

Environmental Science and Pollution Research

Phenylpropanoids are key players in the antioxidant defense to ozone of European ash, *Fraxinus excelsior* --Manuscript Draft--

Manuscript Number:	ESPR-D-16-02709R3	
Full Title:	Phenylpropanoids are key players in the antioxidant defense to ozone of European ash, <i>Fraxinus excelsior</i>	
Article Type:	Research Article	
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Funding Information:	This study was supported by MIUR, Rome, project PRIN 2010-2011 "Planning the green city in the global change era: urban tree functions and suitability for predicted future climates (TreeCity)"	Prof. Giacomo Lorenzini
Abstract:	<p>Physiological and biochemical responses to ozone (O₃) (150 ppb, 5 h d⁻¹, 35 consecutive days) of two Italian provenances (Piedmont and Tuscany) of <i>Fraxinus excelsior</i> L. were evaluated, with special attention to the role of phenylpropanoids. Our results indicate: (i) the high O₃-sensitivity especially of Piedmont provenance (in terms of visible injury, water status and photosynthetic apparatus); (ii) although the intra-specific sensitivity to O₃ between provenances (mainly due to different stomatal behaviors since only Tuscany plants partially avoided the uptake of the pollutant gas), both provenances showed detoxification and defense mechanisms; (iii) the crucial participation of phenylpropanoids, with a key role played by flavonoids (especially quercitrin): among this class of metabolites, isoquercitrin is the principal player in the lower O₃-sensitivity of Tuscany plants, together with lignins; (iv) although coumarins (typical compounds of <i>Fraxinus</i>) were severely depressed by O₃, isofraxidin were triggered suggesting a key role in reactive oxygen species (ROS) detoxification, as well as trans-chalcone. Furthermore, the different behavior of verbascoside and oleuropein among provenances lead us to speculate on their influence in the tentatively repair or acclimation shown by Piedmont plants at the end of the exposure. Finally, the intra-specific O₃-sensitivity may be also due to de-novo peaks triggered by O₃ not yet associated to some chemicals.</p>	
Response to Reviewers:	Pisa, 25 November, 2016	
	Ms. Ref. No.: ESPR-D-16-02709R2	

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Associate Professor of Plant Pathology



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Phenylpropanoids are key players in the antioxidant defense to ozone of European ash, *Fraxinus excelsior*

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Abstract

Physiological and biochemical responses to ozone (O₃) (150 ppb, 5 h d⁻¹, 35 consecutive days) of two Italian provenances (Piedmont and Tuscany) of *Fraxinus excelsior* L. were evaluated, with special attention to the role of phenylpropanoids. Our results indicate: (i) the high O₃-sensitivity especially of Piedmont provenance (in terms of visible injury, water status and photosynthetic apparatus); (ii) although the intra-specific sensitivity to O₃ between provenances (mainly due to different stomatal behaviors since only Tuscany plants partially avoided the uptake of the pollutant gas), both provenances showed detoxification and defense mechanisms; (iii) the crucial participation of phenylpropanoids, with a key role played by flavonoids (especially quercitrin): among this class of metabolites, isoquercitrin is the principal player in the lower O₃-sensitivity of Tuscany plants, together with lignins; (iv) although coumarins (typical compounds of *Fraxinus*) were severely depressed by O₃, isofraxidin were triggered suggesting a key role in reactive oxygen species (ROS) detoxification, as well as trans-chalcone. Furthermore, the different behavior of verbascoside and oleuropein among provenances lead us to speculate on their influence in the tentatively repair or acclimation shown by Piedmont plants at the end of the exposure. Finally, the intra-specific O₃-sensitivity may be also due to *de-novo* peaks triggered by O₃ not yet associated to some chemicals.

Keywords

Air pollution, coumarins, flavonoids, oleuropein, oxidative stress, verbascoside.

Introduction

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Climate change is a serious and urgent complex issue being identified as one of the major issues that affect the European society (EEA 2015). Mediterranean climates are extensively affected by atmospheric pollutants, showing uncommon temperature and precipitation patterns (Nali et al. 2001; Cotrozzi et al. 2016). Tree species can respond differently to climate change depending on several features, such as morphological, physiological, and chemical functional traits of leaves (factors that have demonstrable links to the plant's function or functioning), phenotypic plasticity (the range of phenotypes a single genotype can express as a function of its environment) and phenology of plant, as well as environmental conditions (Bussotti and Pollastrini 2015).

Furthermore, several biotic and abiotic stressors in the various geographic areas are involved in the intra-specific selection of plants that survive the environmental stress by stress avoidance or tolerance (Kline et al. 2009).

O₃ is by far the most widespread and harmful pollutant to trees, and its concentration has been raising in the Northern Hemisphere since the pre-industrial time (Carriero et al. 2016). This gas results in (i) contraction of photosynthesis and growth, (ii) partial stomatal closure, (iii) cell dehydration, (iv) excess excitation energy, and (v) accelerated leaf senescence or even appearance of necrotic leaf injuries (Cotrozzi et al. 2016, Guidi et al. 2016). The ability to avoid (such as by stomatal regulation) and/or turn on tolerance mechanisms (such as activation of scavenging mechanisms) is diverse and specific among plant species, and it characterizes the response to O₃-oxidative stress (Bussotti et al. 2007). Plants have very efficient enzymatic (e.g. superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase) and non-enzymatic (e.g. ascorbic acid, glutathione, alkaloids, α -tocopherols and phenylpropanoids) antioxidant systems which work in cooperation to control the cascades of uncontrolled oxidation and protect plant cells from oxidative damage by scavenging ROS (Gill and Tuteja 2010).

Among non-enzymatic antioxidant systems, it has been affirmed that phenylpropanoids (one of the three main classes of phenolic compounds) may have a great efficacy (Cheynier et al. 2013). The general phenylpropanoid metabolism develops a huge array of secondary metabolites based on the few intermediates of the shikimate pathway as the core unit. The resulting hydroxycinnamic acids and esters are intensified in several cascades by a combination of reductases, oxygenases, and transferases to result in an organized and developmentally specific arrangement of metabolites, peculiar for each plant species (Vogt 2010). Correspondingly, high O₃ levels elicit defense related signaling pathways, leading to accumulation of beneficial compounds for defense and cell wall fortifying like flavonoids, phenolic acids and monolignols, which act as ROS scavengers (Mikkelsen et al. 2015). Furthermore, phenylpropanoids also have been involved in more direct

1 interactions with transport and signal transduction pathways, conferring various physiological
2 functions for plants to cope with environmental constraints (Cheynier et al. 2013).
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4 Although the sensitivity/resistance of trees to O₃ has received attention (e.g. Nali et al. 2004;
5 Paoletti 2009; Carriero et al. 2016), our knowledge of the mechanisms conferring plant capacity to
6 tolerate or avoid oxidative stress is still scarce and fragmentary. Another issue is that different
7 provenances might respond differently to O₃. Actually, in comparison with the huge literature
8 focusing on the responses of plants to O₃, there are few studies concerning the effects of the
9 pollutant on trees of the same species from different geographic areas (e.g. Paludan-Müller et al.
10 1999; Contran and Paoletti 2007; Nali et al. 2010; Gottardini et al. 2014). Moreover, to the best of
11 our knowledge, there is no study that evaluates the role(s) of phenylpropanoid compounds in
12 modulating the responses to O₃ of provenances of a single species.
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22 European (or Common) ash (*Fraxinus excelsior* L., Oleaceae family) is a large deciduous
23 broadleaved tree species, very common in Europe due to its capacity in growing in disparate
24 environmental conditions. Its drought tolerance would have made ash tree species potentially
25 favoured by the expected climate warming and drying (Pautasso et al. 2013). Furthermore, common
26 ash is contemplated one of the best native O₃-bioindicators in terms of visible injury (Innes et al.
27 2001), and its sensitivity to the pollutant has been observed also in relation to morphological and
28 physiological features (e.g. Gerosa et al. 2003; Bussotti et al. 2005; Contran and Paoletti 2007;
29 Paoletti et al. 2007; Sicard et al. 2016). However, only Paoletti et al. (2008) evaluated its O₃-
30 sensitivity at biochemical level, and no study in terms of phenylpropanoid compounds was done.
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40 Therefore, here we summarize the results of an innovative study exploring the responses to O₃ of
41 two Italian provenances of *F. excelsior* in terms of phenylpropanoid metabolism. Moreover, relative
42 water content and photosynthetic pigments were evaluated, whereas O₃-induced visible foliar injury
43 and physiological responses were monitored throughout the fumigation. Our primary objectives
44 were (i) to test the hypothesis of a relationship between plants' sensitivity to O₃ and a geographical
45 origin, and (ii) to evaluate the role of phenylpropanoid compounds in the antioxidant defense.
46 Specifically, we predicted that (i) Piedmont plants are more O₃-sensitive than Tuscany one since
47 they are not subjected to drought summers (ii) phenylpropanoids have a crucial role in
48 detoxification and defense of plants to the oxidative stress, (iii) among phenylpropanoids,
49 antioxidant flavonoids are the most triggered by O₃, and (iv) specific phenylpropanoid compounds
50 have a crucial role in the responses of the provenances.
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Materials and methods

