ORIGINAL ARTICLE

Chitosan oligosaccharides affect xanthone and VOC biosynthesis in *Hypericum perforatum* root cultures and enhance the antifungal activity of root extracts

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Abstract

Key message Water-soluble chitosan oligosaccharides (COS) affect xanthone and volatile organic compound content, as well as antifungal activity against human pathogenic fungi of extracts obtained from *Hypericum perforatum* root cultures.

Abstract Several studies have demonstrated the elicitor power of chitosan on xanthone biosynthesis in root cultures of *H. perforatum*. One of the major limitations to the use of chitosan, both for basic and applied research, is the need to use acidified water for solubilization. To overcome this problem, the elicitor effect of water-soluble COS on the biosynthesis of both xanthones and volatile organic compounds (VOCs) was evaluated in the present study. The analysis of xanthones and VOCs was performed by HPLC and GC–MS headspace analysis. The obtained results showed that COS are very effective in enhancing xanthone biosynthesis. With 400 mg L^{-1} COS, a xanthone content of about 30 mg g^{-1} DW was obtained. The antifungal activity of extracts obtained with 400 mg L^{-1} COS was the highest, with MIC_{50} of 32 µg mL^{-1} against *Candida albicans* and 32–64 µg mL^{-1} against dermatophytes, depending on the microorganism. Histochemical investigations suggested the accumulation of isoprenoids in the secretory ducts of *H. perforatum* roots. The presence of monoterpenes and sesquiterpenes was confirmed by the headspace analysis. Other volatile hydrocarbons have been identified. The biosynthesis of most VOCs showed significant changes in response to COS, suggesting their involvement in plant–fungus interactions.

Keywords *Hypericum perforatum* · Root cultures · Chitooligosaccharides · Xanthones · Volatile organic compounds

Introduction

Hypericum perforatum L. (Hypericaceae), popularly known as St. John's wort, has been one of the most investigated medicinal plants during the past two decades (Wölfle et al. 2014). The interest of scientific community towards *H. perforatum* mainly resides in its antidepressant activity (Russo et al. 2014), although it is being studied for a broad range of other biological activities (Marrelli et al. 2016).

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Research on St. John's wort has focused primarily on metabolites accumulated in the aerial part of the plant, such as hypericins (naphthodianthrones) and hyperforins (phloroglucinols), that are believed to be responsible for the antidepressant activity (Russo et al. 2014). A large number of volatile organic compounds (VOCs) such as monoterpenes and sesquiterpenes with antibacterial and antifungal activities have also been detected in the shoot organs of wild plants and in in vitro shoot cultures of *H. perforatum* (Schwob et al. 2004; Maggi et al. 2010; Pintore et al. 2005; Guedes 2009). The root has not been recognized as a valuable source of bioactive compounds of pharmacological interest until last years, when several studies revealed the presence of bioactive polyphenols in the root of the plant and in in vitro-regenerated roots (Bertoli et al. 2008; Cui et al. 2010a, b, c, 2011; Tocci et al. 2011, 2012, 2013a, b; Tusevski et al. 2013; Brasili et al. 2014; Zubrická et al. 2015; Simonetti et al. 2016; Valletta et al. 2016).

We have demonstrated that in vitro root cultures of St. John's wort produce xanthones at higher levels than the root of the plant (Tocci et al. 2011, 2012, 2013a; Simonetti et al. 2016; Valletta et al. 2016). Xanthones are a large and diverse group of non-flavonoid polyphenols produced by certain plants, fungi, lichens and bacteria (El-Seedi et al. 2009; Masters and Bräse 2012). These metabolites arouse great interest in the research community because of their numerous pharmacological properties (for a review, see Negi et al. 2013). Xanthone-rich extracts obtained from *H. perforatum* in vitro root cultures exhibited antifungal activity against several common human pathogenic fungi, such as *Candida* spp., *Cryptococcus neoformans*, dermatophytes and *Malassezia furfur* (Tocci et al. 2011, 2012, 2013a; Zubrická et al. 2015; Simonetti et al. 2016). In a recent study it has demonstrated that root endodermis and exodermis are the cellular sites of xanthone biosynthesis in *H. perforatum* in vitro-cultured roots (Tocci et al. 2018).

Chitosan (CHIT) is a natural non-toxic biopolymer, composed of randomly distributed β-(1 → 4)-linked D-glucosamine (deacetylated unit) and *N*-acetyl-D-glucosamine (acetylated unit), produced by the partial deacetylation of chitin, a major component of arthropod exoskeleton and fungal cell wall. Among elicitors, CHIT is one of the most commonly used to increase the biosynthesis of plant secondary metabolites of pharmacological interest. Several studies have shown the high effectiveness of CHIT in enhancing xanthone production in *H. perforatum* root cultures (Tocci et al. 2011, 2012, 2013a; Brasili et al. 2014; Simonetti et al. 2016); however, no studies on the impact of this elicitor on the production of VOCs are currently available.

Chitosan is poorly soluble in neutral water as well as in most organic solvents; therefore, it is commonly dissolved in water acidified with acetic acid, which greatly limits its application (Kim and Rajapakse 2005). In this regard, we have recently found that short-chain monocarboxylic acids, as acetic acid, could alter the xanthone profile, masking the effect of CHIT (Valletta et al. 2016). This problem could be overcome using water-soluble CHIT derivatives, also known as chitosan oligosaccharides or chitooligosaccharides (COS), which can be obtained by enzymatic and/or chemical hydrolysis of CHIT (Kim and Rajapakse 2005; Yin et al. 2010).

COS have been commercialized as low-calorie bulking agents since the 1980s and, more recently, they have gained interest in different fields, including food, agriculture, and medicine-related industries. As regards their application in the agri-food sector, most of the available studies focus on the administration of COS in vivo, in the field or in post-harvest, to fight microorganisms responsible for plant diseases, as well as for biodeterioration and mycotoxin contamination of food (Yin et al. 2010 and literature cited therein).

To date, only a few studies are available on the use of COS as elicitors to enhance the production of phytochemicals in plant in vitro cultures, and most of them have been performed on cell cultures (Cabrera et al. 2006; Wang et al. 2008). To the best of our knowledge, no studies have been published on in vitro root cultures.

The main objective of this study was to evaluate the elicitor power of COS on the biosynthesis of xanthones and VOCs in root cultures of *H. perforatum*. HPLC was used for the identification and quantification of six different xanthones, while GC–MS headspace analysis was adopted to determine the VOC profile. To compare the effect of COS with that of CHIT, COS were initially administered to the root cultures by following the same experimental design used in the previous studies for CHIT elicitation; subsequently, time- and concentration-dependent effects of COS on xanthone biosynthesis was investigated. The methanol extracts obtained from control and elicited roots were tested for their antifungal activity against human pathogens i.d. *Candida albicans, Trichophyton mentagrophytes*, and *Microsporum gypseum*.

Materials and methods

Plant material and root cultures

In vitro-regenerated roots of *H. perforatum* were obtained as previously described by Valletta et al. (2016). Liquid cultures were established as described by Valletta et al. (2016) with slight modifications. Briefly, 0.250 g fresh weight (FW) of roots was inoculated in 100 mL flasks containing 50 mL half-strength MS basal salts and vitamins (Murashige and Skoog 1962), supplemented with 1 mg L^{-1} IBA and 1.5% (w/v) sucrose. The MS medium and sucrose were purchased from Duchefa (Haarlem, The

Netherlands), while the growth regulators were obtained from Sigma-Aldrich (Milan, Italy). The cultures were shaken at 100 rpm at 26 ± 1 °C and maintained in continuous darkness.

