Signalling molecules responsive to ozone-induced oxidative stress in *Salvia officinalis*

Alessandra Marchica[(a)], Giacomo Lorenzini[(a,b,c)], Romina Papini[(a)], Rodolfo Bernardi[(a,c)], Cristina Nali[(a,b,c)1], Elisa Pellegrini[(a,b,c)]

[(a)] Department of Agriculture, Food and Environment, University of Pisa, Via del Borghetto 80, Pisa, 56124, Italy

[(b)] CIRSEC, Centre for Climatic Change Impact, University of Pisa, Via del Borghetto 80, Pisa, 56124, Italy

[(c)] Nutrafood Research Center, University of Pisa, Via del Borghetto 80, 56124 Pisa, Italy

ABSTRACT

Tropospheric ozone (O_3) is the most important gaseous pollutant and induces a mass of negative impacts on vegetation at functional and genic levels. The aim of the present study was to investigate the role of reactive oxygen species and signalling molecules in sage plants exposed to O_3 (200 ppb, 5 h). Ozone exposure induced only a transient oxidative burst, as confirmed by the rapid peak of anion superoxide during the first hours of exposure (+16% compared to controls). The spontaneous reaction of O_3 with membrane fatty acids stimulates peroxidative processes, as demonstrated by the rise of thiobarbituric acid reactive substances concentration starting after 1 h of exposure (+25%). The formation of lipid-based signalling molecules (e.g. jasmonic acid) may be regarded as a sort of O_3-perception. The concomitant accumulation of salicylic acid suggests that sage responds early to O_3 by inducing cellular antioxidants mechanisms in order to minimize O_3-oxidative burst. The transient increase of abscisic acid (+25% at the end of the treatment) twinned with the maximal ethylene emission (about two-fold higher than controls) could be interpreted as a first attempt by plants to regulate the signalling responses induced by O_3. In order to investigate the involvement of transcription factors in managing oxidative protection, BLASTX analysis against the *Salvia miltiorrhiza* sequence genome was carried out using *Arabidopsis thaliana WRKY* sequences as

[1] Corresponding author

queries. Six gene sequences were identified for sage *WRKY*s and their relative gene expression analyses were characterized. *WRKY4*, *WRKY5*, *WRKY11* and *WRKY46* were up-regulated by O_3 at 2 and 5 h of exposure and they showed similarity with *AtWRKY48*, *AtWRKY22* and *AtWRKY53* in *A. thaliana*. These results suggest that *WRKY*s could play a pivotal role in the signalling mechanisms during the responses of plants to O_3.

Keywords

Sage, oxidative burst, reactive oxygen species, phytohormones, *WRKY*, qRT-PCR

1. Introduction

To counteract the adverse effect of hostile environmental conditions, plants have evolved defense mechanisms through physical adaptations and/or integrated cellular and molecular regulations (Knight and Knight, 2001). It is established that the perception and transduction of stress signals is necessary to switch on adaptive responses for plant survival (Smékalová et al., 2014).

Tropospheric ozone (O_3) is the most meaningful gaseous pollutant with levels expected to increase in many parts of the world due to changing climatic conditions and to human activities (Mills et al., 2018). Specifically, the Mediterranean area is broadly affected by O_3, due to peculiar temperature, solar irradiance and precipitation patterns, twinned to the plentiful of precursors gases (Pellegrini et al., 2007). Depending on its concentration and on environmental conditions, O_3 affects plants at different degrees by inducing specific biochemical and molecular responses (Vaultier and Jolivet, 2015). For plants acclimated to O_3 stress, a pulse of O_3 (i.e. high concentration within a short period) have to be recognized at the cellular level and transmitted to the nucleus for inducing

47 cell reprogramming. According to Apel and Hirt (2004), this is a physiological process that
48 specifically selects and removes unwanted cells in response to an array of abiotic and biotic signals.

49 In response to O_3 stress, phytohormones [i.e. ethylene (ET), salicylic (SA) and jasmonic acid
50 (JA)] and reactive oxygen species (ROS) can initiate the signals. They then induce downstream
51 signalling cascades and transcription controls, and notify parallel pathways (Waszczak et al., 2018).
52 Specific cross-talks among signalling molecules can diverge in the molecules involved, as well as in
53 the timing and magnitudes of their accumulations, and may be a key factor leading to the different
54 degree of O_3-sensitivity among species and genotypes (Puckette et al., 2009).

55 Several tools have been used to determine the molecular bases of O_3-induced responses in
56 woody and herbaceous plants (Francini et al., 2008; Puckette et al., 2009; Gottardini et al., 2016;
57 Pellegrini et al., 2018a). So, the defense response includes the activation of O_3-inducible genes and
58 their products, which are either regulatory or functional to determine O_3 tolerance directly or
59 through a downstream signal transduction pathway. About 7% of the genome-coding sequences of
60 plants includes transcription factors (TFs), that are among the major master regulatory proteins of
61 abiotic stress responses (Lindemose et al., 2013). TF-mediated regulation of plant signalling and
62 regulatory pathways of stress adaptation have been widely reported (Khan et al., 2018). However,
63 current knowledge concerning the involvement of TFs in the regulation of O_3 responses is limited
64 and has been primarily elucidated in model plants (such as *Arabidopsis thaliana*, Tosti et al., 2006;
65 Xu et al., 2015; *Medicago truncatula*, Puckette et al., 2008), in crops (i.e., *Oryza sativa*, Cho et al.,
66 2013) and woody plants (*Populus* spp., Rizzo et al., 2007; *Viburnum lantana,* Gottardini et al.,
67 2016).

68 WRKY TFs are a large family of regulatory proteins in plants and are classified into three
69 groups on the basis of the number of *WRKY* domains and the features of the associated zinc finger-
70 like motif (Eulgem et al., 2000). Most WRKY proteins play a key role in plant tolerance to biotic
71 and abiotic stress (Chen et al., 2012; Yokotani et al., 2013; Khan et al., 2018), inducing genes

involved both in stress perception and signal transduction, and in redox regulation. Li et al. (2009) reported the overexpression of *WRKY*s genes in *A. thaliana*, as a component of the signal set of transcriptional reprogramming when plants (at different stages of growth) respond to heat stress treatments. However, far less information is available about the role of WRKY proteins in plant responses to O_3 (Ernst et al. 1999; Ernst and Aarts, 2004).

