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Marco Ferretti[a,*,1], Maxime Cailleret[a,b,1], Matthias Haeni[a], Volodymyr Trotsiuk[a], Vladislav Apuhtin[c], Valda Araminiene[d], Václav Buriánek[e], Sébastien Cecchini[f], Laurence Dalstein-Richier[g], Iva Hůnová[h], Tamara Jakovljević[i], Konstantinos Kaoukis[j], Johan Neirynck[k], Manuel Nicolas[f], Anne-Katrin Prescher[l], Radek Novotný[e], Hana Pavlendova[m], Nenad Potočić[i], Matej Rupel[n], Alexander Russ[o], Vidas Stakėnas[d], Arne Verstraeten[k], Pierre Vollenweider[a], Daniel Zlindra[n], Diana Pitar[p], Vicent Calatayud[q], Elena Gottardini[r,2], Marcus Schaub[a,2]

[a] Swiss Federal Institute for Forest, Snow and Landscape Research WSL, Zürcherstrasse 111, 8903 Birmensdorf, Switzerland
[b] INRAE, Aix-Marseille University, UMR RECOVER, 3275 route de Cézanne, CS 40061, F- 13182 Aix-en-Provence Cedex 5, France
[c] Estonian Environment Agency, Mustamäe tee 33, Tallinn 10616, Estonia
[d] Lithuanian Research Centre for Agriculture and Forestry, Institute of Forestry, Liepu st. 1, LT-53101 Girionys, Lithuania
[e] Forestry and Game Management Research Institute (FGMRI), Strnady 136, 252 02 Jíloviště, Czechia
[f] Office National des Forêts, Département recherche, développement, innovation - Bâtiment B, Boulevard de Constance, 77300 Fontainebleau, France
[g] HDM, 18 rue Rubens, Paris 75013, France
[h] Czech Hydrometeorological Institute, Na Šabatce 17, 143 06 Prague 4, Czechia
[i] Croatian Forest Research Institute CFRI Cvjetno naselje 41, 10450 Jastrebarsko, Croatia
[j] Hellenic Agricultural Organization "DEMETER", Terma Alkmanos, 11528 Ilissia, Athens, Greece
[k] Research Institute for Nature and Forest (INBO), Gaverstraat 4, 9500 Geraardsbergen, Belgium
[l] Thünen Institute of Forest Ecosystems, Alfred-Möller-Str. 1, 16225 Eberswalde, Germany
[m] National Forest Centre - Forest Research Institute, T.G. Masaryka 22, 960 01 Zvolen, Slovakia
[n] Slovenian Forestry Institute (SFI), Večna pot 2, 1000 Ljubljana, Slovenia
[o] State Forestry Research Centre Eberswalde (LFE), Alfred-Möller-Str. 1, 16225 Eberswalde, Germany
[p] National Institute for Research and Development in Forestry "Marin Drăcea" (INCDS), Bd. Eroilor 128, 077190 Voluntari, Judetul Ilfov, Romania
[q] Fundación CEAM, Parque Tecnológico C/ Charles R. Darwin, 14 46980, Paterna, Valencia, Spain
[r] Research and Innovation Centre, Fondazione Edmund Mach (FEM), Via E. Mach 1, 38010 San Michele all'Adige, Italy

A R T I C L E I N F O

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A B S T R A C T

Tropospheric ozone (O_3) increased globally in the 20th century, contributes to climate change and can have adverse effects on terrestrial ecosystems. The response of forest vegetation to ozone is modulated by species- and site-specific factors and visible foliar symptoms (VFS) are the only direct evidence of ozone effects on vegetation. VFS have been observed and reproduced under (semi-) controlled conditions and their field assessment has been largely harmonized in Europe. We analyzed ozone concentration and VFS data as measured at (respectively) 118 and 91 intensive monitoring sites of the International Co-Operative Programme on Assessment and Monitoring of

* Corresponding author.
E-mail addresses: marco.ferretti@wsl.ch (M. Ferretti), maxime.cailleret@inrae.fr (M. Cailleret), matthias.haeni@wsl.ch (M. Haeni), volodymyr.trotsiuk@wsl.ch (V. Trotsiuk), vladislav.apuhtin@envir.ee (V. Apuhtin), valda.araminiene@lammc.lt (V. Araminiene), burianek@vulhm.cz (V. Buriánek), sebastien.cecchini@onf.fr (S. Cecchini), ldalstein@aol.com (L. Dalstein-Richier), iva.hunova@chmi.cz (I. Hůnová), tamaraj@sumins.hr (T. Jakovljević), kako@fria.gr (K. Kaoukis), johan.neirynck@inbo.be (J. Neirynck), manuel.nicolas@onf.fr (M. Nicolas), anne.prescher@thuenen.de (A.-K. Prescher), novotny@vulhm.cz (R. Novotný), hana.pavlendova@nlcsk.org (H. Pavlendova), nenadp@sumins.hr (N. Potočić), matej.rupel@gozdis.si (M. Rupel), alexander.russ@lfb.brandenburg.de (A. Russ), vidas.stakenas@lammc.lt (V. Stakėnas), arne.verstraeten@inbo.be (A. Verstraeten), pierre.vollenweider@wsl.ch (P. Vollenweider), daniel.zlindra@gozdis.si (D. Zlindra), Diana.Silaghi@icas.ro (D. Pitar), calatayud_viclor@gva.es (V. Calatayud), elena.gottardini@fmach.it (E. Gottardini), marcus.schaub@wsl.ch (M. Schaub).
[1] Shared first author role.
[2] Shared last author role.

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Air Pollution Effects on Forests (ICP Forests) spanning over five European biogeographic regions from 2005 to 2018. Average values for VFS were calculated accounting for the number of species present and their observed frequency. Spatial and temporal variation of ozone concentrations, VFS, and their relationships across Europe were then investigated by applying Generalized Linear Mixed Models (GLMMs) and combined GLMMs. Ozone concentrations exceeded 40 ppb on 37.3 % of the sites and were significantly higher ($p < 0.05$) in the Alpine and the Mediterranean regions. Over the 2005–2018 period there was a substantial stagnation of ozone concentrations with a tendency towards decreasing values in the Alpine-Boreal sites and increasing values in the Atlantic sites. Ozone left a "fingerprint" in terms of VFS on 38 % of the observed broadleaved woody species across Europe, with no significant difference among biogeographic regions. Overall, and again with the exception of an increase at the Atlantic sites, the frequency of VFS remained unchanged or has been slightly declining over the investigated period. We found positive relationship between ozone concentrations and VFS across Europe ($p < 0.05$), while their temporal trends (both insignificant) were not related. The species with the highest frequency of VFS were those classified as sensitive species under controlled/semi-controlled experimental conditions. Frequency of VFS tends to be modulated by vegetation traits such as specific leaf area and leaf thickness ($p < 0.10$). Our results showed that, although ozone levels suggested a North-to-South gradient of increasing potential risk to vegetation with hot spots in the Alps and in the Mediterranean, VFS observed on the actual species assemblage at the sites modifies this picture. According to frequency of VFS, ozone risk for vegetation may be higher in parts of the Alpine and Continental Europe than in the Mediterranean region.

1. Introduction

1.1. Tropospheric ozone and vegetation

Increased emissions of ozone (O_3) precursors such as nitrogen oxides (NOx), carbon monoxide (CO), and volatile organic compounds (VOC) during the 20th century led to an augmented net chemical production of ozone in the troposphere (The Royal Society, 2008), with a global increase of ozone concentrations (Vingarzan, 2004; Cooper et al., 2014). In Europe, concentrations nearly doubled in rural environments, moving from about 10 –15 ppb at the end of the 19th century to 20–30 ppb in the 1980s (Volz and Kley, 1988; Vingarzan, 2004), with subsequent changes over the last decades more differentiated by regions (Staehelin and Poberaj, 2008; Torseth et al., 2012; Cooper et al., 2014; Yan et al., 2018; EMEP, 2021) and largely driven by meteorological variability (e.g., EEA - European Environment Agency, 2020). While there is evidence that annual mean ozone concentrations in Europe are more influenced by emissions from non-European sources due to intercontinental transport, high concentrations in summer are mainly influenced by precursors emitted in European countries (Nisbet et al., 2019; Jonson et al., 2018). There, despite the considerable reduction in precursor emission in Europe and the evidence that mean summer concentrations have leveled off or decreased slightly since the year 2000 (EEA - European Environment Agency, 2020), ozone exposures in many rural and remote sites continue to be potentially harmful for forest vegetation (Schaub et al., 2018).

Ozone is a strong oxidant, a greenhouse gas and can cause a variety of detrimental effects on forest vegetation, including foliar symptoms (e.g., Novak et al., 2003), effects at physiological and bio-chemical level (e.g. Rao and Davis, 2001; Hůnová et al., 2010), reduction in productivity (e.g., Ashmore, 2005; Braun et al., 2022; Innes et al., 2001; Karlsson et al., 2007; Matyssek et al., 2007; Šrámek et al., 2012; but see Cailleret et al., 2018) and may ultimately affect the entire ecosystem (e.g. Agathokleous et al., 2020). All these effects have been observed at ozone concentrations that are common under ambient conditions (e.g., Wittig et al., 2009). Much attention has been paid to potential ozone effects on tree growth due to its relation with wood production, its implications for carbon sequestration and storage (e.g., Felzer et al., 2004; Unger et al., 2020) and the ease of its measurement through forest inventory and dendroecological methods. Yet, tree growth is a rather unspecific response indicator as it is driven by a variety of interacting past- and present, biotic and abiotic factors which are difficult to disentangle when moving from experiments with individual seedlings and saplings to real forests with mature trees (Cailleret et al., 2018). In field-based observational studies, disentangling the effect of ozone is generally attempted by statistical modelling, where - for example - the overall variance is partitioned among the selected predictors. In this context, however, ecosystem dynamics and ageing processes, interactions among environmental and tree-related factors, and competition among species and individuals can substantially alter the dose–response relationship derived from controlled experiments with individual juvenile trees (e.g., Cailleret et al., 2018; Ferretti et al., 2018; Wang et al., 2016). This may potentially offset the impact of ozone (e.g., Etzold et al., 2020, but see Braun et al., 2022). Even when considering biochemical and physiological processes in plants, there is no specific indicator of ozone impact: ozone leaves no elemental residue in plant tissues that can be detected by analytical techniques.