Chitosan oligosaccharides (COS) preparation and identification

Chitosan (molecular weight 300–500 kDa, minimum 95% deacetylated) was purchased from Jinan Haidebei Marine Bioengineering Co., Ltd. (Shandong, China). COS with a degree of polymerization (DP) of 2–10 were prepared through enzymatic hydrolysis of CHIT according to Zhang et al. (1999). In brief, chitosan was dissolved in 2% acetic acid. Enzyme mixture in 0.05 mol L^{-1} acetate buffer was added and the mixture was incubated for 30 min at 40 °C. The hydrolyzates were filtered on a hollow-fiber membrane. These crude COS were added to ethanol and the mixture was stirred, thus forming a supersaturated solution, and stored at 4 °C overnight. The insoluble precipitate was removed using filter paper. The received COS solution was vacuum dried to obtain COS powder. The DP of the obtained COS was analyzed using hydrophilic interaction liquid chromatography combined with CAD detector. The DP of the COS was from 2 to 10, the mean molecular weight was around 1 kDa, and the acetylation was less than 5%.

Elicitation

Elicitation with COS

The roots were elicited using COS dissolved in deionized water. Different COS concentrations (50–400 mg L^{-1}) were applied to the root cultures. The stock solutions were prepared with COS concentrations ranging from 10 to 80 g L^{-1} to add 250 μL to each flask. Elicitation was always carried out on day 8 of culture. The stock solutions were sterilized with a 0.2 μm syringe filter before being added to the liquid culture medium. Control samples were added with 250 μL deionized sterile water. Three different elicitation protocols were carried out, as described below and in Fig. 1.

Time-dependent xanthone biosynthesis To investigate the xanthone biosynthesis in response to COS elicitation over time, the roots were elicited with 200 mg L^{-1} COS. Root samples were harvested by vacuum filtration on days 5, 10, 15, 20, and 25 post-elicitation, corresponding to days 13, 18, 23, 28, and 33 of culture (Fig. 1a).

Concentration-dependent xanthone biosynthesis To determine the optimal elicitor concentration, the roots were elicited with 50, 100, 200, and 400 mg L^{-1} COS, and then harvested by vacuum filtration on day 25 post-elicitation, corresponding to day 33 of culture (Fig. 1b).
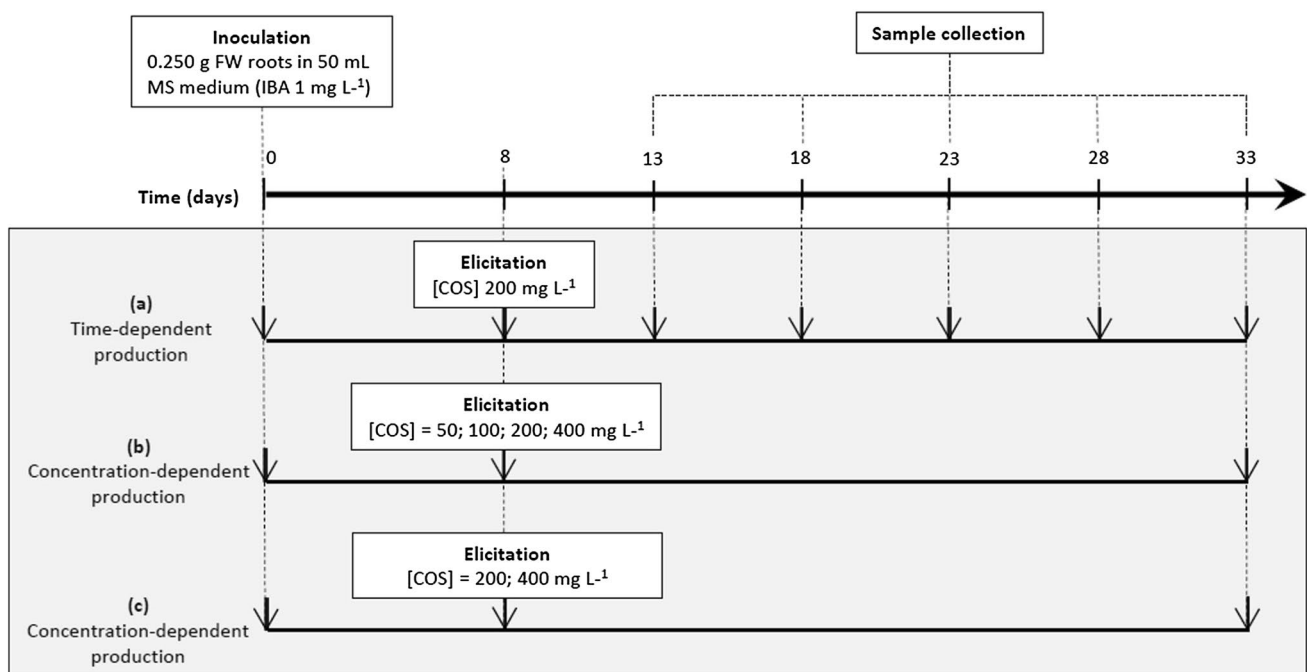

Fig. 1 Diagram representing protocols used to elicit *H. perforatum* root cultures

Concentration-dependent VOC biosynthesis To investigate the effect of different COS concentrations on the volatile compounds profile, the roots were elicited with 200 and 400 mg L^{-1} COS. Root samples were harvested on day 15 after the elicitation (Fig. 1c).

Determination of root biomass

Growth curve of the *H. perforatum* roots, elicited with different COS concentrations (200 or 400 mg L^{-1}) and not elicited, was determined gravimetrically by measuring dry weight increases on days 5, 10, 15, 20, and 25 after the elicitation. The initial weight of all samples was 0.250 g FW of roots. The growth index (GI) was calculated as follows:

GI = Final weight − initial weight.

Xanthone quantification

The roots were dried in an oven at 70 °C until a constant weight was obtained; then, they were powdered with pestle and mortar and extracted three times (each 24 h) with methanol at room temperature. The ratio root dry biomass/methanol was 100/5 (mg:mL). The extracts were dried with a rotavapor (Buchi, Milan, Italy) at 35 °C and redissolved in HPLC-grade methanol (Carlo Erba, Milan, Italy) at the ratio initial biomass DW/methanol of 100/1 (mg:mL). The extracts were analyzed by high-performance liquid chromatography (HPLC), as described by Tocci et al. (2013a) and Valletta et al. (2016). Different xanthones were identified and quantified: (1) mangiferin; (2) 1,3,6,7-tetrahydroxyxanthone; (3) 1,3,5,6-tetrahydroxyxanthone; (4) kielcorin; (5) cadensin G; (6) 1,7-dihydroxyxanthone; (7) toxiloxanthone, (8) paxanthone; and (9) 5-*O*-methyl-2-deprenylrheediaxanthone.

