Ecophysiological and antioxidant traits of *Salvia officinalis* under ozone stress

Elisa Pellegrini[1], Alessandra Francini[1], Giacomo Lorenzini[1,2], Cristina Nali[1*]

[1]*Department of Agriculture, Food and Environment, University of Pisa, Via del Borghetto 80, 56124 Pisa, Italy*

[2]*Interdepartmental Research Center Nutrafood, University of Pisa, via del Borghetto 80, 56124 Pisa - Italy*

Abstract Ecophysiological and antioxidant traits were evaluated in sage (*Salvia officinalis*) plants exposed to 120 ppb of ozone for 90 consecutive days (5 h d^{-1}). At the end of fumigation, plants showed slight leaf yellowing that could be considered the first visual symptom of leaf senescence. Ozone-stressed leaves showed (i) reduced photosynthetic activity (-70% at the end of exposure), (ii) chlorophyll loss (-59 and -56% of chlorophyll *a* and *b* concentrations, starting from 30 days from the beginning of exposure) and (iii) cellular water deficit (-12% of the relative water content at the end of the fumigation). These phenomena are indicative of oxidative stress in the chloroplasts (as confirmed by the strong degradation of β-carotene) despite the photoprotection conferred by xanthophylls cycle [as demonstrated by the significant rise of de-epoxidation index, reaching the maximum value at the end of the treatment (+69%)], antioxidant compounds [as confirmed by the increase of phenols (in particular caffeic acid and rosmarinic acid) and water soluble carbohydrates (especially monosaccharides)]. By means of combined ecophysiological and biochemical approaches, this study demonstrates that *S. officinalis* is able to activate an adaptive survival mechanism allowing the plant to complete its life cycle even under oxidative stressful conditions.

Keywords carbohydrates, carotenoids, medicinal plants, oxidative stress, phenolic acids, xanthophyll cycle

* Corresponding author. Tel.: +39 0502210552; fax: +39 0502210559.

E-mail address: cristina.nali@unipi.it (C. Nali)

Abbreviations

%D Fraction of absorbed light that was thermally dissipated in PSII antennae; Φ_{PSII} Actual quantum yield of PSII; A Photosynthetic activity; ANOVA Analysis of variance; A+V Anteraxanthin+Violaxanthin; CEO_3 Cumulative Exposure to O_3; C_i Intercellular CO_2 concentration; Chl Chlorophyll; CUOs Cumulative O_3 Uptakes; DEPS De-epoxidation index value; F_0 Minimal fluorescence; F'_0 Minimal fluorescence in the light-adapted state; FBE From the beginning of exposure; F_m Maximal fluorescence; F'_m Maximal fluorescence in the light-adapted state; F_s Steady-state fluorescence yield in the light-adapted state; FW Fresh weight; G_w Stomatal conductance to water vapour; LHC Light harvesting complex; O_3 Ozone; PFD, photon flux density; PSI Photosystem I; PSII Photosystem II; qNP No-photochemical quenching; qP Photochemical quenching; ROS Reactive Oxygen Species; RWC Relative Water Content; WUE_i Intrinsic Water Use Efficiency

Introduction

According to the European Environment Agency (EEA 2014), 167 exceedance days of the long-term objective [8-h average of 60 ppb for ozone (O_3)] were reported by Spain and Italy in the summer 2013. Maximum hourly mean was 136 ppb (detected in Italy). Climatic models predict that record-breaking temperature anomalies (heat waves) will become more intense, longer lasting and/or more frequent. As a consequence, exposure to high levels of O_3 of sensitive targets is to be expected (Pellegrini et al. 2007; Lorenzini et al. 2014). Several projections indicate O_3 concentrations reaching 75 ppb over much of Europe by 2100 (Mills et al. 2013). The issue is a priority not only in urban areas (where the greatest production of anthropogenic precursors takes place, mainly as a consequence of road traffic), but also in rural and remote areas (Sicard et al. 2013). Europe's sustained ambient O_3 concentrations continue to adversely affect vegetation growth

and crop productivity (quantity and quality), reducing plant's uptake of carbon dioxide (CO_2) and resulting in serious damage and an increased economic burden for Europe.