1.2. Ozone induced visible foliar symptoms

For these reasons, and despite some inherent limitations (e.g., Bussotti et al., 2003, 2006a, b) ozone-induced visible injury on foliage (hereafter referred to as Visible Foliar Symptoms, VFS) is considered to be the only readily detectable, direct indicator of ozone impact on vegetation (e.g., Benham et al., 2010; Gottardini et al., 2018; Innes et al., 2001; Matoušková et al., 2010; Novak et al., 2003; VanderHeyden et al., 2001; Moura et al., 2022). Indeed, ozone causes highly specific injury by inducing hypersensitive responses-like (HR-like) in light-exposed cells of leaf mesophyll, in addition to unspecific degenerative alterations in the structure of assimilative cells (e.g. Günthardt-Goerg and Vollenweider, 2007; Turc et al., 2023; Vollenweider et al., 2019). The occurrence of ozone-induced VFS has been known since a long time for a variety of species (e.g., Haagen-Smit, 1958; Manning et al., 1970; Heggestad, 1991; Ghosh et al., 1998; Skelly et al., 1999) and VFS assessment has been incorporated into large-scale forest monitoring programs since the 1990s (Smith et al., 2003; Schaub et al., 2018). On broadleaved vegetation, which is considered most sensitive to ozone and for which several experimental results are available (e.g., Novak et al., 2003; VanderHeyden et al., 2001; Moura et al., 2022), VFS are typically interveinal, with thin light-green, reddish or dark-brown spots (stipple) on the upper leaf surface, and are more severe on older and light-exposed leaves (age and shade effects, respectively; see Innes et al., 2001) (Fig. 1). While it is known that adverse effects on growth and physiology can occur before the onset of VFS (e.g., Gravano et al., 2003; Turc et al., 2021), such effects are difficult to be observed in the field and remain in general undetected. As any other visual measurement/observation, VFS assessment is subject to errors (misclassification, subjectivity, e.g. Bussotti et al., 2003; 2006) and appropriate Quality Assurance/Quality Control procedures are therefore needed (see below).

1.3. Differentiated VFS response

Besides ozone concentration, the expression of VFS at a given site is known to be modulated by several factors related to vegetation (species composition, inter- and intra-specific variability), atmosphere (e.g., Vapour Pressure Deficit) and soil (e.g., soil moisture). As for vegetation, species diversity, plant traits, morphological characteristics, detoxification capacity, and microclimate can all play a considerable role in modulating the expression of VFS (e.g., Bussotti, 2008; Bussotti and Ferretti, 2009; Bussotti et al., 2005; Faralli et al., 2022; Moura et al., 2022; Novak et al., 2005; Schaub et al., 2003), even for the same species at a given site (Faralli et al., 2022). We can therefore expect that the same species exposed to similar ozone concentrations at different sites may not always display the same frequency of VFS. In the same line, different species exposed at similar ozone concentrations at the same site may also have differentiated VFS responses depending on their functional traits. Under this perspective, the expression of VFS can integrate the various factors mentioned above, and – therefore - the occurrence of VFS can be considered as a sort of "fingerprint", a useful and ecologically meaningful indicator for detecting the risk that ozone may pose to the forest vegetation at a given site.

Here we seek answer to the following three categories of questions:

(i) Ozone levels: what are the ozone concentrations at European forest sites during the growing season? Is there any significant change over time? How does this trend vary across Europe?
(ii) VFS development: how frequent is the occurrence of VFS at European forest sites and for different species? Is there any significant change over time? How does this trend vary across Europe?
(iii) Relationship between ozone exposure, VFS, and species: what is the relationship between the frequency of VFS and ozone concentrations over time and space? Which species show the highest frequency of VFS and are most sensitive to ozone? Is there a link between frequency of VFS and plant traits?

To address the above questions at the large-scale and over the long-term, harmonized and co-located measurements of ozone concentrations and VFS are a crucial precondition. Although there is always the option to use modeled ozone concentrations and fluxes (Simpson et al., 2012), this has been considered sub-optimal: for example, Gottardini et al. (2010b) found that, although ozone concentrations (a key measurement in whatever risk assessment approach adopted) showed limited variability at the 1 km^2 scale, they showed significant differences at larger scales (e.g., 10^2 to 10^3 km^2) typical of ozone concentrations and flux models. Hence, when downscaled to individual sites, large-scale model estimates may introduce considerable noise in the analysis. The intensive monitoring (Level II) sites installed under the framework of the United Nation Economic Commission for Europe (UNECE) International Co-operative Programme on Assessment and Monitoring of Air Pollution Effects on Forests (ICP Forests, see http://icp-forests.net) are – to our knowledge – the only set of sites responding to the characteristics of co-location, harmonization, large-scale and long-term coverage mentioned above. At these sites, measurements of ozone concentrations and observation of VFS on woody plant species were carried out according to Standard Operating Procedures (SOPs; Schaub et al., 2020a, b) (see below). We used the data collected at the ICP Forests Level II sites to conduct a European-scale study on ozone concentration and VFS over the period 2005–2018. To do so, we accessed data obtained from 222 sites distributed in 20 countries, representing a wide range of plant

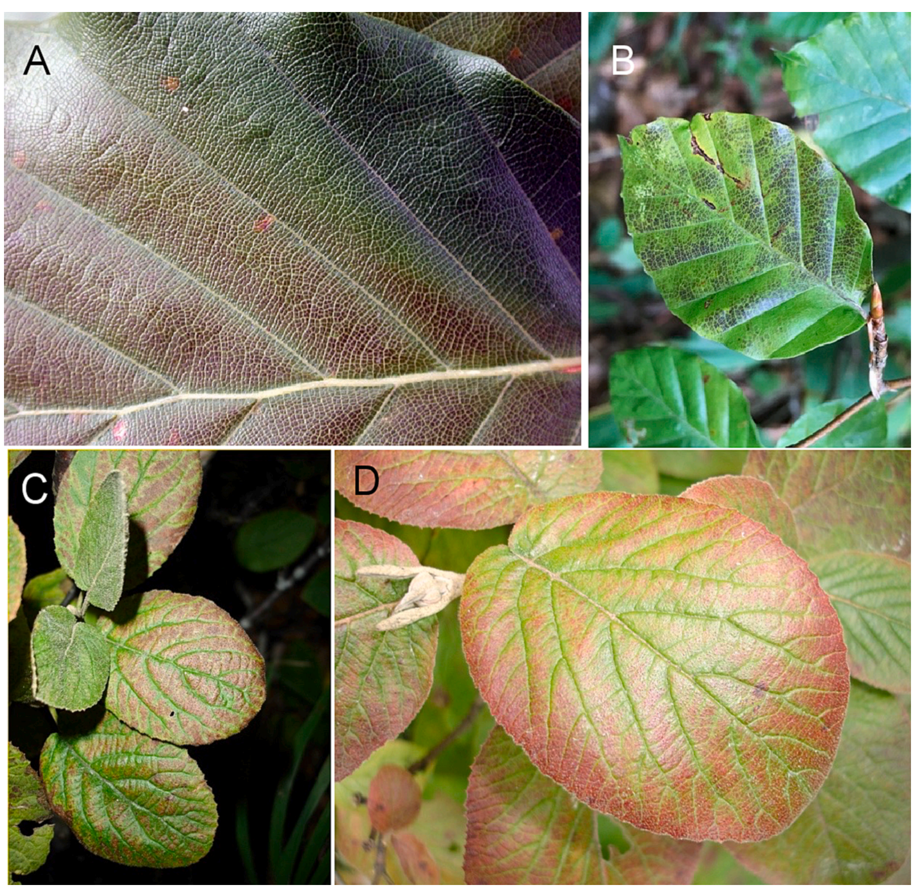


Fig. 1. Typical examples of ozone induced VFS. Beech (Fagus sylvatica, top) and Wayfaring tree (Viburnum lantana, bottom) plants encountered during field surveys (A, C) and in experiments under semi-controlled conditions (B, D).

species, biogeographic regions, and differing exposures to ozone that have occurred over the past decades. We deliberately excluded all measurements carried out between 2000 and 2004 as these early survey years were mostly dedicated to adjusting the methodology and providing trainings to field crews across Europe (see Table A1, Figure A1). While VFS attributable to ozone have been identified and reported also for conifers (e.g. Shashikumar et al., 2023), here we concentrated on broadleaved vegetation because this was particularly in focus during the training and intercalibration sessions carried out over the years. We used a series of Generalized Linear Mixed Models (GLMMs) and we then combined GLMMs results to account for multiple and counteracting pathways and non-linearity. To our knowledge, when considering its spatial, temporal, and ecological domains, this is the largest study of this type carried out in Europe. We expect that our findings will allow to identify temporal and spatial patterns of ozone concentrations and VFS, and to detect potential areas at risk for ozone impact on forest vegetation.

2. Materials and methods

2.1. Field measurements and leaf traits

Measurements of ozone concentration and VFS were carried out at ICP Forests Level II sites. Level II sites were originally selected on a purposive basis to cover the most frequent European forest types. They consist of a forest plot, a buffer zone and an open-field plot, and include in-plot and off-plot measurements (Ferretti et al., 2020). Off-plot measurements are typically carried out in an open area close to the very plot allowing proper conditions for recording meteorological variables, deposition chemistry, ozone concentrations and VFS (Schaub et al., 2020a, b). Ozone concentrations were measured by passive sampling on weekly or bi-weekly basis during the period April-September (Schaub et al., 2020b). Passive samplers were installed in the Level II off-plot open areas, at 2 m above soil level. Data are reported as parts per billion (ppb; 1 ppb = 1.962 μg m^{-3} at the standard conditions of 25 °C and 1′013 mbar). Individual countries were free to use different manufactures and methodologies for passive sampling, provided that quality assurance procedures were applied, in order to ensure the comparability of data. Thus, data were checked for plausibility (lower limit: 5 μg m^{-3} = 2.55 ppb; upper limit: 200 μg m^{-3} = 101.94 ppb), completeness (80 % within the April-September measurement period) and variability among replicates; in addition, data from active samplers and blanks were used for data validation (e.g., Sanz et al., 2007). After filtering, ozone concentration data considered for this study were those collected from 2005 to 2018 at a total of 195 plots located in 20 countries, for a total of 14,246 ozone concentration values (see below).

