTTLES and FO$_3$X samples.

**Stomatal ozone uptake for visible injury onset at the FO$_3$X**

New stomatal conductance model parameters for species (*A. unedo*, *P. angustifolia*, *P. halepensis*, *P. pinaster*, *P. pinea*, *R. ulmifolius*) are shown in Table S2 and Fig. S2, and the $g_{\text{max}}$ values were dependent on species ranging from 95 (*A. unedo*) to 165 mmol O$_3$ m$^{-2}$ PLA s$^{-1}$

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Table 4. Optimal linear model structures relating the percentage of symptomatic species (symptomatic_sps) over the LESS (Light Exposed Sampling Site) and the percentage of ozone visible foliar injury (O$_3$ VFI) in the plot (ITP). Response variables to the total number of species in the LESS (SR_LESS), the country, and the year [R syntax of the starting model: y ~ SR_LESS + year + country + elevation + aspect + dominant species + biogeographical region]. Values for the predictor variables: SR_LESS, country (level Italy and Romania compared with France), are parameter estimates. R$^2$ refers to the fraction of the variation explained by the model structure, and R$^2$ adjusted takes into account the number of independent variables used for predicting the target variable.

| SR_LESS  | Symptomatic_sps | O$_3$ VFI |
|----------|----------------|-----------|
|          |                |           |
| Constant | 0.584***       | 51.619*** |
|          | (0.072)        | (10.321)  |
| Observations | 61          | 23       |
| R$^2$    | 0.426         | 0.231    |
| Adjusted R$^2$ | 0.374   | 0.191    |
| Residual Std. Error | 0.158 (df = 55) | 14.878 (df = 19) |
| F Statistic | 8.165*** (df = 5; 55) | 5.710** (df = 1; 19) |

Significance level: *p < 0.05, **p < 0.01, ***p < 0.001.
Figure 3. Visual results of the selected linear model for the percentage of ozone visible foliar injury (O₃-VFI) per plot, presented in table 3. Significant effect on O₃-VFI determined by the total number of species in the Light Exposed Sampling Site – LESS (SR_LESS) plotted as linear regression with confidence interval.

(P. halepensis, and R. ulmifolius). The stomatal light response function (f_{light}) followed a typical light-saturation curve with a saturation point above 1000 μmol m⁻² s⁻¹. The gₛ response to temperature (f_{temp}) had a bell-shaped curve where gₛ reached the maximum at the optimal temperature (22–26 °C). The f_{VPD} function indicated that stomatal closure was caused by VPD higher than 1.2 to 1.5 kPa regardless of the species. However, the VPD attaining minimum gₛ (VPD_{min}) was relatively low in P. halepensis compared to the other species.

Two representative O₃ indices, i.e., POD₁ and AOT40, were calculated to find the values corresponding to the onset of the first O₃ VFI in the 12 species at the FO₃ X experiment (Table 6). The AOT40 threshold for the onset of O₃ VFI was different among the symptomatic species (3.9 to 50.9 ppm h AOT40). The Mediterranean evergreen A. unedo required 50.9 ppm h AOT40 to show the first O₃ VFI, while the first O₃ VFI appeared on both deciduous A. glutinosa and S. aucuparia from low AOT40 values (3.9 to 6.5 ppm h AOT40). On the other hand, the onset of O₃ VFI was observed from 5 mmol m⁻² POD₁ for A. glutinosa and V. myrtillus, and from 18.1 mmol m⁻² POD₁ for Q. pubescens.

We then applied these FO₃X-derived thresholds to the MOTTLES conditions. In theory, O₃ VFI should be present when POD₁ exceeds the threshold for the onset of O₃ VFI, whereas no O₃ VFI should be found when POD₁ is lower than this threshold. In fact, in the MOTTLES sites, the results were in line with this theory for A. glutinosa and Q. pubescens (Figure 5). However, plants were often asymptomatic for Q. robur, S. aucuparia and V. myrtillus even though POD₁ was higher than this threshold. On the contrary, R. ulmifolius was symptomatic even in most cases when POD₁ was lower than the threshold.

### Discussion

**Color analysis and comparisons of the foliar symptoms between FO₃X and actual forests**

This first attempt to diagnose O₃ VFI by comparing actual field and manipulative experimental conditions based on the symptom color composition appeared promising, especially for two species where 75% of the O₃ VFI/Color were shared between MOTTLES and FO₃X samples. A leaf color chart is defined as a series of color swatches used to identify leaf physiological status and characteristics (Takebe and Yoneyama 0000). Color composition in terms of the color chart has been used in several studies, especially to estimate leaf and canopy chlorophyll content and their variation in field conditions (Nguy-Robertson et al. 2015) and for adjusting nitrogen (N) fertilization rate, thus improving N management in field conditions on the basis of leaf color.

