Ozone-elicited secondary metabolites in shoot cultures of *Melissa officinalis* L.

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Key message **The study focuses on the responsiveness of *in vitro*-cultivated *Melissa officinalis* L. shoots subjected to ozone in order to define a new experimental tool for improving the yield of secondary metabolites**

Abstract The effects of ozone treatment (200 ppb, 3 h) on the accumulation of main secondary metabolites have been investigated in *Melissa officinalis* (lemon balm) aseptic shoot cultures in order to evaluate the biotechnological application of this gas for improving the yield of secondary metabolites of medicinal plants. During the treatment, we found (*i*) an activation of enzymes involved in phenolic metabolism [as confirmed by the increase of shikimate dehydrogenase, phenylalanine ammonia-lyase and cinnamyl alcohol dehydrogenase activities (about twofold higher than controls)], (*ii*) a development of cellular barriers with a higher degree of polymerization of monolignols [as indicated by the increase of lignin (+23% compared to controls)], (*iii*) an accumulation of phenolic compounds, in particular rosmarinic acid (about fourfold compared to control plants cultivated in filtered air) and (*iv*) an increase of antioxidant capacity [as documented by the improved 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) scavenging activity]. The effect of ozone as elicitor of the production of secondary metabolites in lemon balm shoot cultures was dependent on the specific regime, the time of exposure and the concentration of the stressor. After the end of the treatment, we found cell death and hydrogen peroxide (H_2O_2) deposition concomitant with a prolonged superoxide anion-generation suggesting that a transient oxidative burst had occurred.

Keywords: Elicitors, Lemon balm, Shoot cultures, Oxidative stress, Phenylpropanoid pathway, ROS

Introduction

Lemon balm (*Melissa officinalis* L.), a member of the family Lamiaceae, is a perennial herb native to southern parts of Europe, Western Asia and North America. In Italy, it grows spontaneously along hedges and shady areas, but it is also found in ornamental gardens for its fragrance (Zargari 1990). *M. officinalis* is used as aromatic culinary herb in different food and beverage products and it is still an old important medicinal plant. Dried leaves are used as herbal tea for their scent and for other beneficial effects, such as anti-bacterial (Mencherini et al. 2007), sedative, spasmolytic or memory improving (Perry et al. 1999; Dastmalchi et al. 2008). Moreover, lemon balm is reported to reduce stress, gastrointestinal disorders, excitability, anxiety and sleep disturbance. Aqueous lemon balm extracts are used for the treatment of *Herpes simplex* infections (Wölbling and Leonhardt 1994; Mazzanti et al. 2008; Astani et al. 2012). In past years, the attention was focused on the ingestion of natural phenolic antioxidants and essential oils that may decrease the risk of cardiovascular disease, cancer, and inflammation (Arts and Hollman 2005) and, in general, exhibit good antioxidant activities (Marongiu et al. 2004; Kamdem et al. 2013). Lemon balm oil furthermore is very effective against various human cancer cell lines and a mouse cell line (de Sousa et al. 2004).

Some of the beneficial activities of lemon balm are ascribed to the phenolic compounds present in its extracts,

such as rosmarinic acid (RA), tannins and flavonoids (Szollosi and Szollosi Varga 2002; Patora et al. 2003; Petersen and Simmonds 2003). Phenols are natural antioxidants, widely distributed in most of the organs of higher plants, that show good activity to scavenge reactive oxygen species (ROS) (Lin et al. 2012). RA, a caffeic acid derivative, is the main antioxidant compound of the sub-family Nepetoideae of the Lamiaceae family. It is constitutively accumulated in field-grown plants as antimicrobial compound and as protection against herbivores (Szabo et al. 1999; Petersen 2013). RA can be found in all organs of *M. officinalis* with a level of about 6% of the dry weight in leaves (Parnham and Kesselring 1985).

RA production can be enhanced by the use of biotechnological approaches, such as *in vitro* solid or liquid cultures (Barberini et al. 2013; Petersen 2013). In *in vitro* cultures of several *Salvia* spp. (i.e. callus, cell suspension and root cultures), the yield of RA was up to tenfold higher than the yield found in organs of field-grown plants (Hippolyte et al. 1992; Karam et al. 2003). In the last years, many reports documented the use of plants as cell factories (Oksman-Caldentey and Inzé 2004) with the aim to increase the production of secondary metabolites in medicinal plants, e.g. as nutraceutical compounds (Pistelli et al. 2012; Jacobo-Velázquez and Cisneros-Zevallos 2012) or for other industrial purposes. However, the *in vitro* cultures require the optimization of growth and production conditions to maximize the yield of metabolites (Ruffoni et al. 2010). Appropriate culture media guarantee good results, but the generation of stress conditions often induces the plants to further increase the synthesis of their secondary metabolites. The attention has been focused on the stimulation by biotic elicitors. RA was accumulated to a higher yield in *Mentha piperita* after the treatment with methyl jasmonate (MeJa) or jasmonic acid (Krzyzanowska et al. 2012), in *Coleus blumei* after supplementation with MeJa or sterile fungal preparations (*Pythium aphanidermatum*, Szabo et al. 1999) and in *Lithospermum erythrorhizon* after exposure to yeast extract or MeJa (Ogata et al. 2004).

Abiotic stresses such as drought, salinity, UV radiation and ozone (O_3) are known to change the normal environmental conditions and therefore can modify the production of secondary metabolites. From an ecological point of view, these compounds are antioxidants and chemical signals. During the last years, we have learned that plants have an enormous self-defense potential and this would allow a natural disease control with positive effects on environmental safeguard and human health. In the open field, abiotic stress is the most common condition for e.g. the increase of the production of essential oils and antioxidants. Salt stress was shown to trigger the stimulation of essential oil production in field grown lemon balm (Ozturk et al. 2004) and other plants such as *Ocimum basilicum* (El-Shafy et al. 1991), *Salvia officinalis* (Hendawy and Khalid 2005; Taarit et al. 2010), *Rosmarinus officinalis* (Salinas and Deiana 1996) and *Matricaria chamomilla* (Razmjoo et al. 2008). Drought stress in *Thymus vulgaris* (Letchamo et al. 1995) or UV-B

irradiation in *Nepeta cataria*, *M. officinalis* and *S. officinalis* grown in controlled conditions (greenhouses) resulted in similar effects (Manukyan 2013).

O_3 is well-known as tropospheric pollutant (EEA 2013) and its adverse effects on the vegetation are of considerable concern (van Goethem et al. 2013). Because of its strong oxidative potential, it causes negative effects on plant metabolism, physiology and growth. For the same reason, O_3 has a large spectrum of biocidal activities and is used in various forms in (*i*) agriculture for food decontamination (Guzel-Seydim et al. 2004), (*ii*) odontology (Gopalakrishnan and Parthiban 2012), (*iii*) beverage industry (Nishijima et al. 2014) and (*iv*) clinical settings (e.g. in the treatment of infected wounds, Fontes et al. 2012). O_3 has been found to resemble fungal elicitors (Sandermann et al. 1998). Consequently, it can induce plant signal molecules that can mediate the stimulation of secondary answers, that are associated with antioxidant and pathogen defense pathways, at genetic, metabolic and hormonal level. Our knowledge on the biochemical mechanisms which are involved is still limited. Recently, Pellegrini et al. (2013) reported that single square O_3 exposure (200 ppb, 5 h) results in the activation of programmed cell death (PCD) in leaves of *M. officinalis* that resembles the hypersensitive response observed in plant-pathogen interactions. Xu et al. (2011) found that several doses of O_3 (60-180 ppb, 3 h) stimulated hypericin synthesis in *Hypericum perforatum* suspension cultures. Similarly, Sun et al. (2012) reported that O_3 can be considered an efficient elicitor of puerarin production in a plant cell culture of *Pueraria thomsnii*.

Since the major role of plant secondary metabolites is to protect plants from biotic and abiotic stress some strategies based on this principle have been developed to improve their production in *in vitro* culture. These include treatment with various elicitors, signal compounds and abiotic stress. Some reports documented the application of abiotic stress in *in vitro* cultures, such as salt stress for *Myrtus communis* (Di Cori et al. 2013), UV treatment for elicitation of purple basil (Bertoli et al. 2013) and acetylsalicylic acid and UV-B in hairy root cultures of *Anisodus luridus* (Qin et al. 2014).

In the present work, we studied the responsiveness of *M. officinalis* shoot cultures to O_3 treatment in order to define a new method for increasing the synthesis of secondary metabolites, in particular RA. To verify the functionality of *in vitro* shoots exposed to O_3 stress, *in vivo* cellular vitality and H_2O_2 determination have been performed. Chlorophyll (chl) *a* fluorescence, a reliable methodology for assessing the *in vitro* photosynthetic performance (Costa et al. 2014) was also measured.