Histochemical detection of isoprenoids

Fresh sections (thickness ≈ 30 μm) of control roots and roots treated with 50, 100, 200, 400, and 800 mg L^{-1} COS and collected on day 18 of culture (corresponding to day 10 post-elicitation) (Fig. 1a), and were obtained by microtome (Vibratome Series 1000). The histochemical test with Nadi reagent was performed as previously reported by Monacelli et al. (2005).

Viability test

To assess the cytotoxicity of COS, a viability test with fluorescein diacetate (FDA) was carried out as previously reported by Santamaria et al. (2011) on *H. perforatum* cultured roots treated with 50, 100, 200, 400, and 800 mg L^{-1} COS on day 8 and collected on day 33 of culture. Non-treated roots were used as positive control and roots killed with liquid nitrogen were used as negative control. Roots

were analyzed with a Zeiss microscope (Axioscop 2 Plus) fitted with a digital camera (Zeiss AxioCam MRc5) and a blue filter ($\lambda_{excitation}$ 386 nm; $\lambda_{emission}$ 490 nm). Roots that emitted a green fluorescence under blue light were considered viable.

Analysis of volatile organic compounds (VOCs)

The VOCs of roots treated with 200 or 400 mg L^{-1} COS and harvested on day 15 of culture were determined by solid-phase-micro-extraction (SPME) that consists in catching the VOCs contained in the headspace above a sample in an SPME vial with a fiber coated with adapted stationary phases and inject them into a gas chromatograph mass spectrometer (GC–MS) with posterior data analyses.

SPME procedure

A divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS, 50 μm) fiber with manual holder from Supelco (Bellefonte, PA, USA) was used for the extraction of volatile compounds. The SPME fiber was preconditioned before the analyses, according to the instructions of the manufacturer.

The samples were ground using pestle and mortar under liquid nitrogen. A total of 3 g of roots was homogenized with 30% sodium chloride solution (Merck) and placed (10 g) into a headspace vial sealed with a septum. The samples were kept under agitation with a magnetic stir bar and heated to 40 °C. The headspace equilibrium time was 30 min. Volatiles were extracted by exposing the SPME fiber to the headspace of the sample vial that was maintained at 40 °C for 60 min. For thermal adsorption, the SPME fiber was immediately inserted into the GC–MS injector and held for 2 min at 250 °C in splitless mode.

GC–MS conditions

Chromatographic analysis was performed in a Hewlett-Packard 6890 (Agilent Technologies Inc., Santa Clara, USA) GC–MS.

The injector temperature was 200 °C. Components were then separated using a capillary column Supelcowax 10 (30 m × 0.25 mm × 0.25 μm) and the oven temperature was programmed to ramp from 40 to 150 °C at 2 °C min^{-1} and hold for 5 min. Helium was used as a carrier gas with a constant column flow rate of 1 mL min^{-1}. The mass detector operated in electron impact (EI)-mode at 70 eV in a range of 15–210 amu. Volatile compounds were identified by comparison with the NIST database (NIST11, version 2.0, Gaithersburg, USA) and then confirmed with the Kovats retention indexes (RI). Further identification was carried out by calculating non-isothermal retention indices. The RI values were obtained by injecting saturated *n*-alkane standard solution

(C_7–C_{30} 1,000 µg mL^{-1} in hexane, Supelco, Belgium) under the same chromatographic conditions and compared with those described in the literature determined under the same conditions for matching the compounds. The mass spectra data of all volatiles were also confirmed by comparison with the spectral data available at the MassBank of North America (MoNA—http://mona.fiehnlab.ucdavis.edu/).

Antifungal susceptibility testing

The evaluation of the antifungal activity was carried out on *C. albicans* ATCC 10231 coming from American-Type Culture Collection (ATCC, Manassas, VA, USA), *T. mentagrophytes* DSM 4870, and *M. gypseum* DSM 3824 coming from German Collection of Microorganisms (DSMZ, Braunschweig, Germany). To evaluate the minimal inhibitory concentration (MIC), the susceptibility in vitro assay was performed on *C. albicans* according to standardized methods for yeast using the broth microdilution method (CLSI M27-A3 2008b; CLSI 2012) and on dermatophytes according to standardized methods for filamentous fungi (CLSI M38-A2, 2008a). Dermatophytes were grown on potato dextrose agar (Sigma-Aldrich, St. Louis, MO, USA) at 28–30 °C until good conidial growth was present. The conidia suspension was prepared at the final concentration of 1×10^3 to 3×10^3 CFU mL^{-1} (CLSI M38-A2 2008a). *C. albicans* was grown on Sabouraud dextrose agar at 35 °C for 24 h. The final concentration of the inoculum was 0.5×10^3–2.5×10^3 CFU mL^{-1}. The in vitro antifungal susceptibility was evaluated using extracts. The concentration of extracts ranged from 512 to 0.5 µg mL^{-1}. The MIC$_{50}$ was the lowest concentration of extracts or reference drugs that caused $\geq 50\%$ growth inhibition and the MIC$_{100}$ was the lowest concentration that inhibited 100% of growth. Results were expressed as median of three experiments performed in duplicate.

Statistical analysis

All measurements were made at least in triplicate and the results were expressed as means ± SD. Statistical analysis was carried out using SigmaPlot 13.0. Two-way analysis of variance (ANOVA), followed by Holm–Sidak tests, was applied to test differences between groups. Statistical significance of the results was also evaluated, also by paired Student's *t* test, and differences with a *p* value ≤ 0.05 were considered significant. A total of six biological replicates of samples for each treatment were analyzed by GC–MS. Volatile organic data set was imported into Metaboanalyst 3.0 (http://www.metaboanalyst.ca) for multivariate statistical analysis. All imported data were Pareto-scaled. A principal component analysis (PCA) was conducted on GC–MS data to discern inherent similarities in volatiles profiles. Next, a PLS-DA model was used to maximize

covariance between the measured data (concentrations in GC–MS spectra, *X* matrix) and the response variable (predictive classifications, *Y* matrix). The variable importance in the projection (VIP) plot was then used to identify which volatile compound contributes most to clustering or trends observed in the data.

Results

Elicitation with COS

Root biomass growth

The increase in root biomass growth was monitored on days 0 and 8 of culture and every 5 days after elicitation with different COS concentrations (Fig. 2). During the first 8 days, a doubling of root biomass was observed in all analyzed roots. In control roots, the exponential growth phase began on day 8 and continued until day 18; a decrease in biomass growth was observed from day 18 to day 23 (non-significant differences); starting from day 23 to day 28, a plateau was recorded (non-significant differences); another exponential growth phase took place from day 28; and the elicitation on day 8 causes a remarkable decrease of growth in treated roots. The main difference between roots elicited with 200 and 400 mg L^{-1} took place at day 33, after which, the roots treated with the lower COS concentration slowly started to grow again.
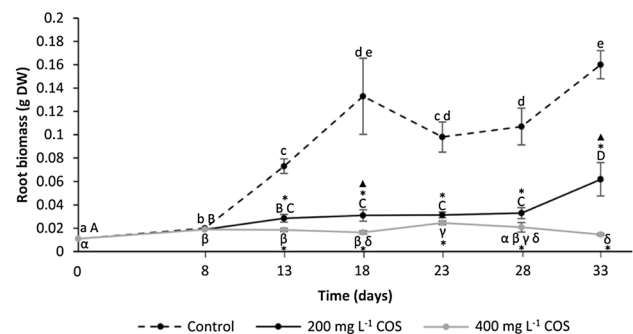