Comparing the O₃ VFI between the field and experimental conditions using the color composition was here demonstrated to be a potential tool for the O₃ VFI diagnosis. For the three species compared in the presented study, 50% to 75% of the colors in the final 8-color pallets were commonly present in the field and under manipulative experimental conditions. This

| Species          | Date for the onset of O₃ visible foliar injury |
|------------------|-----------------------------------------------|
| A. glutinosa     | AA: 31 May, 1.5×AA: 31 May, 2.0×AA: 18 May    |
| A. unedo         | AA: no symptoms, 1.5×AA: 25 September, 2.0×AA: 18 September |
| P. angustifolia  | No symptoms                                    |
| P. halepensis    | AA: no symptoms, 1.5×AA: no symptoms, 2.0×AA: 28 June |
| P. pinaster      | No symptoms                                    |
| P. pinea         | No symptoms                                    |
| Q. ilex          | No symptoms                                    |
| Q. pubescens     | AA: no symptoms, 1.2×AA: 3 September, 1.4×AA: 22 September |
| Q. robur         | AA: 19 August, 1.2×AA: 5 August, 1.4×AA: 28 July |
| R. ulmifolius    | AA: no symptoms, 1.5×AA: 8 July, 2.0×AA: 28 June |
| S. aucuparia     | AA: 21 June, 1.5×AA: 21 June, 2.0×AA: 21 May    |
| V. myrtillus     | AA: 21 June, 1.5×AA: 21 June, 2.0×AA: 26 May    |
indicates that the O₃ VFI/Color are similar between field and FO₃X conditions, suggesting that it is possible to make successful validation of field-observed O₃ symptoms by using manipulative fumigation studies. However, it is important to follow standard procedures for the shooting of pictures used in the image analysis, as lighting conditions and picture resolution may affect the colors. The ICP-forest manual (Schaub et al. 2016) listed a series of indications for taking photographs of O₃ VFI, that must be followed for the quality assurance.

Table 6. Phytotoxic Ozone Dose (POD₁) and accumulated exposure over a threshold of 40 ppb (AOT40) values corresponding to the occurrence of the O₃ visible foliar injury (O₃ VFI) onset at the Free-air O₃ eXposure (FO₃X).

| Species        | POD₁ (mmol m⁻²) | AOT40 (ppm·h) |
|----------------|-----------------|---------------|
| A. glutinosa   | 4.9             | 3.9           |
| A. unedo       | 5.3             | 50.9          |
| S. aucuparia   | 8.6             | 6.5           |
| V. myrtillus   | 5.6             | 8.4           |
| P. angustifolia| no injury       | no injury     |
| P. halepensis  | 9.2             | 25.3          |
| P. pinaster    | no injury       | no injury     |
| P. domestica   | no injury       | no injury     |
| R. ulmifolium  | 11.4            | 23.5          |
| Q. ilex        | no injury       | no injury     |
| Q. pubescens   | 18.1            | 28.6          |
| Q. robur       | 12.0            | 16.4          |

Figure 4. Examples of color composition in adaxial leaf blades with ozone visible foliar injury (O₃ VFI) from Monitoring Ozone injury for seTTting new critical Levels (MOTTLES) sites and the Free-air O₃ eXposure (FO₃X), A and C Homogeneously distributed interveinal reddish (Rd); B and C Reddish interveinal stippling (RdSt) B) Dark brownish interveinal stippling (BrwSt) and Homogeneously distributed interveinal brownish (Brw). Colors better describing the O₃ VFI (O₃ VFI/Color) or Leaf typical chlorophyll pigments (LTCP).
The O₃ VFI provides clear indications of O₃-induced oxidative stress and is reliable when verified by a combination of approaches, once the symptoms expression can be similar even when the stress factors are different (Vollenweider and Gunthardt-Goerg 2006; Guerrero et al. 2013; Alves et al. 2016; Moura et al. 2018; Vollenweider et al. 2019). For instance, the adaxial St can appear following several other sources of oxidative stress (Vollenweider and Gunthardt-Goerg 2006), and CI is a common symptom that needs careful validation to be diagnosed as O₃ VFI (Vollenweider et al. 2019). Furthermore, usually observed O₃ VFI in field surveys are aspecific and difficult to interpret (Bussotti et al. 2006) because leaf colors may also be affected by the plant phenological nutritional and physiological status and other abiotic causes (Bussotti and Ferretti 2009). However, it should be noted that such a field-specific O₃ VFI/Color was found to be always lower than 25% of color composition.