Common sage (*Salvia officinalis* L.) is one of the most well-known aromatic herbs used in the pharmaceutical and food industries. Native of southern Europe, it is largely cultivated in the Mediterranean countries due to its high ability to cope environmental stressors. Some experimental studies demonstrated that this species can (i) withstand moderate dose of salt stress (Tounekti et al., 2012), (ii) counteract mild-severe drought (Munné-Bosch and Alegre, 2003), and (iii) complete their life cycle even under conditions of O_3-oxidative stress (Pellegrini et al., 2015), by activating several mechanisms of photo- and antioxidant protection. However, a literature survey revealed a poorness of data on the programmed cell death (PCD) and hormonal response of aromatic herbs (e.g. *Melissa officinalis*, *Hypericum perforatum* and *Pueraria thomsnii*) to acute O_3 exposure (Xu et al., 2011; Sun et al., 2012; Pellegrini et al., 2013; 2018b).

The present study reports the time course relationships among oxidative burst and biosynthesis of signalling molecules in *S. officinalis* exposed to O_3 in controlled environmental conditions. Here, we wanted to address the following questions: (i) Can an episode of O_3 initiate cell reprogramming? (ii) What is the role of phytohormones and signalling molecules in the perception and transduction of O_3 stress? (iii) What is the potential role of *WRKY* TFs in regulating oxidative protection and signaling responses to O_3?

2. Materials and Methods

2.1. Plant material and experimental design

Salvia officinalis seedlings (four-month old) were grown under field conditions in plastic pots containing a mixture of steam-sterilized soil and peat (1:1, v/v). Two days before the beginning of the O_3 exposure, uniformed-size plants (ca. 22 cm tall) were moved into four controlled fumigation chambers (temperature 20 ± 1 °C, relative humidity 85 ± 5%, and photon flux density of 500 μmol photons m^{-2} s^{-1} at plant height provided by incandescent lamps with light/dark 14:10 photoperiod; lights were switched on from 5:00 a.m. to 7:00 p.m.). Ozone was generated by electrical discharge using a Fisher 500 air-cooled apparatus (Zurich, Switzerland) supplied with pure oxygen, and mixed with the inlet air entering the fumigation chambers. Its concentration at plant height was continuously monitored with a photometric analyzer (Ecotech Acoem Group, mod. Serinus® 10, Milan, Italy). Plants were exposed to 200 ppb of O_3 for 5 h, in form of a square wave between 9:00 a.m. and 2:00 p.m., solar time (1 ppb O_3= 1.96 μg m^{-3}, at 25 °C and 101.325 kPa). Controls were kept under charcoal-filtered air (the O_3 concentration was less than 5 ppb). After the end of fumigation, plants were kept in the growth chamber under O_3-free air to recover. During the exposure, environmental settings were maintained as reported above. The entire methodology has been performed according to Cotrozzi et al. (2017).

Analyses were performed on the second fully expanded mature leaf at 0, 1, 2, 5, 8 and 24 h from the beginning of the exposure (FBE). The leaf material was immediately frozen in liquid nitrogen, stored at -80 °C, and subsequently dried by lyophilization for 72 h until biochemical analyses.

2.2. Biochemical analyses

Oxidative damage was assessed spectrophotometrically in terms of lipid peroxidation by determining the thiobarbituric acid reactive substances (TBARS), following the method of Hodge et al. (1999) with some minor modifications as reported by Guidi et al. (2017). The antioxidant properties were assessed spectrofluorimetrically by the Oxygen Radical Absorption Capacity (ORAC) and Hydroxyl Radical Antioxidant Capacity (HORAC) assays. The HORAC activity

120 method is based on the oxidation-mediated quenching of a fluorescent probe by hydroxyl radicals
121 produced by a hydroxyl radical initiator and Fenton reagent (Ou et al. 2001). The ORAC activity
122 method is based on the oxidation of a fluorescent probe by peroxyl radicals produced by a free
123 radical initiator, 2,20-azobis(2amidino-propane) dihydrochloride (Ou et al. 2002). In particular,
124 HORAC and ORAC assays measure two different, but equally important, aspects of antioxidant
125 properties (radical chain breaking and radical prevention, respectively). Hydrogen peroxide (H_2O_2)
126 and superoxide radical ($O_2^{\bullet-}$) concentrations were measured spectrophotometrically according to
127 Cotrozzi et al. (2017). Ethylene was measured by gas chromatography with the analytical
128 conditions reported by Mensuali Sodi et al. (1992). According to Cotrozzi et al. (2017), conjugated
129 and free SA, and JA were determined by High-Performance Liquid Chromatography. Abscisic acid
130 (ABA) analysis was performed by an indirect ELISA determination, according to Trivellini et al.
131 (2011). Further details of biochemical analyses are reported in the supplementary material.

132 *2.3. Molecular analyses*

133 The total RNA from leaf tissue was extracted using RNeasy® Mini Plants Kit (Qiagen, Hilden,
134 Germany). The final concentration of the isolated RNA was measured spectrophotometrically at
135 260 nm (WPA Biowave, Biochrom, Cambridge, England) using Qubit RNA HS Assay Kit
136 (Invitrogen, Carlsbad, CA, USA). The RNA quality was assessed by separation on agarose gel
137 electrophoresis. The RNA was treated with Amplification Grade DNase I kit (Sigma-Aldrich Saint
138 Louis, MO, USA) and reverse-transcribed into cDNA by using Maxima First Strand cDNA
139 Synthesis kit for RT-qPCR (Thermo Fisher Scientific, Waltham, MA, USA), following the
140 manufacturer's instructions. The products were stored at -80 °C until analyzed. The selected
141 housekeeping genes were *18S rRNA*, *ubiquitin* and *β-actin* (Table 1). Although all the endogenous
142 control genes tested exhibited stable expression, *β-actin* was chosen to normalize gene expression
143 data for its high transcriptional stability. Moreover, the right transcription to the cDNA template
144 was analyzed by PCR reaction using as primer Universal 18S ribosomal gene (QuantumRNA,