O_3 penetrates leaves through the open stomata and, inside the plant, it produces several reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), superoxide ($O_2^{\bullet}$), hydroxyl ($OH^{\bullet}$) and hydroperoxyl ($HOO^{\bullet}$) radicals. These compounds cause many changes in the plant tissues, altering metabolic activity, causing protein and chlorophyll (chl) degradation and reduction in biomass production (Saitanis et al. 2014). Moreover, injury to membrane integrity is a common effect of this stress: ROS have a role in lipid peroxidation and in accelerated senescence (Peñarrubia and Moreno, 1999). The recognized primary O_3 target is the photosynthetic apparatus (Fiscus et al. 2005). O_3-related effects to photosynthetic processes increase the need to dissipate excessive energy due to overreduction of the photosystems. Carotenoids act as protectors of photosynthetic membranes against ROS and a stimulation of the xanthophyll cycle activity was observed especially in O_3-sensitive species, indicating enhanced non-photochemical dissipation processes (Pellegrini et al. 2011).

The production of antioxidants compounds - including vitamins, oligopeptides, phenolic acids, flavonoids and carotenoids - represents a prominent defense trait under O_3 stress and is related to leaf carbon economy. Phenol biosynthesis may be stimulated in response to abiotic constraints and O_3-stressed plants might represent potential source of phenols (Tonelli et al. 2014). In small phenolic molecules, the basic structure requirement responsible for antioxidant capacity is the existence of a phenyl ring with one or two hydroxyl groups in ortho- or meta-positions; in more complex molecules, the structure arrangements imparting the greatest antioxidant activity are: (a) ortho-30,40-dihydroxy moiety in ring B, (b) meta-5,7-dihydroxy arrangements in ring A, (c) 2,3-double bound in combination with both 4-keto group and 3-hydroxyl group in ring C. The more of these structural characteristics a molecule has, the more powerful is its antioxidant power (Severino et al. 2007).

Less is known about the adaptive biochemical function of compatible solutes in the scavenging of ROS that are overproduced during abiotic stresses. These osmoprotectants can accumulate to high levels without disturbing intracellular biochemistry. Carbohydrates are a major category of compatible solutes that include hexoses (mostly fructose and glucose), disaccharides (sucrose, trehalose), sugar alcohols (inositol, mannitol) and complex sugars (raffinose and stachyose), all of which accumulate during stress (Morsy et al. 2007).

During the last decades, medicinal and aromatic plants have been extensively studied and found be excellent sources of bioactive and health-promoting compounds (Ben Salem et al. 2013). Some compounds (i.e. flavonoids) are also beneficial for the plant itself by their significant role in plant resistance (Treutter 2006). In spite of this, medicinal plants can be adversely affected by several stress factors (Bettaieb et al. 2009). The impact of O_3 on medicinal plants remains poorly understood and a small number of species have so far been assessed for sensitivity (Dawnay and Mills 2009). *Salvia officinalis* L. (sage) is one of the most well-known aromatic herbs. Native of the southern Europe, is largely cultivated in the Mediterranean countries, where its leaves are used as raw materials in medicine, perfume and the food industry. The growing demand in the market for health-care products has revalorized species with antioxidant components such as the sage (Corell et al. 2012). Previous analytical investigations showed that health-promoting potential of sage leaves, i.e. antinflammatory, antiallergic, antifungal and antiseptic properties are thought to be due to their biologically active phenols (Ben Salem et al. 2013). Species of *Salvia* are considerate xerophytes and *S. officinalis* is a drought-susceptible species (Munné-Bosch et al. 2001). In Mediterranean areas, the crop is subjected to summer drought stress, high temperature, soil salinity and enhanced O_3 levels. Studies on sage have overall focused on the volatile fraction and phenolic compounds (Ben Taârit et al. 2012). However, a literature survey revealed a poorness of data on the physiological behavior of *S. officinalis* to oxidative stress.

Thus, the primary objective was to study the behavior of sage to oxidative stress, imposed by O_3, giving much emphasis to the role of photochemical and biochemical features that may integrate to avoiding or reducing the oxidative injury, using gas exchange and chl *a* fluorescence techniques. In addition, the significance of carotenoids and the xanthophyll cycle in the protection from photoinhibitory damage was investigated. Furthermore, phenolic and carbohydrate contents of leaf extracts from plants grown under O_3 conditions, that may also act as ROS scavengers, have been evaluated.