The successful assessments of O₃ VFI are mainly dependent on the observer’s experience, and knowledge and color images such as photo guides of O₃ VFI are considered important tools, especially in field conditions where observation is required for several species and a large number of individuals. The validation of O₃ VFI is therefore crucial and must be conducted under experimental conditions and using microscopy observation (Vollenweider, Ottiger, and Gunthardt-Goerg 2003; Bussotti et al. 2005, 2006; Moura et al. 2018) to ascertain an O₃ VFI diagnosis by providing a mechanistic understanding of O₃ effect (Gunthardt-Goerg and Vollenweider 2007; Faoro and Iriti 2009). However, the new methodology proposed here based on color composition can informatize well-trained observer’s experience and digitalizes their visual information and knowledge of how to identify O₃ VFI. For example, the arithmetic manipulation of the RGB color channels has been used to detect a wide variety of plant disease symptoms (Barbedo 2017). Although further analyses for the specificity of O₃ VFI are still needed, a proper visual information for the O₃ VFI can be a simple informatic tool to establish an automated O₃ VFI identifier using a RGB image alternative to the traditional method of a time-consuming
validation by microscopy, thus improving the assessment and identification of O₃ VFI.

**Comparison of flux-based threshold for the ozone visible injury onset between the FO₃X and actual forests**

In the 2000s, several field studies reported that O₃ VFI was shown in forest trees when AOT40 reached 10 ppm h (Vollenweider et al. 2019; Vanderheyden et al. 2001). However, as Vanderheyden et al. (2001) pointed out for forest tree species in Switzerland, the AOT40 threshold showing O₃ VFI varied from year to year. In fact, O₃ damage is closely related to stomatal O₃ uptake rather than O₃ exposure only (CLRTAP, 2017; Watanabe et al. 2021). Therefore, a consensus has increased in the scientific community to recommend the flux-based O₃ risk assessment on forest trees (Paolletti et al. 2022) suggesting that an equivalent stomatal O₃ dose results in a similar O₃ damage over various species with a different sensitivity to O₃ (Reich 1987; Feng et al. 2018). In fact, the AOT40-based threshold for the onset of O₃ VFI for the O₃ resistant evergreen species, A. unedo, is 10-fold higher than that for the O₃ sensitive deciduous species, A. glutinosa. Interestingly, the flux-based threshold corresponding to the first symptom onset for A. unedo was rather similar to that for A. glutinosa. In fact, A. unedo, which has a low $g_{\text{max}}$ limiting stomatal O₃ uptake, showed O₃ VFI only in the autumn season, while A. glutinosa showed O₃ VFI in early summer even under ambient O₃ conditions because it shows a very high stomatal conductance and stomatal O₃ uptake easily exceeds the critical range of O₃ dose that can be detoxified.