Material and methods

Plant material, culture conditions and ozone treatment

Cuttings of *M. officinalis*, growing in plastic pots in a mixture of steam-sterilized soil and peat (1:1), have been kept for 4 months in a greenhouse in air filtered through active charcoal. Afterwards, apical portions, 10 mm length, were submerged in 2% (v/v) Tween-20® for 10 min, then in 70% (v/v) ethanol for 30 s, subsequently sterilised with a 15% (v/v) sodium hypochlorite solution for 10 min and then rinsed 5 times (10 min) in sterile distilled water. The explants were placed on MS (Murashige and Skoog 1962) medium supplemented with 0.5 mg l^{-1} 6-benzylaminopurine (BAP), 3% (w/v) sucrose and 0.8% (w/v) agar. Shoot proliferation was rapidly obtained and successive subcultures performed at 4-weekly intervals. Before the O_3 treatment, 3 week-old shoots were placed on MS medium deprived of BAP for one week to avoid any interaction of phytoregulators. Cultures were maintained in a growth chamber at 22±1 °C under 16 h photoperiod provided by cool white fluorescent tubes (Philips TLM 40W/33RS) with 80 µmol m^{-2} s^{-1} photosynthetic active radiation (PAR). Cultures were monitored regularly for shoot production.

Uniformly sized shoots (one month old) were placed in a controlled environment fumigation facility (Nali et al. 2005) under the same climatic conditions as in the growth chamber. O_3 fumigation was performed in Perspex mini chambers, measuring 23 x 18 x 19 cm, continuously ventilated with charcoal-filtered air (two complete air changes/min). Adequate mixing of incoming air was assured. O_3 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 (Monitor Labs, mod. 8810, San Diego, CA, USA) connected to a computer. Plants were exposed for 3 h to a target O_3 concentration of 200 ppb in form of a square wave (for O_3 1 ppb = 1.96 µg m^{-3}, at 20 °C and 101.325 kPa) from to the 2^{nd} to the 5^{th} hour of the light period. After the end of fumigation, plants were left in the growth chamber under O_3-free air to recover. Shoot samples were taken at 0, 2, 3, 8 and 24 h from the beginning of exposure (FBE). Control shoots were exposed only to charcoal-filtered air in Perspex chambers identical to those mentioned above. The material was stored at -80 °C until the time of analysis.

In vivo markers of ozone stress

For visualization of dead cells, Evans Blue staining was used according to the method of Keogh et al. (1980) with slight modifications. Leaves were boiled for 1 min in a mixture of phenol, lactic acid, glycerol and distilled water containing 20 mg l^{-1} Evans Blue (1:1:1:1), prepared immediately before use. Tissues were then clarified overnight in a solution of 2.5 g l^{-1} chloral hydrate in water.

For determination of H_2O_2, fresh leaf samples were stained with 3,3-diaminobenzidine (DAB) using a modification of the procedure described by Thordal-Christensen et al. (1997). Fresh samples were submerged for 8 h in a DAB solution

(1 mg ml^{-1}, pH 5.6) prepared in distilled water. After that, the samples were soaked in boiling 70% ethanol and clarified overnight in a solution of 2.5 g l^{-1} chloral hydrate in water. Observations were performed under a light microscope (DM 4000 B, Leica, Wetzlar, Germany).

Measurements of the modulated chl *a* fluorescence and of the status of the electron transport of PSII were carried out with a PAM-2000 fluorometer (Walz) on leaves that were dark-adapted for 15 min essentially as described by Döring et al. (2014a). Minimal fluorescence (F_0, all PSII reaction centers open) was determined using the measuring modulated light which was sufficiently low (<1 µmol m^{-2} s^{-1}) without inducing any significant variable fluorescence. The maximal fluorescence level (F_m, all PSII reaction centers closed) was determined by applying a saturating light pulse (0.8 s) at 8000 µmol m^{-2} s^{-1} in dark-adapted leaves. 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} PFD) at 20 s intervals to determine maximal fluorescence in the light-adapted state (F_m). The value of F_v/F_m, that is an estimation of the efficiency of excitation energy transfer to open PSII traps, was computed $F_v/F_m = [(F_m - F_0) / F_m]$ (where F_m is the maximal fluorescence, F_0 is the minimal one and F_v is the difference between F_m and F_0 in the light-adapted state).

Spectrophotometric assays

Peroxidation was determined by the TBARS (thiobarbituric acid reactive substances) method (Heath and Packer 1968). Shoots collected at each time point were pulverized in liquid N_2 and 400 mg suspended in 1 ml 0.1% trichloroacetic acid and centrifuged at 12,000 *g* for 10 min at 4 °C. The supernatant was collected and 400 µl was mixed with 1600 µl 20% trichloroacetic acid with 0.5% thiobarbituric acid. The mixture was heated at 95 °C (25 min), cooled quickly and centrifuged at 12,000 *g* for 10 min at 4 °C. The supernatant was used to determine the malondialdehyde (MDA) concentration at 532 nm corrected for nonspecific turbidity by subtracting the absorbance at 600 nm using a spectrophotometer (6505 UV-Vis, Jenway, UK). The amount of MDA was calculated by using an extinction coefficient of 155 mM^{-1} cm^{-1}.

Superoxide radical production was measured according to the method of Able et al. (1998). This assay is based on the reduction of a tetrazolium dye (sodium 3'-(1-[phenylamino-carbonyl]-3,4-tetrazolium)-bis(4-methoxy-6-nitro) benzene-sulfonic acid hydrate, XTT) by O_2 to a soluble XTT formazan that can be readily quantified in solution by recording the absorbance at 470 nm. Shoots collected at each time point were frozen in liquid N_2, ground with mortar and pestle and 100 mg immediately added to 1500 µl 50 mM Tris-HCl buffer (pH 7.5) and centrifuged (12,000 *g* for 15 min at 4 °C). 50 µl of the supernatant were incubated in a reaction mixture of 0.5 mM XTT in 50 mM Tris-HCl buffer (pH 7.5) at room temperature for 15 min. The XTT formazan was quantified spectrophotometrically and the

background absorbance due to the buffer and the assay reagents subtracted. The quantity of $O_2^{\cdot -}$ produced was calculated using the molar extinction coefficient 2.16 x 10^4 M^{-1} cm^{-1}.

For extraction of soluble phenolics and analysis of total phenolics samples were ground in liquid N_2 and 100 mg extracted with 5 ml methanol acidified with 1% HCl (v/v) for 12 h in the dark at 4 °C. Extracts were centrifuged for 15 min at 12000 *g* at 4 °C and the supernatants were filtered through 0.2 µm Minisart SRT 15 filters and stored in test tubes at -20 °C. Supernatants were used for phenolic, flavonoid, tannin and anthocyanin analyses, and the resulting pellet from the above centrifugation was dried at 35 °C for 24 h and was used for lignin analyses. The content of total phenolics was determined by the method described by Waterhouse (2002) with slight modifications. 25 µl diluted (1:10) extract was mixed with 1.225 µl deionized water and 125 µl Folin-Ciocalteu's reagent. After incubation at room temperature for 6 min, 375 µl 7.5% (w/v) sodium carbonate and 250 µl deionized water were added and mixed. After incubation at room temperature for 120 min, the absorbance was measured at 760 nm. The content of total phenolics was determined using a standard curve of gallic acid (0-1 mg ml^{-1}).

Condensed tannins were determined by a modification of the vanillin method of Morrison et al. (1995). 200 µl diluted (1:4) methanolic extract were pipetted into a test tube and 1 ml vanillin reagent (2% vanillin (w/v) and 4% concentrated HCl (v/v) in methanol) were added and the tubes incubated in a water bath for 20 min at 20-22 °C. The absorbance was read at 500 nm. Absorbances were used to calculate catechin equivalents with help of a five point catechin standard curve (0-1 mg ml^{-1}).

The analysis of total anthocyanins was adapted from Cevallos-Casals and Cisneros-Zevallos (2003) measuring directly the absorbance of a diluted (1:5) methanolic extract. The absorbance was read at 535 nm. The anthocyanin content was expressed as mg cyanidin 3-glucoside equivalents g^{-1} fresh weight, using a molar extinction coefficient of 25.956 M^{-1} cm^{-1} and a molecular weight of 449 g mol^{-1}.