VFS were measured along the forest edge facing the Level II off-plot measurement sites (Schaub et al., 2020a). Here, Light Exposed Sampling

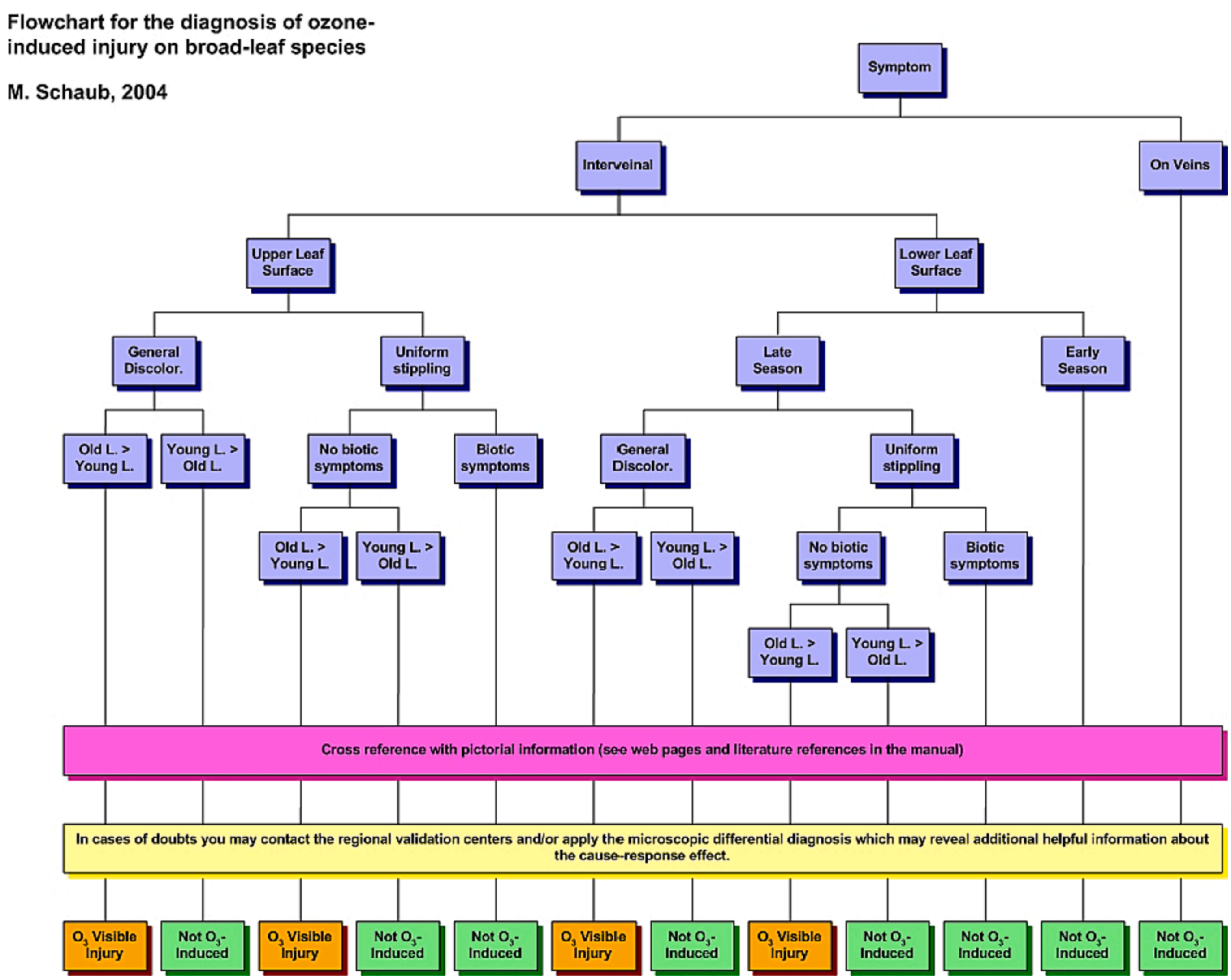


Fig. 2. Flowchart for the diagnosis of ozone symptoms on broad-leaf species (from Schaub et al., 2020a).

Sites (LESS) were established nearby following a randomized design. A LESS consists of a number of 2 x 1 m quadrates randomly selected along the forest edge. The number of quadrates depends on the size (length) of the forest edge and the accepted sampling error. Details on how to calculate the number of quadrates and conduct the assessment are outlined in Schaub et al., (2020a). Assessment within the LESS is carried out to provide estimates of VFS frequency on vegetation at the forest edge closest to ozone measurement device. For this purpose, (i) the woody species (tree, shrub and vines) for each quadrate of the LESS are identified; (ii) individuals of each species within each quadrate are observed for the presence/absence of VFS following a well-established flowchart (Schaub et al., 2020a; Fig. 2). VFS assessment has been carried out once a year during late summer and before natural leaf discoloration. Overall, the dataset on VFS collected between 2005 and 2018 consists of 46,677 records collected at a total of 162 plots located in 18 countries.

Data on Specific Leaf Area (SLA) and Leaf Thickness (LT) for all woody species considered were obtained from TRY Database (Kattge et al., 2020).

2.2. Quality assurance

Standard QA/QC procedures of the ICP Forests have been adopted (Schaub et al., 2020a, b). Intercalibration courses on the assessment of VFS have been implemented on a routine basis among national experts to check and document data quality across Europe and over the period examined (e.g., Bussotti et al., 2003; Gottardini et al., 2019) (see Table A1, Figure A1), with most of the involved countries attending. Although uncertainty and subjectivity cannot be completely eliminated, they can be controlled: early results from European field crews suggest that Data Quality Limits set by Schaub et al., (2020a) were achieved in most cases (Ferretti et al., 2013; Gottardini et al., 2019). Additional VFS validation procedures, such as microscopical analysis, were implemented on a limited number of cases (872 out of 46,677 records). To ensure higher quality and consistency, data collected from 2001 to 2004 were considered as test phase and not included in the analysis. The choice of this test period is due to: (i) adjusted methodology: the last important adjustment was in 2004, when the randomly selected sampling unit "quadrate" was introduced; (ii) the participation to intercalibration courses: by 2004, all the countries that were submitting data had attended at least one course (see Figure A1; Table A1). Data used in this study were extracted from the ICP Forests database on 21 January 2021 for validation purposes within the activity of the ICP Forests Expert Panel on Ambient Air Quality. Data completeness (number of quadrates reported vs. expected) of at least 80 % was mandatory for the field survey. On such a dataset, the distinction between broadleaves and conifers, deciduous and evergreen species have been applied. We concentrated on the subset of broadleaved species (see Introduction): rare species with less than 10 observations were excluded from the analyses, as well as Rubus spp. because highly likelihood of confounding symptoms.

Results were presented for biogeographic regions as determined according to the EEA stratification (https://www.eea.europa.eu/data-and-maps/data/biogeographical-regions-europe-3). Due to their low frequency, "Pannonian" sites were grouped within "Continental"; the same was done for the "Boreal" site with the "Alpine" ones. Species were assigned to one or more biogeographic regions in relation to their actual occurrence in the plots of the region considered.

2.3. Statistical analysis

Considering that both ozone concentration and VFS data (i) are heterogeneous, i.e., that sample size differs among countries and among plots, (ii) have a temporal extent of 14 years, (iii) and are highly variable in terms of completeness for individual sites (e.g., some sites were observed 10 times while others only once or twice; the time between two observations vary between one year and >5 years; Figure A2), we followed a generalized mixed-effects modelling (GLMM) approach, which is more appropriate to analyze incomplete datasets than classical regression models.

For the study questions related to ozone concentrations, we assumed that the temporal trend in ozone concentration varies among plots. Thus, in the first GLMM (GLMM1), the averaged ozone concentrations depend on the year, and random effects were estimated for the year (i.e., the slope) with study plot as the grouping variable ($O_p \sim \text{year} + \text{year}|\text{plot} + \varepsilon$; assuming a Gaussian distribution). Then, based on the Akaike Information Criterion (AIC), we choose if the random effects should be included or not in the model. To analyze the temporal trends for each plot, we looked at their random effects estimated on the fixed effect of the year. We also estimated the mean ozone concentration for every individual plot while taking into account the differences in temporal trends and in the years of surveys among plots (e.g., some plots were studied in 2005–2008 while others in 2012–2016) by predicting the concentration for every year with the GLMM1, and using the average. Differences in the mean values and in the temporal trends of ozone concentration among biogeographical regions were tested with a TukeyHSD test.

For the study questions related to VFS occurrence, the mean annual frequency of VFS for each species *s* in a given plot *p* at year *y* ($Sf_{s,p,y}$) was calculated as follows:

$$Sf_{s,p,y} = \left(\sum_{i=1}^{i=n_{s,p,y}} S_i\right) \Big/ n_{s,p,y} \quad (1)$$

where:

$Sf_{s,p,y}$ is the frequency of VFS for the species *s* in plot *p* at year *y*, *i* is the number *n* of quadrates in which the species *s* has been recorded at year *y*, and S_i is a binary variable representing the presence ($S_i = 1$) or absence of leaf symptoms ($S_i = 0$) for the quadrate *i*. An example of a calculation for a plot with three species with differentiated response is provided in Table A2. When averaged across all the species present at the plot, such a mean value accounts for the number of species present and their observed frequency.

We assumed that general temporal trend in VFS varies among species and among plots. Thus, in this second GLMM (GLMM2), the averaged symptom frequency depends on the year, and random effects were estimated for the year, with study species nested into study plot as the grouping variables ($Sf_p \sim \text{year} + \text{year}|\text{plot/species} + \varepsilon$). Considering the skewed distribution of VFS frequencies (i.e., most of the data are around zero), we assumed that the response function followed a binomial distribution (in the model the response variable is the number of individuals with observed symptoms, weighted by the total number of quadrates observed for a given year, species, and plot; checked with QQ plots drawn on the residuals). Then, we applied the same procedure as for ozone concentration: based on AIC, we determined if the random effects should be included or not in the model. To analyze the temporal trends for each plot, we looked at the random effects of the plot estimated on the fixed effect of the year. Based on the GLMM2, we also estimated the mean frequency of ozone symptoms for every plot while taking into account the differences in temporal trends, and in the years of surveys among plots (e.g., some plots were surveyed in 2005–2008 while others in 2012–2016). To do so, we predicted symptoms frequency for every year with the GLMM2, and used the average.

For the study questions related to the relationship between ozone and VFS at species and plot levels, we quantified the relationship between the frequency of VFS and the ozone concentration at plot scale with the GLMM3. The effect of ozone on VFS (fixed effect) is assumed to vary among species (random effects were estimated for the effect of ozone with species as grouping variable). The random effect of the plot is included on the intercept to consider for a potential observer effect (e.g., VFS attributed to ozone but that is actually caused by pathogens or another factor), but not on the fixed effect ($Sf_p \sim O_p + O_p|\text{species} + 1|$

plot + ε; binomial response function). Consequently, for this model, we assumed that the species sensitivity to ozone does not change over sites (~no intra-specific variability). We then used the GLMM3 to predict the mean frequency of VFS for each species at a given ozone concentration. This was done to standardize the data, i.e., to consider that some species may be over-represented in sites with high ozone concentration, and vice-versa. We finally compared these results among biogeographical regions (Tukey HSD test) and by grouping species into two groups (VFS for species validated in dedicated experiments vs. non-validated species, based on literature review; See Table A3). We also analyzed how the predicted mean frequency of VFS changes according to the species leaf traits SLA and LT.