universal 18S Internal Standard; Applied Biosystems/Ambion, Foster City, CA; USA; *data not shown*). Before qRT-PCR analyses, the PCR efficiency for each primers pair (Table 1) was calculated with a linear regression analysis of serial dilutions of cDNA following Real-time PCR handbook (Carlsbad, CA, USA). BLASTX analysis against the current assembly of the *S. miltiorrhiza* sequence genome was performed using *Arabidopsis WRKY* nucleotide coding sequences as queries [identified by Chen et al., (2012) and Banerjee and Roychoudhury, (2015)]. A total of 6 gene sequences were identified for sage *WRKY*s. Specific primers for sage *WRKY4*, *WRKY5*, *WRKY11*, *WRKY21*, *WRKY23* and *WRKY46* (Table 1) were designed using Primer3 software (Applied Biosystems, Foster City, CA, USA). Details of qRT-PCR reactions are reported in the supplementary material.

2.4. Statistical analysis

A minimum of three replicates (plants) per treatment and per time were used in each of the three repeated experiments. The Shapiro-Wilk W test was used to preliminary test the normality of data. Statistical differences between measurements at different times were analyzed by a two-way Analysis of Variance (ANOVA) with O_3 and time as variability factors. Tukey's test was used as post-hoc test, with a significance level of $P \leq 0.05$. Analyses were performed by JMP 11 Statistical Analyses System Software (SAS Institute Inc., Cary, NC, USA). The relative abundance of transcripts was calculated by using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

3. Results

3.1. Visible injury, oxidative damage and antioxidant capacity

No symptoms were showed by control and treated plants. Membrane integrity was significantly affected by O_3 (Fig. 1A). According to the two-way ANOVA, the effects of O_3, time and their interaction were significant for both parameters. In treated plants, TBARS levels increased throughout the entire period of treatment (except at 8 h FBE), reaching their maximum at 1 h FBE

(+25% in comparison to controls). The antioxidant activity (expressed as the HORAC and ORAC values) of treated and untreated leaves is shown in Fig. 1 B-C. According to the two-way ANOVA test, the effects of O_3, time and their interaction were significant for both parameters. O_3 increased the antioxidant activity starting from 1 h onwards. The ORAC values were always significantly higher in treated plants than in controls, reaching a maximum at the end of the treatment (+32%). During the recovery phase, they decreased to near constitutive levels (Fig. 1 B). HORAC values remained high during the whole period of exposure, peaking at 8 and 24 h FBE (+54 and +51% when compared to untreated material; Fig. 1 C).

3.2. ROS production

A gradual decrease of H_2O_2 values was observed during the first hours of treatment, reaching a minimum at 2 h FBE (-51% in comparison to controls, Fig. 2 A). They then significantly increased starting to 5 h FBE onwards (+47%), and remained high during the whole period of recovery. The concentration of $O_2^{\bullet-}$ did not show a clear trend (Fig. 2 B), revealing no significant differences between treated and untreated plants at 1 h FBE. It then was higher than control already after 2 h FBE (+16%), and decreased starting to the end of treatment, reaching the minimum values at 8 h FBE (-32%).

3.3. Phytohormones

According to the two-way ANOVA test, the effects of O_3, time and their interaction, were significant for all the examined phytohormones (except O_3 in the case of SA; Fig. 3 A-D). In treated plants, an evident increase of ET emission was observed from 2 h FBE (+71% in comparison to controls), and it peaked at the end of treatment (about two-fold higher than controls, Fig. 3 A). The concentration of SA did not show a clear trend (Fig. 3 B): it significantly increased at 1 h FBE (+62% in comparison to controls), revealing no significant differences between treated and untreated plants at 2 and 5 h FBE; there after it was lower than control during the recovery phase,

reaching the minimum values at 24 h FBE (-37%). In treated plants, a slight increase of JA concentrations was observed at 1 h FBE (+9% in comparison to controls, Fig. 3 C). Starting to 2 h FBE onwards, the levels of JA significantly decreased, reaching a minimum at the end of treatment (-32%) and remaining low throughout the entire period of recovery. ABA content did not show change when compared to controls throughout the entire period of the experiment, except at 5 h FBE (Fig. 3 D). At this time point, it significantly increased, reaching the maximum (+23%).

3.4. WRKY*s gene expression analyses*

At the molecular level, the effect of O_3 was investigated by monitoring the accumulation of 6 *WRKY*s genes at 2, 5 and 24 h FBE (Fig. 4 A-F). According to the two-way ANOVA test, the interaction between O_3 and time was significant for all the *WRKY*s genes examined (except *WRKY21*), as well as separate factors (except O_3 in the case of *WRKY4*). In treated plants, an increase of the transcript levels was observed at 2 h FBE for *WRKY11* and *WRKY46* (about 2- and 3- fold higher), indicating that they were upregulated by O_3 (Fig. 4 C-D). By contrast, *WRKY23* gene expression was downregulated during the first hours of treatment (-25% compared to control, Fig. 4 E). After 5 h FBE, only the expression levels of *WRKY4* and *WRKY5* were significantly increased (about 2- fold higher than controls; Fig. 4 A-B). No significant differences were observed in the expression levels of all the *WRKY*s genes at the end of the recovery phase.

4. Discussion

The O_3 sensitivity of plants is strictly correlated with the activation of various signalling pathways including several messengers that interact with each other (Vainonen and Kangasjärvi, 2015; Pellegrini et al., 2016), just as it is equally well known that plants challenge by adverse environmental conditions activate proper modifications of genic expression to raise their tolerance (Rizzo et al., 2007). Oxidative stress triggers the activation of some families of TF such as *WRKY*s (Tosti et al., 2006), which may operate as positive or negative regulators of genic expression.