According to the field observations in MOTTLES sites, Sicard et al. (2020) proposed flux-based thresholds for O₃ VFI of 5 and 12 mmol m⁻² POD₁ in dominant conifers and broadleaved trees, respectively, while 11 mmol m⁻² POD₁ was required for the presence of O₃ VFI in various sensitive tree species present within the LESS (Sicard et al. 2021). Although categorizing plant types is useful for setting the critical standard for forest protection, plant responses to O₃ are rather species-specific, as confirmed by the high flux-based threshold reported for Pinus halepensis (e.g., 8.2 mmol m⁻² POD₁) in Southeastern France (Sicard et al. 2016). At the FO₃X, the flux-based threshold for the O₃ VFI onset was ranged from 4.9 to 18.1 mmol m⁻² POD₁. It seems that the FO₃X-derived threshold also explained well the presence of O₃ VFI for A. glutinosa and Q. pubescens in the MOTTLES conditions. However, the threshold observed for the injury onset at the FO₃ X did not often match the presence of O₃ VFI in actual forests. The incidence of the VFI was lower for S. aucuparia and V. myrtillus in actual forests compared to the FO₃X condition. Indeed, S. aucuparia, and *V. myrtillus* showed O₃ VFI in early summer at FO₃ X even under AA conditions, while they were not always found symptomatic in actual forests. Also, for the less sensitive species such as *Q. robur*, very limited O₃ VFI were found under the MOTTLES conditions even though a relatively high POD₁ was observed (>20 mmol m⁻² POD₁). There is a possibility that $g_s$ of field plants would be different with that of pot plants. In fact, Beikircher et al. (2021) suggested a lower $g_s$ in field-grown mature *Acer pseudoplatanus* trees than in potted-seedlings. On contrary, Samuelson and Kelly (1997) indicate a greater $g_s$ in mature *Quercus rubra* trees than seedlings. Actually, a site-specific $g_s$ parameter may provide a more precise estimation of POD₁ in the MOTTLES sites. In addition, in a manipulative experiment, plants are usually potted, well-watered, isolated, and totally exposed to the atmospheric O₃ conditions; therefore, the higher incidence of symptoms can be related to the lack of stress compensation by an interaction of leaves within a canopy crown (Löw et al. 2006). Otherwise, a complex structure of forests with species mixtures may decrease herbivory, disease and other abiotic stresses, and increasing nutrient supply rates over the long term (Tilman, Isbell, and Cowles 2014). Interestingly, according to the multivariate analysis on MOTTLES datasets, O₃ VFI decreased with an increasing number of species in the LESS. LESS is a forest edge, and therefore it is generally characterized by a high number of species with different growth forms determining a multi-layered structure (Ranney, Bruner, and Levenson 2000; Łuczaj and Sadowska 1997). As a vertical O₃ gradient can be observed in a forest canopy (Ollinger, Aber, and Reich 1997), the presence of nonsensitive species in the upper layer can establish a protective mechanism for more sensitive species in the bottom layer. Such a hypothesis is in line with the assumption that mixed stands are more resilient to environmental stress than monospecific ones (Grossiord et al. 2014). Eichhorn et al. (2005) and Pollastrini et al. (2016) identified species diversity as a relevant factor that positively influences the crown conditions (i.e., reduced defoliation) in North European and Mediterranean forests.

Only *R. ulmifolius* showed the opposite behavior, i.e., it was symptomatic in the field even with a relatively low POD₁ below the level in which the VFI was observed at the FO₃X. Such differences can be explained by the strong competitive habit of the species for light (Gaudio, Balandier, and Marquier 2008). *R. ulmifolius* shrubs tend to overwhelm other species and, therefore, are more exposed to atmospheric conditions.

**Conclusions**

The results confirmed that a new generation free-air O₃ fumigation facility such as FO₃X is an appropriate tool
for the validation activities of field-observed O₃ VFI. New imaging analysis of color composition for O₃ VFI indicates that a major part of the colors were similar between the field and the FOₓX. Such digitized information will provide an advancement in the approach for O₃ VFI assessment from the bio-informatic point of view such as a mobile application as diagnostic tool for field scientists. A further calibration for estimating O₃ VFI by color composition (i.e., differences between O₃ VFI and other visible injury, color distribution in the leaves) is recommended for a more extensive range of O₃-sensitive species because other abiotic stress factors may sometimes mask the O₃ VFI.

The flux-based threshold for the O₃ VFI onset at the FOₓX was ranged from 4.9 to 18.1 mmol m⁻² POD₁. Although this FOₓX-derived threshold also explained the presence of O₃ VFI for A. glutinosa and Q. pubescens under real-world conditions, it did not always match with the observation for the other species. Q. robur, S. aucuparia and V. myrtillus were relatively resistant in actual forests rather than the FOₓX experiment, given that they were not visibly affected in the MOTTLES sites even though POD₁ exceeded the FOₓX-derived threshold. On the other hand, R. ulmifolius exhibited the O₃ VFI even at a relatively low POD₁. Although the mechanisms are still unknown, the multivariate analysis indicated an interaction of the presence of various species on O₃ VFI and suggested the importance of biodiversity and continuous monitoring activities in the field.

European forests are considerable areas of the world terrestrial biodiversity. In MOTTLES, we investigated 23 tree species. The ozone FACE (Free Air Controlled Exposure) experiments need to be expanded to more species to realize a proper and representative assessment of forest health under O₃ pollution.

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References

Alves, E. S., B. B. Moura, A. N. V. Pedroso, F. Tesmondo, and S. R. Machado. 2016. “Cellular Markers Indicative of Ozone Stress on Bioindicator Plants Growing in a Tropical Environment.” Ecological Indicators 67: 417–424. doi:10.1016/j.ecolind.2016.03.011.

Barbedo, J. G. A. 2017. “A New Automatic Method for Disease Symptom Segmentation in Digital Photographs of Plant Leaves.” European Journal of Plant Pathology 147 (2): 349–364. doi:10.1007/s10658-016-1007-6.