The lignin amount was determined by the acetylbromide method adapted from Brinkmann et al. (2002). Aliquots of about 5 mg of dry pellet (3 replicates), previously obtained from the supernatants used for phenolic, flavonoid, tannin and anthocyanin analyses, were mixed with 500 µl 25% acetylbromide (v/v in glacial acetic acid) and incubated for 30 min at 70 °C. Samples were rapidly cooled on ice, mixed with 500 µl 2 N NaOH and centrifuged for 5 min at 12,000 *g* at 4 °C. 125 µl supernatant were mixed with 2.5 µl 15 N NH_4OH and 1247.5 µl glacial acetic acid. The absorbance of the solution was measured at 280 nm. Calibration curves were generated by subjecting 0-4.1 mg of commercial lignin (alkaline spruce lignin, Sigma, USA) to the same procedure.

For the DPPH (1,1-diphenyl-2-picrylhydrazyl radical) assay, the method reported by Hanato et al. (1988) was followed. 100, 150 and 200 µl extract were adjusted to 500 µl with 70% ethanol and added to 500 µl of an ethanolic 0.2

mmol l^{-1} DPPH solution. The mixture was left at room temperature for 30 min in the dark. The absorbance of the resulting solution was then measured at 517 nm. The antiradical activity was expressed as EC_{50} (mg ml^{-1}), the efficient concentration required to cause a 50% DPPH inhibition. The ability to scavenge the DPPH radical was calculated using the following equation: DPPH scavenging effect (%) = $(A_0-A_1)/A_0x100$, where A_0 is the absorbance of the DPPH, and A_1 is the absorbance of the sample at 30 min.

Enzyme assays

For the shikimate dehydrogenase (SKDH) assay, the method reported by Diaz et al. (1997) was followed. Samples (100 mg) were homogenized in the presence of 0.1 M potassium phosphate buffer (pH 7.4) containing 0.5 mM dithiotreitol (DTT), 2 mM L-cysteine, 2 mM EDTA, 8 mM 2-mercaptoethanol and 100 mg polyvinylpolypyrrolidone (PVPP) and centrifuged for 5 min at 12,000 *g* at 4 °C. The spectrophotometric assay for SKDH was performed at 25 °C in a reaction medium containing 4 mM shikimic acid and 2 mM $NADP^+$ in 0.1 M Tris-HCl buffer (pH 9). The reaction was initiated by adding the protein extract (50 µl), and the NADP reduction was followed at 340 nm for 5 min.

The phenylalanine ammonia-lyase (PAL) activity was assayed in samples (100 mg) ground in a pre-chilled mortar with liquid N_2. The powder was immediately added to 1 ml 100 mM potassium phosphate buffer (pH 8.0) containing 2 mM EDTA, 1.4 mM 2-mercaptoethanol and 0.1% PVPP. The homogenate was then centrifuged at 12,000 *g* for 30 min at 4 °C, and the supernatant was used as enzyme extract. The PAL assay was performed using a reaction mixture containing 2% (w/v) L-phenylalanine in 50 mM Tris-HCl at pH 8.8 and enzyme extract. The reaction was incubated at 37 °C for 120 min. The cinnamic acid produced was measured at 290 nm and the PAL activity calculated using the molar extinction coefficient for t-cinnamic acid 17,400 M^{-1} cm^{-1} (Gadzovska et al. 2007).

Cinnamyl alcohol dehydrogenase (CAD) activity was determined by measuring the increase in absorbance at 400 nm when coniferyl alcohol was oxidized to coniferaldehyde (Wyrambik and Grisebach 1975). The assay was performed for 30 min at 30 °C in a total volume of 500 µl containing 100 mM Tris-HCl (pH 8.8), 0.5 mM coniferyl alcohol, 1 mM NADP and 100 µl enzyme extract.

Protein concentration was determined by the method of Bradford (1976) using the dye-binding reagent (Bio-Rad).

Spectrofluorimetric assay of hydrogen peroxide

Hydrogen peroxide (H_2O_2) production was measured using the Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Molecular Probes, Invitrogen, USA) according to Shin et al. (2005). This assay is based on the reaction of 10-acetyl-3,7-dihydrophenoxazine (Amplex Red reagent) with H_2O_2 in a 1:1 stoichiometry to produce the red-fluorescent

oxidation product resorufin (Mohanty et al. 1997). Shoots were frozen in liquid N_2, ground with mortar and pestle and 10 mg frozen powder added to 400 µl 20 mM potassium phosphate buffer pH 6.5. After centrifugation (12,000 *g* for 20 min at 4 °C), 50 µl of the supernatant were incubated with 50 µl of the mixture of 0.5 µl 10-acetyl-3,7-dihydrophenoxazine (10 mM), 1 µl horseradish peroxidase (10 U ml^{-1}) and 48.5 µl buffer at 25 °C for 30 min in the dark. The resorufin fluorescence (Ex/Em = 530/590 nm) was quantified with a fluorescence/absorbance microplate reader (Victor3 1420 Multilabel Counter, Perkin Elmer, USA), after subtracting the background fluorescence of blank reactions without plant extract. Results were calculated with help of a H_2O_2-standard curve (0-100 µM).

Extraction and HPLC analysis of rosmarinic acid

Frozen plant material was pulverized in liquid N_2 and 10 mg suspended in 1 ml 70% ethanol and mixed vigorously. Extraction was conducted by sonicating the samples at 70 °C for 10 min twice with vigorous mixing in between. After centrifugation for 10 min at 6,000 *g* at 4 °C the supernatant was diluted 1:10 with 40% methanol acidified with 0.01% H_3PO_4, followed by another centrifugation (5 min) before HPLC analysis. The HPLC analysis was performed at room temperature with a reverse-phase column (Dionex Acclaim 120, C18, 5 µm particle size, 4.6 mm internal diameter × 150 mm length). The eluent was 40% methanol/0.01% H_3PO_4 at a flow rate of 1 ml min^{-1} with detection at 333 nm.

Statistical analysis

A minimum of 20 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) and comparison among means was determined by Fisher's LSD Multiple-Comparison Test. Where no significant variation was found, data were then analyzed by Student's t-test to highlight the differences due to O_3 application. Linear correlations were applied to DPPH vs phenolic compounds or RA data. All analyses were performed by the NCSS 2000 Statistical Analysis System Software.

Results

At the end of the O_3 treatment, shoots appeared symptomless, by microscopic observation, however, the O_3-treated leaves showed (after Evan's blue staining) some blue stained areas, identifying cell damage and dead cells. Blue stained cells were absent in control plants maintained in filtered air (Fig 1a-b). Histological staining for H_2O_2 showed local accumulation of this molecule evidenced by dark zones only in treated material and not in control leaves (Fig. 1c-d). At 1 h FBE, a significant peroxidation was detected, confirmed by the marked increase of TBARS levels (+19% in

comparison to controls). At the end of fumigation the percentage rose to 26%. The content of MDA decreased to values below the constitutive levels during the recovery time (Fig. 2).

The decrease of F_v/F_m (providing an estimate of the maximum quantum efficiency of PSII photochemistry, Bussotti et al. 2011) was 7% 1 h FBE. At the end of the treatment, the ratio F_v/F_m decreased about 20%, indicating that O_3 partially impaired the efficiency of PSII. However, at 8 and 24 h FBE no significant differences between treated and control plants were measurable (Fig. 3).

Analysis of the ROS content indicated a high H_2O_2 production in response to O_3 (Fig. 4a): H_2O_2 levels showed a peak in the first three hours during the fumigation (about twofold in comparison to untreated plants) and then declined to control level. O_3 induced a first increase of $O_2^{\cdot -}$ levels at 1 h FBE (+34%) and a slight decline at 2 h FBE (Fig. 4b). Then, values of $O_2^{\cdot -}$ showed a rise at 3 h FBE (59% more than filtered-air controls) and remained high during the recovery period (+53 and 57% at 8 and 24 h FBE).

Changes in the concentration of phenolic compounds are shown in Fig. 5. Total phenol levels showed a massive accumulation that peaked at 2 h FBE (+82%, Fig. 5a). They were always significantly higher in treated individuals than in untreated control plants. Anthocyanin and tannin levels increased at 1 h FBE (+81 and +110%, respectively), reaching a maximum value at 3 h FBE. At that time-point anthocyanin and tannin accumulation was more than twofold compared to the control (Fig. 5b-c). In treated leaves, levels of lignin significantly increased at 1, 3 and 24 h FBE (+27, +23 and +27%, respectively) in comparison to controls (Fig. 5d).