Fig. 2 Growth curves of *H. perforatum* root cultures elicited with 200 and 400 mg L^{-1} COS. Control roots (black dashed line); treated roots with 200 mg L^{-1} COS (black line); treated roots with 400 mg L^{-1} COS (grey line). Mean values were based on three replicates from two separate experiments. Bars represent standard deviations of the means. Different letters represent significant differences between samples ($p \leq 0.05$); asterisk represents significant differences between samples compared with control at the same day of culture; black up-pointing triangle represents significant differences between samples compared with 400 mg L^{-1} COS at the same day of culture

Time-dependent xanthone biosynthesis

The HPLC analysis showed that the addition of 200 mg L^{-1} COS to *H. perforatum* root cultures significantly enhances xanthone *biosynthesis*. All extracts obtained from COS-elicited roots and collected at different times' post-elicitation (days 5, 10, 15, 20, and 25) (Fig. 1a) showed a significant increase in total xanthones compared to their respective controls (Fig. 3). In the control roots, total xanthone content increased with an increasing culture time from about 3.42 to 7.87 mg g^{-1} DW. In COS-elicited roots, the highest xanthone levels were observed after a short time (day 5) and after a long-time (day 25) post-elicitation, with 13.25 e 14.31 mg g^{-1} DW of total xanthones, respectively. On days 10, 15, and 20, xanthone content remained almost constant with an average value of about 9.8 mg g^{-1} DW.

The levels of individual xanthones were measured at all experimental times (supplementary material), and in Fig. 4, the data acquired at times of maximum production (days 5 and 25) are shown. On day 5 (Fig. 4a), all the analyzed xanthones were present in control roots, with the exception of kielcorin (Kiel) and 1,7-dihydroxyxanthone (Dihydroxy). Mangiferin (Mang) and toxyloxanthone (Toxy) were accumulated at relatively low levels (0.22–0.18 mg g^{-1} DW). Other xanthones were accumulated at levels ranging from 0.55 for paxanthone (Pax) to 1.02 mg g^{-1} DW for 5-*O*-methyl-2-deprenylrheediaxanthone (Rheedia). The levels of all xanthones significantly increased in response to COS elicitation, with the exception of mangiferin (Mang) and cadensin G (Cad). The highest increases were observed for tetrahydroxixanthones (Tetra), Toxy, Pax, and Rheedia (4.6-, 17.0-, 5.6-, and 2.8-fold increase, respectively). On day 25 (Fig. 4b), all
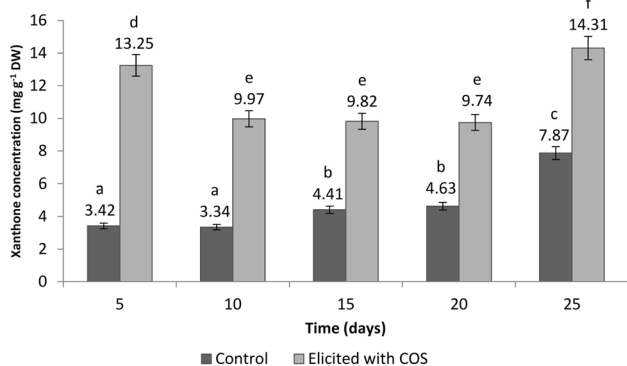

Fig. 4 Xanthone production in *H. perforatum* root cultures on days 5 (**a**) and 25 (**b**) after elicitation with 200 mg L^{-1} COS. The numbers next to the bars represent the concentration of xanthones (mg g^{-1} DW). The data shown are mean of three replicates from two separate experiments. Bars represent standard deviations of the means. Asterisks indicate statistically significant differences ($p \leq 0.05$) between COS-elicited roots and control roots. *Mang* mangiferin, *Tetra* 1,3,5,6-tetrahydroxyxanthone and 1,3,6,7-tetrahydroxyxanthone, *Cad* cadensin G, *Toxy* toxyloxanthone, *Pax* paxanthone, *Rheedia* 5-*O*-methyl-2-deprenylrheediaxanthone

analyzed xanthones were detected in control roots, with the exception of Kiel and Dihydroxy. Mang, Tetra, and Cad were accumulated at relatively low levels (0.31, 0.29, and 0.51 mg g^{-1} DW). Toxy, Pax, and Rheedia were accumulated at higher levels (1.02, 1.04, and 4.7 mg g^{-1} DW). In response to COS, the levels of all analyzed xanthones increased, with the exception of the Mang, the level of which decreased significantly. These increases were statistically significant for Tetra, Cad, Pax, and Rheedia (3.8-, 0.22-, 4.2-, and 1.3-fold increase, respectively).

Fig. 3 Time-dependent xanthone production in *H. perforatum* root cultures in response to elicitation with 200 mg L^{-1} COS. The numbers next to the bars represent the exact concentration of total xanthones (mg g^{-1} DW). The data shown are mean of three replicates from two separate experiments. Bars represent standard deviations of the means. Different letters represent significant differences between samples ($p \leq 0.05$)
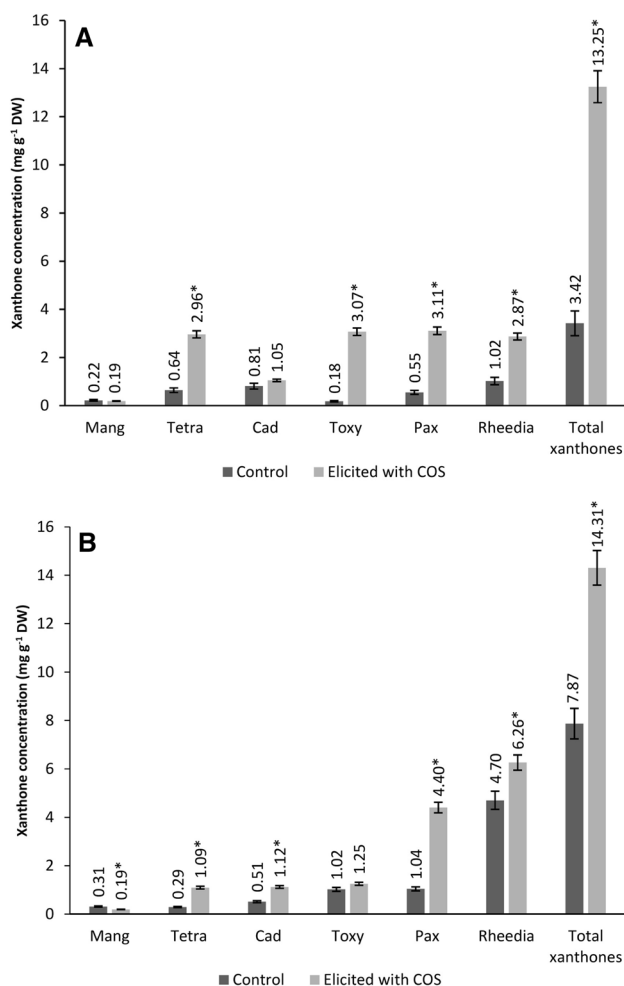
Concentration-dependent xanthone biosynthesis