Finally, to detect the potential link between the temporal change in VFS frequency and the temporal change in ozone concentration, we combined the outputs of the GLMM1 (ozone) and GLMM2 (VFS) and analyzed the relationship between the plot-specific random effects of both GLMMs.

2.4. Datasets used and resampling procedures

To investigate the spatial and temporal trends in ozone concentrations and VFS (questions under categories (i) and (ii)), we focused on the plots with at least three years of respective data, and named hereafter P3O for ozone data (118 plots and 804 plots*years) and P3S for VFS data (91 plots and 1390 plots*years; 149 species; Table 1). The combined analysis of ozone concentrations and VFS (study questions under category (iii)) was carried out using the plots with at least three years with both data, named hereafter P3SO (49 plots, see Table 1). We considered this choice as the best compromise between sample size and robustness of results. Nevertheless, we used three different procedures to assess the dependency of our results on the characteristics of input datasets. First, we tested more restrictive criteria by considering only the plots with at least five years of VFS and ozone data (hereafter P5S, P5O and P5SO; see Table A4). Second, we used bootstrap resampling procedures to assess the uncertainty in the GLMM estimates (200 re-samplings of the plot*-year data). Third, we used a one-delete jackknife procedure to assess the uncertainty related to the short extent of the temporal data used, i.e., we generated 14 different samples each of them excluding one year of data (e.g., sample without data from 2005; sample without data from 2006 and so on) and then fitted a GLMM to the resulting dataset (Figures A3 and A4).

Finally, the relationship between temporal change in frequency of leaf symptoms and temporal change in ozone concentration is potentially affected by those species that are insensitive to ozone. As a consequence, we combined the outputs of the GLMM1 and GLMM2 fitted on the datasets P3SO, but also on the dataset V-P3SO, which only includes broadleaved species with validated symptoms (Table 1).

3. Results

3.1. High ozone concentrations and widespread visible foliar symptoms

We observed high mean April-September ozone concentrations and widespread VFS over the entire continent and the period examined (Fig. 3 A, B). Mean ozone concentrations were higher than 40 ppb on 37.3 % of the plots. A distinct spatial pattern of increasing concentrations from North to South emerged from the total of 118 forest sites across 10 European countries from 2005 to 2018. Mean values above 40 ppb were mainly recorded in France, Spain, Italy, Switzerland, Slovak Republic, and Romania. Concentrations were significantly ($p < 0.05$) higher in the Alpine/Boreal and Mediterranean biogeographic regions as compared to Continental/Pannonian and Atlantic ones ("2 groups"; TukeyHSD test; $p < 0.05$) (Fig. 3 A).

VFS were found on 57 out of the 149 species observed (38.3 %) and at 57 out of the 91 plots examined (62.6 %), with high variability and a less clear spatial pattern than for concentrations (Fig. 3B). Clusters of highly symptomatic sites (mean plot annual frequency > 5 %) were found mainly in France, Germany, Switzerland (Continental and Alpine region), Lithuania (Continental and Alpine-Boreal) and Greece (Mediterranean). Low symptomatic sites were frequent mainly in the Mediterranean countries (Croatia, Spain, Italy) and parts of central and Eastern Europe. Differences among biogeographic regions were, however, non-significant. Species composition at the monitoring sites varies largely across Europe, with only 14 species being common among the biogeographic regions considered (Table 2; Fig. 4; see also Table A5). Within these common species, beech accounts for the largest part of observation and the highest frequency of VFS (21.0 %, Fig. 4), while the frequency was much lower for the other species (0.0–12.4 %, Fig. 4). The highest number of exclusive species was observed in the Mediterranean region (42), and the lowest in the Atlantic (6) (Table 2). The species that were exclusive for the Mediterranean contains many sclerophyll species such as *Arbutus unedo, Myrtus communis, Phyllirea latifolia, Rhamnus alaternus* and – in general – species for which foliar characteristics (e.g., low SLA, high LT) are not conducive to VFS.

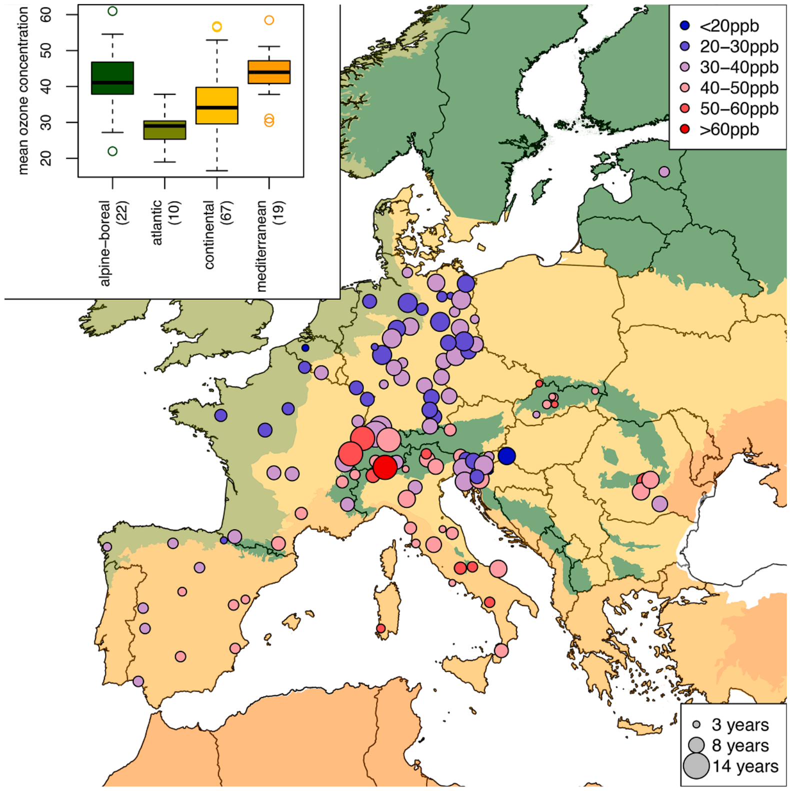
The example of Greece is particularly relevant here: at the three Greek sites, a total of 19 species were observed and VFS mostly detected on beech (*Fagus sylvatica*) and checker trees (*Sorbus torminalis*) in mountainous region (Michopoulos et al., 2022). In particular, VFS were detected on 46.3 % of the beech observations (n = 136). The cluster of symptomatic sites in Greece was therefore almost entirely due to a non-Mediterranean species like beech.

3.2. Temporal trends in ozone concentrations and VFS are slightly declining

Although mean ozone concentrations tend to decrease at European scale from ca. 43 ppb to ca. 35 ppb (boxplot in Fig. 5A), the trend is not significant and highly dependent on the dataset used (GLMM1; Figure A3) (see detailed model results in Table A6). Alpine-Boreal sites show more instances of significant negative trend in ozone concentrations than Continental and Mediterranean sites (Fig. 6A) where the trends are both positive and negative, and generally non-significant. Opposite, still not significant, trends towards higher concentrations can be observed for most of the Atlantic sites (Fig. 6A). Focusing on the plots with at least three years of data (P3S), we observed a slightly declining, non-significant trend in VFS mean annual frequency from ca.

Table 1
General characteristics of the sampling for each main subset. "P3S" and "P3O" indicate plots with at least three years of leaf symptoms (VFS) and ozone data, respectively. The other subsets are detailed in Table A3 and A4.

Variable of interest	Code	P3S	P3O	P3SO	V-P3SO
Visible Leaf Symptoms (VFS)	No. of observations (quadrates*plots*years)	29128		18623	8049
	No. of plots*years	1390		1038	317
	No. of plots	91		49	36
	No. of species	149		117	27
	No. of countries	13		7	6
Ozone concentration	No. of plots*years		804	320	277
	No. of plots		118	49	36
	No. of countries		10	7	6



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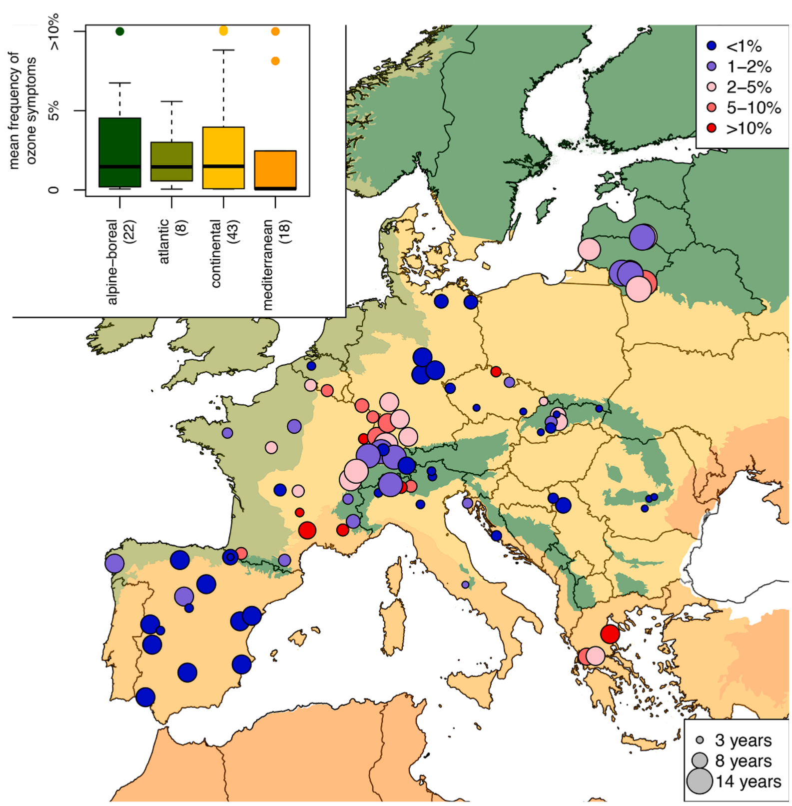


B

Fig. 3. Mean April-September concentrations of ozone (A, 118 sites) and mean frequency of VFS (B, 91 sites) across different biogeographic regions (inset boxplots diagrams) in Europe from 2005 to 2018. The size of the dots on the maps indicates the number of years with data availability for each plot (from 3 to 14). The upper-left boxplot indicates the variation among biogeographic regions and the color keys to the map. The number of plots per biogeographic region is indicated in brackets.