Changes in the activity of two key enzymes for the formation of phenolic compounds, SKDH and PAL, and of a key enzyme in lignin biosynthesis, CAD, are shown in Fig. 6. SKDH displayed a high peak at 1 h FBE (+165% in comparison to control material) (Fig. 6a). This enzyme maintained a higher activity in protein extracts from O_3-treated compared to control plants until the end of fumigation (+110%) and during the recovery period (+35%). At 3 h FBE, there also was a strong increase in PAL activity (+152%), that dropped back to control level at 8 h FBE (Fig. 6b). CAD showed a maximum of activity at 2 h FBE (+76%); as reported for SKDH, levels remained higher than the control at the end of fumigation (+71%) and during the recovery period (+53 and +74% at 8 and 24 h FBE, respectively) (Fig. 6c).

RA amounts increased with a peak at 2 h FBE (fourfold higher than filtered-air controls). They remained higher throughout the recovery period (+71 and 76% at 8 and 24 h FBE, respectively) (Fig. 7).

O_3-treated plants showed a prominent increase in antioxidant capacity as indicated by a very significant drop in the DPPH content at 2 h FBE (about threefold). At 1, 3 and 24 h FBE the respective levels were lower by 15, 16 and 33%, respectively (Fig. 8). In fumigated material, the antioxidant capacity was correlated to the concentration of phenolic compounds ($y = -0.020x + 0.434$, $R^2 = 0.49$) and to the RA level ($y = -0.007x + 0.351$, $R^2 = 0.69$).

Discussion

The importance of lemon balm for traditional and modern medicine has promoted the long-lasting research interest on its antioxidant activity in infusions and various kinds of other extracts. This plant is reported as one of the most interesting sources of antioxidant compounds (Döring et al. 2014b). Some of the bioactive compounds in *M. officinalis* belong to the secondary metabolites which can be stimulated by biotic and abiotic elicitors. In the recent past, there has been a growing interest in the degree of sensitivity/resistance of this species to oxidative stress. Lemon balm has been proposed as (*i*) a model O_3-bioindicator candidate for different regimes of this pollutant [such as changes in background concentrations (Döring et al. 2014a) and peak episodes (Pellegrini et al. 2011] and (*ii*) one of the most O_3-sensitive species belonging to the Lamiaceae family (Asensi-Fabado et al. 2013).

Plant tissue cultures can be considered a useful and convenient experimental system for examining various factors influencing the biosynthesis of desired products and for exploring effective measures to enhance their production without interference with pathogens and other microbes. Nevertheless, there are few reports on *in vitro* cultured lemon balm (Weitzel and Petersen 2010, 2011; Dias et al. 2012). The treatment of plant tissue cultures with elicitors are an effective strategy for improving the yield of secondary metabolites. In previous studies, the most common elicitors used include the components of microbial cells, especially poly- and oligosaccharides, heavy metal ions, hyperosmotic stress, UV radiation and signalling compounds in plant defence response (Campbell et al. 1992; Baque et al. 2010; Cai et al. 2012; Jacobo-Velázquez and Cisneros-Zevallos 2012; Bertoli et al. 2013). It is well established that, upon the challenge by biotic elicitors and certain signal molecules (for example salicylic and jasmonic acid), cultured cells can trigger an array of defence or stress responses (Gadzovska et al. 2007; Dogo et al. 2010; Zhao et al. 2010; Krzyzanowska et al. 2012). However, until now, very few reports focused on the effects of O_3 on the growth and accumulation of bioactive compounds in *in vitro* cultures (Sudhakar et al. 2007; Kadono et al. 2010; Sun et al. 2012). Well-known markers characterize this kind of response: e.g. (*i*) activation of the phenylpropanoid pathway and (*ii*) induction of cellular barriers (Pandey and Pandey-Rai 2014).

The current paper represents the first attempt to assess O_3 as elicitor of antioxidant compounds in *in vitro*-cultured shoots of *M. officinalis*. We document here that O_3 treatment induces an activation of some enzymes involved in phenolic metabolism, as confirmed by the large, but transient rise of SKDH and PAL activities. SKDH catalyses the conversion of dehydroshikimate to shikimate in the shikimate pathway that converts carbohydrates to aromatic amino acids, such as phenylalanine, which is the starting material for the phenylpropanoid pathway. Phenylalanine is required for the synthesis of phenolic secondary metabolites with a broad spectrum of antioxidant activities, and it's activity often induced in whole leaves (Francini et al. 2008, Döring et al. 2014b) and cultured cells (Sgarbi et al. 2003; Dogo et

al. 2010) after biotic and abiotic stress. Ali et al. (2006) documented that copper stress induced an evident increase of SKDH and PAL activities in root cultures of *Panax ginseng*. Dogo et al. (2010) observed that the treatment with salicylic acid (3.125-25.0 mg l^{-1}) induced an accumulation of phenolic compounds and a stimulation of PAL activity in *Salvia miltiorrhiza* cell cultures. Similar findings have been reported by Sgarbi et al. (2003) in two differentially O_3-sensitive *Vitis vinifera* cell lines exposed to a single O_3 treatment (300 ppb for 2 h). In our study, the time course of PAL was similar to typical PAL stimulation by plant pathogens or wounding with a maximum activity in the first hours (Sudha and Ravishankar 2002). For this reason, these data suggest that the induction of PAL by O_3 may be mechanistically similar to pathogen defense responses (Kangasjärvi et al. 1994). Under oxidative stress, *M. officinalis* shoots exhibited a stimulation of CAD activity associated with lignin biosynthesis. Usually lignification occurs upon wounding and fungal infection. Campbell and Ellis (1992) documented that lignin-like polymers were induced in pine and spruce cell cultures upon treatment with fungal elicitors, and CAD activity has been found to increase upon this challenge. Our results support the hypothesis that O_3 might stimulate the development of cellular barriers with a higher polymerization of cinnamyl alcohols. Similar findings have been reported by Sudhakar et al. (2007) in *in vitro* propagated *Rhinacanthus nasutus* plants exposed to a single square O_3 treatment (100 ppb for 30 min day^{-1} for 7 consecutive days). O_3 treatment induced not only an activation of enzymes controlling the phenylpropanoid pathway, but also an accumulation of metabolites derived from this pathway. We documented an increase of tannins, anthocyanins and phenols during the entire period of the treatment; in particular a massive increase was observed at 2-3 h after the onset of O_3 fumigation. These phenolic compounds are known to be effective antioxidants (Gill and Tuteja 2010) and play a variety of roles, e.g. defense against herbivores and pathogens and absorption of high energy radiation (Taiz and Zeiger 2002). They have protective properties against ROS and it has been found that there is a considerable increase in their levels in whole leaves (Kováčik et al. 2010; Saviranta et al. 2010) and in cultured cells (El-Beltagi et al. 2011) following biotic and abiotic stress. For this reason, our results suggest that the active phenol defense system induced by O_3 may be mechanistically similar to the responses against pathogens or herbivores. Oxidative stress affects not only the total phenolic content, but also the amount of single phenols. In particular, the level of RA significantly increased during and post fumigation. In cultured plant cells, the biosynthesis and the production of this metabolite have been extensively studied (Petersen 2013). The stimulation of RA by biotic (such as yeast elicitor and methyl jasmonate) and abiotic elicitors (e.g. silver ions) has been observed in cell cultures of e.g. *Lithospermum erythrorhizon* (Ogata et al. 2004), *Coleus blumei* (Petersen et al. 1994; Szabo et al. 1999) and *Salvia miltiorrhiza* (Yan et al. 2006, Zhao et al. 2010). Recently, the accumulation of RA in *M. officinalis* cell cultures was investigated (Weitzel and Petersen 2011), but the responsiveness of this species to abiotic or biotic elicitors was not reported. We observed a massive formation of

RA at 2 h after the onset of O_3 fumigation, which is concomitant with the maximum activity of free radical scavenging, as confirmed by the analysis of the DPPH radical scavenging activity of the extract from *M. officinalis* shoot cultures. In this assay, antioxidant activity mainly depends on the dissociation of hydrogen radicals from phenolic substances to form a stable compound with DPPH radicals. Some phenolic compounds can act as antioxidants by retarding protein oxidation or by binding to the proteins. The antiradical mechanism in phenol-protein aggregates may be due to ability of phenolic compounds to transfer oxidative damage from one phenolic site to another, protecting proteins from oxidation. According to other results, high salt strength enhanced DPPH radical scavenging activity in adventitious roots of *Morinda citrifolia* and a positive correlation was observed between DPPH radical scavenging activity and accumulation of phenolic compounds (Baque et al. 2010). In root cultures of *Hypericum perforatum*, elevated levels of phenolics in the roots grown in a sucrose-rich medium correlate with improved DPPH radical scavenging activity (Cui et al. 2010). Recently, Cai et al. (2012) reported similar findings in cell suspension cultures of *Vitis vinifera*. Our results indicate a close relationship between the concentration of phenolic compounds, the RA level in particular, and their free radical scavenging capacity.