Although ROS are toxic by-products of stress metabolism, they can play a pivotal role in intracellular communication which induces plant acclimation, and indirectly orchestrates PCD (Choudhury et al., 2017). In treated *S. officinalis*, only a slight increase of $O_2^{\bullet-}$ was observed in the first hours of exposure. Some authors reported a similar temporal trend in the O_3-tolerant tobacco cultivar Bel-B when exposed to a pulse of O_3 (150-400 ppb, 5 h; Schraudner et al., 1998; Wohlgemuth et al., 2002). Starting to 5 h FBE onwards, a significant decrease in $O_2^{\bullet-}$ content was observed suggesting that it may act as a precursor of toxic radical derivatives (Pellegrini et al., 2013), as confirmed by the concurrent production of H_2O_2. These results indicate that O_3 did not elicit cellular ROS production, but induced only a transient oxidative burst (Kangasjärvi et al., 2005). This self-propagating ROS generation in the apoplast and the spontaneous reaction of O_3 with unsaturated lipids of the plasma membrane leads to the production of peroxidative processes, as confirmed by the significant increase of TBARS levels during the exposure. The formation of lipid hydroperoxides and other lipid-based signalling molecules may also be regarded as O_3-perception (Kangasjärvi et al., 2005). In treated plants, JA showed only a rapid and transient peak at 1 h FBE. The concomitant accumulation of SA suggests that this species responds early to O_3 by inducing cellular antioxidants defense mechanisms (e.g. phenylpropanoid pathway and glutathione-based compounds, Munné-Bosch and Peñuelas, 2003; Munné-Bosch et al., 2008), in order to minimize the O_3-oxidative burst (Rao et al., 2000). A similar spatial and functional correlation between JA and SA has been observed in *Quercus ilex* plants exposed to a pulse of O_3 (200 ppb, 5 h; Cotrozzi et al., 2017). These results indicate that JA and SA can serve as mediator of cell survival by providing better defense reactions rather than part of the signalling pathway (Koch et al., 2000; Tamaoki, 2008). We are also conscious that this topic is in its infancy and deserves intensive future research.

In the timing of signalling molecules induced by O_3, the transient accumulation of these biologically active substances comes before ET and ABA. In treated plants, an intense ET evolution

242 was observed starting from 2 h FBE until the end of exposure and it was accompanied by enhanced
243 lipid peroxidation (as confirmed by the significant increase of TBARS levels). However, no leaf
244 damage was reported during the entire period of the experiment, suggesting that the transient ET
245 induction could be sufficient to induce a protective response, but under the level that would spread
246 the cell death (Mehlhorn, 1990). A similar "pro-survival" role of ET was described by Vahala et al.
247 (2003) in an O_3-tolerant birch clone treated with a pulse of O_3 (200 ppb, 8 h). In addition, Tamaoki
248 et al. (2003) documented that ET signalling in the O_3-tolerant *Arabidopsis* ecotype Col-0 exposed to
249 a single pulse of O_3 (200 ppb, 12 h) might lead to O_3 tolerance by the induction of several defense
250 genes.

251 In relation to the magnitude of synthesis and the temporal patterns, it is possible to conclude
252 that the requirement of JA, SA and ET for the initiation, propagation and containment of O_3-lesions
253 had less significance (Vahala et al., 2003; Diara et al., 2005). These signalling molecules can serve
254 as mediators of cell survival by (i) providing better antioxidant defenses (as confirmed by the partial
255 control of ROS production) and (ii) regenerating active reduced forms (as confirmed by the
256 significant increase of the total antioxidant capacity reported during the whole the experiment). In
257 response to the initial key questions, we can conclude that, firstly, *S. officinalis* did not activate a
258 PCD in response to an episode of O_3 (200 ppb, 5 h). Secondly, phytohormones (e.g. ET and ABA)
259 and signalling molecules (e.g. SA and JA) are not involved in the perception and transduction of O_3
260 stress. Our results highlight the need to further investigate possible alteration of sensing, signalling
261 and defensive mechanism of aromatic herbs to predict their behavior in the future conditions.

262 Plants have developed complex responses at biochemical and molecular levels to increase
263 their tolerance and to adapt to unfavorable environmental conditions. It is known that some TFs are
264 involved in switching on/off whole pathways due to their capacity to control specific downstream
265 responses by regulating the transcription of target genes. Integrated signal transduction cascades are
266 activated when one (or more) TF(s) interact(s) with specific cis-acting elements located in the

promoters of stress-inducible genes, playing, therefore, a key role in plant stress tolerance. Ozone alters the expression of a set of TFs and thus influences plant metabolism (Ludwikow and Sadowski, 2008). Members of the plant-specific *WRKY* TF family are involved in several developmental and physiological processes of plants such as disease resistance, growth, and senescence. They can influence and regulate several responses pertaining to cell cycle, signal perception/transduction, redox regulation, and even secondary metabolism (Rushton et al., 2010). Several abiotic stresses such as high/low temperature, drought, salinity, nutrient deficiency, and wounding activate many WRKY proteins conferring resistance to a particular stress or even providing a resistance to multiple stresses (Chen et al., 2012). Many *WRKY*s genes in *A. thaliana* involved in phytohormones pathways mediate biological processes (Shang et al., 2010; Li et al., 2009). They belong to a superfamily of TF and represent a basic constitutive fraction of the signals which regulate several response processes (Rushton et al., 2010). These observations suggest that studying *WRKY* gene families may provide valuable insights into the mechanism underlying abiotic stress tolerance in plants. Although the function of *WRKY* genes is becoming clear in model plants such as *A. thaliana* (Xu et al., 2006; Shang et al., 2010), knowledge of these genes in other plants, for instance aromatic herbs, is lacking (Ma et al., 2009; Li et al., 2013). Furthermore, very little is known about the identity and functions of *WRKY* genes in *Salvia* spp. plants subjected to oxidative stress. Recently, isolated 6 *WRKY* genes from *S. miltiorrhiza* by Li et al. (2015) were induced in a differential way by O_3.