Plant material and ozone exposure

Two-year-old potted uniform-sized plants of *F. excelsior* were obtained from forest nurseries of Piedmont (Valle Pesio, Cuneo, Northern Italy, UTM-WGS84: E 377446 – N 4919307, 675 m a.s.l.) and Tuscany (Alta Val Tiberina, Arezzo, Central Italy, UTM-WGS84: E 265205 – N 4825645, 764 m a.s.l.). According to the Köppen and Geiger climate classification, the Piedmont nursery is located in an area classified as Cfa (temperate-without dry summer-hot summer) and the Tuscan one is located in an area classified as Csa (temperate-dry summer-hot summer) (Peel et al. 2007). In both nurseries, plants were grown from seeds of mother trees hailed from the two locations and the same nursery techniques were adopted.

Plants were transferred in the field-station of San Piero a Grado, Pisa, Tuscany, Italy (UTM-WGS84: E 608510 – N 4837241, 3 m a.s.l.), where they were grown for the last one year (one complete vegetative cycle) in plastic pots (3.7 l) placed on a bed of wet perlite, containing a mix of organic soil:sand:clay (2:1:1 in volume). One week before O₃ exposure, plants were located in a controlled environment facility at 20±1 °C, relative humidity (RH) of 85±5% and photon flux density (PFD) at plant height of 500 μmol photon m⁻² s⁻¹ provided by incandescent lamps, during a 15 h photoperiod. Plants were exposed to 150 ppb O₃ (1 ppb = 1.96 μg m⁻³, at 25 °C and 101.325 kPa) for 35 consecutive days (8 h d⁻¹, in form of a square wave between 9:00 a.m. and 5:00 p.m.). During the exposure, environmental factors were kept as above. Control individuals were exposed to charcoal-filtered air under the same experimental conditions as O₃-treated plants. Further details are in Lorenzini et al. (1994). Ecophysiological measurements were performed weekly on the subapical leaflets of two fully expanded leaves per plant. Target leaves were free of any symptom. At the end of exposure, fully expanded leaves (same size as those used for the ecophysiological analyses) were divided into aliquots, instantly immersed in liquid nitrogen and stored at -80 °C until biochemical analyses.

Visible injury

Every day, visible foliar injury was assessed on each plant, in order to detect the time of onset of the first visible symptom. Leaf injury mature symptoms were evaluated manually at the end of the fumigation, on the basis of the percentage of necrotic area on the adaxial surface (Pellegrini et al. 2011). Further details are in Supplementary Material.

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3 *Relative water content*
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5 At the end of exposure, relative water content (RWC) was measured according to Pellegrini et al.
6 2015a. Further details are in Supplementary Material.
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13 *Ecophysiological measurements*
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16 CO₂ assimilation rate (A), stomatal conductance to water vapor (g_s) and intercellular CO₂
17 concentration (C_i) were measured with an open infra-red gas analyzer (CIRAS-1, PP-Systems)
18 equipped with a Parkinson leaf chamber (2 cm² of single leaf portion). Measurements were
19 performed at ambient CO₂ concentrations (340 ppm) and 1.3±0.19 kPa vapour pressure deficit. The
20 chamber was illuminated by a quartz halogen lamp at 1300±20 μmol m⁻² s⁻¹ and leaf temperature
21 was maintained at 24±2.3 °C. The calculation of C_i was based on the equations described by von
22 Caemmerer and Farquhar (1981).
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30 A PAM-2000 fluorometer (Walz) was used to evaluate the modulated chlorophyll *a* fluorescence
31 and the status of the electron transport of photosystem II (PSII) on the same leaves used for gas
32 exchange after dark-adapting for 40 min. The maximum efficiency of PSII photochemistry was
33 determined as $F_v/F_m = (F_m - F_0)/F_m$. The actual quantum yield of PSII (Φ_{PSII}) was computed as $(F'_m$
34 $- F_s) / F'_m$, where F_s achieved ($F_t - F'_0$), is the steady-state fluorescence yield in the light-adapted
35 state. Maximal fluorescence (F_m), minimal fluorescence (F₀), maximal fluorescence in the light-
36 adapted state (F'_m), and minimal fluorescence in the light-adapted state (F'₀) were determined as
37 reported by Pellegrini et al. 2011. The saturation pulse method was used for analyzing the
38 quenching components as described by Schreiber et al. (1986).
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50 *Biochemical analyses*
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53 Pigments and phenylpropanoids were analyzed by high pressure liquid chromatography (HPLC,
54 P680 HPLC Pump, UVD170U UV-Vis detector, Dionex) at room temperature with a reverse-phase
55 Dionex column (Acclaim 120, C18, 5 μm particle size, 4.6 mm internal diameter × 150 mm length).
56 Pigments analysis was performed according to Döring et al. (2014). Further details are in
57 Supplementary Material.
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1 Phenylpropanoids were extracted as follows: leaf samples were ground in liquid nitrogen, weighed
2 to 100 mg FW and homogenized in 1 ml of acidified methanol (v/v in HCl 4%) for 12 h in the dark
3 at 4 °C. Extracts were centrifuged for 15 min at 12,000g at 4 °C and the supernatants were filtered
4 through 0.2 µm Minisart SRT 15 filters and preserved in test tubes at -20 °C. Supernatants were
5 used for HPLC analyses, and the pellets, dried for 24 h at 35 °C, were used for lignin analyses.
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10 Phenylpropanoids were analyzed according to Fini et al. (2012), with some minor modifications.
11 Lignins were determined according to Brinkmann et al. (2002). Further details are in
12 Supplementary Material.
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19 *Statistics*

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22 A minimum of three replicates (plants) per treatment was used in each of the three repeated
23 experiments. The normality of data was preliminary tested by the Shapiro-Wilk *W* test. If
24 measurements were carried out for more than two time-points, data were analyzed using one-way
25 repeated measures ANOVA and comparison among means was determined by least significant
26 difference (LSD) Fisher's multiple comparison test ($P \leq 0.05$). All the other data were analyzed by
27 Student's *t*-test. Analyses were performed by NCSS 2000 Statistical Analysis System Software.
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37 **Results**

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40 *Visible foliar injury and leaf water status*

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43 Both provenances showed visible foliar injury in form of a widespread chlorosis which developed
44 in minute (1-2 mm Ø) roundish dark-reddish necrosis scattered among the leaf veins of the adaxial
45 surface of completely expanded leaves. In Piedmont provenance, onset was after 12 days of
46 treatment, corresponding to an AOT40 [accumulated ozone exposure over a threshold of 40 ppb,
47 08.00 a.m. - 08.00 p.m. – sensu de Leeuw and van Zantwoort (1997)] of 10,560 ppb h; in Tuscany
48 provenance, onset was after 21 days of exposure (AOT40 = 18,480 ppb h). No injury was detected
49 in charcoal-filtered controls. No significant differences were observed in the percentage of injured
50 leaflets between provenances (76.3%±2.23, Piedmont vs 75.0%±13.6, Tuscany; $P > 0.05$). In
51 contrast, the percentage of injured surface per symptomatic leaflet was higher in Piedmont plants
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2 than in Tuscany ones ($15.1\% \pm 11.02$ vs $6.7\% \pm 2.30$, $P \leq 0.001$). Compared to controls, O_3 exposure
3 significantly decreased RWC only in Piedmont provenance (86 ± 7.9 vs 57 ± 2.9 , $P \leq 0.01$; -34%).
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7 *Weekly profiles of gas exchange and chlorophyll a fluorescence*