Barton, D. N., S. Kuikka, O. Varis, L. Uusitalo, H. J. Henriksen, M. Borsuk, A. D. L. Hera, R. Farmani, S. Johnson, and J. D. C. Linnell. 2012. “Bayesian Networks in Environmental and Resource Management.” Integrated Environmental Assessment and Management 8 (3): 418–429. doi:10.1002/ieam.1327.

Beikircher, B., L. Sack, A. Ganthaler, A. Losso, and S. Mayr. 2021. “Hydraulic-Stomatal Coordination in Tree Seedlings: Tight Correlation Across Environments and Ontogeny in Acer Pseudoplatanus.” The New Physiologist 232: 1297–1310. doi:10.1111/nph.17585.

Bičárová, S., Z. Sitková, H. Pavlendová, P. Bynerowicz, P. Fleischer, and A. Fleischer. 2019. “The Role of Environmental Factors in Ozone Uptake of Pinus Mugo Turra.” Atmospheric Pollution Research 10 (1): 283–293. doi:10.1016/j.apr.2018.08.003.

Büker, P., Z. Feng, J. Uddling, A. Briolat, R. Alonso, S. Braun, S. Elvira, et al. 2015. “New Flux Based Dose–Response Relationships for Ozone for European Forest Tree Species.” Environmental Pollution 206: 163–174. doi:10.1016/j.envpol.2015.06.033.

Bussotti, F., G. Agati, R. Desotgiu, P. Matteini, and C. Tani. 2005. “Ozone Foliar Symptoms in Woody Plant Species Assessed with Ultrastructural and Fluorescence Analysis.” The New Physiologist 166 (3): 941–955. doi:10.1111/j.1469-8137.2005.01385.x.

Bussotti, F. and M. Ferretti. 2009. “Visible Injury, Crown Condition, and Growth Responses of Selected Italian Forests in Relation to Ozone Exposure.” Environmental Pollution 157 (5): 1427–1437. doi:10.1016/j.envpol.2008.09.034.

Bussotti, F., M. Schaub, A. Cozzi, G. Gerosa, K. Novak, and C. Hug. 2006. “Sources of Errors in Assessing Ozone Visible Symptoms on Native Vegetation.” Environmental Pollution 140 (2): 257–268. doi:10.1016/j.envpol.2005.07.012.

Bussotti, F., M. Schaub, A. Cozzi, K. Krauchi, M. Ferretti, K. Novak, and J. M. Skelly. 2003. “Assessment of Ozone Visible Symptoms in the Field: Perspectives of Quality Control.” Environmental Pollution 125 (1): 81–89. doi:10.1016/S0269-7491(03)00095-2.

Calatayud, V., F. Marco, J. Cerveró, G. Sánchez-Peña, and M. J. Sanz. 2010. “Comparing Ozone Sensitivity in Related Evergreen and Deciduous Shrubs.” Environmental Pollution 158 (12): 3580–3587. doi:10.1016/j.envpol.2010.08.013.

CLRTAP. 2017. Mapping Critical Levels for Vegetation, Chapter III. Manual on Methodologies and Criteria for Modelling and Mapping Critical Loads and Levels and Air Pollution Effects, Risks and Trends. In UNECE Convention on Long-Range Transboundary Air Pollution. Geneva, Switzerland: UNECE.
Dalstein, L. N. Vas, F. Tagliferro, A. Ferrara, and F. Spaziani. 2005. “Effets de l’ozone sur la forêt et la végétation dans les alpes Franco-Italiennes.” *Forêt Méditerranéenne, Forêt Méditerranéenne XXVII* (2): 149–156.

de Marco, A., C. Proietti, A. Anay, L. Ciancarella, I. D’elia, S. Fares, M. F. Fornasier, et al. 2019. “Impacts of Air Pollution on Human and Ecosystem Health, and Implications for the National Emission Ceilings Directive: Insights from Italy.” *Environment International* 125: 330–333. doi:10.1016/j.envint.2019.01.064.

Eichhorn, J., R. Icke, A. Isenberg, U. Paar, and E. Schönfelder. 2005. “Temporal Development of Crown Condition of Beech and Oak as a Response Variable for Integrated Evaluations.” *European Journal of Forest Research* 124 (4): 335–347. doi:10.1007/s10342-005-0097-z.

Elvira, S., E. Bermejo, E. Manrique, and B. S. Gimen. 2004. “On the Response of Two Populations of Quercus Coccifera to Ozone and Its Relationship with Ozone Uptake.” *Atmospheric Environment* 38 (15): 2305–2311.