A peak episode of O_3 (200 ppb, 3 h) was effective for stimulating a variety of secondary metabolites in *M. officinalis* shoot cultures. Previous investigations reported that O_3 can be considered as a major factor in the vulnerability of lemon balm whole leaves (Pellegrini et al. 2011). For this reason, we analyzed physiological and biochemical biomarkers that may help in better understanding the mechanisms involved in the response of *M. officinalis* shoots to O_3 regimes. Chlorophyll fluorescence is an intriguing indicator to assess photochemical efficiency and photoinhibition. It has been widely used in monitoring plant responses to environmental stress (Mohammed et al. 1995; Maxwell and Johnson 2000). A decline in photochemical efficiency is easily induced even by mild stress conditions. The F_v/F_m ratio is therefore a sensitive and early indicator of a change in photosynthesis and the physiological status of the plant in general. In dark-adapted untreated *M. officinalis* shoots, the mean value of this ratio was 0.789. This value is lower than that reported by Björkman and Demming (1987) for healthy plants ($0.800 \leq F_v/F_m \leq 0.860$). Often low rates of photosynthetic activity of *in vitro* shoots were observed, as expected from the culture conditions, such as low light intensity and CO_2 concentration in the headspace (During and Harst 1996; Dŭrkovič et al. 2010) and the feeding of sugar in the culture medium. Under oxidative stress, the overall quantum yield of primary photochemistry was slightly reduced and at the end of the recovery time, the F_v/F_m ratio reached constitutive values.

According to previous investigations conducted on whole leaves of naturally grown lemon balm (Döring et al. 2014a), O_3 slightly impaired the efficiency of PSII and, in particular, this damage was reversible. In the absence of visible injury, DAB staining and Evan's blue incorporation indicated that H_2O_2 deposition and cell death occurred only

382 at the end of exposure. Similar findings have been obtained with membrane denaturation measurements. In treated *M.*
383 *officinalis* shoots, the content of malondialdehyde [its production can be considered a signal of peroxidation of
384 polyunsaturated fatty acids (Del Rio et al. 2005)] raised to constitutive values during the recovery period, suggesting
385 that a partial control of ROS production was observed at the end of the O_3 treatment. The missing migration of these
386 secondary oxidation products from injured to relatively healthy neighboring cells showed that an early response of *M.*
387 *officinalis* shoots to short-term O_3 exposure is a transient oxidative burst leading to an endogenous, active and self-
388 propagating ROS generation. H_2O_2 exhibited a peak only during the treatment although a prolonged $O_2^{\cdot-}$ generation
389 occurred during and post fumigation.
390 In conclusion, we found (i) an activation of enzymes involved in phenolic metabolism; (ii) a development of
391 cellular barriers with a greater polymerization of cinnamyl alcohols; (iii) an accumulation of phenolic compounds, in
392 particular rosmarinic acid and (iv) an increase of antioxidant ability. As previously observed by Beauchamp et al.
393 (2005), O_3 is a good plant stress 'model' agent for several reasons: (i) exposure can be conducted under well-defined
394 conditions; (ii) experiments may be easily repeated mimicking the same conditions; (iii) doses of O_3 can be varied over
395 a wide range. Furthermore, O_3 has a great advantage compared to other biotic/abiotic elicitors, because it can be
396 degraded to oxygen during the treatment without toxic traces (Nishijima et al. 2014). Biotechnological applications of
397 O_3 in the field of medicinal plants for improving the secondary metabolites production deserve attention.
398
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404 **References**
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618

FIGURE CAPTIONS

Fig. 1 Localization of dead cells visualized with Evans blue staining (a, b) and of hydrogen peroxide (H_2O_2) visualized with the 3,3'-6 diaminobenzidine (DAB) uptake method (c, d) in *in vitro Melissa officinalis* shoots: a, c maintained in filtered air; b, d exposed to ozone (200 ppb, 3 h). Bars = 50 μm.

Fig. 2 Time course of the content of thiobarbituric acid reactive substances (TBARS) in *in vitro Melissa officinalis* shoots maintained in filtered air (open circle) or exposed to ozone (200 ppb, 3 h, closed circle). Data are shown as mean ± standard deviation. The measurements were carried out 0, 1, 2, 3, 8 and 24 hours from the beginning of exposure to ozone. Different letters indicate significant differences ($P \leq 0.05$). Boxes show the results of two-way ANOVA, asterisks show the significance of factors/interaction for: *** = $P \leq 0.001$; ns = $P > 0.05$. The hatched bar indicates the time (3 h) of ozone exposure.

Fig. 3 Time course of variable and maximal fluorescence ratio (F_v/F_m) in *in vitro Melissa officinalis* shoots maintained in filtered air (open circle) or exposed to ozone (200 ppb, 3 h, closed circle). Data are shown as mean ± standard deviation. The measurements were carried out 1, 2, 3, 8 and 24 hours from the beginning of exposure to ozone. Different letters indicate significant differences ($P \leq 0.05$). Boxes show the results of two-way ANOVA, asterisks show the significance of factors/interaction for: *** = $P \leq 0.001$. The hatched bar indicates the time (3 h) of ozone exposure.

Fig. 4 Time course of hydrogen peroxide (H_2O_2, a) content and rate of superoxide anion (O_2^-, b) generation in *in vitro Melissa officinalis* shoots maintained in filtered air (open circle) or exposed to ozone (200 ppb, 3 h, closed circle). Data are shown as mean ± standard deviation. The measurements were carried out 1, 2, 3, 8 and 24 hours from the beginning of exposure. Different letters indicate significant differences ($P \leq 0.05$). Boxes show the results of two-way ANOVA, asterisks show the significance of factors/interaction for: *** = $P \leq 0.001$; ** = $P \leq 0.01$. The hatched bar indicates the time (3 h) of ozone exposure.

Fig. 5 Time course of contents of total phenols (a), anthocyanins (b) and tannins (c) in *in vitro Melissa officinalis* shoots maintained in filtered air (open circle) or exposed to ozone (200 ppb, 3 h, closed circle). Data are shown as mean ± standard deviation. The measurements were carried out 1, 2, 3, 8 and 24 hours from the beginning of exposure. Boxes show the results of two-way ANOVA, asterisks show the significance of factors/interaction for: *** = $P \leq 0.001$.