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10 According to the one-way repeated measures ANOVA, the interaction between O_3 and time was
11 significant for all the ecophysiological parameters in both provenances, with the exception of g_s and
12 qNP in Piedmont plants (Figures 1 and 2). No significant differences were observed between
13 qNP in Piedmont plants (Figures 1 and 2). No significant differences were observed between
14 controls of the two provenances at the beginning of the exposure ($P > 0.05$).
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18 In O_3 -exposed plants, A decreased in both provenances after 7 days from the beginning of exposure
19 (FBE) and remained lower than in the controls until the end of the treatment (Figure 1A, B).
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21 Compared to the controls, A reached a minimum of -58% and -80% in Piedmont and Tuscany
22 treated plants after 7 and 14 days FBE, respectively. Concerning g_s , the interaction $O_3 \times$ time was
23 only significant in Tuscany plants: this parameter decreased throughout the whole experiment (-
24 64% after 7 days, reaching -71% after 28 days FBE; Figure 1C, D). In treated plants of both
25 provenances, C_i showed unchanged or increased values in comparison to controls throughout the
26 whole fumigation, with the exception of the Piedmont plants at the end of the treatment (-25%,
27 Figure 1E) and the Tuscany ones after 28 days FBE (-14%, Figure 1F).
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36 F_v/F_m ratio was significantly lower in treated Piedmont plants in comparison to the controls: from -
37 3% to -10% after 7 days and 35 days FBE (Figure 2A). In exposed Tuscany plants, the ratio slightly
38 and intermittently decreased (-3, -2 and -5% after 7, 21 and 35 days FBE, respectively), but
39 remaining always close to 0.800 (Figure 2B). In both provenances, Φ_{PSII} decreased 14 days FBE (-
40 45% and -21% in Piedmont and Tuscany plants, respectively). In Piedmont plants, this parameter
41 remained at values lower than the controls until the end of the treatment (-26%), while in those from
42 Tuscany it reached values similar to the controls at treatment's end (Figure 2C, D). After 14 days
43 FBE, O_3 significantly decreased qP levels in both provenances (-28% and -10% in Piedmont and
44 Tuscany plants, respectively), reaching a minimum after 21 days (-50% and -18%, respectively). At
45 the end of the fumigation, in Piedmont provenance the values came back similar to those of the
46 controls (Figure 2E, F). Regarding qNP, the interaction $O_3 \times$ time was significant only in Tuscany
47 plants (Figure 2G, H). This parameter increased at 28 days (+22%, compared to controls) and then
48 decreased at the end of the treatment (-23%).
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Leaf pigments

O₃ significantly decreased leaf pigments levels in Piedmont plants: chlorophyll (*a + b*) (-33%), total carotenoids (-36%), and β -carotene (-38%). In Tuscany plants, only total carotenoids were affected by the exposure (-28%). The de-epoxidation index (DEPS) did not change in treated plants of both provenances (Table 1).

Phenylpropanoids

After O₃ treatment, lignins showed a marked increase (2-fold higher than controls) only in Tuscany plants (Piedmont: 92 ± 18.1 vs 82 ± 8.6 , $P > 0.05$; Tuscany: 74 ± 4.7 vs 148 ± 9.0 , $P \leq 0.001$).

A total of 18 phenylpropanoids detected in both provenances are presented in Table 2. Among coumarins, esculin decreased in both provenances (-51% and -30% in Piedmont and Tuscany plants, respectively), as well as esculetin (-41% and -88%) and fraxetin (-40% and -43%). Scopoletin decreased only in Tuscany plants (-39%). Isofraxidin increased in both provenances (+57% and +70%). Verbascoside and oleuropein showed opposite responses between provenances. The former decreased in comparison to controls only in Tuscany plants (-61%) and the latter increased up to more than 8-fold just in Piedmont plants. Trans-chalcone increased up to 4 times the controls in treated Piedmont plants and by 43% in Tuscany ones. In Piedmont provenance, flavonoids showed the same levels between control and exposed material, with exception of quercetrin which showed higher values in treated plants (+73%). Rutin decreased by 43% in Tuscany provenance where, in contrast, isoquercetrin and quercetrin showed higher values in treated plants (about 3-fold higher). The flavon apigenin increased in Piedmont provenance (5-fold higher than controls) and even more in Tuscany one (10-fold higher than controls). Among the hydroxybenzoic acids, the exposure decreased the vanillic acid in both provenances (-69% and -76%) and the syringic one only in Piedmont one (-50%). The hydroxycinnamic acids showed different responses to exposure: *p*-coumaric increased only in Tuscany plants (3-fold higher than controls) and ferulic acid raised in both provenances (+68% and more than 4-fold than controls, in Piedmont and Tuscany plants respectively).

Also profiles of unknown compounds identity peaks showed interesting responses to fumigation and some differences between the provenances (Supplementary material, Table 1). Peak B was not observed in control plants of Tuscany provenance while in controls of Piedmont it was at the same levels as treated plants. Peak E was observed only in controls of Tuscany provenance and increased

1 (4-fold higher than controls) after O₃ exposure. Peaks F, I, J, K and L were not observed in any
2 control plants, but occurred only after the treatment. Ozone exposure decreased the peak A only in
3 Tuscany plants (-45%), and the peak H in both provenances (-20% and -42% in Piedmont and
4 Tuscany plants, respectively). In contrast, it increased peak C only in Tuscany provenance (+26%)
5 and peak D only in Piedmont one (more than 4-fold, in comparison to control plants). Peak G
6 decreased in Piedmont provenance (-65%) and increased in Tuscany ones (+21%).
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14 **Discussion**