Table 2
Number and frequency (%) of observed species (species), observed symptomatic species (Sy), observations (Obs.) and symptomatic observations (Sy): in total, in common among biogeographic regions, and exclusive of individual biogeographic regions.

	Species, n	Sy species, n	Sy species, %	Tot obs, n	Sy obs, n	Sy obs, %
Total broadleaves	149	57	38.3	29,128	1317	4.62
Common in four biogeographic regions	14	12	85.7	7592	653	8.60
Exclusive Alpine-Boreal	14	5	35.7	592	12	2.03
Exclusive Atlantic	6	0	0.0	405	0	0.00
Exclusive Continental	10	2	20.0	494	2	0.40
Exclusive Mediterranean	42	3	7.1	4560	16	0.35

15 % to ca. 5 % over the period examined (Fig. 5B), a tendency that is consistent with the decreasing trend in ozone concentrations (Fig. 5A) (see detailed model results in Table A6 and Figure A4).

While at plot scale the majority of VFS trends was non-significant, when comparing the distribution of trends in ozone concentrations and VFS across biogeographic regions (insets in Fig. 6A, B) we can observe some consistency. The Atlantic sites show frequent positive trends of VFS, which is consistent with the pattern observed in ozone concentration in the same region. On the opposite, the frequent decreasing trends in ozone concentrations observed in the Alpine-Boreal biogeographic regions is accompanied by a decrease in VFS in the same region. Plots in the Continental and Mediterranean biogeographic regions do not show clear trends for ozone concentrations. A certain tendency towards a decreasing trends for VFS can be observed, mainly in the Mediterraneans.

3.3. Relationships between ozone concentration, VFS, species and traits

We found a significant positive effect from ozone concentration on VFS (GLMM3; $p < 0.05$), with differences among species (Fig. 7). Species for which symptoms were validated under controlled conditions ("validated species") show the highest levels of VFS (red dots vs. grey dots in Fig. 7). They are apparently more sensitive to ozone than non-validated ones (*t*-test comparison of the random effects species from the GLMM3; $p < 0.05$).

There are no significant differences in the random effects of the species on the intercept of the GLMM3 between the two species groups (Fig. 7), indicating that the frequency of VFS not attributable to ozone by the GLMM3 is similar among them. In other words, if there is a bias due to, e.g., misclassification of e.g. pathogens- or drought-induced leaf symptoms, it is probably in the same order of magnitude for both validated and non-validated species.

Predicted frequency of VFS (Fig. 8) is positively related to specific leaf area (SLA) and negatively related leaf thickness (LT) (Fig. 9). Although only marginally significant ($p < 0.074$ and $p < 0.077$, respectively), these correlations are consistent with the negative relationship between species sensitivity to ozone and LMA (leaf dry mass per unit leaf area, the inverse of SLA) at a given ozone exposure obtained by

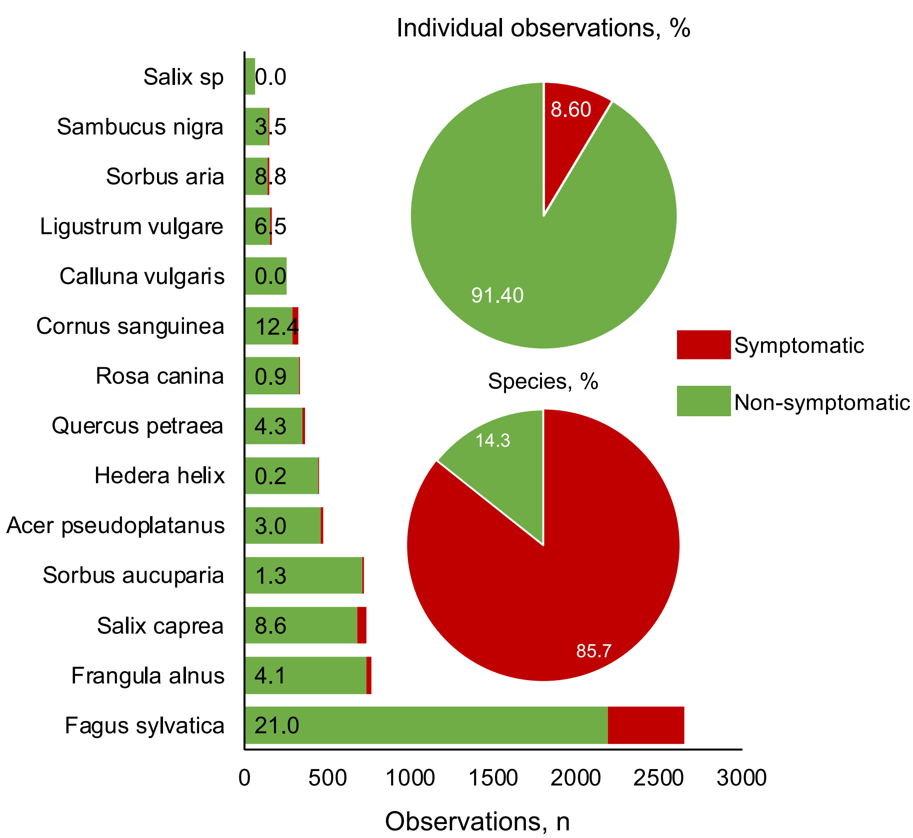


Fig. 4. Common species in the four biogeographic regions and frequency of VFS. The graph illustrates the number of observations per species (green: non-symptomatic; purple: symptomatic. The percentage of symptomatic observation is provided (data labels). Inset: frequency (%) of non-symptomatic and symptomatic observation on the entire set of common species.

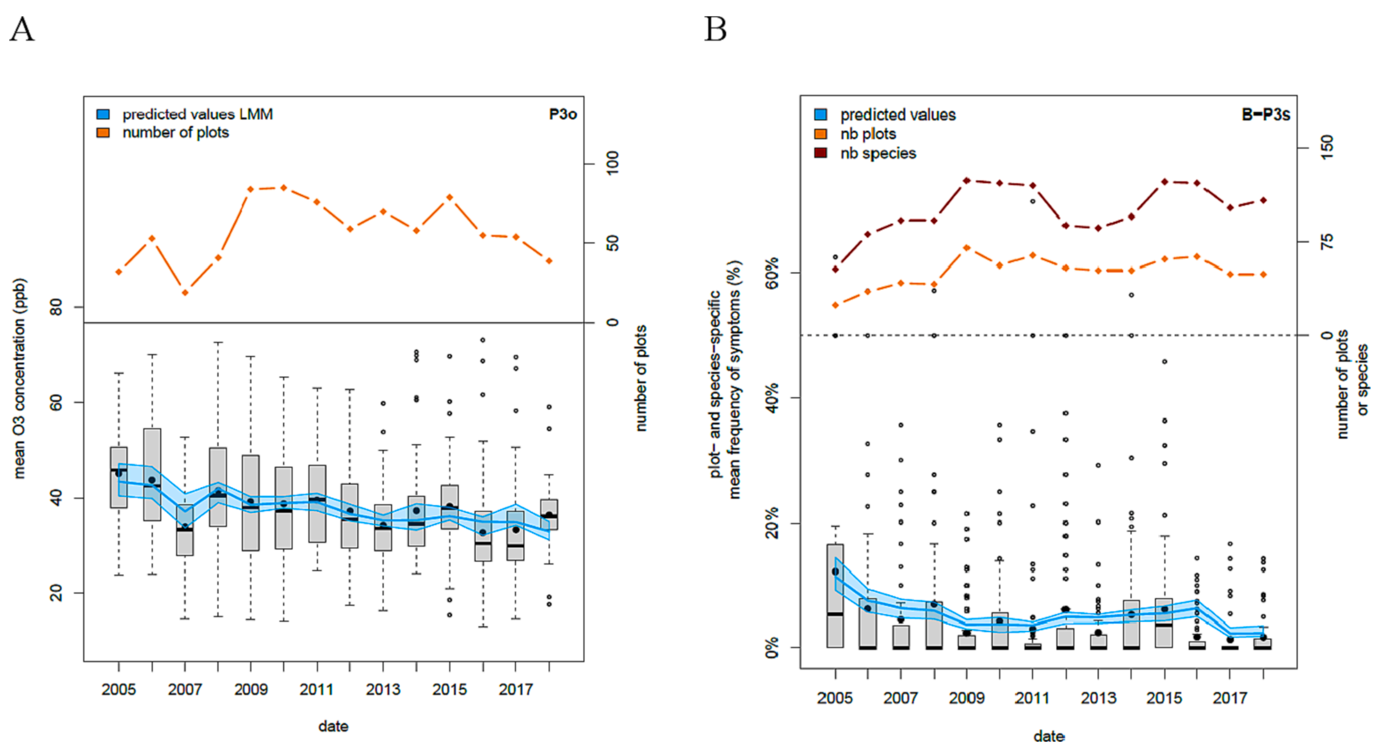


Fig. 5. Temporal changes in ozone concentrations and VFS. Temporal change in (A) ozone mean April-September concentrations (P3O subset) and (B) in frequency of VFS for the plots with at least 3 years of data (P3S subset). Medians, quartiles, and individual plots are indicated with boxplots; mean values are represented with the black full dots. The mean values predicted by the GLMM1 (A) and GLMM2 (B) and their 95 % confidence intervals (from bootstrapping procedures; n = 100) are indicated with the blue curve and polygon. Sample size in terms of the number of plots and species considered are indicated in the upper diagram.

Feng et al. (2018).

When considering the temporal development, and by combining the outputs of the GLMM1 and GLMM2 fitted on the plots and years with both symptoms and ozone data, we did not find any relationship between the change in ozone concentrations over time at a given plot and the change in VFS. This was true for both the entire set of species (Fig. 10A) and the species with validated symptoms only (Fig. 10B).