Materials and methods

Plant material, cultivation and ozone exposure

Four-month-old seedlings of *S. officinalis*, grown in plastic pots containing a mix of steam sterilized soil and peat (1:1), were placed for two weeks in a controlled environment facility at a temperature of 20±1 °C, a RH of 85±5% and a photon flux density at plant height of 500 µmol photon m^{-2} s^{-1} provided by incandescent lamps, during a 12 h photoperiod. Uniform-sized plants (20 cm tall) were placed in a controlled environment fumigation facility under the same climatic conditions as the growth chamber. The entire methodology has been performed according to Pellegrini et al. (2011). Plants were exposed to 120±13 ppb of O_3 (for O_3, 1 ppb = 1.96 µg m^{-3}, at 20 °C and 101.325 kPa) for 90 consecutive days (5 h d^{-1}, in form of a square wave between 09:00 and 14:00, solar time). Analyses were performed on fully expanded leaves at 30, 60 and 90 days from the beginning of exposure (FBE), corresponding to cumulative O_3 uptakes (CUOs) of: 1.62, 3.50 and 5.55 mmol m^{-2}, respectively. The calculation for CUO was performed utilizing the equation, according to Lombardozzi et al. (2013): CUO (mmol m^{-2}) = CEO_3 g_s k_{O3} 3600 × 10^{-6}, where k_{O3} = 1.67 is the ratio of the leaf resistance for O_3 to the leaf resistance to water, g_s is the leaf-level stomatal resistance (in units of mol H_2O m^{-2} s^{-1}) and CEO_3 is the cumulative exposure to O_3 calculated as: CEO_3 (nmol mol^{-1} h) = [O_3] H D, where H is the number of daytime hours the plant was exposed to

O_3, D is the total number of days, and [O_3] is the external O_3 concentration in ppb that plants were exposed to during daytime hours of the entire experimental period. 3600 is the number of seconds per hour and 10^{-6} is the conversion from nmol to mmol.

Visual assessment and plant water status

No O_3-specific visible injury symptoms such as chlorotic or necrotic speckling (Pellegrini et al. 2011) were observed throughout the fumigation period. At the end of exposure, O_3 effects were manifested as leaf yellowing which gradually spread from the distal tip of the leaf to the proximal region. As these symptoms appeared to be an enhancement of natural senescence, they were described here as 'senescence'. Whole plant senescence was assessed by a single trained assessor as the percentage of leaves with ≥ 25% senescence (Dawnay and Mills 2009).

Plant water status was determined by measuring the relative water content (RWC) in leaf discs (Ø 1 cm) excised, avoiding the midrib, according to Nali et al. (2005).

Ecophysiological measurements

Foliar CO_2 and water vapour exchanges were measured with an open infra-red gas exchange system (CIRAS-1, PP-Systems) equipped with a Parkinson leaf chamber, able to clamp single leaves. Details are reported in Pellegrini et al. (2011). Measurements were performed at ambient CO_2 concentrations (340-360 ppm) at 80% RH. The chamber was illuminated by a quartz halogen lamp and the leaf temperature was maintained at 26±0.4 °C. Photosynthetic activity (A) was measured at 800 μmol photons^{-2} s^{-1}. The calculation of intercellular CO_2 concentration (C_i) was based on the equations described in von Caemmerer and Farquhar (1981). Intrinsic water use efficiency (WUE_i) was determined as the ratio between A and stomatal conductance to water vapour (G_w) (according to Volkova et al. 2011).

Modulated chl *a* fluorescence measurements were carried out with a PAM-2000 fluorometer (Walz) on the same leaves used for gas exchange dark-adapted for 40 min. Minimal fluorescence (F_0), when all photosystem II (PSII) reaction centres were open, was determined using the

measuring modulated light; the maximal fluorescence level (F_m), when all PSII reaction centres were closed, was determined by applying a saturating light pulse (0.8 s) at 8,000 μmol m^{-2} s^{-1}. Fluorescence induction was started with actinic light (about 400 μmol m^{-2} s^{-1}) and superimposed with 800 ms saturating pulses (10,000 mol m^{-2} s^{-1} photon flux density, PFD) at 20 s intervals to determine maximal fluorescence in the light-adapted state (F'_m). Minimal fluorescence in the light-adapted state (F'_0) was determined in the presence of a far-red (>710 nm) background for 10 s. The saturation pulse method was used for analysis of photochemical (qP) and no-photochemical quenching (qNP) components as described by Schreiber et al. (1986). The actual quantum yield of PSII (Φ_{PSII}) was computed as $(F'_m - F_s)/F'_m$, where F_s is the steady-state fluorescence yield in the light-adapted state, as in Rohacek (2002).