European Parliament and Council DIRECTIVE (EU) 2016/2284 on the Reduction of National Emissions of Certain Atmospheric Pollutants. *Official Journal of the European Union*. 2016.344. https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A52016DC0266.

Faoro, F., and M. Iriti. 2009. “Plant Cell Death and Cellular Alterations Induced by Ozone: Key Studies in Mediterranean Conditions.” *Environmental Pollution* 157 (5): 1470–1477. doi:10.1016/j.envpol.2008.09.026.

Feng, Z., P. Bükker, H. Pleijel, L. Emberson, P. E. Karlsson, and J. Uddling. 2018. “A Unifying Explanation for Variation in Ozone Sensitivity Among Woody Plants.” *Global Change Biology* 24 (1): 78–84. doi:10.1111/gcb.13824.

Feng, Z. J. Sun, W. Wan, E. Hu, and V. Calatayud. 2014. “Evidence of Widespread Ozone-Induced Visible Injury on Plants in Beijing, China.” *Environmental Pollution* 193: 296–301. doi:10.1016/j.envpol.2014.06.004.

Gaudio, N., P. Balandier, and A. Marquier. 2008. “Croissance de Deux Espèces Compétitrices (Rubus Idaeus, Cytisus Scoparius) Colonisatrices des Trouées En Forêt Tempérée selon la Disponibilité En Lumière.” *Annals of Forest Science* 65 (1): 104. doi:10.1051/forest:2007076.

Grossiord, C., A. Granier, S. Ratcliff, O. Bouriaud, H. Bruelheide, E. Checko, D. I. Forrester, et al. 2014. “Tree Diversity Does Not Always Improve Resistance of Forest Ecosystems to Drought.” *Proceedings of the National Academy of Sciences* 111 (41): 14812–14815. doi:10.1073/pnas.1411970111.

Guerrero, C. C., M. S. Günthardt-Goerg, P. Vollenweider, and D. Ballhorn. 2013. “Foliar Symptoms Triggered by Ozone Stress in Irrigated Holm Oaks from the City of Madrid, Spain.” *PLoS One* 8 (7): e69917. doi:10.1371/journal.pone.0069171.

Günthardt-Goerg, M. S., and P. Vollenweider. 2007. “Linking Stress with Macroscopic and Microscopic Leaf Response in Trees: New Diagnostic Perspectives.” *Environmental Pollution* 147 (3): 467–488. doi:10.1016/j.envpol.2006.08.033.

Hoshika, Y., E. Carrari, B. Mariotti, S. Martini, A. D. Marco, P. Sicard, and E. Paoletti. 2020. “Flux-Based Ozone Risk Assessment for a Plant Injury Index (PII) in Three European Cool-Temperate Deciduous Tree Species.” *Forests* 11 (1): 82. doi:10.3390/f11010082.

Hoshika, Y., E. Carrari, L. Zhang, G. Carrieri, S. Pignatelli, G. Fasano, A. Materassi, and E. Paoletti. 2018. “Testing a Ratio of Photosynthesis to O3 Uptake as an Index for Assessing O3-Induced Foliar Visible Injury in Poplar Trees.” *Environmental Science and Pollution Research* 25: 8113–8124. doi:10.1007/s11356-017-9475-6.

Hoshika, Y., E. Paoletti, E. Agathokleous, T. Sugai, and T. Koike. 2020. “Developing Ozone Risk Assessment for Larch Species.” *Frontiers in Forests and Global Change* 3. doi:10.3389/fgc.2020.00045.

Hoshika, Y., E. Paoletti, and K. Omasa. 2012. “Parameterization of Zelkova serrata stomatal conductance model to estimate stomatal ozone uptake in Japan.” *Atmospheric Environment* 55: 271–278. doi:10.1016/j.atmosenv.2012.02.083.

Hoshika, Y., F. Pecori, I. Conese, T. Bardelli, E. Marchi, W. J. Manning, O. Badea, and E. Paoletti. 2013. “Effects of a Three-Year Exposure to Ambient Ozone on Biomass Allocation in Poplar Using EthyleneUrea.” *Environmental Pollution* 180: 299–303. doi:10.1016/j.envpol.2013.05.041.

