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Chitosan Oligosaccharides Promote the Content of Polyphenols in Greek Oregano (*Origanum vulgare* ssp. *hirtum*)

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ABSTRACT: Greek oregano is commonly used as a spice and in traditional medicine in Eurasia. The plant is rich in secondary metabolites, such as volatile organic compounds (VOC) and polyphenols. Chitosan oligosaccharides (COS) are used as a plant elicitor. The objectives of this study were to determine the effects of COS on the growth and content of secondary metabolites in Greek oregano. Four COS treatments (50, 200, 500, and 1000 ppm) were used in a field experiment. The 200 and 500 ppm COS treatments promoted plant height growth, whereas 50 and 200 ppm COS upregulated the content of polyphenols significantly (38 and 29%, respectively). The COS treatments induced H_2O_2 generation in Greek oregano leaves; thus, the effect of H_2O_2 treatment was studied to investigate the possible role of H_2O_2 in growth and polyphenol production. A low concentration of H_2O_2 also promoted plant height growth, but only tendencies to higher polyphenol content were seen.

KEYWORDS: *Chitosan oligosaccharides (COS), hydrogen peroxide, secondary metabolites, volatile organic compounds (VOC), phenolic acids, flavonoids, plant development*

INTRODUCTION

Greek oregano (*Origanum vulgare* L. ssp. *hirtum*, Lamiaceae) is a perennial herb distributed in Eurasia and north Africa. It is widely used as a flavoring in meat products, salads, soups, and sauces but is also commonly used in traditional medicine to treat various diseases, such as cold, cough, and digestive disorders.[1] Greek oregano is known for its powerful antimicrobial and antioxidant activity, which may to some extent explain its use in traditional medicine.

The culinary function of Greek oregano is due to its relatively high concentration of essential oils, which normally consist of the monoterpenes carvacrol and thymol, accompanied by their biosynthetic precursor's *p*-cymene and *γ*-terpinene as the primary components. These compounds are responsible for the aroma and flavor of this herb but also significantly contribute to its antimicrobial activity.[2,3] Although carvacrol and thymol have oxidant-scavenging properties,[4,5] the antioxidant activity of Greek oregano is mainly due to flavonoids and phenolic acids, which are also abundantly present in this herb.[2,6] Rosmarinic acid and derivatives of lithospermic acid are the dominant phenolic acids in Greek oregano, whereas apigenin and luteolin derivatives constitute the major group of flavonoids in this plant (Figure 1).[2,6,7] Dietary phenolic antioxidants, such as phenolic acids and flavonoids, play important roles in delaying the development of chronic diseases, such as cardiovascular diseases and inflammatory bowel syndrome, and, hence, are believed to be important contributors to the health effects of Greek oregano as well as in other plant species where they occur.[8,9] Enhancement of the content of essential oil components and polyphenols in Greek oregano may therefore improve the health effects of this herb as well as its culinary function.

The composition and content of secondary metabolites is very variable in different *Origanum* species and within the same *Origanum* species; for example, in Greek oregano collected from different localities in Turkey, the content of essential oils varied from 3.1 to 6.1% in dry plant material.[3] Furthermore, geographic and climatic factors, such as duration of daylight and temperature, as well as cultivation factors, such as water stress and harvesting time, all affect the content of secondary metabolites in plants, including *Origanum* species.[2,10−14] For example, it has been shown that the development stage has a significant impact on the content and composition of both volatile terpenes and polyphenols in Greek oregano and that an optimal harvest time of aerial parts of the plant depends upon the secondary metabolites of interest.[2] Numerous studies have focused on optimizing the cultivation phase in an attempt to promote the production of secondary metabolites in oregano and other plant species. The primary methods employed to manipulate the production of secondary metabolites in *Origanum* species include the application of nitrogen fertilizers[11,12] and altering irrigation strategies.[13,14] Although several reports are available that focused on upregulating the level of secondary metabolites in *Origanum* species using purely agronomic approaches as described above, there are limited studies available on the effect of plant elicitors on the accumulation of secondary metabolites in these plants.