A significant increase of the expression of *WRKY4* and *WRKY5* was observed after 2 h FBE (concomitant with the peak of $O_2^{\bullet-}$). *WRKY11* and *WRKY46* showed a marked rise at the end of the treatment (concomitant with the peak of H_2O_2, ET and ABA). They show similarity with *AtWRKY48*, *AtWRKY22* and *AtWRKY53* in *A. thaliana* (Vanderauwera et al., 2005; Li et al., 2015; Banerjee and Roychoudhury, 2015; Chen et al., 2012). An increase of the expression of these TFs during the first phases of exposure to O_3 (300 ppb, 6 h) has been reported by Tosti et al. (2006) in *A.*

292 *thaliana*. Moreover, again in *A. thaliana*, Banerjee and Roychoudhury (2015) and Chen et al.
293 (2012) have described a huge activation of *AtWRKY48*, as a response to a treatment with H_2O_2,
294 which implies ROS production. *AtWRKY48* is likely involved in plant-biotic stress interaction (Xing
295 et al., 2008). *AtWRKY22* is also induced by a treatment with H_2O_2 (Bhattacharjee, 2005) and is
296 likely involved in leaf senescence and PCD induced by several stressors (Asai et al., 2002; Yoshida,
297 2003; Zhou et al., 2011; Banerjee and Roychoudhury, 2015). Moreover, in *A. thaliana AtWRKY22*
298 and *AtWRKY29* are fundamental components of the transductional signal mediated by protein
299 kinases (MAPKs), which bring resistance to bacterial and fungal pathogens, as well as to abiotic
300 stresses (Asai et al., 2002). An activation of MAPKs by O_3 has been reported in tobacco plants
301 (Samuel et al., 2000; Samuel and Ellis, 2002), rice (Kim et al., 2003) and *A. thaliana* (Ahlfors et al.,
302 2004). *AtWRKY53* is a member of Group IIIb of these TFs, which are likely involved in defense
303 responses of plants to stress. Besseau et al. (2012) have used H_2O_2 and O_3 as elicitors and a positive
304 modulation has been observed.

305 For *WRKY23* a significant down regulation has been observed after 2 h FBE (concomitant
306 with the minimum value of O_2^- and JA). Afterwards the expression levels come back to the control
307 values. It shows similarity with *WRKY39*, induced in *A. thaliana* in the presence of oxidative stress,
308 according to Banerjee and Roychoudhury (2015) and Chen et al. (2012). This is in full agreement
309 with Tosti et al. (2006), who put in evidence a down regulation in the presence of an oxidative
310 stress. The *AtWRKY39* gene is induced as a response to high temperatures and the WRKY protein
311 coded by this gene positively modulates the signalling cascades of SA and JA (Li et al., 2010). This
312 allows to hypothesize a role for *WRKY39* in the signalling cascades triggered in plants as a response
313 to both abiotic and biotic stressors (Banerjee and Roychoudhury, 2015).

314 The mechanisms responsible for the direct activation of the WRKY proteins in the initial
315 stages of the exposure to O_3 are still under debate. It has been suggested that ROS interact in a
316 selective way with the target molecule which detect the increase in ROS concentration. Afterwards,

these info are transformed into modifications of genic expression. As the *WRKYs* have a linkage dominium of DNA zinc-finger sensitive to redox, they are excellent candidates for the regulation of the redox state inside the cell. These results suggest that *WRKY*s could be pivotal components in the intricate signalling processes involved in the responses of plants to O_3. In particular, they could act as redox-responsive sequences and, consequently, as promoter elements responsive to general apoplastic ROS due to abiotic stress. In response to initial key question, we can conclude that *WRKY* TFs are interesting genes for regulating oxidative protection and providing O_3-stress tolerance. Other TFs (e.g. MYB) could be investigated in order to evaluate their role in response to oxidative stress due to O_3.

In conclusion, this research can be considered as an useful basis to better understand the response to O_3 exposure in a non-model species that evolved several biochemical mechanisms to cope with adverse environmental conditions. Undoubtable, more studies are needed to better elucidate the involvement of signalling molecules at molecular level.

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Table 1. Housekeeping genes and specific primers for *Salvia officinalis*.

SoACT = β-actin; *SmWRKY4 = WRKY4*; *SoWRKY5 = WRKY5*; *SoWRKY11 = WRKY11*; *SoWRKY21 = WRKY21*; *SoWRKY23 = WRKY23*; *SoWRKY46 = WRKY46*. Abbreviations: Sm = primers from da Li et al. (2015) analyzed with OligoAnalyzer 3.1 (http://eu.idtdna.com/calc/analyzer); So = new primers designed, using the Primer Express 3.0 software program (Applied Biosystems, Foster City, CA, USA);

Primers	Sequences	Accession number
SoACT	F: 5'- GGTGCCCTGAGGTCCTGTT-3'	DQ243702
	R:5'- CATGAATTCCAGCAGCTTCCA-3'	
SmWRKY4	F: 5'-TTCTAGGGTTCCTCCTCCAT-3'	KM823127
	R:5'-TTTGGCTCCTTCAACATCTC-3'	
SoWRKY5	F:5'-CCGTTTGAGCCCAGAAGAAG-3'	KM823128
	R:5'-GGGAAGAAAGGTTTCCAAAGCT-3'	
SoWRKY11	F:5'-GGAACTGTTGGGCAACGAA-3'	KM823134
	R:5'-ATCTCAAATACGGTGGCATCTTC-3'	
SoWRKY21	F:5'-TGCGTTATTTGCTAATCTCTTTCAA-3'	KM823144
	R:5'-CGACGGCAGCATGTCTTG-3'	
SoWRKY23	F:5'-TGCTTGGAAGATTCCTCGAT-3'	KM823146
	R:5'-TGTTAGCCGACTGTGAAG-3'	
SoWRKY46	F:5'-AGCGATGCTTGGAAGATCCTT-3'	KM823169
	R:5'-GCCTCGGGTGGTTGTGTTC-3'	