Innes, J. L., J. M. Skelly, and M. Schaub. 2001. “Ozone and Broadleaved Species: A Guide to the Identification of Ozone-Induced Foliar Injury/Ozon.” In Laubholz- Und Krautpfanizen: Ein Fuhrer Zum Bestimmen von Oszymptoms; *Birmensdorf, Eidgossnische Forschungsanstalt WSL Haupt*. 136, Bern, Stuttgart, Wien: Haupt (January 1, 2001).

Lefohn, A. S., C. S. Malley, L. Smith, B. Wells, M. Hazucha, H. Simon, V. Naik, et al. 2018. “Tropospheric Ozone Assessment Report: Global Ozone Metrics for Climate Change, Human Health, and Crop/Ecosystem Research.” *Elementa: Science of the Anthropocene* 6. doi:10.1525/elementa.279.

Löw, M., K. Herberinger, A. J. Nunn, K. H. Häberle, M. Leuchner, C. Heerdt, H. Werner, et al. 2006. “Extraordinary Drought of 2003 Overrules Ozone Impact on Adult Beech Trees (Fagus Sylvatica).” *Trees - Structure and Function* 20 (5): 539–548. doi:10.1007/s00468-006-0059-z.

Łuczaj, Ł., and B. Sadowska. 1997. “Edge Effect in Different Groups of Organisms: Vascular Plant, Bryophyte and Fungi Species Richness Across a Forest-Grassland Border.” *Folia Geobotanica Et Phytotaxonomica* 32 (4): 343–353. doi:10.1007/BF02821940.

Moura, B. B., E. S. Alves, M. A. Marabesi, S. R. de Souza, M. Schaub, and P. Vollenweider. 2018. “Ozone Affects Leaf Physiology and Causes Injury to Foliage of Native Tree Species from the Tropical Atlantic Forest of Southern Brazil.” *The Science of the Total Environment* 610–611: 912–925. doi:10.1016/j.scitotenv.2017.08.130.

Nguy-Robertson, A., Y. Peng, T. Arkebauer, D. Scoby, J. Schepers, and A. Gitelson. 2015. “Using a Simple Leaf Color Chart to Estimate Leaf and Canopy Chlorophyll a Content in Maize (Zea Mays).” *Communications in Soil Science and Plant Analysis* 46 (21): 2734–2745. doi:10.1080/00103624.2015.1093639.

Nussbaum, S., and J. Fuhrer. 2000. “Difference in Ozone Uptake in Grassland Species Between Open-Top Chambers and Ambient Air.” *Environmental Pollution* 19 (3): 463–471.

Ollinger, S. V., J. D. Aber, and P. B. Reich. 1997. “Simulating Ozone Effects on Forest Productivity: Interactions Among Leaf-, Canopy-, and Stand-Level Processes.” *Ecological Applications* 7 (4): 1237–1251. doi:10.1890/1051-0761(1997)007[1237:SOOEF2.0.CO;2.

Paoletti, E. A., A. Allivernini, A. Anav, O. Badea, E. Carrari, S. Chivulescu, A. Conte, et al. 2019. “Toward Stomatal-Flux Based Forest Protection Against Ozone: The MOTTLES Approach.” *The Science of the Total Environment* 691: 516–527. doi:10.1016/j.scitotenv.2019.06.025.

Paoletti, E. G. Carrario, G. Fasano, Y. Hoshika, G. Carrier, D. Silaghi, and O. Badea. 2017. “A New-Generation 3D
Ozone FACE (Free Air Controlled Exposure). Science of the Total Environment." The Science of the Total Environment 2016; 21–25. doi:10.1016/j.scitotenv.2016.09.217.

Paoletti, E., M. Schaub, R. Matyssek, G. Wieser, A. Augustitis, A. M. Bastrup-Birk, A. Bytnerowicz, M. S. Günthardt-Goerg, G. Müller-Starck, and Y. Serengil. 2010. “Advances of Air Pollution Science: From Forest Decline to Multiple-Stress Effects on Forest Ecosystem Services.” Environmental Pollution 158 (6): 1986–1989. doi:10.1016/j.envpol.2009.11.023.

Paoletti, E., P. Sicard, Y. Hoshika, S. Fares, O. Badea, D. Pitar, I. Popa, A, Anav, B. B. Moura, and A. de Marco. 2022. “Towards Long-Term Sustainability of Stomatal Ozone Flux Monitoring at Forest Sites.” Sustainable Horizons 2: 100018. doi:10.1016/horiz.2022.100018.

Pollastrini, M., M. Fediucci, D. Bonal, M. Fotelli, A. Gessler, C. Grossiord, V. Guyot, et al. 2016. “Physiological Significance of Forest Tree Defoliation: Results from a Survey in a Mixed Forest in Tuscany (Central Italy).” Forest Ecology and Management 361: 170–178. doi:10.1016/j.foreco.2015.11.018.