COS at different concentrations were tested on *H. perforatum* root cultures. Chemical analyses were performed on roots collected on day 25 post-elicitation, which gave the best results in the previous experiments. The roots treated with 400 mg L^{-1} COS were the most productive in term of total xanthones (Fig. 5), the content of which was about 12 times higher than in control roots (30.8 and 2.5 mg g^{-1} DW, respectively). At higher concentrations, the xanthone content significantly decreased (data not shown) and the roots

showed symptoms of necrosis (Fig. 8). In the roots elicited with 400 mg L^{-1} COS, all analyzed xanthones were produced at much higher levels than roots treated with lower COS concentrations, with the exception of Mang (Fig. 6). The highest levels of Mang were detected in roots treated with 50 e 100 mg L^{-1} COS (1.95–1.71 mg g^{-1} DW). As regards Tetra, Cad, Pax, and Rheedia levels in elicited roots, it was 15.4-, 23.4-, 41.3-, and 15.4-fold higher than in control roots. Toxy was not detected in untreated roots, while it was produced at relatively high levels (7.06 mg g^{-1} DW) in roots treated with 400 mg L^{-1} COS.

Histochemical detection of isoprenoids

The treatment with Nadi reagent revealed secretory ducts in both control and COS-treated roots, each delimited by four secretory cells (Fig. 7). Both the secretory cells and the lumen of the ducts reacted positively with Nadi reagent, which revealed the presence of isoprenoid compounds. As regards the signal intensity, no differences were observed between control roots and roots treated with different concentrations of COS (Fig. S1).

Viability test

H. perforatum cultured roots subjected to COS concentrations ≤ 200 mg L^{-1} (Fig. 8a) showed a macroscopic appearance similar to non-treated roots (Fig. 8b, c). Symptoms of suffering in the form of tissue darkening and morphological alterations were observed in roots treated with COS
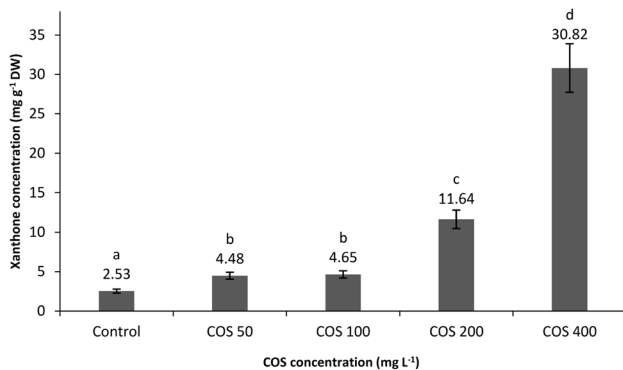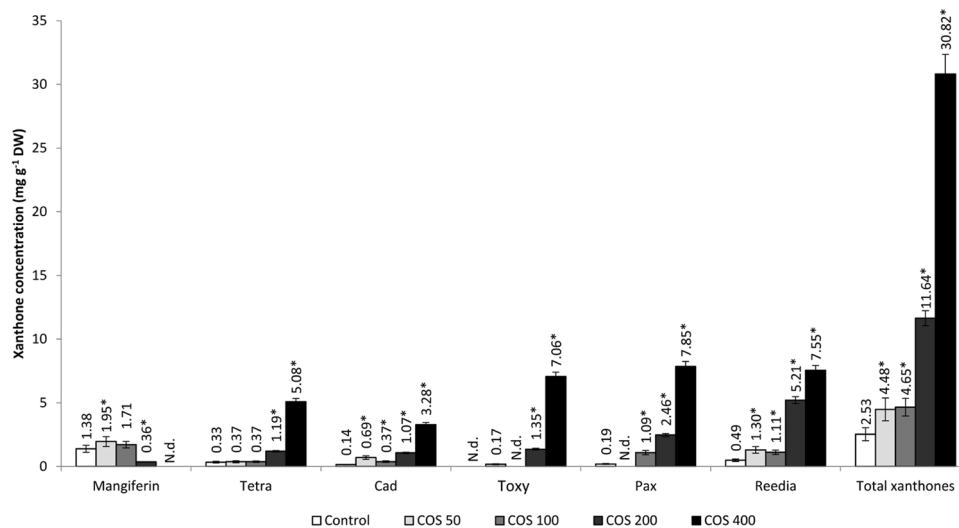
Fig. 5 Total xanthone content in *H. perforatum* root cultures not subjected to COS elicitation (control) or elicited with different COS concentrations. The numbers next to the bars represent the exact xanthone content (mg g^{-1} DW). Each value is the mean of three independent determinations ± SD. Different letters represent significant differences between samples ($p \leq 0.05$)

Fig. 6 Xanthone content in *H. perforatum* root cultures not subjected to COS elicitation (control) or elicited with different COS concentrations. The numbers next to the bars represent the exact xanthone content (mg g^{-1} DW). Each value is the mean of three independent determinations ± SD. Asterisks indicate that the differences between COS-elicited roots and the corresponding control roots are statistically significant ($p < 0.05$). *Mang* mangiferin, *Tetra* 1,3,5,6-tetrahydroxyxanthone and 1,3,6,7-tetrahydroxyxanthone, *Cad* cadensin G, *Toxy* toxyloxanthone, *Pax* paxanthone, *Rheedia* 5-*O*-methyl-2-deprenylrheediaxanthone, *N.d.* non-detected
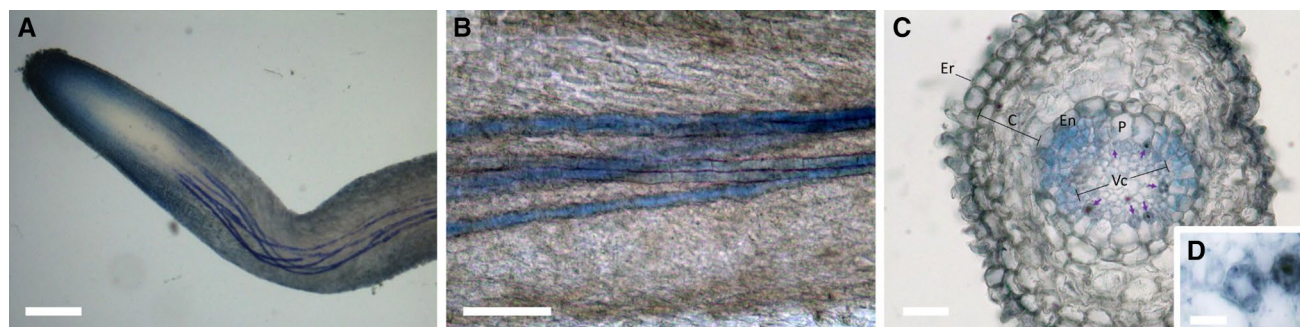
Fig. 7 In vitro roots of *H. perforatum* elicited with 200 mg L^{-1} COS, treated with Nadi reagent and observed under bright field. Intact root at different magnifications (**a b**); root cross section (**c**); detail of the root section in which two close secretory ducts are visible (**d**). *Er* root epidermis, *C* cortex, *En* endodermis, *P* pericycle, *Vc* vascular cylinder. Bars represent 500 (**a**), 200 (**b**), 100 (**c**) and 25 µm (**d**)
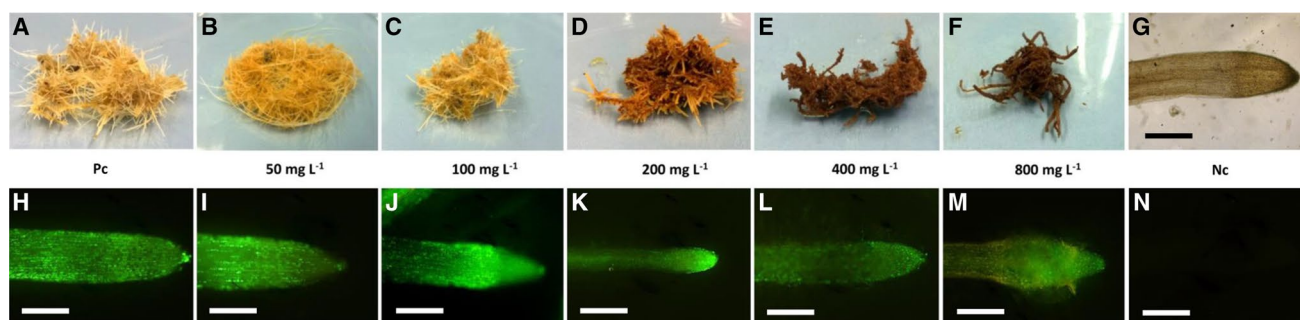