Initially, plant elicitors are compounds, which can induce plant defense reactions to pathogens and may result in the

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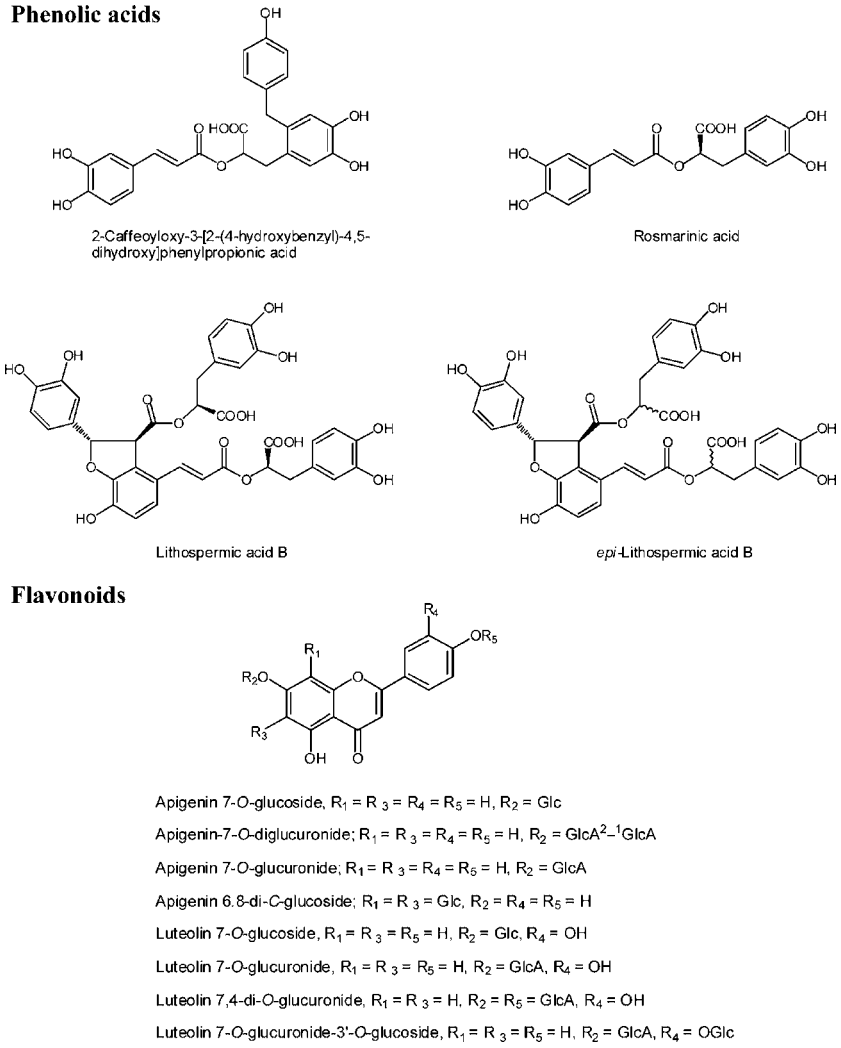

Figure 1. Chemical structures of flavonoids and phenolic acids identified in aerial parts of *O. vulgare* ssp. *hirtum* in the present investigation. Glc, glucose; GlcA, glucuronic acid.

production of defense-related secondary metabolites, such as phytoanticipins and/or phytoalexins.[15] Currently, elicitors are extensively used to enhance the yield of secondary metabolites in medicinal plants.[16] The most widely used elicitors are fungal carbohydrates, yeast extracts, and chitosan.[17] Chitosan is frequently used in cell cultures of medicinal plants as well as plant organs for inducing the accumulation of bioactive secondary metabolites.[18] For example, it has been demonstrated that the total amounts of phenolics and terpenoids in sweet basil (*Ocimum basilicum* L.) increased after chitosan treatment.[19] Chitosan oligosaccharides (COS), obtained through the decomposition of chitosan, retains the function of chitosan but has improved aqueous solubility, and therefore, COS is considered to be an improved and more convenient substitute of chitosan.[20] Hydrogen peroxide (H_2O_2) is known to be a significant secondary signal messenger, which plays an important role in many plant activities, including plant growth and secondary metabolite production.[21,22]

The hypothesis of this study is that COS may be used as an additional method to the purely agronomic approaches to enhance the content of bioactive secondary metabolites in Greek oregano. In this study, we used COS in water in different concentrations to treat Greek oregano plants and to study the effect of COS on plant growth, yield, secondary metabolite concentration, and composition in aerial parts. In addition, we studied the effect of COS treatment on H_2O_2 production in plant epidermis cells to determine a possible correlation between H_2O_2 and the production of secondary metabolites in Greek oregano.