Biochemical analyses

Pigment analysis was performed by HPLC according to Döring et al. (2014). Thirty mg of leaves were homogenised in 3 ml of 100% HPLC-grade methanol overnight. The supernatant was filtered through 0.2 μm Minisart SRT 15 filters and immediately analysed. The extraction was carried out as quickly possible, in dimmed green light. HPLC separation was performed at room temperature with a Dionex column (Acclaim 120, C18, 5 μm particle size, 4.6 mm internal diameter x 150 mm length). The pigments were eluted using 100% solvent A (acetonitrile/methanol, 75/25, v/v) for the first 12 min to elute all xanthophylls, including the resolution of lutein from zeaxanthin, followed by a 3 min linear gradient to 100% solvent B (methanol/ethylacetate, 68/32, v/v), 15 min with 100% solvent B, which was pumped for 15 min to elute chl *b* and chl *a* and β-carotene, followed by 2 min linear gradient to 100% solvent A. The flow-rate was 1 ml min^{-1}. The column was allowed to re-equilibrate in 100% solvent A for 10 min before the next injection. The pigments were detected by their absorbance at 445 nm. To quantify the pigment content, known amounts of pure standard were injected into the HPLC system and an equation, correlating peak area to pigment concentration, was formulated.

For carbohydrate analyses (Keutgen et al. 2005), 300 mg of leaves were ground and homogenized in 5 ml of demineralized water for HPLC and heated for 60 min in a water bath at 60 °C. Then, samples were centrifuged for 20 min at 5,000*g* at room temperature. Monosaccharides (glucose and fructose), disaccharides (sucrose and maltose) and alcohols (sorbitol and mannitol) were determined from the supernatant by injection of 20 µl sample volume into an HPLC system using a refill separation column (Sugar SC1011, 8 mm internal diameter × 300 mm length) equipped with a Showa Denko pre-column (Sugar SC-LG, 6 mm internal diameter × 50 mm length). Column temperature was 70 °C and distilled water for HPLC was used as mobile phase (flow rate 1 ml min^{-1}). Carbohydrates were detected with a differential refractometer (LC 30 RI, Perkin Elmer, UK) and quantified with known amounts of pure standard that were injected into the HPLC system and an equation, correlating peak area to carbohydrates concentration, was formulated.

Phenols analysis was performed by HPLC according to Sgarbi et al. (2003). Forty mg of leaves were homogenised in 3 ml of 100% HPLC-grade methanol and 1% HCl overnight. The supernatant was filtered through 0.2 µm Minisart SRT 15 filters and immediately analysed. HPLC separation was performed at room temperature with a Dionex column described above. Phenols were eluted using 100% solvent A (methanol/water/acetic acid, 30/67.5/2.5, v/v) which was pumped with a linear gradient to 50% solvent B (methanol) for 40 min. The flow-rate was 0.3 ml min^{-1}. The column was allowed to re-equilibrate in 100% solvent A for 10 min before the next injection. Phenols were detected by their absorbance at 280 nm. To quantify the phenolic content, known amounts of pure standard were injected into the HPLC system and an equation, correlating peak area to phenolic concentration, was formulated.

The choice of a reference basis for metabolite contents is important because it can influence the nature of trends (e.g. Lichtenthaler et al. 2007). Biochemical data have been calculated both per leaf

area and fresh weight (FW). Since the trend was the same for both leaf area and FW bases, data have been expressed in terms of leaf FW, according to what reported by Logan et al. (1998).

Statistical analysis

A minimum of three plants per treatment were used in each of the three repeated experiments. Following performance of the Shapiro-Wilk W test, data were analyzed using two-way analysis of variance (ANOVA) or repeated-measures ANOVA (where appropriate) and comparison among means was determined by Tukey's test ($P<0.05$). Linear correlations were applied to: A vs G_w, fraction of absorbed light that was thermally dissipated in PSII antennae (%D) vs de-epoxidation index value (DEPS) and β-carotene/total chls vs RWC data. Analyses were performed by NCSS 2000 Statistical Analysis System Software.

Results

Visible injury and plant water status

At the end of exposure, increased senescence was observed (+23% in comparison to controls): plants showed leaf yellowing and sometimes even abscission. Plant water status was affected by O_3 (Fig. 1a), as demonstrated by lower - albeit slightly - values of the RWC starting from 30 days FBE (-7, -9 and -12%, after 30, 60 and 90 days FBE, respectively, when compared to air filtered material).