649 Different letters indicate significant differences ($P \leq 0.05$) (a, b, c); for each time, significant differences are for *** =
650 $P \leq 0.001$ and * = $P \leq 0.05$ (d). The hatched bar indicates the time (3 h) of ozone exposure.
651
652 **Fig. 6** Time course of specific activities of shikimate dehydrogenase (SKDH, a), phenylalanine ammonia-lyase (PAL,
653 b) and cinnamyl alcohol dehydrogenase (CAD, c) in protein extracts from *in vitro Melissa officinalis* shoots maintained
654 in filtered air (open circle) or exposed to ozone (200 ppb, 3 h, closed circle). Data are shown as mean ± standard
655 deviation. The measurements were carried out 1, 2, 3, 8 and 24 hours from the beginning of exposure. Different letters
656 indicate significant differences ($P \leq 0.05$). Boxes show the results of two-way ANOVA, asterisks show the significance
657 of factors/interaction for: *** = $P \leq 0.001$. The hatched bar indicates the time (3 h) of ozone exposure.
658
659 **Fig. 7** Time course of rosmarinic acid (RA) content in *in vitro Melissa officinalis* shoots maintained in filtered air (open
660 circle) or exposed to ozone (200 ppb, 3 h, closed circle). Data are shown as mean ± standard deviation. The
661 measurements were carried out 1, 2, 3, 8 and 24 hours from the beginning of exposure. Different letters indicate
662 significant differences ($P \leq 0.05$). Boxes show the results of two-way ANOVA, asterisks show the significance of
663 factors/interaction for: *** = $P \leq 0.001$. The hatched bar indicates the time (3 h) of ozone exposure.
664
665 **Fig. 8** Time course of antioxidant capacity calculated as EC_{50} (efficient concentration required to cause a 50% DPPH
666 inhibition) values in extracts from *in vitro Melissa officinalis* shoots maintained in filtered air (open circle) or exposed
667 to ozone (200 ppb, 3 h, closed circle). Data are shown as mean ± standard deviation. The measurements were carried
668 out 1, 2, 3, 8 and 24 hours from the beginning of exposure. Different letters indicate significant differences ($P \leq 0.05$).
669 Boxes show the results of two-way ANOVA, asterisks show the significance of factors/interaction for: *** = $P \leq 0.001$.
670 The hatched bar indicates the time (3 h) of ozone exposure.
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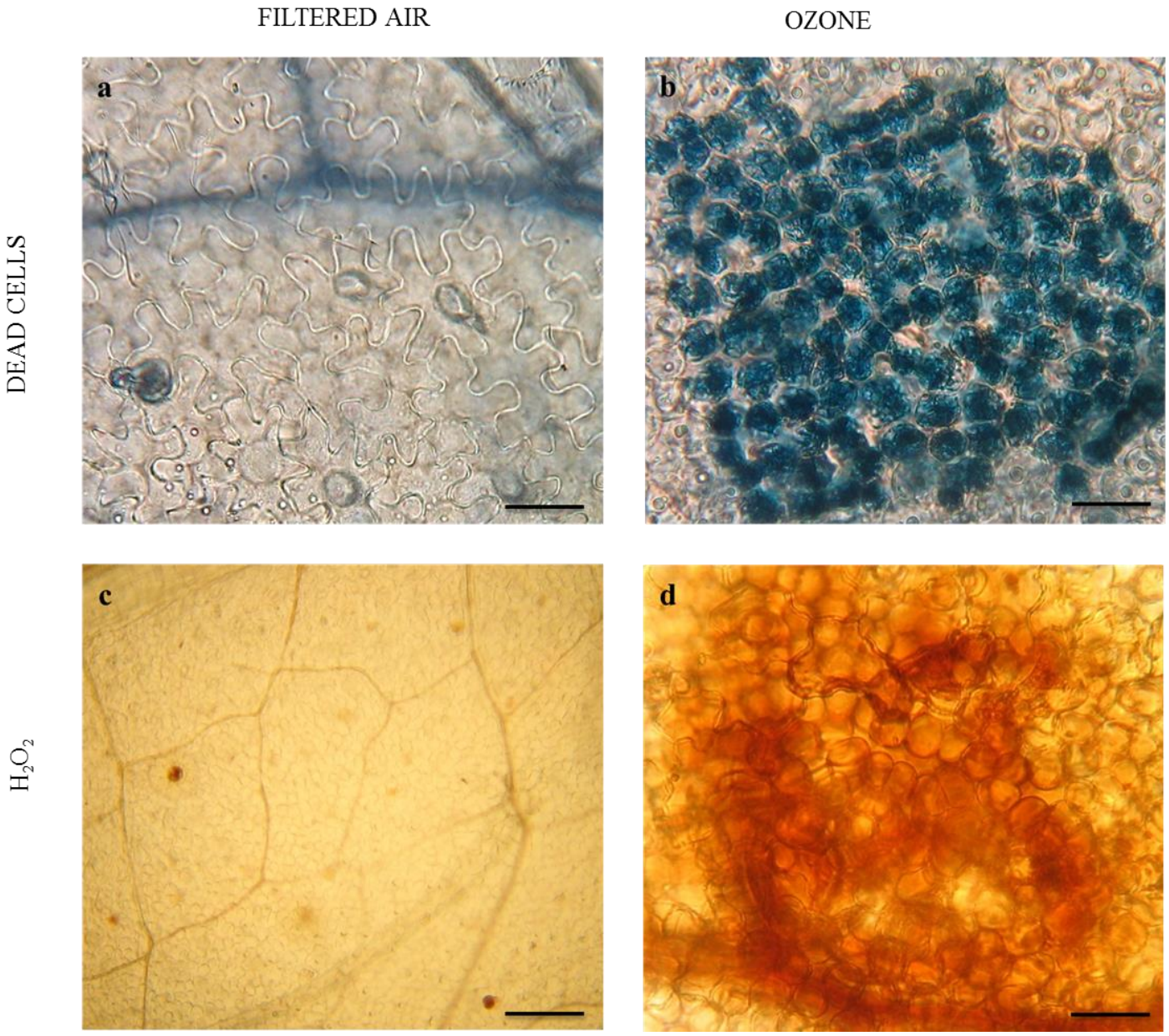
674 Figure 1



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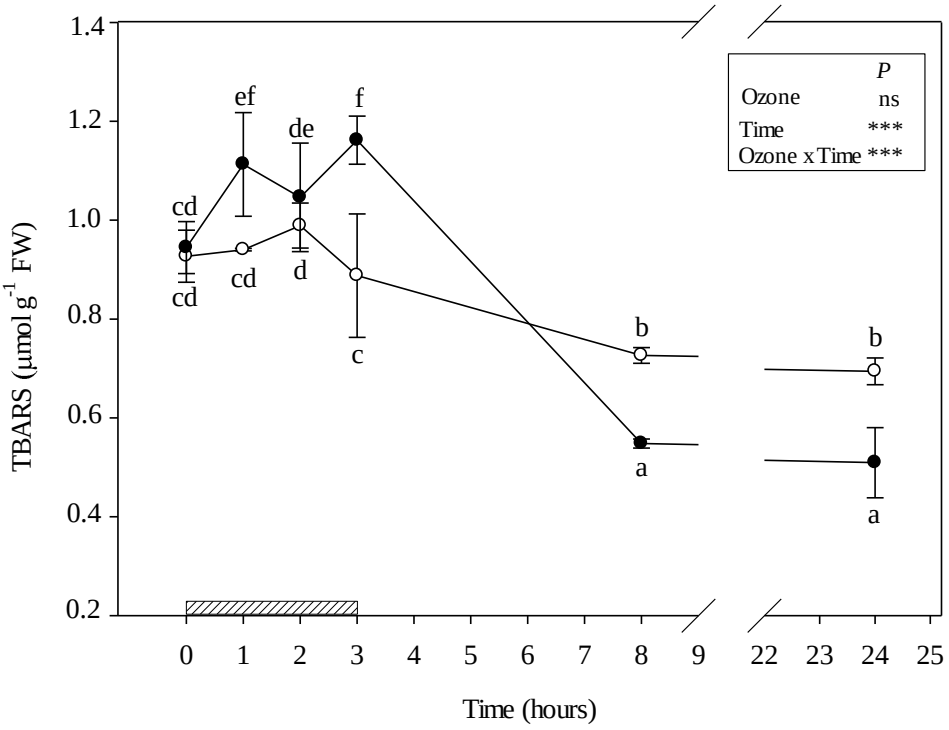
680 Figure 2



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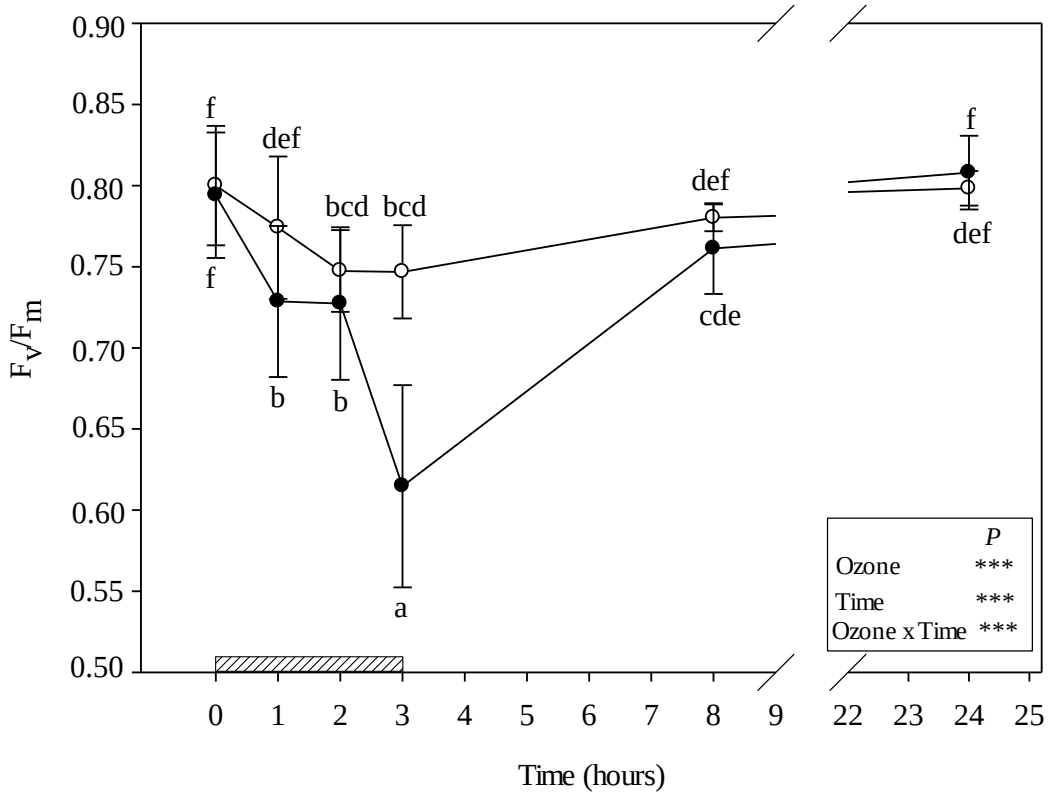