MATERIALS AND METHODS

Plant Material. *O. vulgare* ssp. *hirtum* seeds were purchased from the seed company Pharmasaat (Arten, Germany). In 2009, transplants were produced in a glasshouse in soil plugs (5 × 4 cm), and in May 2009, transplants were established in the field (Sandy loam soil at Aarslev, Denmark; coordinates: 55.3° N, 10.45° E) in a bed system 1.5 m wide with three rows, with row distance of 0.5 m and in-row plant spacing of 0.25 m. The spacing between individual beds was 0.5 m, and this area was held as bare soil. The oregano field was cut to 5 cm above the ground prior to winter in 2009, and the elicitor treatments were performed on fresh regrowth in July 2010.

The individual plots for elicitor treatment consisted of 2 m long single rows. The statistical design of the field experiment was in a randomized complete block design with four replicates for each treatment. Three plants for height growth measurements were randomly chosen and marked in all treated and control plots.

Reagents and Standards. COS powder (degree of polymerization = 2−10; degree of deacetylation > 95%) was obtained from Dalian GlycoBio Co., Ltd. (Dalian, China). 4-Methyl-1-pentanol, 5-*O*-caffeoylquinic acid, eriodictyol, luteolin, hydrogen peroxide (H_2O_2),

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Table 1. Characteristic Ions of Identified Phenolic Acids and Flavonoids in Extracts of Aerial Parts of *O. vulgare* ssp. *hirtum* as Determined by LC−MS (APCI; Negative Mode; Capillary Voltage of 200 V) and Their UV Spectra as Determined by PDA

R_t (min)[a]	compounds	PDA, UV spectrum λ_{max} (nm)	LC−MS APCI m/z (% base peak)
		Phenolic Acids	
12.3	2-caffeoyloxy-3-[2-(4-hydroxybenzyl)-4,5-dihydroxy] phenylpropionic acid[b]	298sh,[c] 323	465 $[M - H]^-$ (15), 303 (10), 179 (100)
24.8	rosmarinic acid[d]	294sh, 329	359 $[M - H]^-$ (11), 197 (58), 179 (21), 161 (100)
26.1	*epi*-lithospermic acid B[b]	254, 286, 308sh, 330sh	717 $[M - H]^-$ (79), 539 (30), 519 (100), 339 (52)
29.1	lithospermic acid B[d]	254, 286, 308sh, 330sh	717 $[M - H]^-$ (65), 537 (14), 519 (100), 339 (46), 267 (12)
		Flavonoids	
16.1	apigenin 6,8-di-*C*-glucoside (vicenin-2)[d]	271, 335	593 $[M - H]^-$ (100), 575 (5), 503 (16), 473 (28)
18.7	luteolin 7,4′-di-*O*-glucuronide[b]	257, 264sh, 346	637 $[M - H]^-$ (59), 461 (15), 351 (7), 285 (100)
19.7	luteolin 7-*O*-glucuronide-3′-*O*-glucoside[b]	258, 265sh, 287sh, 347	623 $[M - H]^-$ (24), 609 (100), 447 (2), 285 (47)
21.3	apigenin 7-*O*-diglucuronide[d]	267, 336	621 $[M - H]^-$ (18), 431 (100), 269 (12)
22.1	luteolin 7-*O*-glucuronide[d]	255, 265sh, 347	461 $[M - H]^-$ (65), 285 (100)
22.5	luteolin 7-*O*-glucoside[d]	254, 265sh, 347	447 $[M - H]^-$ (100), 285 (6)
23.2	apigenin 7-*O*-glucoside[d]	266, 337	431 $[M - H]^-$ (100), 269 (4)
24.6	apigenin 7-*O*-glucuronide[b]	266, 336	445 $[M - H]^-$ (34), 269 (100)

[a] R_t = retention time on HPLC. [b] Identification based on R_t, UV, and LC−MS data compared to data from the literature.[34,35] [c] sh = shoulder. [d] Identified by a comparison to an authentic standard (see the Materials and Methods).