Ranney, J. W., M. C. Bruner, and J. B. Levenson. The Importance of Edge in the Structure and Dynamics of Forest Islands. Forest Island Dynamics in Man-Dominated Landscapes 1981.10.1007/978-1-4612-5936-7_6

R Core Team. 2018. A Language and Environment for Statistical Computing. Vienna, Austria: R Foundation for Statistical Computing. https://cran.r-project.org/doc/FAQ/R-FAQ.html#Citing-R

Reich, P. B. 1987. “Quantifying Plant Response to Ozone: A Unifying Theory.” Tree Physiology 3: 63–91. doi:10.1093/treephys/3.1.63.

Samuelson, L. J., and J. M. Kelly. 1997. “Ozone Uptake in Prunus Serotina, Acer Rubrum and Quercus Rubra Forest Trees of Different Sizes.” The New Phytologist 136: 255–264. doi:10.1046/j.1469-8137.1997.00734.x.

Schaub, M., and V. Calatayud. 2013. “Assessment of Visible Foliar Injury Induced by Ozone.” Developments in Environmental Science 12: 205–221.

Schaub, M., V. Calatayud, M. Ferretti, G. Brunialti, G. Lövblad, G. S. M. Krause, and M. J. Sanz. 2016. “Part VIII: Monitoring of Ozone Injury.” In Manual on Methods and Criteria for Harmonized Sampling, Assessment, Monitoring and Analysis of the Effects of Air Pollution on Forests; UNECE ICP Forests Programme Coordinating Centre, ed. UNECE ICP Forests Programme Coordinating Centre, 14. Eberswalde, Germany: Thünen Institute of Forest Ecosystems.

Sicard, P., A. de Marco, E. Carrari, L. Dalstein-Richier, Y. Hoshika, O. Badea, D. Pitar, et al. 2020. “Epidemiological Derivation of Flux-Based Critical Levels for Visible Ozone Injury in European Forests.” Journal of Forestry Research 31 (5): 1509–1519. doi:10.1007/s11676-020-01191-x.

Sicard, P., A. de Marco, L. Dalstein-Richier, F. Tagliaferro, C. Renou, and E. Paoletti. 2016. “An Epidemiological Assessment of Stomatal Ozone Flux-Based Critical Levels for Visible Ozone Injury in Southern European Forests.” The Science of the Total Environment 541. doi:10.1016/j.scitotenv.2015.09.113.

Sicard, P., Y. Hoshika, E. Carrari, A. de Marco, and E. Paoletti. 2021. “Testing Visible Ozone Injury Within a Light Exposed Sampling Site as a Proxy for Ozone Risk Assessment for European Forests.” Journal of Forestry Research 32 (4): 1351–1359. doi:10.11766/0269-7491(00)00060-9.

Vollenweider, P., and M. Günthardt-Goerg. 2006. “Erratum to “Diagnosis of Abiotic and Biotic Stress Factors Using the Visible Symptoms in Foliage” [Environ. Pollut. 137 (2005) 455–465].” Environmental Pollution 140 (140): 562–571. doi:10.1016/j.envpol.2006.01.002.

Vollenweider, P., M. S. Günthardt-Goerg, T. Menard, M. Baumgarten, R. Matyssek, and M. Schaub. 2019. “Macro- and Microscopic Leaf Injury Triggered by Ozone Stress in Beech Foliage (Fagus Sylvatica L.).” Annals of Forest Science 76 (3). doi:10.1007/s13595-019-0856-5.

Vollenweider, P., M. Ottiger, and M. S. Günthardt-Goerg. 2003. “Validation of Leaf Ozone Symptoms in Natural Vegetation Using Microscopic Methods.” Environmental Pollution 124 (1): 101–118. doi:10.1016/S0269-7491(02)00412-8.

Watanabe, M., E. Agathokleous, A. Anav, V. Araminiene, E. Carrari, A. De Marco, Y. Hoshika, C. Proietti, P. Sicard, and E. Paoletti. “Impacts of Ozone on the Ecophysiology of Forest Tree Species.” In Tropospheric Ozone - A Hazard for Vegetation and Human Health, edited by S. B. Agrawal, M. Agrawal, A. Singh, S. B. Agrawal, M. Agrawal, and A. Singh 277–306. Newcastle, UK: Publishing, Cambridge Scholars. 2021.