Dynamics of photosynthetic gas exchange and chl *a* fluorescence

Starting from 30 days FBE, A significantly decreased (-26% compared to controls) and this reduction was maintained during the entire period of fumigation with values in the range between -55 and -70% (Fig. 1b). This decrease was twinned with lower values of G_w (-49, -72 and -76%, 30, 60, and 90 days FBE, respectively) (Fig. 1c). A decrease in C_i (Fig. 1d) was also prolonged during the exposure period (-7, -10 and -8%, respectively, 30, 60 and 90 days FBE). Photosynthetic rates

were 42% lower in O_3-treated plants at a CUO of 1.62 mmol m^{-2} and 55% lower at a CUO of 3.50 mmol m^{-2} (Fig. 2). G_w in treated plants decreased by 52% and was 70% lower at CUO of 3.50 mmol m^{-2}. At the end of the treatment (CUO of 5.55 mmol m^{-2}), O_3-induced decline in A (-72%) was twinned with a severe depression in G_w (-79%), confirming that stomatal limitations to photosynthesis prevailed over biochemical ones. WUE_i ranged between 0.056 and 0.069 in treated plants and between 0.043 and 0.046 in controls (*data not shown*). Relationships between A and G_w showed $R^2 > 0.40$ in both untreated and treated materials ($y = 24.23x - 6.46$, $R^2 = 0.60$, $P < 0.0001$, untreated; $y = 13.91x + 6.21$, $R^2 = 0.78$, $P < 0.0001$, treated).

In fumigated plants, the ratio F_v/F_m was not depressed by the oxidative stress, indicating that O_3 did not impair the efficiency of PSII (*data not shown*). Φ_{PSII} values were significantly reduced in treated plants already 30 days FBE (-23%, in comparison to controls) and this reduction was maintained during the entire period of fumigation (-59 and -64% at 60 and 90 days FBE, respectively, Fig. 3a). Referring to quenching analysis, 1 – qP increased, starting 30 days FBE (+72% in comparison to controls) and reaching the maximum value at the end of exposure (about 4-fold higher than controls) (Fig. 3b). After 60 days FBE qNP increased (+74%) and a similar trend was observed until the end of the treatment (+72%, Fig. 3c).

Pigments and carbohydrates contents

O_3 induced a marked decrease in the content of both chl *a* (Fig. 4a) and *b* (Fig. 4b), starting from 30 days FBE (-59 and -56% in comparison to controls, respectively), reaching the maximum value of -71 and -73% at the end of fumigation. Lutein (Fig. 4c) and β-carotene (Fig. 4d) followed the same trend of chlorophylls: the levels of these pigments showed significant differences between treated plants and controls 30 days FBE (-42 and -51%, respectively), with the highest differences at 90 days FBE (-58 and -57%, respectively). At the end of the exposure, the ratio β-carotene/total chls significantly increased in treated leaves (the mean value of controls was 0.94±0.081 vs 1.13±0.043 of fumigated ones, $P = 0.020$). Relationship between RWC and β-carotene concentration per

amount of total chls was close and linear in control plants ($y = -2.74x + 277.64$, $R^2 = 0.41$, $P = 0.0423$). This close correlation was lost when leaves were exposed to O_3 ($y = +1.02x - 56.13$, $R^2 = 0.15$, $P = 0.3020$). The amount of anteraxanthin and violaxanthin (Fig. 4e) also showed a marked decrease starting from 30 days FBE (-68%), with the value of -45% at the end of fumigation. DEPS significantly increased, reaching the maximum value at the end of the treatment (+69%, in comparison to controls) with, consequently, a marked activation of the cycle (Fig. 4f). The indication of the role played by xanthophylls cycle in dissipating excess light energy could be explained by the significant correlation in both treated and untreated materials between the efficiency of thermal energy dissipation (%D) and DEPS ($y = 2.72x - 10.55$, $R^2 = 0.93$, $P < 0.0001$, untreated; $y = 1.95x - 5.59$, $R^2 = 0.83$, $P < 0.0001$, treated).

The monosaccharide concentration showed an increasing trend (Fig. 5a), the maximum value occurring at 30 days FBE (the treated material was about 4 times higher than the control one). Similar behavior was shown by disaccharide and sugar alcohols levels (about 3 and 2 times higher in fumigated plants, respectively) (Fig. 5b, c) and by total carbohydrates also (Fig. 5d).