fluorescent probe 2′,7′-dichloroluorescin diacetate (H2DCF-DA), high-performance liquid chromatography (HPLC)-grade methanol (MeOH), dichloromethane (DCM), and H_2O_2 (30%) were purchased from Sigma-Aldrich (Steinheim, Germany). Standards of volatile organic compounds (VOC) were purchased from Sigma-Aldrich or TCI Tokyo Organic Chemicals (Tokyo, Japan). Authentic samples of apigenin 7-*O*-glucoside and luteolin 7-*O*-glucoside were purchased from Extrasynthese (Genay, France). Rosmarinic acid, lithospermic acid B, apigenin 6,8-di-*C*-glucoside (vicenin-2), and apigenin 7-*O*-diglucuronide were isolated from a MeOH extract of Greek oregano and identified by nuclear magnetic resonance (NMR) spectroscopy and liquid chromatography−photodiode array−mass spectrometry (LC−PDA−MS) as described previously.[2]

Elicitor and Reagent Treatments. Plants were treated with elicitors 2 weeks prior to the anticipated flowering time (mid-July 2010) with 50, 200, 500, and 1000 ppm COS or 1 and 10 mM H_2O_2 water solution sprayed on until runoff. Neighboring plants were screened by a 1.0 × 0.5 × 1.0 m aluminum box during treatment. The aluminum box was washed with pure water before treatment with a new elicitor. Control plants were treated with the same pure water as used for reagents. For secondary metabolite analysis, plants were harvested 1 week after treatment. Another set of plants were subjected to COS treatments (in the same concentrations as above) 3 weeks prior to flowering to investigate the effect of the COS treatments on plant height and growth over time.

Plant Growth Determination and Sample Harvest for Chemical Analysis. Plant growth based on plant height was determined as an effect of elicitor treatment at 1 and 2 weeks prior to harvest time. The height of three plants in every plot was measured before treatment (H) and 1 week after treatment (H_t). The growth rate was calculated [growth rate = $(H_t - H)/H \times 100\%$]. At 1 week after the last treatment, all plant material in a plot was harvested. The aerial parts were cut off 5 cm above ground, and the yield of fresh plant material was recorded by weighing directly after harvest. At each harvest time, a sample was collected per plot (about 100 g), weighed, and packed in an airtight plastic bag for subsequent DM analysis. A second sample (about 200 g) was collected per plot and placed in an airtight box for analysis of secondary metabolites. All samples were quickly frozen and stored at −20 °C until analysis.

Extraction of Secondary Metabolites. Secondary metabolites were extracted from fresh frozen plant material. The plant material (60−70 g) was finely cut in a blender, and a 5 g sub-sample was thereafter immediately homogenized in a glass bottle (Corning, 100 mL) using an Ultra Turrax blender (model T18; IKA Labortechnik, Buch and Halm, Denmark) for 1 min together with 40 mL of DCM (for extraction of VOC) or 40 mL of 80% aqueous MeOH (for extraction of polyphenols) and then extracted for 16 h under constant stirring at room temperature in the dark. The extracts were then filtered into a 100 mL volumetric flask, and the plant material in the filters was washed several times with DCM or 80% aqueous MeOH. The filtered extracts were adjusted to a final volume of 100 mL. The DCM and the 80% aqueous MeOH extracts were filtered (nylon 0.45 μm, Cameo 25P syringe filter) into a gas chromatography (GC) and HPLC vial, respectively, before analysis. The extraction procedures described above ensured almost complete extraction (>95%) of VOC and polyphenols, as shown by sequential extraction experiments on the same plant material (data not shown). All extractions were made in three replicates (i.e., 3 × 5 g for VOC and 3 × 5 g for polyphenols).

Analysis of VOC. A Thermo Scientific DSQ II ion-trap mass spectrometer, coupled to a Trace GC Ultra gas chromatograph (Thermo Fisher Scientific, Waltham, MA), was used for identification of VOC in DCM extracts as described previously.[2] Compounds suggested by the National Institute of Standards and Technology (NIST) MS database were verified by comparison of GC retention indices (RI) and mass spectra of authentic reference compounds. For quantification of VOC, a Hewlett-Packard 6890 Series Plus gas chromatograph (Hewlett-Packard, Avondale, PA) with a flame ionization detector (FID) was used. Individual VOC were quantified from the FID peak areas relative to that of the internal standard (4-methyl-1-pentanol) as described previously.[2]