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17 The degree to which plants develop visible injury is usually utilized in many intra- and inter-
18 species comparisons as an indicator of their O₃ sensitivity (Bussotti et al. 2007). In agreement with
19 previous studies performed both in natural and controlled conditions (e.g. Novak et al. 2007; Gerosa
20 et al. 2009; Sicard et al. 2016), our results confirm the high O₃-sensitivity of *F. excelsior* in terms of
21 visible injury. Furthermore, on the basis of onset and diffusion of these manifestations, the
22 Piedmont provenance seems to be more sensitive than the Tuscany one. These outcomes are
23 reported also in previous studies of our research groups (Contran and Paoletti 2007; Nali et al.
24 2010). Specifically, Nali et al. (2010) observed that stippling showed ultrastructural modifications
25 of the mesophyll such as the collapse of the palisade cells and the formation of callose layers close
26 to the cell membrane. Similar ultrastructural responses were also reported by Bussotti et al. (2005)
27 and Gravano et al. (2004) which explained this hypersensitive response as a result of the
28 accumulation of ROS, responsible for the activation of genes determining the programmed cell
29 death, as defense mechanisms against the spreading injury. Thus, the higher sensitivity of Piedmont
30 plants in terms of visible injury seems to be connected with the disorders in water status (reduced
31 RWC) observed only in this provenance, probably due to membrane oxidative damage caused by
32 O₃.
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48 Alterations of chlorenchyma, usually expressed by chlorosis, yellowish spots and necrosis, are often
49 associated to alterations of the photosynthetic performance (Pellegrini et al. 2011). In our study, O₃
50 exposure reduced photosynthetic efficiency, as already reported in previous reports (e.g. Gravano et
51 al. 2004; Contran and Paoletti 2007; Nali et al. 2010). Although all treated plants showed a
52 reduction of CO₂ assimilation, the provenances had different physiological mechanisms mainly
53 diverging in stomatal regulation, an important factor in controlling O₃ uptake. These divergences in
54 stomatal mechanisms might be ascribable to passed adaptations to the different climatic conditions
55 of their geographical areas (Piedmont, without dry-summer vs Tuscany, with dry-summer).
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1 In Piedmont plants, O₃ exposure did not induce stomatal closure. Missing contaminant's avoidance
2 of these plants seems to confirm and explain their high sensitivity in terms of visible injury and
3 water status described above. However, these plants showed mesophyllic alterations (higher C_i)
4 only in the first two weeks of treatment (when the onset of stippling happened), suggesting that
5 some repair or acclimation mechanisms might be established in the second period of the exposure.
6 Thus, the reduced photoassimilation was mainly related to impairments of PSII (reduced F_v/F_m,
7 Φ_{PSII} and qP) due to the strong oxidative stress caused by the missing stomatal closure throughout
8 the whole fumigation. Indeed, at the end of the treatment O₃ induced a decrease in chlorophylls and
9 carotenoids contents in accordance with recent reports on several tree species (e.g. Pellegrini et al.
10 2013; Pellegrini 2014). Although Piedmont leaves did not partially avoid O₃ uptake, they recovered
11 also in terms of qP at the end of the treatment (28-35 days FBE), and their heat dissipations were
12 not affected throughout the whole experiment (unchanged qNP). Indeed, DEPS did not change, and
13 this suggests that xanthophyll cycle was not activated. Thus, the tentatively repair or acclimation
14 mechanisms shown by Piedmont plants at the end of the exposure (in terms of mesophyll CO₂
15 fixation ability and qP) could be provided by some antioxidant defense systems, in order to alleviate
16 the excess of excitation pressure (Pellegrini et al. 2015b). At the chloroplast level, an important
17 antioxidant role is played by β-carotene (Castagna et al. 2001). However, in our case the reduction
18 of this leaf pigment was similar to the other carotenoids (*data not shown*), and it leads to evaluate
19 this response as a damage and not as a protective mechanism.
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35 Differently, in Tuscany plants the constrains in photoassimilation were attributable to both stomatal
36 and mesophyllic limitations. Similar responses have been reported before (Pellegrini 2014;
37 Pellegrini et al. 2011; Cotrozzi et al. 2016). In these plants, stomatal closure was regarded as the
38 critical factor in reducing photosynthetic rate (A and g_s changed at the same rates throughout the
39 first four weeks). However, stomatal closure reduced not only the CO₂ in substomatal chamber, but
40 also the incoming O₃ (cumulative O₃ uptake decreased, *data not shown*). These mechanisms
41 presumably reduced the oxidative pressure, leading to the lower sensitivity related to visible injury
42 (in comparison with Piedmont plants) and the tolerance in terms of water status (unchanged RWC)
43 mentioned above, as well as preventing a chronic photoinhibition. We found that the maximal
44 efficiency of PSII photochemistry of Tuscany leaves was not affected throughout the whole
45 treatment (although F_v/F_m decreased a couple of times, plants recovered and this parameter was
46 always close to the range reported for healthy plants by Björkman and Demming 1987), similarly to
47 other studies on *F. excelsior* (Contran et al. 2009; Paoletti et al. 2008). This is in agreement with the
48 unchanged chlorophyll concentrations observed in this provenance. However, lowered O₃ levels
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2 incoming into the leaves led to a significant decrease of qP reducing the capacity for reoxidizing
3 Q_A during actinic illumination (Pellegrini et al. 2015b). Furthermore, the reduction of Φ_{PSII} suggests
4 that there was a tendency to reduce the light energy used in photochemistry at the expense of the
5 capacity to dissipate the excess of excitation energy, as indicated by the higher values of qNP after
6 28 days. Similar results have been reported in *Quercus ilex* by Cotrozzi et al. (2016). However, the
7 strategy adopted by Tuscan provenance was lost at the end of the treatment when plants were not
8 more able to partially avoid the O_3 incoming by stomatal closure which led to a slight decrease
9 of F_v/F_m . Interestingly, plants responded to the increased oxidative pressure removing the negative
10 effects on Φ_{PSII} , qP and qNP observed the week before, plausibly due to the activation of some
11 photoprotective mechanisms. Probably, they were still able to turn on these responses by virtue of
12 the reduced oxidative stress suffered in the weeks before. However, also in this provenance the
13 defensive responses were not twinned to the activation of xanthophyll cycle (reduced total
14 carotenoids and unchanged DEPS) or to β -carotene (unchanged).

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16 Therefore, although the intra-specific sensitivity to O_3 among provenances mainly differing in
17 stomatal behaviors and consequently in the oxidative pressure levels, both provenances showed
18 detoxification and defense mechanisms. We speculate that these responses were presumably due to
19 a crucial participation of some secondary metabolites (the reprogramming of metabolism toward
20 secondary metabolites synthesis could represent a substantial rerouting of carbon skeletons,
21 suggesting a possible additional explanation of the observed reductions in net CO_2 assimilation).
22 The effect of elevated O_3 levels on secondary metabolites in woody plants has mainly been studied
23 in relation to phenylpropanoid compounds, which play a key role in plant defense against different
24 environmental stress conditions thanks to their barrier and antioxidant effects (Richet et al. 2012).
25 The phenylpropanoid pathway is indeed one of the most affected target of O_3 , which is able to
26 induce genes, enzymes and biosynthetic products (Castagna and Ranieri 2009). Several previous
27 studies in woody species have shown increases in total phenols due to O_3 (e.g. Di Baccio et al.
28 2008; Richet et al. 2012; Couture et al. 2014). However, variable trends were observed as far as
29 single metabolites are concerned, probably also because of the extraordinary diversity functions
30 played by phenylpropanoid compounds (Castagna and Ranieri 2009). This is supported by the
31 different behavior of simple phenolic acids (located at the beginning of phenylpropanoid pathway)
32 between provenances: hydroxybenzoic ones tended to drop under O_3 (especially in the more O_3 -
33 sensitive Piedmont plants), whereas hydroxycinnamic ones tended to be triggered by the pollutant
34 (especially in the less O_3 -sensitive Tuscan plants). Among phenylpropanoids, a great deal of effort
35 in ROS scavenging is performed by flavonoids by virtue of their wide localization into the cell and

1 the number and arrangement of their hydroxyl groups attached to ring structures (Gill and Tuteja
2 2010). This function is especially performed by dihydroxy B-ring-substituted flavonoids (Agati et
3 al. 2013). However, several studies indicated that flavonoid composition among plant species and
4 even different tissues can be remarkably different (Gutha et al. 2010). Our chromatographic profiles
5 suggest that these differences may be also among provenances of the same species. Similar
6 responses have been reported also in ash plants in response to the insect *Agrius planipennis* (Chen
7 et al. 2011) and drought (Fini et al. 2012). Differences in flavonoids among species/provenances
8 might be due to their multiple functions and locations in a wide-range of plant organs as well as in
9 different cells and cellular compartments (Agati et al. 2012; 2013). However, regarding the leaf
10 content of specific flavonoids, our results lead us to speculate that the main function of these
11 compounds in *F. excelsior* is antioxidative. In fact, although unaltered levels of quercetin, its
12 glycosides (rutin, isoquercitrin and quercitrin; dihydroxy B-ring-substituted ‘effective antioxidant’
13 flavonols, Agati et al. 2012) were affected by O₃ at least in one provenance, as well as apigenin (a
14 mono-B-ring substituted flavone), whereas the ‘poor antioxidant’ kaempferol rutinoside (Agati et
15 al. 2013) did not change in all plants. Specifically, only quercitrin and apigenin increased in both
16 provenances, suggesting a key role of these compounds as antioxidants. Particularly, quercitrin
17 should be more efficacious since its higher antioxidant capacity compared to apigenin (Agati et al.
18 2012). Furthermore, the increase of isoquercitrin observed only in Tuscany plants, supports our
19 speculation on the role of flavonoids as antioxidants, suggesting a possible involvement of this
20 compound in less O₃-sensitivity of this provenance (in comparison to Piedmont one) described
21 above, despite the presumably increased oxidative pressure experienced at the end of the
22 experiment. The behavior of rutin (it decreased only in Tuscany) might be to capture superoxide
23 anion and drop lipid peroxidation, as reported in Palmer et al. (2002).
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43 Also lignins seem to be responsible of the intra-specific O₃-sensitivity among provenances (they
44 increased only in Tuscany plants). Stimulation of lignin pathway was associated with increased
45 lignin content in the leaves of poplar and beech exposed to O₃ (Cabané et al. 2004; Richet et al.
46 2012). In both species, the lignins synthesized under O₃ displayed a peculiar structure (relative to
47 constitutive lignins), which suggested a possible role in defense against ROS propagation. These
48 results suggest that O₃-induced lignins might contribute to trees tolerance to the contaminant
49 because of their barrier or antioxidant effect toward ROS. Further evidence for a role in defense is
50 that lignins increase in response to abiotic stresses is related to stress intensity and damages (Frei et
51 al. 2011).
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Co-occurring Mediterranean species display very contrasting polyphenol composition and concentration, thus suggesting for polyphenols multiple and species-specific functions in plants inhabiting severely constrained Mediterranean regions (Di Ferdinando et al. 2014). The presence of coumarins, secoiridoids, and phenylethanoids is a characteristic feature of *Fraxinus* species, whereas lignans, flavonoids and simple phenolic are also common, but they appear to have more limited distribution. Specifically, the coumarins distinguish the genus *Fraxinus* from the other genera in Oleaceae (Kostova and Iossifova 2007). Coumarins contribute essentially to the persistence of plants being involved in processes such as defense against phytopathogens, response to abiotic stresses, regulation of oxidative stress, and probably hormonal regulation (Tattini et al. 2014). In our experiment, this class of compounds was severely affected by O₃ (this could be a further explanation of the high O₃ sensitivity of *F. excelsior*). In contrast, isofraxidin, as well as trans-chalcone, were triggered by the contaminant suggesting a key role of these compounds as a secondary antioxidant system, activated when primary antioxidant defenses (e.g. carotenoids) are depleted. Furthermore, the different behaviors of verbascoside (phenylethanoid) and oleuropein (secoiridoid) among provenances lead us to speculate on their influence in the tentatively repair or acclimation mechanisms shown by Piedmont plants at the end of the exposure in terms of mesophyll functionality and qP, despite the high oxidative pressure suffered throughout the whole experiment. However, these compounds were mainly explored in phytotherapy and food bioscience studies (e.g. D'Imperio et al. 2014; Yu et al. 2016) assessing their antioxidant activity, which partially confirm our hypothesis.