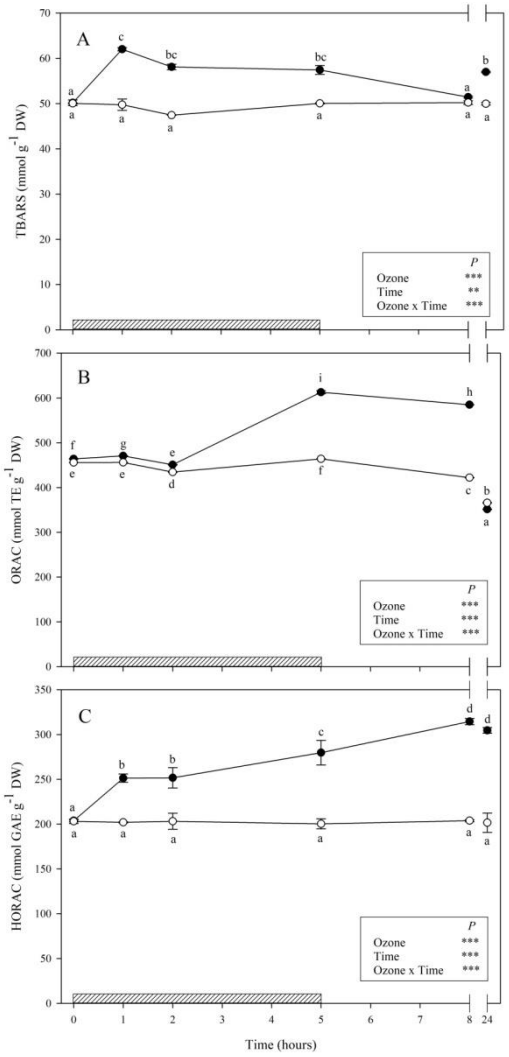

Fig. 1. Time course of thiobarbituric acid reactive substances (TBARS, A) content and antioxidant capacity expressed as oxygen radical absorbance capacity (ORAC, B) and hydroxyl radical antioxidant capacity (HORAC, C) in *Salvia officinalis* plants exposed to ozone (200 ppb, 5 h, closed circle) or maintained in filtered air (open circle). Data are shown as mean ± standard error. The measurements were carried out at 0, 1, 2, 5, 8 and 24 h from the beginning of exposure. Different letters indicate significant differences ($P \leq 0.05$). Boxes show the results of two-way ANOVA (ozone and time as variability factors) with Tukey Post Hoc test. Asterisks show the significance of factors/interaction for: *** = $P \leq 0.001$; ** = $P \leq 0.01$. The hatched bar indicates the time (5 h) of ozone exposure. Abbreviations: DW, dry weight; GAE, gallic acid equivalents; TE, trolox equivalents.

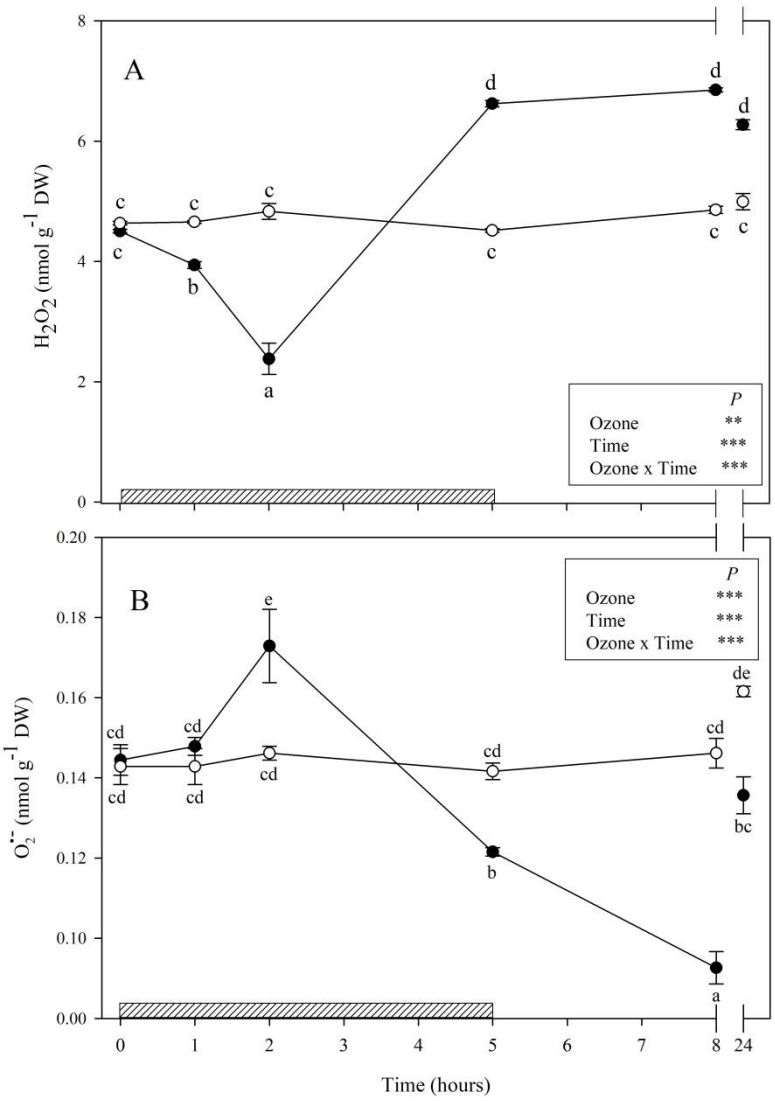

Fig. 2. Time courses of hydrogen peroxide (H_2O_2, A) and superoxide anion ($O_2^{\bullet-}$, B) in *Salvia officinalis* leaves exposed to ozone (200 ppb, 5 h, closed circle) or maintained in filtered air (open circle). Data are shown as mean ± standard error. The measurements were carried out at 0, 1, 2, 5, 8 and 24 h from the beginning of exposure. Different letters indicate significant differences ($P \leq 0.05$). Boxes show the results of two-way ANOVA (ozone and time as variability factors) with Tukey Post Hoc test. Asterisks show the significance of factors/interaction for: *** = $P \leq 0.001$; ** = $P \leq 0.01$. The hatched bar indicates the time (5 h) of ozone exposure. Abbreviations: DW, dry weight.