Analysis of Polyphenols. Identification of polyphenols in 80% aqueous MeOH extracts was based on LC−MS, NMR, and ultraviolet (UV) data, as described in Table 1. A Thermo Scientific Accela HPLC−PDA station hyphenated with a Thermo Scientific LTQ XL ion-trap mass spectrometer (Thermo Fisher Scientific, Waltham, MA) fitted with atmospheric pressure chemical ionization (APCI) was used for LC−MS analysis according to the method described by Grevsen et al.,[2] modified with the following acquisition parameters: vaporizer and capillary temperatures of 450 and 275 °C, respectively, sheath, auxiliary, and sweep gas flow rates of 50, 5, and 5 (arbitrary unit), respectively, discharge current of 5 μA, and capillary and tube lens voltages of −23 and −110 V, respectively. Polyphenols were quantified on an Agilent HPLC 1200 Series system equipped with a PDA detector (G 1315D). Polyphenols were separated by reverse-phase (RP)-HPLC and monitored at 280 and 360 nm.[2] The concentrations of polyphenols were determined by external calibration curves of 5-*O*-caffeoylquinic acid (phenolic acids) and luteolin (flavones) as described previously.[2]

Determination of H_2O_2 Production. The H_2O_2 assay consisted of a H_2O_2-specific fluorescent probe H2DCF-DA as reported previously,[23] with slight modifications. The epidermis was carefully peeled from the abaxial leaf surface of Greek oregano plants and cut into 5 × 15 mm long bands. The bands were incubated in Tris/KCl buffer for 2 h in light and hereafter placed into Tris/KCl buffer containing H2DCF-DA (50 μmol/L) for 30 min at room temperature in the dark. Excess fluorescent probe was removed by 3 rinses with fresh Tris/KCl buffer, and the epidermal bands were then placed in Tris/KCl buffer containing COS (50, 200, 500, and 1000 ppm) for 5 min. The bands were investigated by fluorescence microscopy (Nikon AZ100) at an exciting wavelength of 430−485 nm and an emitting wavelength of 515 nm for a period of more than 2 h. The fluorescent strength was measured with the software program ImageJ (version 1.37; http://rsb.info.nih.gov/ij/). Experiments were repeated at least 3 times for each treatment.

Statistics. Analysis of variance (ANOVA) was performed on each variable using the Statistical Analysis System (SAS Institute, Inc., Cary, NC). The variations [standard errors (SE)], the significances of treatment effects, and interactions (F tests) were calculated and tested using the ANOVA procedure. If the F tests showed significant treatment effects, least significant difference (LSD) values ($p = 0.05$) are used to separate means in tables of treatment effects.

RESULTS

Effect of COS on Growth and Yields. Plant height, fresh weight (FW) yield, and dry matter (DM) yield were determined to evaluate the effect of COS on Greek oregano plant growth. The plant height evaluation experiments were conducted over 2 weeks to validate the effect of COS on different growth periods. After COS treatment in the first week, the growth rates of oregano based on plant height were 11.63, 12.08, 14.08, and 9.73% at 50, 200, 500, and 1000 ppm COS, respectively, whereas in the second week, the growth rates were 3.75, 4.95, 5.55, and 4.15%, respectively, after the COS treatment (Figure 2). These data are all higher than the control plants (water sprayed) with growth rates based on plant height of 9.68 and 2.78%, respectively, in the two growth periods. Growth rates at 200 ppm COS (week 1) and 500 ppm COS (weeks 1 and 2) treated plants showed significant differences ($p < 0.05$) compared to the control plants. These results suggested that 500 ppm is the most effective concentration for COS to induce the promotion of height growth in Greek oregano. However, there was no significant effect on the FW and DW of plants treated with COS (data not shown).

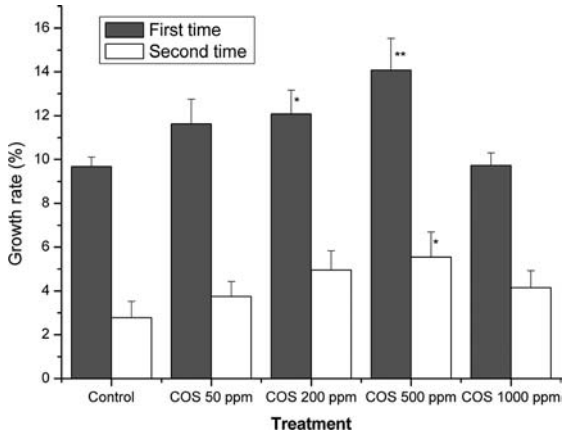

Figure 2. Height growth rate of Greek oregano plants treated with different concentrations of COS. The gray bars indicate the rapid growth period (from 3 to 2 weeks prior to flowering), and the white bars indicate the slower growth period (from 2 to 1 week prior to flowering). Data show the average growth rates ($n = 3 \times 4$, i.e., 12 plants). (*) $p < 0.05$ and (**) $p < 0.001$ significant difference in relation to the control, and the error bars indicate the SE.