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Finally, the different behaviors and O₃-sensitivity among provenances may be also ascribable to the *de-novo* peaks triggered by O₃ not yet associated to some chemical compounds. Their presence is justified since synthesis and accumulation of phytoalexins, antimicrobial compounds in plants exposed to biotic and abiotic stressors, are well known in phytopathology (Iriti and Faoro 2009).

50 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65 **Conclusions**

In this study, the responses of two Italian provenances of *F. excelsior* to O₃ were evaluated. Although plants were monitored throughout the whole treatment in terms of visible foliar injury and photosynthetic performance, the innovative and central issue of our work was the evaluation of phenylpropanoid compounds at quali-quantitative levels performed at the end of exposure (at this time, also the RWC and leaf pigments were assessed). Indeed, to our knowledge, this is the first

1 study to evaluate the effects of O₃ on *F. excelsior* phenylpropanoids, known to play key roles in the
2 detoxification and defense of plants to the oxidative stress.
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4 In conclusion, the results of this study indicate: (i) the high O₃-sensitivity of *F. excelsior*, also
5 reported in previous studies (in terms of visible injury, water status and photosynthetic apparatus),
6 especially of Piedmont provenance in comparison with the Tuscany one; (ii) although the intra-
7 specific sensitivity to O₃ among provenances mainly differing in stomatal behavior (only Tuscany
8 plants closed stomata in order to partially avoid the pollutant gas) and presumably in the oxidative
9 pressure levels, both provenances showed detoxification and defense mechanisms: Piedmont plants
10 recovered in terms of CO₂ fixation ability and qP at the end of the experiment, despite the consistent
11 oxidative pressure; Tuscany plants responded to the increased oxidative pressure when they were
12 not more able to partially avoid the O₃ incoming at the end of the experiment, preserving PSII; (iii)
13 these response mechanisms seemed due to a crucial participation of phenylpropanoids, with a key
14 role played by flavonoids (especially quercitrin); among this class of compounds, isoquercitrin
15 might be a principal actor in the less O₃ sensitivity of Tuscany plants, as well as lignins; (iv) among
16 characteristic compounds of *Fraxinus* species, despite coumarins were severely affected by O₃,
17 isofraxidin were triggered by the contaminant suggesting a key role of this compound in ROS
18 detoxification, as well as trans-chalcone. Furthermore, the different behaviors of verbascoside
19 (phenylethanoid) and oleuropein (secoiridoid) among provenances lead us to speculate on their
20 influence in the tentatively repair or acclimation shown by Piedmont plants at the end of the
21 experiment. Finally, the intra-specific O₃ sensitivity among provenances may be also related to *de-*
22 *novo* peaks triggered by O₃ not yet associated to some chemical compounds.
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40 Additional work is clearly needed, although our work already proved that the expanding knowledge
41 of phenylpropanoids can provide novel insights into the evolution of plant responses to O₃, and
42 more generally to climate change expected in the Mediterranean environment.
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Acknowledgments

This study was supported by MIUR, Rome, project PRIN 2010-2011 “Planning the green city in the global change era: urban tree functions and suitability for predicted future climates (TreeCity)”.

References

- Agati G, Azzarello E, Pollastri S, Tattini M (2012) Flavonoids as antioxidants in plants: location and functional significance. *Plant Sci* 196:67-76.
- Agati A, Brunetti C, Di Ferdinando M, Ferrini F, Pollastri S, Tattini M (2013) Functional roles of flavonoids in photoprotection: new evidence, lessons from the past. *Plant Physiol Biochem* 72:35-45.
- Björkman O, Demming B (1987) Photon yield of O₂ evolution and chlorophyll fluorescence characteristics at 77 K among vascular plants of diverse origin. *Planta* 170:489–504.
- Brinkmann K, Blaschke L, Polle A (2002) Comparison of different methods for lignin determination as a basis for calibration of near-infrared reflectance spectroscopy and implications of lignoproteins. *J Chem Ecol* 28:2483-2501.
- Bussotti F, Agati G, Desotgiu R, Matteini P, Tani C (2005) Ozone foliar symptoms in woody plant species assessed with ultrastructural and fluorescence analysis. *New Phytol* 166:941-955.
- Bussotti F, Desotgiu R, Cascio C, Strasser RJ, Gerosa G, Marzuoli R (2007) Photosynthesis responses to ozone in young trees of three species with different sensitivities, in a 2-year open-top chamber experiment (Curno, Italy). *Physiol Plant* 130:122-135.
- Bussotti F, Pollastrini M (2015) Evaluation of leaf features in forest trees: methods, techniques, obtainable information and limits. *Ecol Indic* 52:219-230
- Cabané M, Pireaux JC, Léger E, Weber E, Dizengremel P, Pollet B, Lapierre C (2004) Condensed lignins are synthesized in poplar leaves exposed to ozone. *Plant Physiol* 134:586-594.
- Carriero G, Brunetti C, Fares S, Hayes F, Hoshika Y, Mills G, Tattini M, Paoletti E (2016) BVOC responses to realistic nitrogen fertilization and ozone exposure in silver birch. *Environ Pollut*, 213: 988-995.

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55
56
57
58
59
60
61
62
63
64
65

Castagna A, Nali C, Ciompi S, Lorenzini G, Soldatini GF, Ranieri A (2001). Ozone exposure affects photosynthesis of pumpkin (*Cucurbita pepo*) plants. *New Phytol* 152:223-229.

Castagna A, Ranieri A (2009) Detoxification and repair process of ozone injury: from O₃ uptake to gene expression adjustment. *Environ Pollut* 157:1461-1469.

Chen Y, Whitehill JGA, Bonello P, Poland TM (2011) Differential response in foliar chemistry of three ash species to emerald ash borer adult feeding. *J Chem Ecol* 37:29-39.

Cheynier V, Comte G, Davies KM, Lattanzio V, Martens S (2013) Plant phenolics: recent advances on their biosynthesis, genetics, and ecophysiology. *Plant Physiol Biochem* 72:1-20.

Contran N, Paoletti E (2007) Visible foliar injury and physiological responses to ozone in Italian provenances of *Fraxinus excelsior* and *F. ornus*. *Scientific World Journal* 7:90-97.

Cotrozzi L, Remorini D, Pellegrini E, Landi M, Massai R, Nali C, Guidi L, Lorenzini G (2016) Variations in physiological and biochemical traits of oak seedlings grown under drought and ozone stress. *Physiol Plant* 157:69-84.

Couture JJ, Holeski LM, Lindroth RL (2014) Long-term exposure to elevated CO₂ and O₃ alters aspen foliar chemistry across developmental stages. *Plant Cell Environ* 37:758-765.

D'Imperio M, Cardinali A, D'Antuono I, Linsalata V, Minervini F, Redan BW, Ferruzzi MG (2014) Stability-activity of verbascoside, a known antioxidant compound, at different pH conditions. *Food Res Int* 66:373-378.

de Leeuw FAAM, van Zantwoort EDG (1997) Mapping of exceedances of ozone critical levels for crops and forest trees in the Netherlands. *Environ Pollut* 96:89-98.

Di Baccio D, Castagna A, Paoletti E, Sebastiani L, Ranieri A (2008) Could the differences in O₃ sensitivity between two poplar clones be related to a difference in antioxidant defense and secondary metabolic response to O₃ influx? *Tree Physiol* 28:1761-1772.