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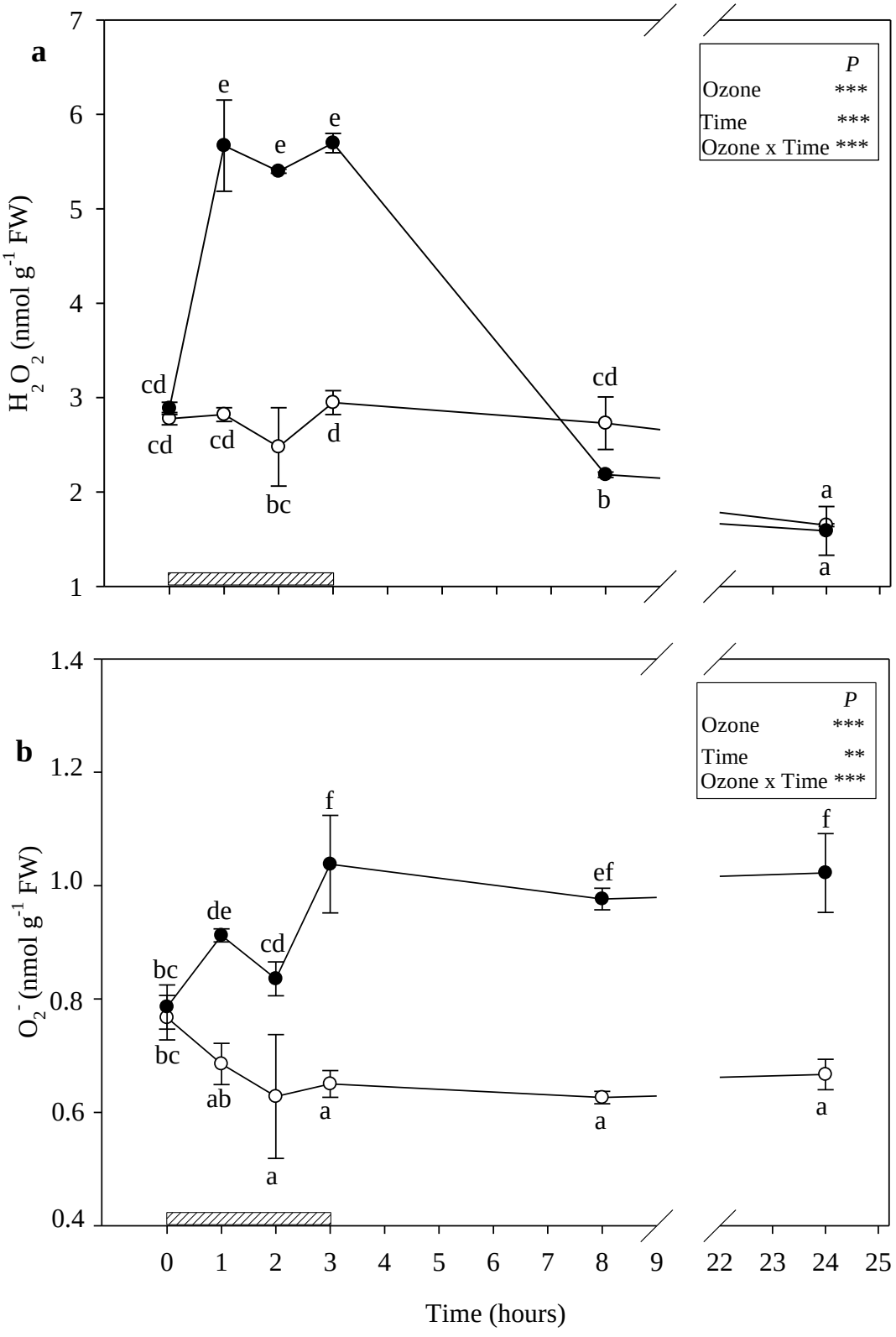
687 Figure 4



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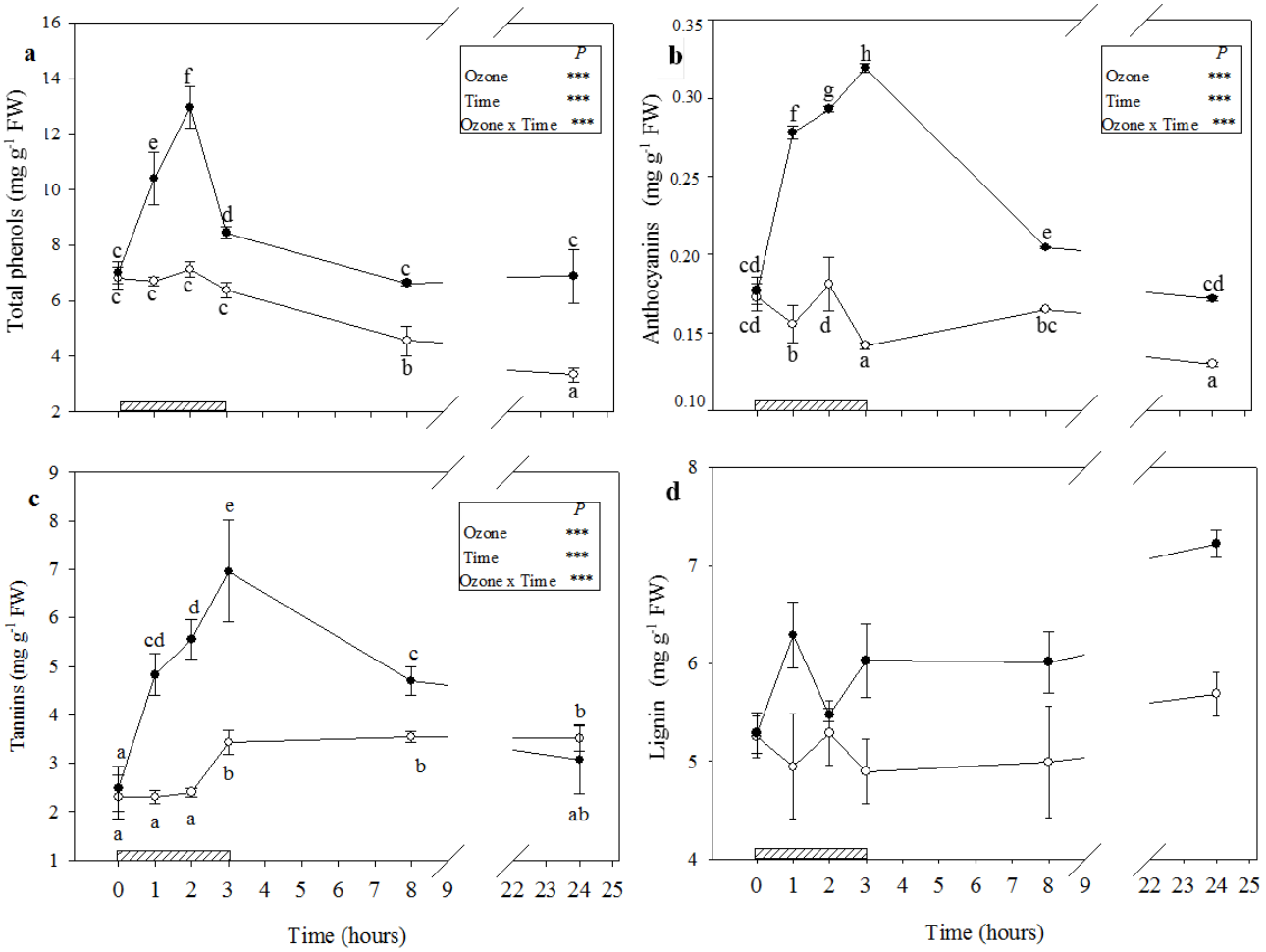
691 Figure 5