Effect of COS on the Content and Composition of VOC. A total of 16 terpenes that constituted >98% of the total content of VOC in the DCM extracts from aerial parts of *O. vulgare* ssp. *hirtum* in both control and treated plants were identified and quantified (Figure 3). The most abundant non-terpenoid compound identified and quantified was 1-octen-3-ol (around 0.3% of total VOC). The thymol content was low (around 0.3% of total VOC), indicating that the Greek oregano plant material used here was of the carvacrol chemotype. The three most abundant terpenes were the monoterpene carvacrol and its biosynthetic precursor's γ-terpinene and *p*-cymene, which together accounted for approximately 90% of the total VOC content. Sesquiterpenes, such as β-caryophyllene, accounted for less than 3% of the total VOC content. No significant differences were determined in the composition of terpenes between the control and the different treatments with COS. The yield of some of the major and/or most important terpenes from a bioactivity point of view, as well as the total VOC content in Greek oregano after different COS treatments, is listed in Table 2. All treatments apparently promoted the total content of terpenes and the concentration of carvacrol, γ-terpinene, and thymol but not enough to result in significant differences (Table 2 and Figure 3).

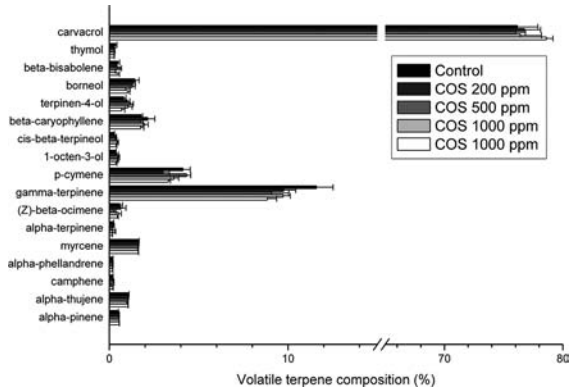

Figure 3. VOC identified and quantified in aerial parts of Greek oregano, as affected by the application of different concentrations of COS. Data are expressed in percent composition of total VOC and represent averages ($n = 4$), and bars represent SE.

Effect of COS on the Content and Composition of Polyphenols. Polyphenols in extracts of aerial parts of Greek oregano were monitored by RP-HPLC−PDA and LC−PDA−MS. The polyphenols were identified by a comparison of their mass spectral data and UV data to those of authentic reference compounds (Table 1) and accounted for >98% of the total polyphenol mass in the extracts. On the basis of their chemical structure, the polyphenols were divided into phenolic acids, including caffeic acid derivatives, rosmarinic acid, and lithospermic acids, as well as flavonoids, including luteolin derivatives and apigenin derivatives (Table 1 and Figure 1).

The contents of the major phenolic acids (rosmarinic acid and lithospermic acid B) and flavonoids, the total contents of flavonoids and phenolic acids, and the total polyphenol content 1 week after COS treatment are listed in Table 3. The total polyphenol content was significantly promoted by the COS

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Table 2. Concentrations of Individual and Total Terpene Content (mg/g of FW) in DCM Extracts of Aerial Parts of Greek Oregano Affected by the Application of Different Concentrations of COS[a]