4. Discussion

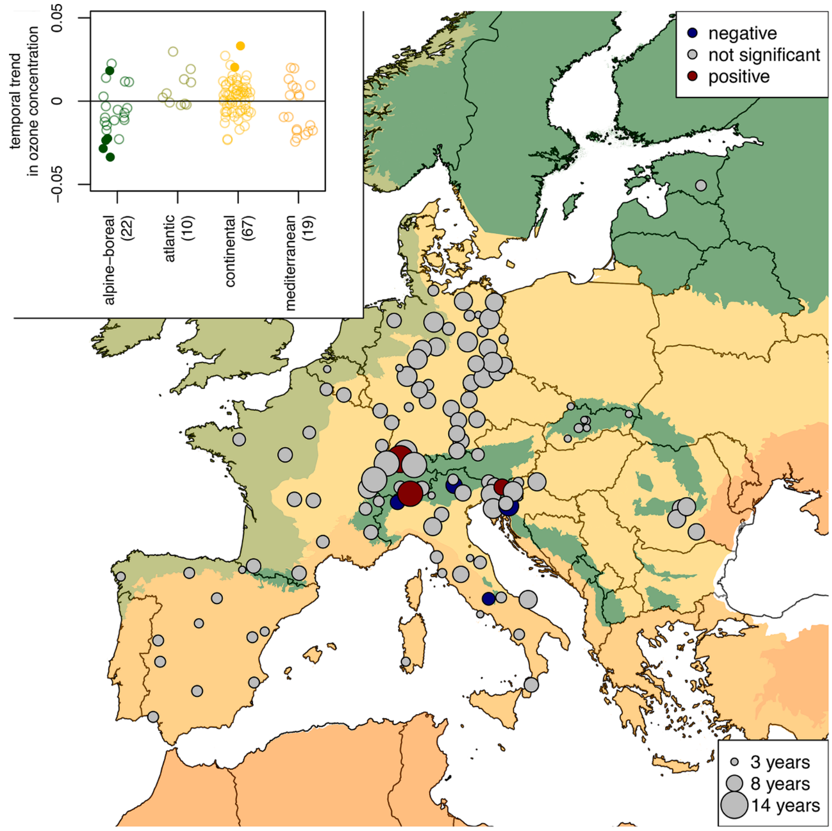
Ozone concentrations in European forests over the period 2005–2018 were frequently exceeding 40 ppb, followed a distinct latitudinal pattern with higher values in the Alps and the Mediterranean, and displayed a generalized, stagnating or slightly declining trend. Although with generally low frequency of occurrence, VFS were identified on a large number of broadleaved species and were partially related to ozone concentrations and to leaf traits. The geographic pattern of VFS differed however from the pattern of ozone concentrations, with higher values recorded in the Alpine and Continental regions and lower ones in the Mediterranean. The overall temporal trend of VFS was also showing a slightly decreasing trend. All together, these findings suggest that (i) ozone is still posing a significant risk to forest vegetation as demonstrated by measured frequency of VFS; that (ii) such a risk is only partially related to ozone concentrations; and that (iii) over the period examined, the risk has slightly declined across most of Europe, with perhaps the exception of the Atlantic sites. These results can be discussed under different perspectives. Here we considered the following four main directions: (i) uncertainties due to measurement methods and data; (ii) relationship between VFS and ozone metrics; (iii) ecological significance; and (iv) current and future relevance of VFS and ozone monitoring at forest sites.

4.1. Methods and dataset

There are some limitations of both VFS and ozone concentration data that may affect the analyses and introduce uncertainty in the results. Measurements of ozone concentration by passive sampling provided time-integrated (weekly to bi-weekly) data that smooth peak values and therefore may not be entirely representative of the stress condition created by ozone, or not capture temporal variations in the overall distribution of concentration values (e.g. Yan et al., 2018). However, while future research may benefit from high-frequency measurements that better capture short-term variations (see below), data from passive sampling were demonstrated to be well correlated to those carried out by real-time ozone monitors, including the derivation of cumulative exposure metrics such as AOT40 (ozone Accumulated Over Threshold 40 ppb, an indicator of vegetation exposure to ozone, e.g., Gerosa et al., 2007; Ferretti et al., 2012).

Although the assessment of VFS is carried out by trained field crews, it may still be subject to different error sources, with misclassification of the symptom (when observed) and missed symptoms. Despite the considerable attention paid to QA/QC procedures (see section 2.2), training, the microscopic validation procedure, and the decision not to use the early surveys (2000–2004) in the study, a certain degree of uncertainty remained and must be taken into account when evaluating the results.

A possible more significant issue in our study may emerge from data completeness. Both data sets (ozone concentration and VFS) are heterogeneous and intermittent, and there were few sites (n = 49, Table 1) with both data series available over on the long-term. In consequence, the results obtained may partly depend on the dataset used, and may affect the assessment of temporal trend. For instance, excluding the year 2005 from the ozone concentration calibration dataset using a jackknife procedure led to an increase in the slope value (trend tends to be positive but still not significant), while the opposite effect was obtained when excluding the year 2015 (trend becomes significantly negative)



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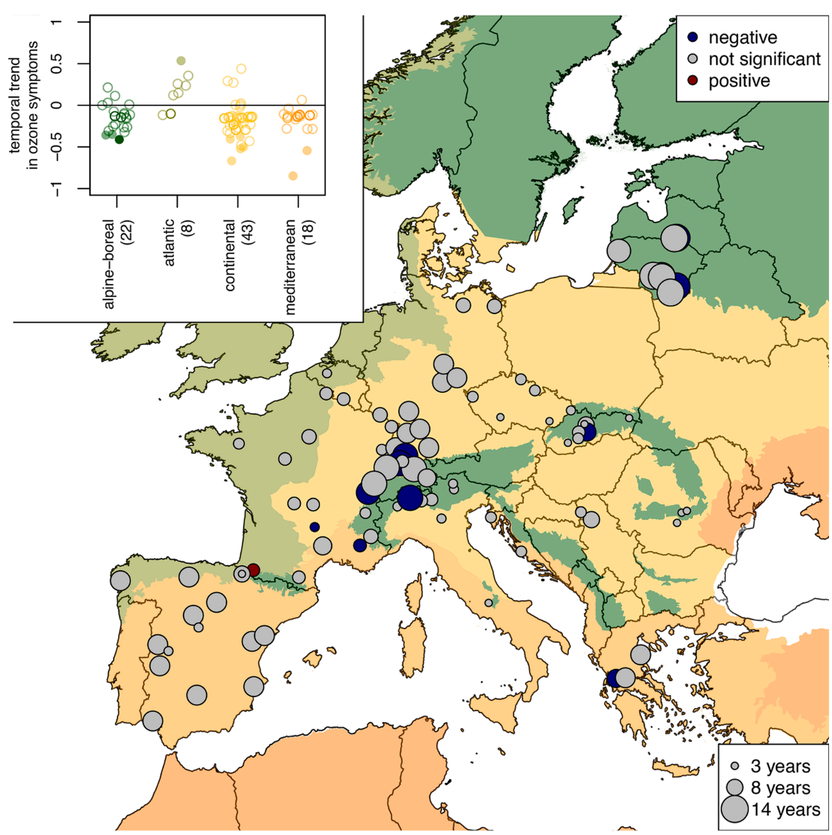

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Fig. 6. Spatial distribution of the temporal changes in ozone concentrations and VFS. Spatial distribution of the temporal trends in (A) ozone concentrations and (B) mean annual frequency of VFS estimated by the GLMM1 and GLMM2 for the 2005–2018 period and for the plots with at least 3 years of data (P3O and P3S subsets). The size of the dots indicates the number of years with data availability for each plot (from 3 to 14). The upper-left scatter plots indicate the variation among biogeographic regions and the color keys to the map. Full and empty dots indicate significant and non-significant plot-specific temporal change in leaf symptom, respectively. The number of plots per biogeographic region is indicated in brackets.

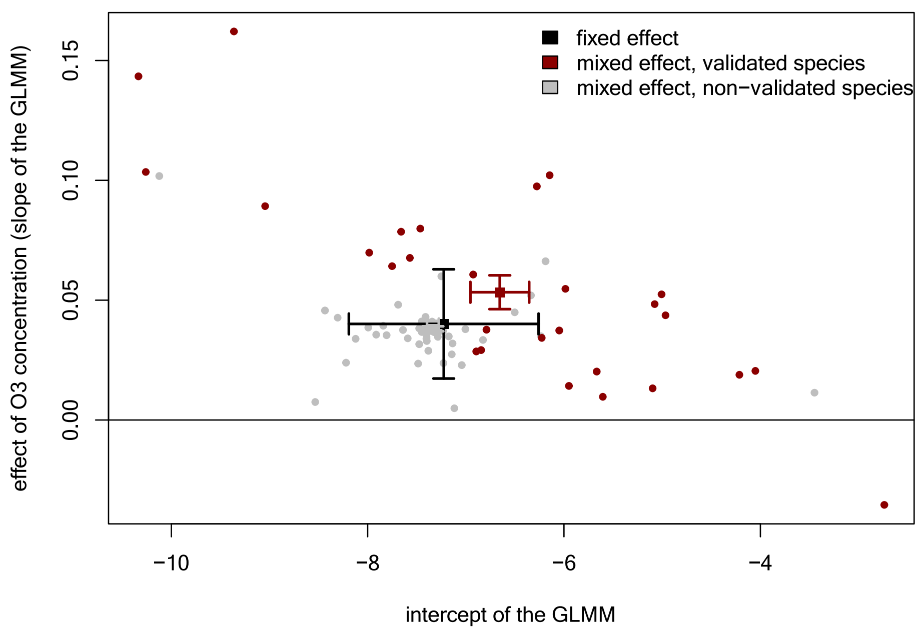

Fig. 7. Summary of the species differences in frequency of VFS. The figure separates species sensitivity to ozone concentration (y-axis) and the presence of VFS unrelated to ozone (x-axis), as estimated from the random effects in the GLMM3. Higher is the random effect on the intercept (x-axis), higher is the frequency of VFS for that species irrespective of the ozone concentration. Most of the species show a positive effect of ozone concentration on the frequency of VFS (y-axis). The same weight is attributed to each species, irrespective of the number of observations.

(Figure A2).

While it is reassuring that slight decline / substantial stagnation since the year 2000 has been also recorded by the automated measurements carried out across Europe (https://www.eea.europa.eu/data-and-maps/indicators/air-pollution-by-ozone-2/assessment), the availability of data may have affected also the study on the relationships between time trends in ozone concentrations and VFS. We did not find any significant link between the two temporal trends, even when considering only the species with validated VFS. However, low sample size and the combination of plots*years for which we have both VFS and ozone data (P5SO and V-P5SO) show temporal trends that differ from the ones obtained with larger datasets (e.g., P3S and P3O; Figures A3 and A4).