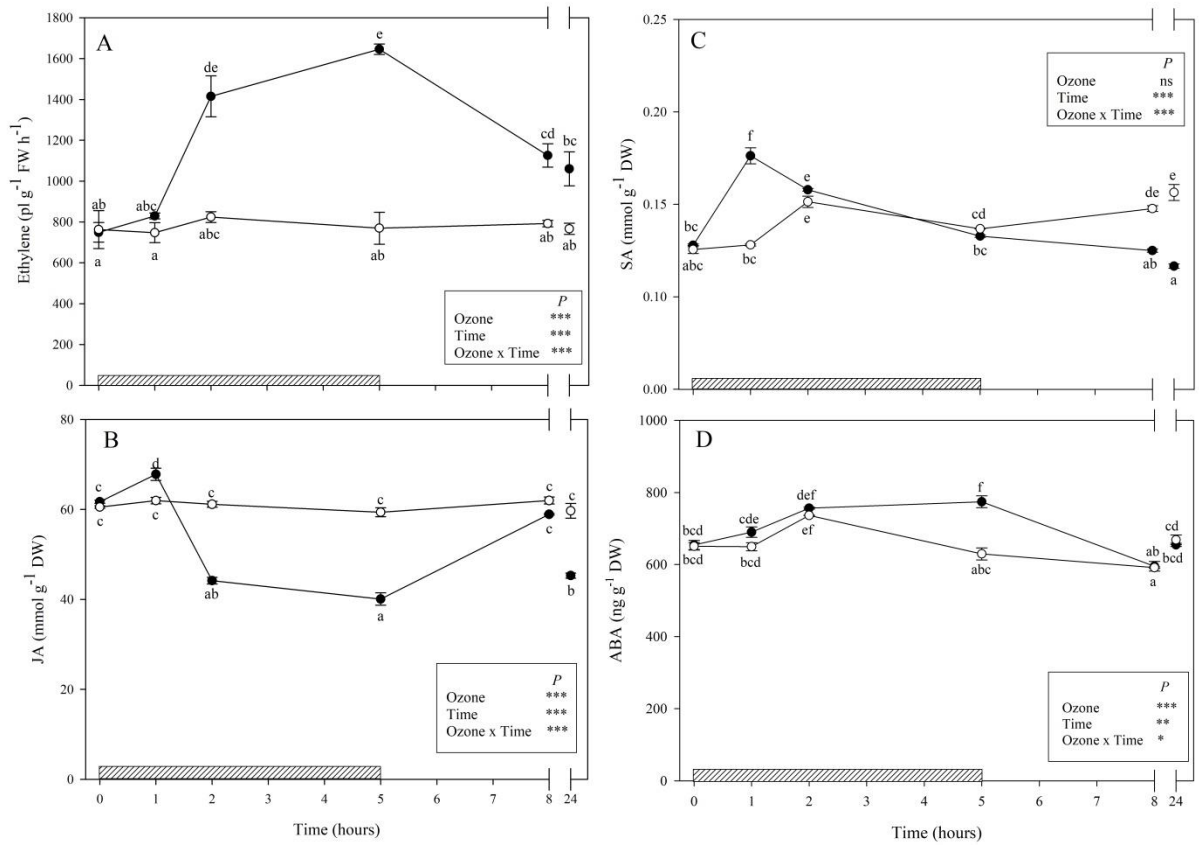

Fig. 3. Time course of ethylene (ET, A), salicylic (SA, B), jasmonic (JA, C) and abscisic (ABA, D) acids in *Salvia officinalis* leaves exposed to ozone (200 ppb, 5 h, closed circle) or maintained in filtered air (open circle). Data are shown as mean ± standard error. The measurements were carried out at 0, 1, 2, 5, 8 and 24 h from the beginning of exposure. Different letters indicate significant differences ($P \leq 0.05$). Boxes show the results of two-way ANOVA (ozone and time as variability factors) with Tukey Post Hoc test. Asterisks show the significance of factors/interaction for: *** = $P \leq 0.001$; ** = $P \leq 0.01$; * = $P \leq 0.05$; ns = $P > 0.05$. The hatched bar indicates the time (5 h) of ozone exposure. Abbreviations: DW, dry weight; FW, fresh weight.