Fig. 8 Fluorescein diacetate (FDA) viability test on *H. perforatum* roots collected on day 33 of culture (corresponding to day 25 post-elicitation). Macroscopic appearance of non-treated roots (**a**) and roots subjected to different COS concentrations (**b–f**). Roots observed through epifluorescent microscopy under blue light to reveal the green signal generated by FDA (**h–n**). Positive control (Pc) represented by non-treated roots (**a, h**) and negative control (Nc) represented by roots killed with liquid nitrogen (**g, n**). Bars represent 300 µm. (Color figure online)

concentrations > 200 mg L^{-1} (Fig. 8d–f). The viability of non-treated roots and roots elicited with COS concentrations ranging from 50 to 800 mg L^{-1} was investigated through FDA-viability test. The cells of non-treated roots and roots treated with 50–800 mg L^{-1} COS emitted a green fluorescence when observed with a microscope under blue light (Fig. 8h–m). No fluorescent signal has been observed in roots treated with liquid nitrogen (Fig. 8n). These results indicate that COS does not cause cell death at the tested concentrations.

Antifungal activity of extracts obtained from *H. perforatum* cultured roots elicited with COS

The antifungal activity of extracts obtained from *H. perforatum* cultured roots elicited with different COS concentrations and collected on different days' post-elicitation has been evaluated against *C. albicans*, *T. mentagrophytes* and *M. gypseum*. As regards MIC$_{50}$ and MIC$_{100}$ of *C. albicans* and MIC$_{50}$ of *M. gypseum*, the best activity was exhibited by extracts of roots collected on days 20 and 25 post-elicitation

(32, 64, and 16 µg mL^{-1}, respectively). As regards MIC$_{100}$ of *M. gypseum*, the best activity was exhibited by extracts of roots collected on days 15 and 20 post-elicitation (32 µg mL^{-1}). Antifungal tests performed against *T. mentagrophytes* with extracts obtained from roots collected at different experimental times, yielded similar MIC values (MIC$_{50}$ 16 µg mL^{-1}; MIC$_{100}$ 32 µg mL^{-1}) (Tables 1 and S1). Moreover, the antifungal activity increased by increasing the COS concentration (Tables 2 and S2). COS did not show any antifungal activity on the investigated fungal strains.

Concentration-dependent volatile organic compounds (VOCs) biosynthesis

As shown in Table S3, VOCs were identified and listed according to their biosynthetic origin. A total of 43 volatile compounds were identified, including fatty acid derived volatiles, phenylpropanoid/benzenoid compounds, acyclic, monocyclic and bicyclic monoterpenes, and sesquiterpenes. The values of relative peak areas (median normalized) obtained by SPME–GC–MS for each compound constitute

Table 1 Antifungal activity of methanol extracts of *H. perforatum* root cultures collected at different days after elicitation with 200 mg L^{-1} COS against *Candida albicans*, *Trichophyton mentagrophytes*, and *Microsporum gypseum*

Extracts	*C. albicans* ATCC 10231		*T. mentagrophytes* DSM 4870		*M. gypseum* DSM 3824	
	MIC$_{50}$ (µg mL^{-1})	MIC$_{100}$ (µg mL^{-1})	MIC$_{50}$ (µg mL^{-1})	MIC$_{100}$ (µg mL^{-1})	MIC$_{50}$ (µg mL^{-1})	MIC$_{100}$ (µg mL^{-1})
Control day 5	256	256	64	128	128	256
EL day 5	64 (*a)	256 (a)	16 (*a)	32 (*a)	32 (*a)	64 (*a)
EL day 10	64 (*a)	128 (*b)	16 (*a)	32 (*a)	32 (*a)	64 (*a)
EL day 15	32 (*b)	128 (*bc)	16 (*a)	32 (*a)	32 (*a)	32 (*b)
EL day 20	32 (*b)	64 (*c)	16 (*a)	32 (*a)	16 (*b)	32 (*b)
EL day 25	32 (*b)	64 (*cd)	16 (*a)	32 (*a)	16 (*b)	64 (*a)
Fluconazole	2 (*c)	64 (*d)	16 (*a)	32 (*a)	16 (*b)	32 (*b)

MIC$_{50}$ and MIC$_{100}$ are the lowest concentration of extracts or reference drugs that caused growth inhibition ≥ 50% and 100%, respectively. Results are expressed as median of three experiments performed in duplicate. Asterisks represent significant differences ($p \leq 0.05$) between MIC values obtained with extracts from COS-elicited roots (EL) and from non-treated roots (control). Different letters represent significant differences ($p \leq 0.05$) between MIC values reported in each column

Table 2 Antifungal activity of methanol extracts of *H. perforatum* root cultures elicited with COS at different concentrations and collected at day 25 post-elicitation against *Candida albicans*, *Trichophyton mentagrophytes*, and *Microsporum gypseum*

Extracts	*C. albicans* ATCC 10231		*T. mentagrophtyes* DSM 4870		*M. gypseum* DSM 3824	
	MIC$_{50}$ (µg mL^{-1})	MIC$_{100}$ (µg mL^{-1})	MIC$_{50}$ (µg mL^{-1})	MIC$_{100}$ (µg mL^{-1})	MIC$_{50}$ (µg mL^{-1})	MIC$_{100}$ (µg mL^{-1})
Control	256	512	128	512	128	256
EL (50 mg L^{-1})	256 (a)	512 (a)	128 (a)	256 (*a)	128 (a)	256 (a)
EL (100 mg L^{-1})	64 (*b)	256 (*b)	64 (*b)	256 (*a)	64 (*b)	128 (*b)
EL (200 mg L^{-1})	32 (*c)	128 (*c)	64 (*b)	128 (*b)	64 (*b)	128 (*b)
EL (400 mg L^{-1})	32 (*c)	128 (*c)	64 (*b)	64 (*c)	32 (*b)	64 (*b)
Fluconazole	2 (*d)	64 (*d)	16 (*c)	32 (*d)	16 (*c)	32 (*c)