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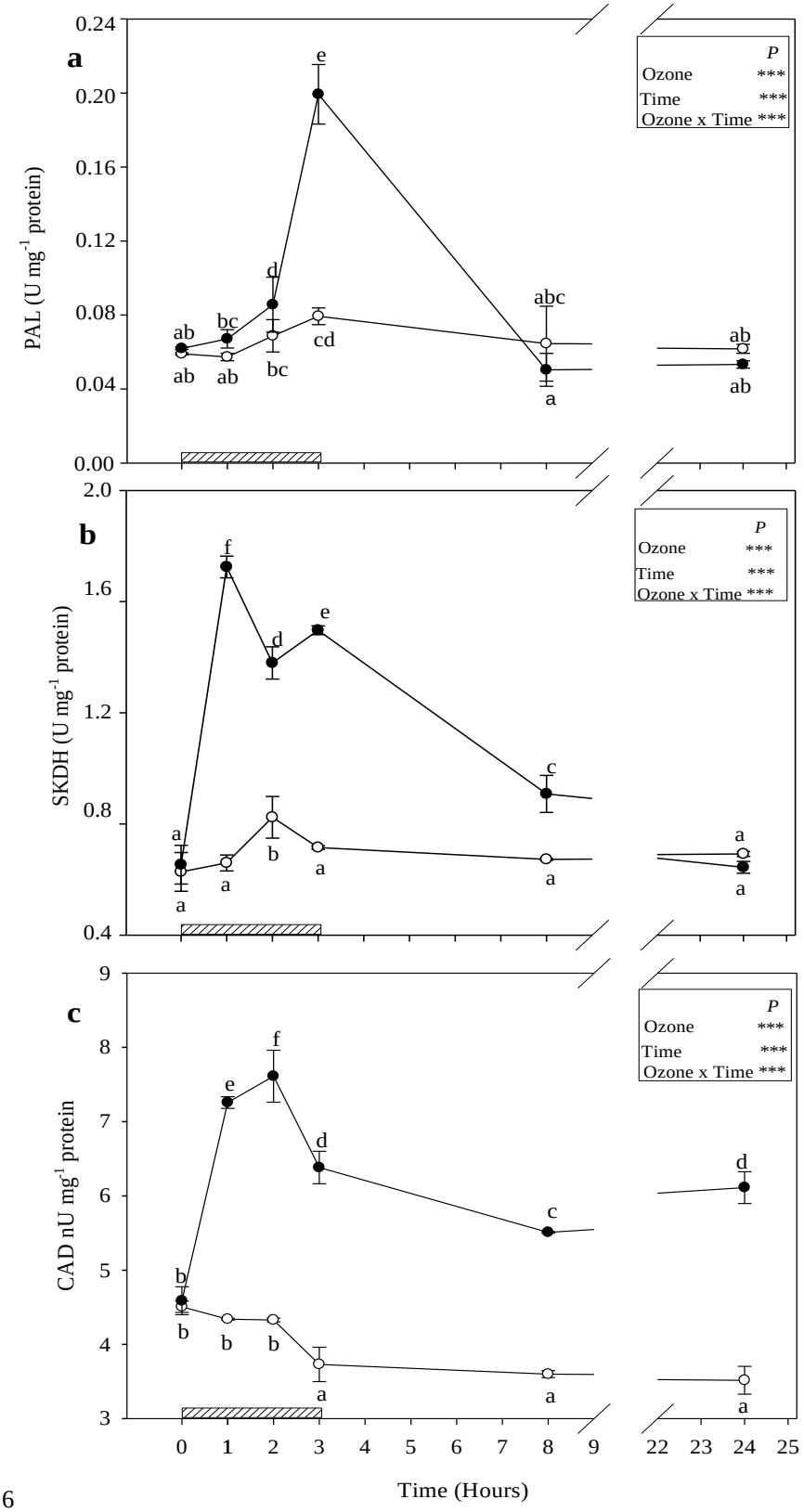

694 Figure 6

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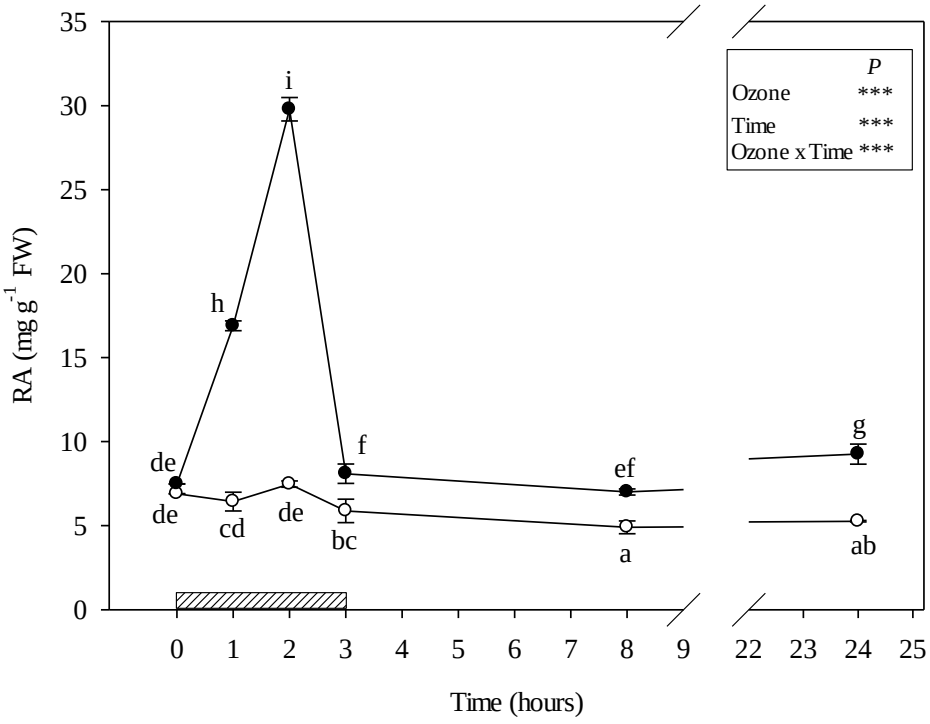

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698 Figure 7

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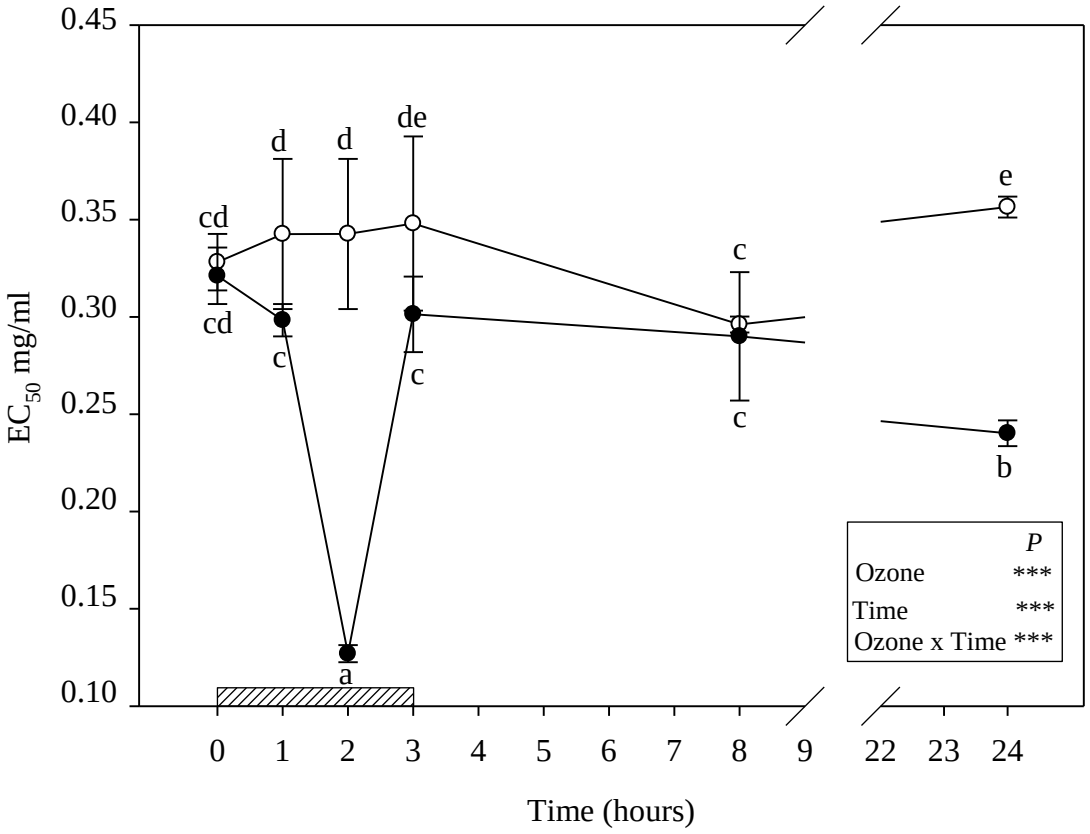
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Time (hours)

Figure 8