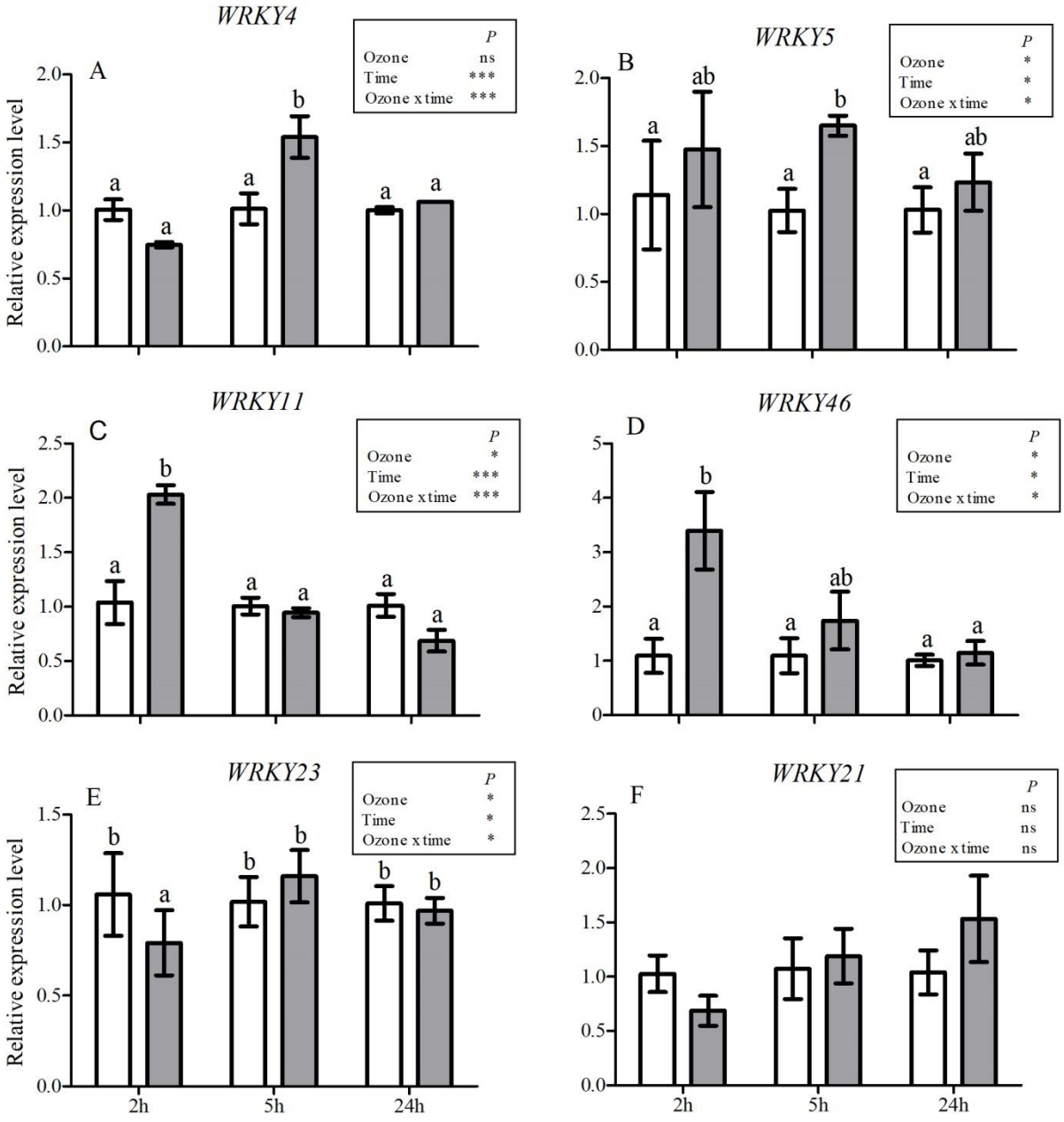

Fig. 4. Abundance of gene transcripts encoding six *WRKYs* genes in *Salvia officinalis* leaves exposed to ozone (200 ppb, 5 h, grey bars) or maintained in filtered air (white bars) Bars represent means ± standard error. The analyses were carried out at 2, 5, and 24 h from the beginning of exposure. Different letters indicate significant differences ($P \leq 0.05$). Boxes show the results of two-way ANOVA (ozone and time as variability factors) with Tukey Post Hoc test. Asterisks show the significance of factors/interaction for: ***= $P \leq 0.001$; * = $P \leq 0.05$, ns= $P > 0.05$.

Supplementary materials

Biochemical analyses

Oxidative damage was assessed in terms of lipid peroxidation by determining the thiobarbituric acid reactive substances (TBARS), according to the method of Hodge et al. (1999), with minor modifications as reported by Guidi et al. (2017). Samples (50 mg dry weight, DW) were extracted with 80:20 (v/v) ethanol/water and used to determine the malondialdehyde (MDA) concentration spectrophotometrically (6505 UV-Vis, Jenway, UK) at 440, 532 and 600 nm. The antioxidant properties were tested by measuring the Oxygen Radical Absorption Capacity (ORAC) and Hydroxyl Radical Antioxidant Capacity (HORAC) (Ou et al. 2001; 2002). For both analyses, samples (400 mg DW) were extracted with 100% cold methanol. The antioxidant activity was quantified with a fluorescence/absorbance microplate reader (Victor3 1420 Multilabel Counter, Perkin Elmer, Waltham, MA, United States; Excitation/Emission = 480/530 nm). Fluorescence/absorbance was expressed in Trolox (T) and gallic acid (GA) equivalents for ORAC and HORAC, respectively. Hydrogen peroxide (H_2O_2) production was measured using the Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Molecular Probes, Invitrogen, Carlsbad, CA, USA), according to Cotrozzi et al. (2017). Samples (25 mg DW) were extracted with 20 mM potassium-phosphate buffer (pH 6.5) and quantified with the fluorescence/absorbance microplate reader described above (Excitation/Emission = 530/590 nm). The content of superoxide ($O_2^{\bullet-}$) was determined according to Cotrozzi et al. (2017) by the reduction of tetrazolium dye sodium by O_2 to soluble formazan. Samples (30 mg DW) were extracted with 50 mM Tris-HCl buffer (pH 7.5). The quantity of O_2^- produced was determined with the spectrophotometer described above at 470 nm and calculated using the molar extinction coefficient $2.16 \times 104\ M^{-1}\ cm^{-1}$. Ethylene was measured by enclosing intact leaves (1.5 g fresh weight, FW) in air-tight glass containers (25 ml). Gas samples (2 ml) were taken from the headspace of containers after 1 h incubation. ET was determined using a gas chromatograph (HP5890, Hewlett-Packard, Minneapolis, MN, USA)

equipped with a stainless steel column (150 × 0.4 cm internal diameter packed with Hysep T) and a flame ionization detector. A detailed description of analytical conditions is reported in Mensuali Sodi et al. (1992). According to Cotrozzi et al. (2017), conjugated and free SA was determined using an High-Performance Liquid Chromatography (HPLC, Dionex, Sunnyvale, CA, United States. Samples (90 mg DW) were extracted with 100% methanol. HPLC separation was performed at room temperature with a reverse-phase Dionex column (Acclaim 120, C18, 5 µm particle size, 4.6 mm internal diameter × 150 mm length). SA was quantified fluorimetrically (RF 2000 Fluorescence Detector, Dionex, Sunnyvale, CA, USA). Jasmonic acid was determined according to Cotrozzi et al. (2017). Samples (150 mg DW) were added to 100% methanol. HPLC separations were performed with the Dionex column described above and a UVD 170U UV/VIS detector. Abscisic acid (ABA) determination was performed according to Trivellini et al. (2011). Samples (50 mg DW) were extracted with distilled water and ABA content was measured by an indirect ELISA determinations, based on the use of DBPA1 monoclonal antibody, raised against S(+)-ABA. Analyses were performed with a microplate reader (MDL 680, Perkin-Elmer, Waltham, MA, United States) at 415 nm.

Molecular analyses

Real-time RT-PCR reactions were carried out with 20 ng of cDNA, 200 nM primers, and 1x Fast SYBR GreenMaster Mix (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions. PCR analyses were performed in a StepOnePlus real-time PCR System (Applied Biosystems, Foster City, CA, USA) by using the suggested thermal-cycling conditions. Before carrying out the relative expression calculation, the performance of each amplification was checked to ensure maximum specificity and efficiency.