Di Ferdinando M, Brunetti C, Agati G, Tattini M (2014) Multiple functions of polyphenols in plants inhabiting unfavorable Mediterranean areas. *Environ Exp Bot* 103:107-116.

Döring AS, Pellegrini E, Campanella A, Trivellini A, Gennai C, Petersen M, Nali C, Lorenzini G (2014) How sensitive is *Melissa officinalis* to realistic ozone concentration? *Plant Physiol Biochem* 74:156-164.

1 EEA (2015) The European environment – state and outlook 2015: synthesis report, European
2 Environment Agency, Copenhagen.

3
4 Fini A, Guidi L, Ferrini F, Brunetti C, Di Ferdinando M, Biricolti S, Pollastri S, Calamai L, Tattini
5 M (2012) Drought stress has contrasting effects on antioxidant enzymes activity and
6 phenylpropanoid biosynthesis in *Fraxinus ornus* leaves: An excess light stress affair? J Plant
7 Physiol 169:929-939.

8
9
10
11
12 Frei M, Kohno Y, Wissuwa M, Makkar HPS, Becker KL (2011) Negative effects of tropospheric
13 ozone on the feed value or rice straw are mitigated by an ozone tolerance QTL. Glob Chang Biol
14 17:2319-2329.

15
16
17
18
19 Gerosa G, Marzuoli R, Bussotti F, Pancrazi M, Ballarin-Denti A (2003) Ozone sensitivity of *Fagus*
20 *sylvatica* and *Fraxinus excelsior* young trees in relation to leaf structure and foliar ozone uptake.
21 Environ Pollut 125:91-98.

22
23
24
25
26 Gerosa G, Marzuoli R, Desotgiu R, Bussotti F, Ballarin-Denti A. (2009) Validation of the stomatal
27 flux approach for the assessment of ozone visible injury in young forest trees. Results from the TOP
28 (transboundary ozone pollution) experiment at Curno, Italy. Environ Pollut 157:1497-1505.

29
30
31
32 Gill SS, Tuteja N (2010) Reactive oxygen species and antioxidant machinery in abiotic stress
33 tolerance in crop plants. Plant Physiol Biochem 48:909-930.

34
35
36
37 Gottardini E, Cristofori A, Cristofolini F, Nali C, Pellegrini E, Bussotti F, Ferretti M (2014)
38 Chlorophyll-related indicators are linked to visible ozone symptoms: evidence from a field study on
39 native *Viburnum lantana* L. plants in northern Italy. Ecol Indic 39:65-74.

40
41
42
43 Gravano E, Bussotti F, Strasser RJ, Schaub M, Novak K, Skelly J, Tani C (2004) Ozone symptoms
44 in leaves of woody plants in open-top chambers: ultrastructural and physiological characteristics.
45 Physiol Plant 121:620-633.

46
47
48
49
50 Guidi L, Remorini D, Cotrozzi L, Giordani T, Lorenzini G, Massai R, Nali C, Natali L, Pellegrini
51 E, Trivellini A, Vangelisti A, Vernieri P, Landi M (2016) The harsh life of an urban tree: the effect
52 of a single pulse of ozone in salt-stressed *Quercus ilex* saplings. Tree Physiol.
53 doi:10.1093/treephys/tpw103.

1
2 Gutha LR, Casassa LF, Habertson JF, Naidu RA (2010) Modulation of flavonoid biosynthetic
3 pathway genes and anthocyanins due to virus infection in grapevine (*Vitis vinifera* L.) leaves. BMC
4 Plant Biol 10:187.
5
6 Innes JL, Skelly JM, Schaub M (2001) Ozone and broadleaved species. A guide to the identification
7 of ozone-induced foliar injury. Eidgenossische Forschungsanstalt WSL, Birmensdorf.
8
9 Iriti M, Faoro F (2009) Chemical diversity and defence metabolism: how plants cope with
10 pathogens and ozone pollution. Int J Mol Sci 10:3371-3399.
11
12 Kline LJ, Davis DD, Skelly JM, Decoteau DR (2009) Variation in ozone sensitivity within Indian
13 hemp and common milkweed selections from the Midwest. Northeast Nat 16:307-313.
14
15 Kostova I, Iossifova T (2007) Chemical components of *Fraxinus* species. Fitoterapia 78:85-106.
16
17 Lorenzini G, Medeghini Bonatti P, Nali C, Baroni Fornasiero R (1994) The protective effect of rust
18 infection against ozone, sulfur-dioxide and paraquat toxicity symptoms in broad bean. Physiol Mol
19 Plant Pathol 45:263-279.
20
21 Mikkelsen BL, Olsen CE, Lyngkjær MF (2015) Accumulation of secondary metabolites in healthy
22 and diseased barley, grown under future climate levels of CO₂, ozone and temperature.
23 Phytochemistry 118:162-173.
24
25 Nali C, Ferretti M, Pellegrini M, Lorenzini G (2001) Monitoring and biomonitoring of surface
26 ozone in Florence, Italy. Environ Monit Assess 69:159-174.
27
28 Nali C, Francini A, Pellegrini E, Santarelli S, Lorenzini G (2010) Ecophysiological and
29 histochemical responses to ozone in tree species are influenced by the provenance. Acta Hort
30 881:535-538.
31
32 Nali C, Paoletti E, Marabottini R, Della Rocca G, Lorenzini G, Paolacci AR, Ciaffi M, Badiani M
33 (2004). Ecophysiological and biochemical strategies of response to ozone in Mediterranean
34 evergreen broadleaf species. Atmos Environ 38:2247-2257.
35
36 Novak K, Cherubini P, Saurer M, Fuhrer J, Skelly JM, Krauchi N, Schaub M (2007) Ozone air
37 pollution effects on tree-ring growth, $\delta^{13}\text{C}$, visible foliar injury and leaf gas exchange in three
38 ozone-sensitive woody plant species. Tree Physiol 27:941-949.
39
40
41
42
43
44
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46
47
48
49
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51
52
53
54
55
56
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61
62
63
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- 1
2 Palmer H, Ohta M, Watanabe M, Suzuki T (2002) Oxidative stress-induced cellular damage caused
3 by UV and methyl viologen in *Euglena gracilis* and its suppression with rutin. J Photoch Photobio
4 B 67:116-129.
5
- 6 Paludan-Müller G, Saxe H, Leverenz JW (1999) Responses to ozone in 12 provenances of
7 European beech (*Fagus sylvatica*): genotypic variation and chamber effects on photosynthesis and
8 dry-matter partitioning. New Phytol 144:261-273.
9
- 10 Paoletti E, Manning WJ, Spaziani F, Tagliaferro F (2007) Gravitational infusion of ethylenediurea
11 (EDU) into trunks protected adult European ash trees (*Fraxinus excelsior* L.) from foliar ozone
12 injury. Environ Pollut 145:869-873.
13
- 14 Paoletti E, Contran N, Manning WJ, Castagna A, Ranieri A, Tagliaferro F (2008) Protection of ash
15 (*Fraxinus excelsior*) trees from ozone injury by ethylenediurea (EDU): Roles of biochemical
16 changes and decreased conductance in enhancement of growth. Environ Pollut 155:464-472.
17
- 18 Paoletti E (2009) Ozone and urban forests in Italy. Environ Pollut 157:1506-1512.
19
- 20 Pautasso M, Aas G, Queloz V, Holdenrieder O (2013) European ash (*Fraxinus excelsior*) dieback –
21 A conservation biology challenge. Biol Conserv 158:37-49.
22
- 23 Peel MC, Finlayson BL, McMahon TA (2007) Updated world map of the Köppen-Geiger climate
24 classification. Hydrol Earth Syst Sc 11:1633–1644.
25
- 26 Pellegrini E, Francini A, Lorenzini G, Nali C (2011) PSII photochemistry and carboxylation
27 efficiency in *Liriodendron tulipifera* under ozone exposure. Environ Exp Bot 70:217-226.
28
- 29 Pellegrini E, Nali C, Lorenzini G (2013) Ecophysiology of *Tilia americana* under ozone
30 fumigation. Atmos Pollut Res 4:142-146.
31
- 32 Pellegrini E (2014) PSII photochemistry is the primary target of oxidative stress imposed by ozone
33 in *Tilia Americana*. Urban For Urban Gree 13:94-102.
34
- 35 Pellegrini E, Campanella A, Paolucci M, Trivellini A, Gennai C, Muganu M, Nali C, Lorenzini G
36 (2015a) Functional leaf traits and diurnal dynamics of photosynthetic parameters predict the
37 behavior of grapevine varieties towards ozone. PLoS ONE 10 (8): e0135056.
38
- 39 Pellegrini E, Francini A, Lorenzini G, Nali C (2015b) Ecophysiological and antioxidant traits of
40 *Salvia officinalis* under ozone stress. Environ Sci Pollut Res 22:13083-13093.
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