Phenolic concentration

Phenolic concentrations are shown in Figure 6, which only reports those compounds which show statistically significant differences between controls and treated materials. Among selected phenolics, rosmarinic acid is found to be the most plentiful in both control and fumigated material. During the exposure, a general rise occurs in the amount of each phenol, with maximum differences between treated and control materials at the end of fumigation for gallic (about 2-fold, Fig. 6a), catechinic (once, Fig. 6b) and caffeic acids (8-fold, Fig. 6d). The content of rosmarinic acid reached the highest level in treated leaves at 60 days FBE (+122% in comparison to control, Fig. 6c).

Discussion

Following O_3 treatment, *S. officinalis* showed leaf yellowing and sometimes even abscission. This is in agreement with Dawnay and Mills (2009), who found a rapidly induced premature senescence in *Anthoxanthum odoratum* plants exposed to O_3 for 12 weeks. In the re-organisation (also termed transdifferentiation or degeneration) phase of leaf senescence, the major metabolic and cell ultrastructural changes include: (i) disassembly of cellular integration, (ii) decrease in photosynthetic activity and (iii) chlorophyll degradation (Munné-Bosch and Alegre 2004). In our case, the appearance of leaf yellowing was twinned with some disorders in plant water status, as a consequence of the slight cellular water deficit, probably due to membrane damage caused by O_3. Generally, membranes are the primary target of this pollutant that can induce a deleterious effect on their function (Guidi et al. 2001), integrity (Calatayud et al. 2003; Francini et al. 2007), conformation (Ranieri et al. 2001) and transport capacity (Płażek et al. 2000).

In treated plants, a consistent decline of photosynthetic activity was observed during and following the treatment and was associated to stomatal closure, that was regarded as the critical factor in reducing photosynthetic rate. The close relationship between A and G_w still showed that the inhibition of CO_2 fixation might be attributed to the stomatal component. Furthermore, photosynthesis and stomatal conductance change at same rates over the same CUO, suggesting that it is unlikely an additional mechanism causing the observed decreases in CO_2 assimilation rate. This is in disagreement with what reported in the literature for chronic O_3 exposure (Lombardozzi et al. 2012; Pellegrini 2014). However, fumigated plants had a greater WUE_i than controls: in the presence of O_3, for the same A, they showed lower G_w values and therefore lost less water through transpiration. This result suggests that the functioning of stomata is not altered by oxidative stress. Munné-Bosch et al. (2001) also obtained a similar result in sage plants exposed to drought: decline in the photosynthetic rate was associated to stomatal closure and to changes in Φ_{PSII}. This behavior has been also observed in other Mediterranean species (Nali et al. 2004). Because CO_2 is the final acceptor in the electron transport chain, an inhibition of photosynthetic rate could be expected to

affect the redox state of the PSII electron acceptor Q_A. In treated plants we found no changes in the maximal efficiency of PSII photochemistry, similarly to other studies (James et al. 2002; Qiu et al. 2003). In treated plants, a significant increase of 1 - q_P indicated that O_3 decreased the capacity for reoxidizing Q_A during actinic illumination, because this pollutant (i) increased the excitation pressure on PSII, and (ii) contributed to the closure of PSII reaction centers, which determined a lower possibility of electron transport from PSII to photosystem I (PSI). The reduction of Φ_{PSII} suggests that there was a tendency to reduce the light energy used in photochemistry at the expense of the capacity to dissipate the excess of excitation energy, as indicated by the higher values of qNP in treated plants. Similar results have been reported in *Lamottea dianae* plants exposed to ambient O_3 concentrations (hourly mean concentrations about 40 ppb) for 113 days (Calatayud et al. 2011). The photoinhibition was not enhanced in treated plants, suggesting that some adaptive defense mechanisms could be activated in order to dissipate the excess of energy. In particular, we have observed (i) a decrease of (A+V) content, and (ii) an increase in the de-epoxidation index (DEPS) during and following the O_3 treatment and this suggests an activation of xanthophyll cycle. The functionality of this photoprotective mechanism was maintained even in O_3-stressed injured leaves, which suggests that one mechanism alone is not enough to protect chloroplast from stress.