MIC$_{50}$ and MIC$_{100}$ are the lowest concentration of extracts or reference drugs that caused growth inhibition ≥ 50% and 100%, respectively. Results are expressed as median of three experiments performed in duplicate. Asterisks represent significant differences ($p \leq 0.05$) between MIC values obtained with extracts from COS-elicited roots (EL) and from non-treated roots (control). Different letters represent significant differences ($p \leq 0.05$) between MIC values reported in each column

an estimate and do not reflect the actual value of volatile compounds in root samples, but are merely a parameter to compare the effect of different COS concentrations on the volatile profile of *H. perforatum* roots. Primarily, a PCA was applied to explore the volatile data set and to highlight the differences. The first two principal components (PC1 and PC2) explained 44.1% of total variability among the samples and showed a separation between root samples (Fig. S2). PC1 separated 200 mg L^{-1} COS-treated roots from 400 mg L^{-1} COS-treated and control roots due to their characteristic volatile profile. Interestingly, roots treated with 400 mg L^{-1} of COS and control roots were not distinguished by PC1.

Next, PLS-DA was applied to minimize the possible contribution of intergroup variability and to improve the separation between the samples. The PLS-DA score plot ($R2Y = 0.89$, $Q2 = 0.80$) showed a clear differentiation between control, the roots treated with COS 200 and 400 mg L^{-1} (Fig. 9).

According to the results obtained by PLS-DA, 15 volatile organic compounds were statistically significant as showed by VIP values (VIP > 1.0) in Table 3. Samples treated with COS 200 mg L^{-1} presented a characteristic volatile profile containing sesquiterpenes such as seychellene, cis-β-farnesene and (+)-α-chamigrene that were not identified in CTRL and COS 400 mg L^{-1} treated roots. Estimations of VOCs content in the roots indicated that only two sesquiterpenes (+)-δ-cadinene and (+)-epi-bicyclo-sesquiphellandrene increased after elicitation with the increasing COS concentration.

Interestingly, the majority of VOCs decreased or increased after elicitation with COS 200 mg L^{-1} and then returned to similar levels as CTRL roots. In particular, α-copaene, α-patchoulene, and cadina-3,5-diene increased after COS 200 mg L^{-1} and decreased after COS 400 mg L^{-1} treatment, while myrtenal, myrtanol, and limonene decreased after COS 200 mg L^{-1} and increased after COS 400 mg L^{-1} treatment. (−)-Zingiberene and
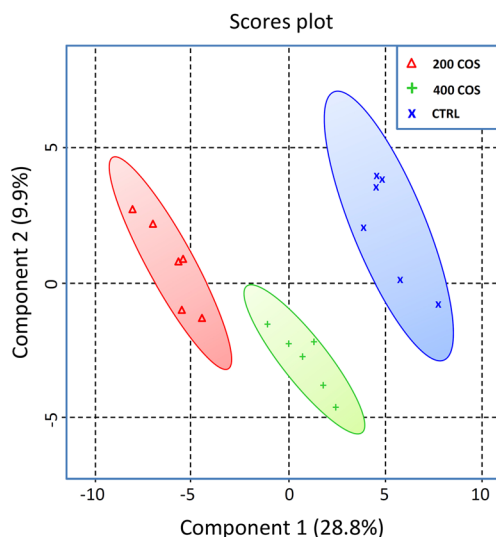
Fig. 9 PLS-DA score plot of the *H. perforatum* roots analyzed by SPME–GC–MS methodology. Control samples (CTRL) are represented in blue; COS-treated samples are represented in red (200 mg L^{-1}) and in green (400 mg L^{-1}). (Color figure online)

1,2,4-trimethylbenzene were not detected after COS 200 mg L^{-1} treatment, but a decrease of them was observed after COS 400 mg L^{-1} compared to CTRL roots. Conversely, naphthalene and α-terpineol were reduced after elicitation with COS 200 mg L^{-1}, but were not detected after elicitation with COS 400 mg L^{-1}.

Discussion

Several studies have demonstrated the elicitor effect of CHIT on plant cells (Vasconsuelo et al. 2003; Fan et al. 2010) and organ cultures (Putalun et al. 2007; Sivanandhan et al. 2012). A limitation to the use of CHIT in in vitro cultures, as well as in field and in post-harvest, is the poor solubility in neutral water and organic solvents, which makes it necessary to use acidified water for solubilization. In most of the elicitation studies, CHIT is dissolved in acetic acid–water solutions. However, we recently demonstrated that mono-carboxylic acids could affect the xanthone profile of *H. perforatum* in vitro-cultured roots and this makes it difficult to discriminate the effect of CHIT to that of solvent (Valletta et al. 2016). For this reason, we evaluated the effect of water-soluble derivatives of CHIT, named chitooligosaccharides (COS), obtained through enzymatic digestion of CHIT in this study. At present, only few studies are available on the impact of COS on secondary metabolite production in plant cell cultures (Linden and Phisalaphong 2000), and to the best of our knowledge, no studies have been performed on root cultures.

In this context, an issue we addressed in the present study was whether COS have an elicitor power comparable to that of CHIT on xanthone biosynthesis in *H. perforatum* root cultures. This is not a trivial query, since it is well known that the effect of CHITs on plant cells is strongly affected by their structural properties, e.g., molecular weight and degree of acetylation (Iriti and Faoro 2009 and literature therein reported). In a previous study we tested different CHITs on *H. perforatum* root cultures and we found significant

Table 3 Volatile organic compounds statistically significant after COS treatment

Compounds	Control	200 mg L^{-1} COS	400 mg L^{-1} COS	VIP
Seychellene	–	18.29 ± 7.31	–	2.01
Naphthalene	3.16 ± 2.26	3.13 ± 1.94	–	1.99
(−)-Zingiberene	2.42 ± 0.62	–	1.53 ± 0.27	1.87
α-Terpineol	2.21 ± 1.69	0.42 ± 0.06	–	1.73
Cis-β-farnesene	–	2.87 ± 1.12	–	1.67
α-Copaene	8.70 ± 0.05	11.59 ± 3.65	9.37 ± 2.98	1.64
1,2,4-Trimethylbenzene	0.33 ± 0.19	–	0.21 ± 0.07	1.52
(+)-α-Chamigrene	–	0.45 ± 0.30	–	1.25
Myrtenal	4.31 ± 3.92	0.66 ± 0.21	0.97 ± 0.36	1.20
Limonene	0.46 ± 0.25	0.15 ± 0.02	0.38 ± 0.11	1.19
α-Patchoulene	2.77 ± 0.32	3.87 ± 1.12	2.11 ± 0.78	1.19
(+)-δ-Cadinene	0.54 ± 0.03	1.30 ± 0.51	2.06 ± 0.66	1.17
Myrtanol	6.64 ± 4.38	1.29 ± 0.26	4.64 ± 1.79	1.05
(+)-Epi-bicyclo-sesquiphellandrene	1.03 ± 0.09	1.51 ± 0.53	2.10 ± 2.22	1.00
Cadina-3,5-diene	1.29 ± 0.16	1.79 ± 0.59	1.34 ± 0.32	1.00