1 Richet N, Tozo K, Afif D, Banvoy J, Lingay S, Dizengremel P, Cabané M (2012) The response to
2 daylight or continuous ozone of phenylpropanoid and lignin biosynthesis pathways in poplar differs
3 between leaves and wood. *Planta* 236:727-737.
4
5

6 Schreiber U, Schilwa U, Bilger W (1986) Continuous recording of photochemical and non-
7 photochemical chlorophyll fluorescence quenching with a new type of modulation fluorometer.
8 *Photosynth Res* 10:51-62.
9
10

11 Sicard P, De Marco A, Dalstein-Richier L, Tagliaferro F, Renou C, Paoletti E (2016) An
12 epidemiological assessment of stomatal ozone flux-based critical levels for visible ozone injury in
13 Southern European forests. *Sci Total Environ* 541:729-741.
14
15
16
17
18

19 Tattini M, Di Ferdinando M, Brunetti C, Goti A, Pollastri S, Bellasio C, Giordano C, Fini A, Agati
20 G (2014) Esculetin and esculin (esculetin 6-O-glucoside) occur as inclusions and are differently
21 distributed in the vacuole of palisade cells in *Fraxinus ornus* leaves: a fluorescence microscopy
22 analysis. *J Photochem Photobio B* 140:28-35.
23
24
25
26

27 Vogt T (2010) Phenylpropanoid biosynthesis. *Mol Plant* 3:2-20.
28
29

30 Von Caemmerer S, Farquhar GD (1981) Some relationship between the biochemistry and
31 photosynthesis and the gas-exchange of leaves. *Planta* 153:376-387.
32
33
34

35 Yu H, Liu P, Tang H, Jing J, Lv X, Chen L, Jiang L, Xu J, Li J (2016) Oleuropein, a natural extract
36 from plants, offers neuroprotection in focal cerebral ischemia/reperfusion injury in mice. *Eur J of*
37 *Pharmacol* 775:113-119.
38
39
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Table 1. Leaf pigment content (mean \pm standard deviation) in *Fraxinus excelsior* plants from Piedmont and Tuscany, exposed to 150 ppb O₃ for 35 days (8 h d⁻¹) or maintained in filtered air. For each parameter, the data were analyzed by Student's *t*-test. The significant differences are for: *** = $P \leq 0.001$, ** = $P \leq 0.01$, * = $P \leq 0.05$, ns = $P > 0.05$.

		Piedmont			Tuscany		
		Control	Ozone	<i>P</i>	Control	Ozone	<i>P</i>
Chlorophyll (<i>a + b</i>)	($\mu\text{g mg}^{-1}$ DW)	63.1 \pm 3.47	42.5 \pm 2.23	***	30.5 \pm 1.80	26.9 \pm 2.62	ns
Total carotenoids	($\mu\text{g mg}^{-1}$ DW)	33.7 \pm 2.87	21.4 \pm 0.95	**	18.8 \pm 2.13	13.5 \pm 2.17	*
β -carotene	($\mu\text{g mg}^{-1}$ DW)	23.9 \pm 2.03	14.9 \pm 0.64	**	12.7 \pm 1.23	9.4 \pm 2.00	ns
DEPS	%	6.1 \pm 0.87	5.5 \pm 0.63	ns	5.4 \pm 1.11	5.2 \pm 0.93	ns

Table 2. HPLC determination of coumarins, phenylethanoids, secoiridoids and chalcones (mean \pm standard deviation) in leaves of *Fraxinus excelsior* plants from Piedmont and Tuscany, exposed to 150 ppb O₃ for 35 days (8 h d⁻¹) or maintained in filtered air. For each parameter, the data were analyzed by Student's *t*-test. The significant differences are for: *** = $P \leq 0.001$, ** = $P \leq 0.01$, * = $P \leq 0.05$, ns = $P > 0.05$.

		Piedmont			Tuscany			
		Control	Ozone	<i>P</i>	Control	Ozone	<i>P</i>	
<i>Coumarins</i>								
	Esculin	(ng g ⁻¹ DW)	6551 \pm 318.1	3213 \pm 101.2	***	4149 \pm 49.8	2893 \pm 85.5	***
	Esculetin	(ng g ⁻¹ DW)	445 \pm 45.5	264 \pm 24.8	**	264 \pm 68.6	32 \pm 10.1	**
	Fraxetin	(ng g ⁻¹ DW)	1157 \pm 250.9	693 \pm 47.4	*	690 \pm 56.4	397 \pm 46.0	**
	Scopoletin	(ng g ⁻¹ DW)	197 \pm 92.8	161 \pm 65.1	ns	71 \pm 10.5	43 \pm 9.4	*
	Isofraxidin	(ng g ⁻¹ DW)	1128 \pm 33.6	1774 \pm 323.5	*	564 \pm 27.9	957 \pm 123.5	**
<i>Phenylethanoids</i>								
	Verbascoside	(ng g ⁻¹ DW)	2104 \pm 584.5	2000 \pm 324.0	ns	1970 \pm 740.7	765 \pm 49.4	*
<i>Secoiridoids</i>								
	Oleuropein	(ng g ⁻¹ DW)	666 \pm 170.1	5151 \pm 336.8	***	2577 \pm 1006.1	4272 \pm 496.9	ns
<i>Chalcones</i>								
	Trans-chalcone	(ng g ⁻¹ DW)	10 \pm 1.2	36 \pm 3.8	***	20 \pm 2.8	28 \pm 0.2	**
<i>Flavonoids</i>								
	Quercetin	(ng g ⁻¹ DW)	42 \pm 7.8	42 \pm 7.4	ns	36 \pm 2.9	43 \pm 11.7	ns
	Rutin	(ng g ⁻¹ DW)	56 \pm 31.1	36 \pm 16.3	ns	443 \pm 44.0	254 \pm 35.9	**
	Isoquercetrin	(ng g ⁻¹ DW)	182 \pm 54.2	190 \pm 17.9	ns	70 \pm 30.8	227 \pm 67.9	*
	Quercitrin	(ng g ⁻¹ DW)	210 \pm 83.2	362 \pm 26.0	*	135 \pm 21.4	390 \pm 41.4	***
	Kaempferol-rutinoside	(ng g ⁻¹ DW)	721 \pm 195.0	737 \pm 100.4	ns	997 \pm 121.7	1056 \pm 153.0	ns
	Apigenin	(ng g ⁻¹ DW)	2 \pm 1.6	8 \pm 2.1	*	5 \pm 1.0	43 \pm 5.1	***
<i>Phenolic acids</i>								
	Vanillic acid	(ng g ⁻¹ DW)	248 \pm 35.7	77 \pm 10.1	**	170 \pm 25.9	41 \pm 6.1	**
	Syringic acid	(ng g ⁻¹ DW)	112 \pm 21.5	56 \pm 2.2	*	62 \pm 20.7	60 \pm 15.1	ns
	<i>p</i> -coumaric acid	(ng g ⁻¹ DW)	94 \pm 10.1	108 \pm 5.5	ns	39 \pm 2.1	113 \pm 10.5	***
	Ferulic acid	(ng g ⁻¹ DW)	534 \pm 33.2	898 \pm 16.7	***	102 \pm 2.1	460 \pm 64.4	***

Figure captions

Fig. 1 Profiles of CO₂ assimilation rate (A) (A-B), stomatal conductance to water vapor (g_s) (C-D) and intercellular CO₂ concentration (C_i) (E-F) in Piedmont (left) and Tuscany (right) provenances of *Fraxinus excelsior* leaves exposed to 150 ppb O₃ (filled symbols) or 0 ppb O₃ (empty symbols) for 35 days (8 h d⁻¹). Data are shown as mean ± standard deviation. For each parameter, different letters indicate significant differences ($P \leq 0.05$). In the boxes, results of repeated measurements ANOVA are reported, asterisks showing the significance of factors (ozone and time) and their interaction for: *** = $P \leq 0.001$, ** = $P \leq 0.01$, * = $P \leq 0.05$, ns = $P > 0.05$.