The need for the plants to alleviate the excess of excitation pressure and to provide antioxidative protection to chloroplasts results also in the increased antioxidant activity. This is confirmed by the rise of phenols in the treated leaves. Some studies report that the production of these secondary metabolites is enhanced in stressful situations, including O_3 exposure (Kangasjärvi et al. 1994; Frei et al. 2011). In particular, the differences in phenol quality could be important: it is known that simple phenols such as caffeic acid [that plays a central role in the biochemistry of *Lamiaceae* and constitute the building block of a variety of metabolites in the genus *Salvia* (Lu and Foo 2002)] sustain *in vivo* and *in vitro* the ascorbate peroxidase activity in response to O_3. Also rosmarinic acid [a caffeic acid dimer that is generally the most abundant phenolic compound in sub-family

Nepetoideae of the *Lamiaceae* (Petersen and Simmonds 2003)] is a strong radical scavenger and is known to be an effective antioxidant (Tepe 2008). In our case, O_3-treated plants showed an increased accumulation of phenols during and following the exposure. Similarly, Saviranta et al. (2011) report that the chronic O_3 exposure (1.5 times the ambient level for 9 weeks) induced a marked increase in the concentrations of antioxidant phenolic compounds in *Trifolium pratense* leaves. The high amounts of these metabolites would indicate a better capacity to scavenge oxidants produced during O_3 stress.

The imbalance in the redox state of our treated plants could be responsible for the degradation of leaf chlorophyll content. During the entire period of exposure, the lower photosynthetic performance was associated with a significant decrease of chlorophylls, indicating that there was an evident effect of the chlorophyll binding proteins of the light harvesting complex (LHC). The chlorophyll loss is not necessarily a symptom of unsuccessful adaptation to photoinhibitory and photoxidative conditions, but it could have a regulatory role when chloroplasts still retain substantial photosynthetic activity (Kyparissis et al. 1995). The decline in light absorption might reduce the potentially damaging heating effects of high solar radiation in O_3-stressed plants whose stomata are closed. The benefits of chlorophyll loss are limited by the extent of O_3-induced oxidative stress. In particular, the decrease in leaf light absorption caused by chlorophyll loss did not compensate for the severe degradation of lipophilic antioxidants in the chloroplast. The concomitant decrease of β-carotene (but to a lesser extent, as shown by the increase of carotenoids to chlorophylls ratio, Biswal 1995) and lutein content confirms that O_3 can induce (i) photoxidation, (ii) a partial breakdown of chlorophylls, and (iii) a reorganization of the photosynthetic apparatus (Mikkelsen et al. 1995; Calatayud and Barreno 2004). A reduced photosynthetic active leaf area, as a result of this, may induce a spiral of steady decline in plant vitality. Some Authors suggest that the decline of photosynthetic pigments which are affected by O_3 is a secondary effect related to processes such as accelerated aging (Köllner and Krause 2000), since it is associated with nutrient

remobilization. In our case, the decline in chlorophyll was concomitant with the accumulation of water soluble carbohydrates, suggesting that the O_3-induced oxidative stress could trigger senescence. The water soluble carbohydrates-regulated senescence is an universal response (Wingler et al. 2006) that can be interpreted in two ways: acclimation, when sugar signaling forms part of the photosynthetic acclimation response of plants to oxidative stress inducing early senescence, or damage, when the sugar transport to the conducting elements is interfered from the cellular compartmentalization (Liu et al. 2013). The marked increase of total soluble carbohydrates (in particular monosaccharides) and the concomitant rise of phenols during and following O_3 exposure suggests that the carbon metabolism of *S. officinalis* leaves was modified by detoxification processes, protection and repair of cell structure. According to Bolouri-Moghaddam et al. (2010), sugars - being the primary carbon and energy source in plants - could produce reducing power for biosynthesis of non-enzymatic antioxidants such as glutathione, ascorbate and phenolic compounds.

This work demonstrates that leaf yellowing, that could be considered the first visual symptom of senescence, is twinned to (i) reduced photosynthetic activity, (ii) chlorophyll loss and (iii) cellular water deficit. These phenomena could be indicative of oxidative stress despite the photoprotection conferred by xanthophylls cycle, antioxidant compounds and water soluble carbohydrates. Combined ecophysiological and biochemical approaches show that *S. officinalis* seems able to activate an adaptive survival mechanism allowing the plant to complete its life cycle even under oxidative stressful conditions. Undoubtedly, further investigations are required in order to verify the behavior of *S. officinalis* in other conditions, such as exposures to lower O_3 concentrations, and using ecotypes with differential response to oxidative stress.

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