4.2. Correlation between ozone metrics and VFS

Correlation between VFS and ozone concentrations was not always clear in our study. This goes in the same direction of previous results that do not always show consistent relationship between observed VFS and various concentration-based or flux-based ozone metrics. A few example will suffice. On one side, Ferretti et al. (2007a) reported a non-significant correlation between ozone AOT40 and VFS in a trans-national study involving France, Italy, Spain and Switzerland, and these results were confirmed in a follow-up study at Italian level (Bussotti and Ferretti, 2009). Similarly, Hůnová et al. (2011) reported that, despite the relatively high ozone phytotoxic potential (AOT40) observed in Czech Republic, VFS on native ozone-sensitive vegetation species resulted scarce. On the same line, Moura et al. (2022) found that experimentally derived POD (Phytotoxic Ozone Dose) can only partially explain the observed VFS on several species at forest monitoring plots.

On the other side, Sicard et al. (2021) evidenced significant correlations between PODY (POD above a threshold Y of stomatal uptake, with Y = 1 nmol O_3*m^{-2}*s^{-1}) and the occurrence, frequency and severity of VFS on the dominant tree species in ICP Forests Level II plots in Italy. Focusing on single species, VFS occurrences related to ozone exposures were observed on, e.g., *Viburnum lantana* L (Gottardini et al., 2010b, 2014, 2017, 2018) and on *Pinus uncinata* Mill. (Kefauver et al., 2014). For the latter species, symptom severity increased with increasing mean annual ozone concentration when summer water availability was high (Diaz-de-Quijano et al., 2016).

We argue that establishing direct and generalized relationship between ozone metrics and vegetation response (whatever the response: VFS, tree vitality, tree growth) was and remain an elusive task for field studies. This is true also when considering flux-based metrics (e.g. POD) that were conceptualized to incorporate the role of factors (e.g. stomatal conductance, phenology, soil water content) that can modify ozone uptake by plants and their response. Even in such a case, however, it is important to consider the substantial differences existing between the controlled experimental condition, where relationships and thresholds were developed, and the actual field condition (species assemblage, plant traits, growing condition, site, ozone concentrations and their combination). Together with the inherent technical and financial difficulty to measure or model all the above factors at the scale of small forest plots, over large scale and the long-term (e.g. Bussotti and Ferretti, 2009; Ferretti et al., 2007b; Gottardini et al., 2010a, 2010b; Moura et al., 2022), this render it difficult to transfer relationship/ thresholds developed in experiments to field survey, and therefore to identify a simple, univocal relationship between ozone metrics and VFS.

By integrating all the above factors, the expression of VFS can offer a useful bioindication operational framework. In such a framework, it is well acceptable that non-sensitive vegetation (like Mediterranean evergreens) may not show VFS even under high ozone concentrations,

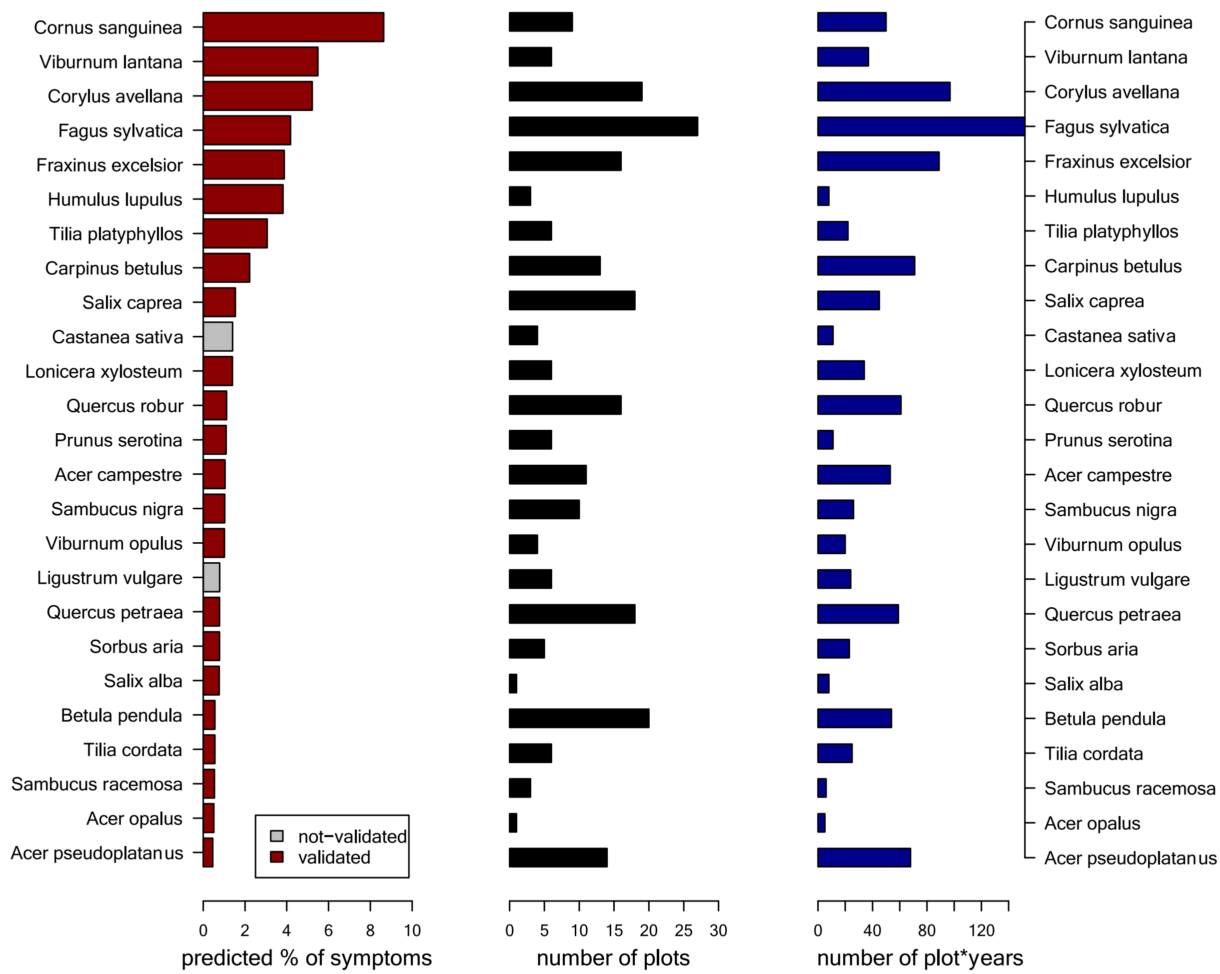

Fig. 8. Classification of the 25 species which show the highest frequency of leaf symptoms, as predicted by the GLMM3 considering an annual ozone concentration of 40.1 ppb (mean of the dataset P3SO). For each species, we reported if they typically show VFS, validated in ozone treatment experiments (left; red color) or not validated (left; grey color), the sample size in terms of the number of plots (center), and number of plots*years (right).

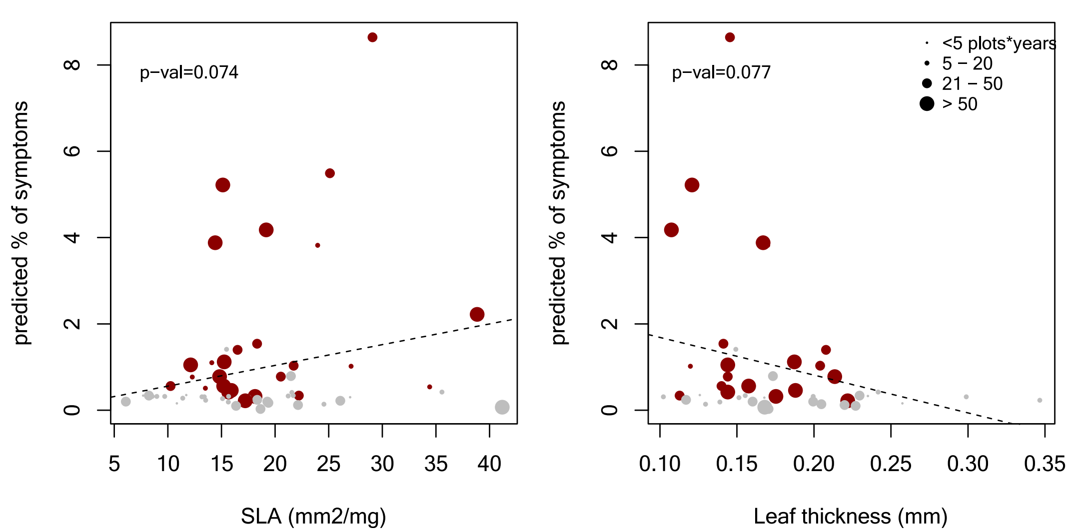

Fig. 9. VFS and leaf traits. Relationship obtained between species SLA (left) and LT (right) and the predicted frequency of VFS. Red: validated species; grey: non-validated species. The size of the dots indicates the number of plots*years where a given species has been observed.

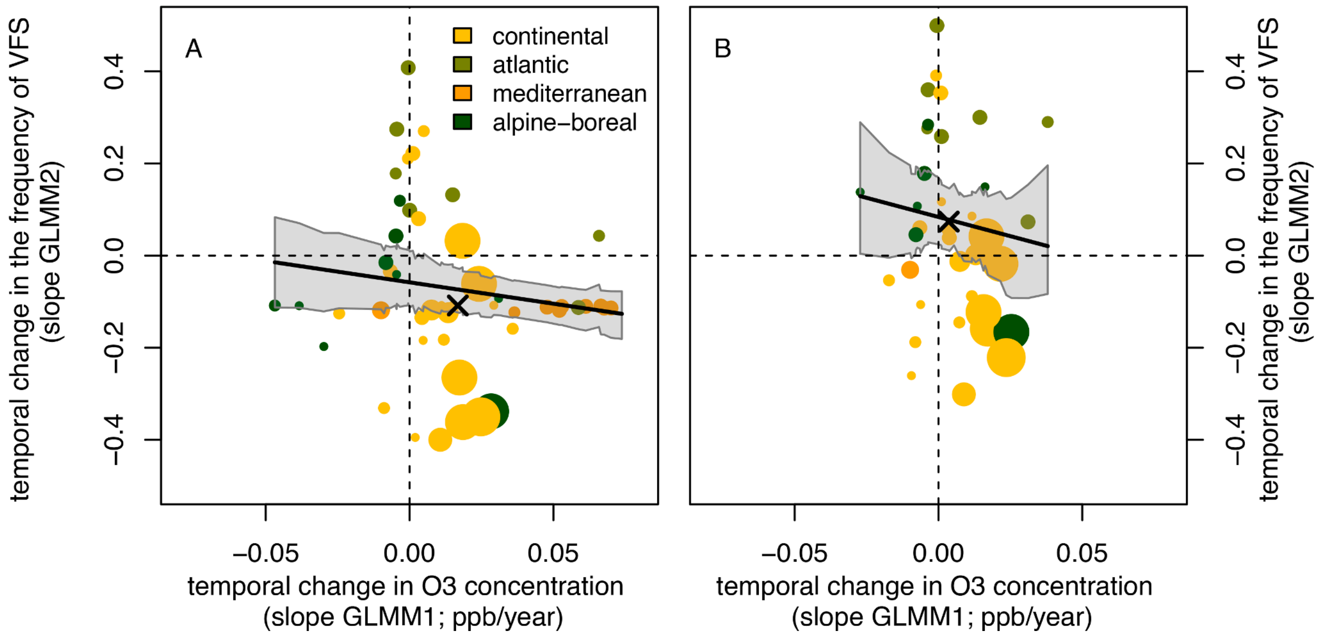

Fig. 10. Relationship between the temporal change in frequency of VFS and the temporal change in mean April-September ozone concentrations calculated considering all species (A; dataset P3SO) and only the validated species (B; dataset V-P3SO). A simple linear regression model was fitted to the data and indicated with the black line ($p > 0.1$), while the grey area is the 95 % CI (from bootstrap). The size of the dots indicates the number of years for which both ozone and symptoms data are available. The black cross indicates the coefficients of the fixed effects of the GLMM1 and GLMM2 (i.e., the average trend at European scale).