	terpenes (mg/g of FW)						
treatment	carvacrol	γ-terpinene	p-cymene	β-caryophyllene	myrcene	thymol	total
50 ppm COS	7.73 ± 0.43 a	0.93 ± 0.13 a	0.44 ± 0.03 a	0.18 ± 0.01 a	0.16 ± 0.01 a	0.029 ± 0.001 a	10.16 ± 0.51 a
200 ppm COS	7.87 ± 1.20 a	1.00 ± 0.18 a	0.27 ± 0.02 b	0.21 ± 0.04 a	0.17 ± 0.03 a	0.027 ± 0.003 a	10.24 ± 1.53 a
500 ppm COS	7.35 ± 0.33 a	1.18 ± 0.09 a	0.40 ± 0.05 a	0.17 ± 0.01 a	0.16 ± 0.01 a	0.035 ± 0.008 a	9.68 ± 0.57 a
1000 ppm COS	8.20 ± 0.62 a	0.92 ± 0.08 a	0.34 ± 0.02 ab	0.18 ± 0.01 a	0.17 ± 0.01 a	0.028 ± 0.002 a	10.41 ± 0.74 a
control	7.03 ± 0.38 a	0.89 ± 0.06 a	0.33 ± 0.03 ab	0.18 ± 0.02 a	0.15 ± 0.01 a	0.025 ± 0.002 a	9.20 ± 0.47 a

[a]Data are presented as averages (n = 4), with ±SE. Different letters within a column (between treatments) indicate a significant difference at $p < 0.05$ with the LSD test.

Table 3. Polyphenol Concentrations (mg/g of FW) in 80% Aqueous MeOH Extracts of Aerial Parts of Greek Oregano Affected by Different Concentrations of COS[a]

	phenolic acids (mg/g of FW)			flavonoids (mg/g of FW)				
treatment	rosmarinic acid	lithospermic acid B	total phenolic acids	Ap-6,8-digluc	Lu-7,4′-diglu	Lu-7-glu	total flavonoids	total polyphenols (mg/g of FW)
50 ppm COS	3.8 ± 1.4 a	2.5 ± 0.4 a	6.7 ± 1.2 a	1.4 ± 0.7 ab	2.4 ± 0.3 a	3.4 ± 1.4 a	9.4 ± 1.1 a	16.1 ± 1.8 a
200 ppm COS	3.8 ± 1.2 a	1.5 ± 0.3 b	6.1 ± 1.2 a	1.5 ± 0.7 a	2.1 ± 0.8 a	3.1 ± 0.6 a	9.0 ± 0.9 ab	15.1 ± 2.0 ab
500 ppm COS	2.6 ± 0.8 a	2.2 ± 0.9 ab	5.1 ± 1.4 a	1.2 ± 0.1 abc	1.7 ± 0.9 a	3.1 ± 1.5 a	7.5 ± 1.1 bc	12.6 ± 2.0 bc
1000 ppm COS	2.8 ± 0.3 a	1.6 ± 0.5 b	4.9 ± 0.8 a	0.9 ± 0.2 bc	1.7 ± 0.1 a	2.5 ± 0.5 a	7.3 ± 0.6 c	12.2 ± 1.3 bc
control	2.9 ± 0.9 a	1.7 ± 0.4 b	4.9 ± 1.3 a	0.8 ± 0.1 c	2.0 ± 0.2 a	2.5 ± 0.2 a	6.8 ± 0.5 c	11.7 ± 1.6 c

[a]Data are presented as averages (n = 4) 1 week after treatment, with ±SE. Different letters within a column (between treatments) indicate a significant difference at $p < 0.05$ with the LSD test. Ap-6,8-digluc, apigenin 6,8-di-C-glucoside (vicenin-2); Lu-7,4′-diglu, luteolin 7,4′-di-O-glucuronide; Lu-7-glu, luteolin 7-O-glucuronide.

solutions lower than 200 ppm, as well as the total flavonoids and lithospermic acid B content. No significant effect as opposed to the control treatment (water) was seen following treatment with 500 and 1000 ppm COS solution. This means that, according to our data, the 50 ppm COS treatment (the lowest concentration) seems to be the most effective concentration to induce polyphenol accumulation in Greek oregano aerial parts.

COS Induced H_2O_2 Production in Epidermal Cells. Application of COS promoted the production of H_2O_2 in the epidermal cells of leaves of Greek oregano shortly after treatment. Results showed, on the basis of measurements of the fluorescence density, that the 50 ppm COS treatment induced the highest accumulation of H_2O_2 relative to control plants. The 50 ppm COS-induced H_2O_2 also lasted for a longer time period. The H_2O_2 generation started within 5 min after treatment, lasted for more than 30 min, and declined after 2 h (Figure 4). The 200 ppm COS-induced H_2O_2 showed a similar pattern but with a lower intensity of fluorescence. The 500 ppm COS induced H_2O_2, but it took a longer time period to generate fluorescence (30 min) relative to both the 50 and 200 ppm COS treatments. The 1000 ppm COS treatment induced H_2O_2 and generated a very rapid (10 min) and intense initial fluorescence, but nearly no fluorescence was visible in the cells after 30 min.