All the values are the mean of three independent analyses ± SD

VIP variable importance in the projection

differences in the elicitor power related to different molecular structures (Tocci et al. 2013a). To compare the effect of COS with those of CHIT, the first elicitation experiment (Fig. 1a) was conducted by treating the roots with the same concentration of COS that was used in previous studies on CHIT elicitation (Tocci et al. 2011, 2013a; Brasili et al. 2014, 2016). By recording the xanthone content over time, two accumulation peaks were detected (Fig. 3), the first one at an early stage and the second one at a later stage, i.e., on days 5 and 25 post-elicitation. Brasili et al. (2014, 2016) investigated the combined effect of CHIT and overcrowding stress on both primary and secondary metabolism in *H. perforatum* root cultures and through an NMR-based metabolomics approach and ANOVA simultaneous component analysis (ASCA), they demonstrated that early responses are mainly caused by the elicitor, while the late responses are generated by the combined effect of the elicitor and the overcrowding stress due to the high root biomass growth in a confined environment. The impact of overcrowding stress clearly emerges by the observation of control roots, the xanthonic content of which progressively increases with culture duration (Fig. 3). The early response leads to neosynthesis of almost all the analyzed xanthones, while in the xanthone profile corresponding to the late response, the dominant compound is paxanthone. In future applications of *H. perforatum* root cultures for the biotechnological production of xanthones, the choice of collecting the roots at a short- or long-time post-elicitation will have to be made on the basis of the molecules of interest.

In the second experiment (Fig. 1b), the effect of different COS concentrations on the xanthone content was investigated. The best results in terms of the total xanthone content were obtained with the highest COS concentration tested in this study (400 mg L^{-1}). This concentration is much higher than those used in most CHIT elicitation experiments, both on cell (Wiktorowska et al. 2010; Chakraborty et al. 2009; Ferri et al. 2009) and root cultures (Udomsuk et al. 2011; Sivanandhan et al. 2012; Shinde et al. 2009; Putalun et al. 2007). With 400 mg L^{-1} COS, the xanthone content reached very high levels (over 30 mg g^{-1} DW), which has never been obtained with CHIT in the previous studies (Tocci et al. 2011, 2012, 2013a; Brasili et al. 2014; Simonetti et al. 2016). It should be emphasized that FDA test showed that these COS concentrations, while causing visible symptoms of suffering to roots (Fig. 8), do not cause death, as opposed to CHIT, which even at lower concentrations cause PCD or necrosis in plant cells (Zuppini et al. 2004; Iriti et al. 2006) and in *H. perforatum* cultured roots (personal observation). Cytotoxicity of CHIT represents a limitation for biotechnological purposes, due to the dramatic morpho-anatomical alterations caused by this elicitor (Brasili et al. 2016) which make in vitro-cultured cells and organs non-reusable for subsequent production cycles. From the analysis of the

individual xanthones, it appears that the administration of 400 mg L^{-1} COS stimulates the biosynthesis of all the analyzed xanthones, except mangiferin.

The effect of COS on *H. perforatum* cultured roots has been also investigated in terms of volatile organic compound (VOC) biosynthesis. In a previous study, we observed a remarkable increase in dimethylallyl-pyrophosphate (DMAPP) levels in *H. perforatum* CHIT-treated roots (Brasili et al. 2014). Since the ^{1}H-NMR analysis failed to reveal the presence of terpenoids, we performed both histochemical and headspace VOCs analysis of *H. perforatum* roots in this study, with the aim to investigate the presence of isoprenoids in root biomass after COS elicitation.

First, the histochemical analysis with Nadi reagent suggested the presence of isoprenoid compounds localized in secretory ducts of both control and treated roots. The presence of essential oils in secretory ducts was previously demonstrated only in the aerial parts of *H. perforatum*, including sepals, petals, stamens, leaf, and stem, but never in roots (Ciccarelli et al. 2001). Second, the GC–MS headspace analysis confirmed the presence of a wide spectrum of isoprenoids in cultured roots. The lack of isoprenoids in the extracts previously analyzed by ^{1}H-NMR (Brasili et al. 2014) was probably due to their volatilization during the Bligh–Dyer extraction, as the identified isoprenoids (mono- and sesqui-terpenes) have a low molecular weight corresponding to a high volatility.

It is well-documented that roots are able to synthesize and release VOCs in the rhizosphere, where act as key mediators in belowground biotic interactions (Delory et al. 2016). In this scenario, VOCs can have negative (phytotoxins, auto-inhibition, and development of associations with parasitic plants) or positive effects (resistance to herbivores and root detection) on neighbouring plants, but also can affect plant growth directly (phytotoxin biosynthesis) or indirectly (alteration of soil chemistry, microbial populations, and nutrient availability) (Weston et al. 2012; Zeng 2014). The majority of the studies published so far focus on root VOC biosynthesis by three major plant models as *Zea mays, Citrus* spp., and *Brassica* spp. in response to nematodes and parasites (Delory et al. 2016). To our knowledge, it is the first study that deals with the biosynthesis of VOCs by *H. perforatum* roots in response to COS elicitation. The obtained results suggest that VOCs play a key role in mediating the interactions between *H. perforatum* root and soil organisms, especially fungi, since their biosynthesis resulted affected by COS, which are fungal elicitors.

As reported in our previous studies, xanthone-rich crude extracts obtained from *H. perforatum* in vitro-cultured roots elicited with CHIT exhibit a high antifungal activity (Tocci et al. 2011, 2012, 2013; Simonetti et al. 2016). In the present study, we observed that elicitation with 200 mg L^{-1} COS leads to a total xanthone content comparable to that obtained

using CHIT at the same concentration; however, a higher antifungal activity was observed compared to the previous results. These results suggest that the extracts contain other metabolites with antifungal activity induced by COS, which may act additively or synergistically with xanthones. An antifungal activity of certain VOCs such as monoterpenes, monoterpenes hydrocarbons, sesquiterpenes, and diterpenes was previously demonstrated in other plant species (Badawy et al. 2017; Fraternale et al. 2016) and against other fungi such as *Fusarium verticillioides*, the major producer of mycotoxin in contaminated aliments (Dambolena et al. 2008).

Conclusions

For the first time in the present study, the elicitor effect of COS was tested on St. John's wort in vitro root cultures. The obtained results showed that COS are very effective elicitors, more powerful of CHIT in stimulating the biosynthesis of xanthones in *H. perforatum* root cultures. They also showed a lower phytotoxicity that allows its usage at high concentrations. In addition to enhancing xanthone biosynthesis, COS caused significant changes in the production of VOCs. The obtained results suggest that xanthones and VOCs are involved in regulating the relationships between root and edaphic microorganisms, especially fungi.

Author contribution statement AV conceived and designed experiments, analyzed data, and prepared figures and tables; AV, GDA, and CB performed experiments and the HPLC analysis of xanthones; EB, ECT, and EP carried out GC–MS headspace analysis; HY synthesized COS; AV, CB, GDA, EB, GS, and GP wrote the manuscript; GP coordinated and guided intellectually all process of the work; GP and GS made a critical revision of the manuscript. All the authors read and approved the manuscript.

Compliance with ethical standards

Conflict of interest The authors have declared that no competing interests exist.

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