while more sensitive species may, even under comparatively lower ozone concentrations: for example, Moura et al. (2022) found that *Arbutus unedo* L., a Mediterranean evergreen species required an AOT40 of 50.9 ppm h to develop VFS, which is ca. eight times higher AOT40 threshold than for *Sorbus aucuparia* L. While confirming the validity of the approach, this warns against the blanket application of results from controlled experiments to the real field conditions.

4.3. Ecological significance

Most observational and modelling studies on ozone impact in European forests are based on individual species (e.g., European beech, Norway spruce - Grünhage et al., 2012), as this is also true for the development of dose response functions. Here, we incorporate the species composition in assessing the frequency of symptoms and in calculating the VFS statistics. While on the one side the variability in floristic composition among the different study areas may reduce the possibility of comparisons among sites (Bussotti et al., 2006), on the other side this offers the possibility to evaluate the actual impact of the measured ozone concentrations on the real vegetation across Europe. Here, we found that the development and frequency of VFS is different among species, and that a forest site may be more or less prone to develop symptoms depending on its species composition – even under the same ozone concentration. We then expect that – given the diversity of European forest vegetation – the frequency of VFS across Europe may vary, reflecting not only ozone exposures, but also the vegetation characteristics (that in turn reflect a number of other ecological characteristics) and thus providing an indication for a possible risk due to ozone. At the same time, changes of VFS over subsequent years can reveal changes in the risk posed by ozone on the actual vegetation over the region of concern. Estimating the actual ozone risk for the vegetation at a given site needs to account also for the overall species functional characteristics (e.g., leaf traits) that can be more or less conducive to the development of VFS. From our data we can show that - despite high ozone concentrations – the ozone risk for vegetation appears lower in the Mediterranean than in central Europe. This is likely due to the different species composition observed, which is in turn determined by different ecological characteristics (site, climate). Results from the Mediterranean are mostly driven by the data from Spain, where the traits of the broadleaved species (e.g., the typical Mediterranean evergreen species) and the environment (e.g., water shortage) are less conducive to VFS. In addition, the highest diversity observed may also play a role in reducing the potential impact of ozone. This is very much in line with the bioindication approach described in the previous chapter: the combination with non-sensitive plant species and site condition not conducive to ozone uptake prevent high ozone concentrations to result into high frequency of VFS.

It is also worth noting that we (i) concentrated on broadleaved species and (ii) deliberately ignored the intraspecific variation in sensitivity to ozone. These are two obvious limitations in our results, as on one side some conifers may be highly sensitive to ozone and – on the other side – within-species variability in ozone sensitivity may substantially affect, for example, the relationship between ozone concentrations and effects.

Finally, in terms of ecological significance, it is important to note that – although generally close to the nearest Level II plot - the forest edge assessed for VFS may have a different floristic composition from the plot itself. This may render somewhat difficult to connect VFS data with other measurements carried out on the main plot, such as forest growth. Therefore, while the occurrence of VFS can be considered as an indicator of the potential impact of ozone on vegetation at a given site, care should be taken in inferring possible effects on forest growth, health, and biodiversity.

4.4. VFS surveys to monitor ozone impact on vegetation: still relevant?

What emerges from our results is that frequency, temporal rend and spatial distribution of VFS on broadleaves in Europe are not always consistent with ozone levels. While this can be taken as the weakness of this (and other) studies, in our opinion it represents also its strength and constitutes also the main motivation to continue such studies. As a matter of fact, outside of controlled experiments it is difficult – for the time being – to estimate an impact on vegetation only on the basis of an ozone metric because the important role of a number of concurring ecological factors. As such, continuous monitoring of VFS represents a powerful bioindication approach to track the impact of tropospheric ozone on vegetation. It is worth noting, however, that the value of such a monitoring is driven by its consistency over time and space. While the ICP Forests did a lot in this respect (Schaub et al., 2020a, b), still much work is necessary to further enhance the value of VFS and ozone monitoring. We identified the following main directions for future work:

(i) Improve the measurements of ozone and other environmental variables at the ICP Forests (and other) sites. Passive sampling represented a valid option for monitoring ozone concentrations when the survey was launched in the early 2000s (Schaub et al., 2020b). In the meantime, new technologies developed that can permit affordable active real-time ozone measurements (e.g. Mueller et al., 2017) as well as the measurements of many others environmental variables (e.g. Zweifel et al., 2023). These should be considered for securing technological readiness while keeping attention to data comparability.

(ii) Augment the quality of VFS assessment and validation in the field. Continuous training and intercomparison of results have been proven essential and should continue to ensure comparability across space and time. At the same, augmenting the possibility for rapid validation of field observation (*sensu* Vollenweider et al., 2003) will greatly improve data quality. This can be done by means of control assessment and by diagnostic tools.

(iii) Secure continue and complete data series. The major challenges we faced when analyzing the data was due to incomplete data sets. Securing data completeness over space and time is a key for a consistent, robust evaluation of ozone impact on our forests.

(iv) Explore connections between VFS, tree growth, vitality, and biodiversity at the forest sites. While many studies addressed the impact of ozone on tree growth, health and biodiversity (see Introduction), less has been done in exploring whether VFS can be indicative of an impact of ozone on the same properties and processes. For example, experimental data with seedling found no or limited relationship between occurrence of VFS and different growth metrics, while the results of the few field studies appear less univocal (see the review by Marzuoli et al., 2019). Similarly, species competition may modulate VFS and growth (e.g. Novak et al., 2008). Therefore, a comprehensive field study on how VFS development over time and space is related to changes in tree growth, vitality and species richness and diversity can represent an important development area.

5. Conclusions

We observed that high ozone concentrations remained pervasive in Europe over the past 20 years, especially in the Mediterranean and Alps and despite some reduction observed in these region (Question (i)). Overall, we found a distinct North-South spatial pattern, and a slightly declining insignificant trend, with some variation among biogeographic regions. Accordingly, we found frequent VFS on European broadleaves, likely the functional group expected to be more sensitive to develop VFS, with a slightly declining, insignificant temporal trend (Question (ii)).

The relationship between ozone, VFS and species (Question (iii)) was not always clear, and this is likely due to the different species composition across Europe (with inherently different leaf traits more or less conducive to VFS) which is in turn driven by climate and site characteristics. This also helps explaining why - despite the significantly higher ozone concentrations in the region – very few of the species exclusive of the Mediterranean was reported symptomatic. Therefore, while the use of individual indicator species can be a valid option for a first screening over a given region, to have a realistic estimate of the potential ecological impact of ozone over the same region, it is important to represent the entire plant community (and not only few selected species) present there.

In conclusion, while ozone concentration showed a North-South gradient, with a potential higher ozone risk to vegetation with hot spots in the Alps and in the Mediterranean, VFS data modified this picture. According to our 2005–2018 data, the risk may be higher in parts of the Alpine and Continental Europe, and lower in the Mediterranean, with perhaps the exception of mountain deciduous forests. We observed some limitation in the data sets that call for the need to continue the long-term monitoring, with continuity of measurements being the essential aspect to be ensured in future. Finally, we focused on the occurrence of VFS to demonstrate that an impact of ozone on European forests can be widespread. Other important effects on growth, carbon sequestration, vitality and biodiversity were not subject of this study and therefore cannot be inferred from our results.

CRediT authorship contribution statement

Marco Ferretti: Conceptualization, Supervision, Writing – original draft, Writing – review & editing. **Maxime Cailleret:** Formal analysis, Methodology, Writing – review & editing. **Matthias Haeni:** Data curation. **Volodymyr Trotsiuk:** Data curation. **Vladislav Apuhtin:** Data curation, Writing – review & editing. **Valda Araminiene:** Data curation, Writing – review & editing. **Václav Buriánek:** Data curation, Writing – review & editing. **Sébastien Cecchini:** Data curation, Writing – review & editing. **Laurence Dalstein-Richier:** Data curation, Writing – review & editing. **Iva Hůnová:** Data curation, Writing – review & editing. **Tamara Jakovljević:** Data curation, Writing – review & editing. **Konstantinos Kaoukis:** Data curation, Writing – review & editing. **Johan Neirynck:** Data curation, Writing – review & editing. **Manuel Nicolas:** Data curation, Writing – review & editing. **Anne-Katrin Prescher:** Data curation, Writing – review & editing. **Radek Novotný:** Data curation, Writing – original draft. **Hana Pavlendova:** Data curation, Writing – review & editing. **Nenad Potočić:** Data curation, Writing – review & editing. **Matej Rupel:** Data curation, Writing – review & editing. **Alexander Russ:** Data curation, Writing – review & editing. **Vidas Stakėnas:** Data curation, Writing – review & editing. **Arne Verstraeten:** Data curation, Writing – review & editing. **Pierre Vollenweider:** Data curation, Writing – review & editing. **Daniel Zlindra:** Data curation, Writing – review & editing. **Diana Pitar:** Data curation, Writing – review & editing. **Vicent Calatayud:** Data curation, Writing – review & editing. **Elena Gottardini:** Data curation, Supervision, Writing – review & editing. **Marcus Schaub:** Methodology, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The authors do not have permission to share data.

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Appendix A. Supplementary data

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