Fig. 2 Profiles of potential PSII photochemical activity (F_v/F_m) (A-B), actual PSII photochemical activity (Φ_{PSII}) (B-C), photochemical (qP) (E-F) and no photochemical quenching (qNP) (G-H) in Piedmont (left) and Tuscany (right) provenances of *Fraxinus excelsior* leaves exposed to 150 ppb O₃ (filled symbols) or 0 ppb O₃ (empty symbols) for 35 days (8 h d⁻¹). Data are shown as mean ± standard deviation. For each parameter, different letters indicate significant differences ($P \leq 0.05$). In the boxes, results of repeated measurements ANOVA are reported, asterisks showing the significance of factors (ozone and time) and their interaction for: *** = $P \leq 0.001$, ** = $P \leq 0.01$, * = $P \leq 0.05$, ns = $P > 0.05$.

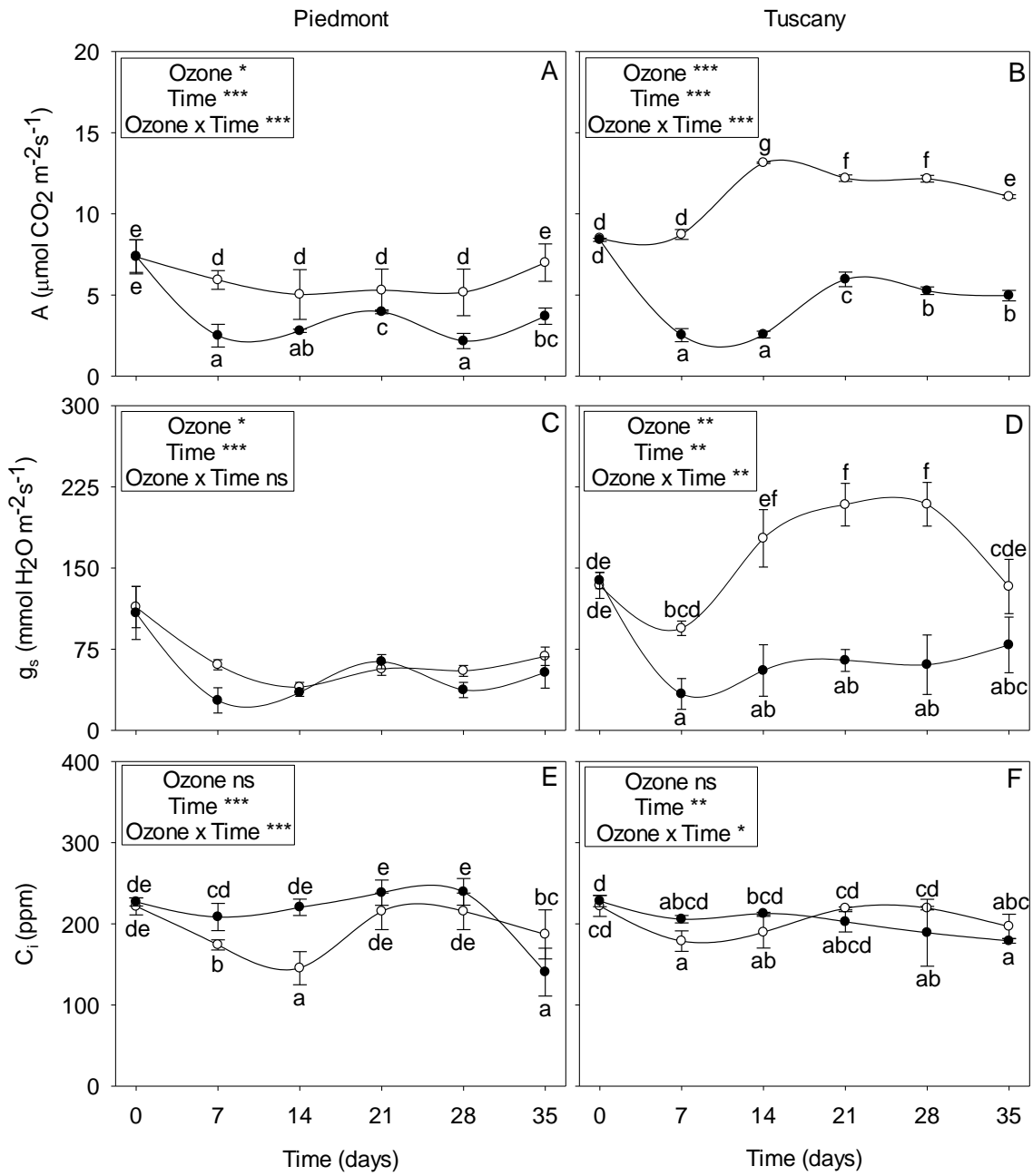


Figure 1

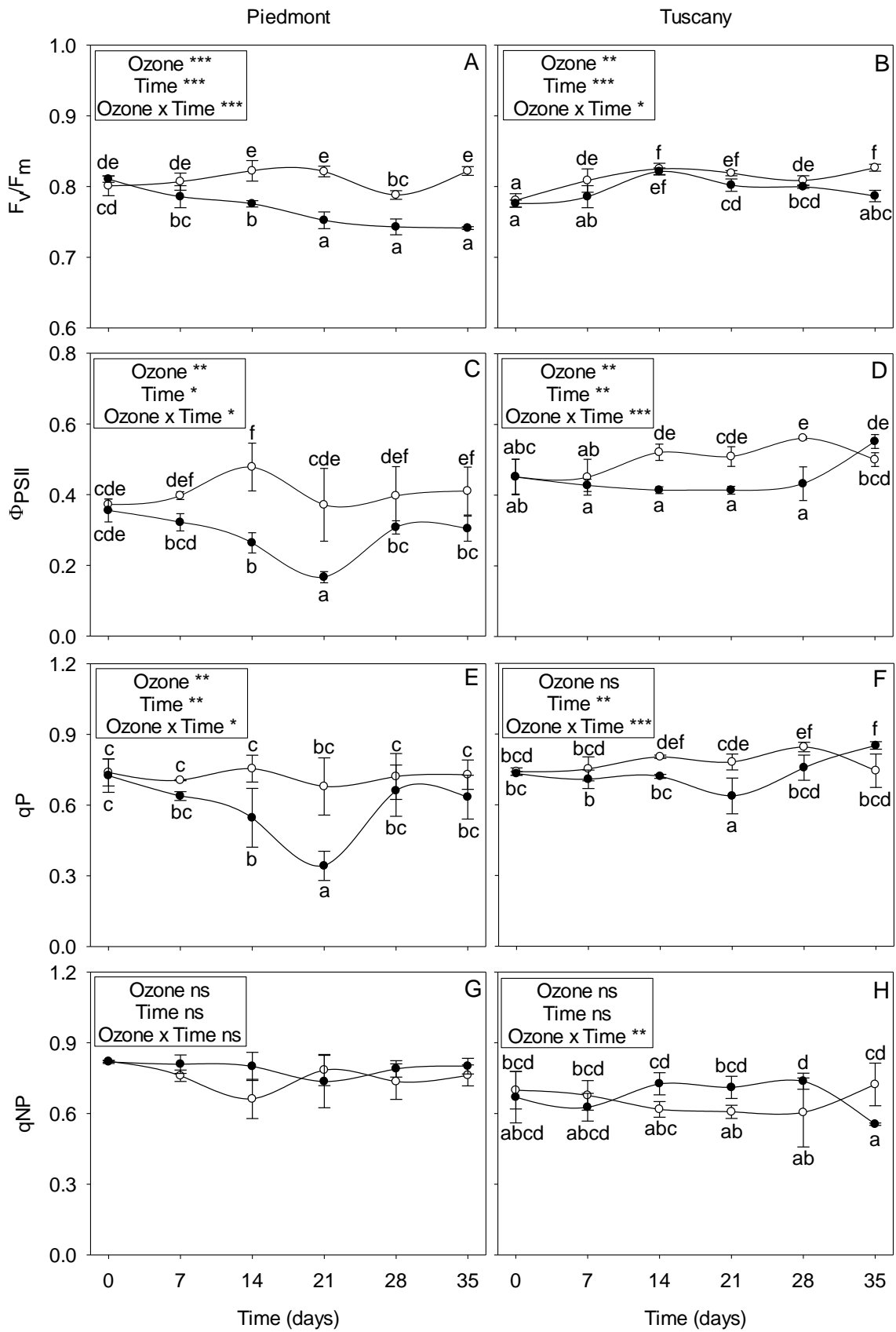


Figure 2



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Supplementary Material

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