Effect of H_2O_2 on Growth and Production of Secondary Metabolites. As a consequence of the fact that COS apparently can induce H_2O_2 accumulation in leaves, we investigated the effect of H_2O_2 on Greek oregano height growth and secondary metabolite contents to study the function of H_2O_2 on Greek oregano (Table 4). Results showed that only the low concentration of H_2O_2 (1 mM) and not the high concentration of H_2O_2 (10 mM) promoted the height growth of Greek oregano. Both H_2O_2 treatments had no significant effect on the production of secondary metabolites (VOC and polyphenols), as compared to the control plants, although a trend for higher content could be seen for carvacrol, lithospermic acid B, and both the content of total VOC and total polyphenols (Table 4).

DISCUSSION

Biomass Yield. The plant biomass yield is an important factor related to the production of plant secondary metabolites. A recent study showed that nitrogen fertilization with 40 kg/ha significantly increased the number of stems, branches, and the DM yield of Greek oregano.[11] Oligosaccharides are also considered to be a broad-spectrum plant growth promoter,[24] where application of 60 ppm COS increased both the height (increased 33.5%) of coffee seedlings and the biomass yield (increased 9.2%).[25] Studies on oligosaccharides, such as COS, on the biomass yield and secondary metabolites of oregano have not previously been investigated. In the present study, treatments with COS solutions (200 and 500 ppm) significantly promoted the plant height growth rate in Greek oregano. However, there was no statistically significant effect on the fresh matter and DM yield of oregano aerial parts following treatment with COS in the field experiment. A controlled experiment under greenhouse conditions could be a good alternative to reduce the impact of biological variation on the results.

Accumulation of Secondary Metabolites by COS Treatments. COS and its polymer chitosan are considered to be potent elicitors of secondary metabolite accumulation in plants, but the majority of elicitation experiments reported in the literature were conducted on suspension cell culture. Only a few reports describe the effect of elicitor application on plants *in vivo*. Obara et al.[26] reported that a 10% COS treatment (i.e., about 100 times stronger than our highest concentration)

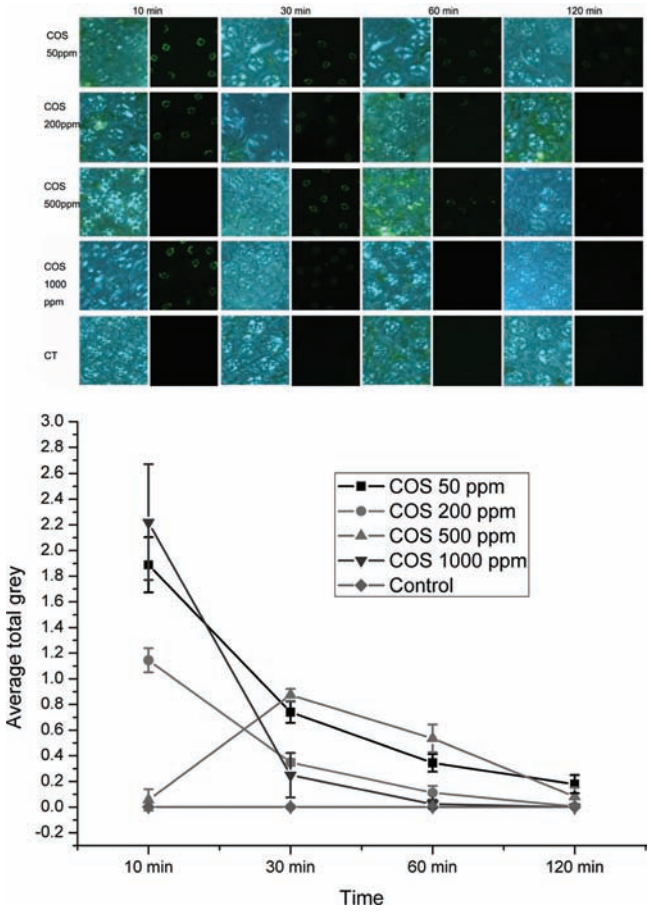

Figure 4. COS-induced production of H_2O_2 in oregano leaf epidermis. (Above) Time series of fluorescence from epidermal peel. This picture is representative of one of three replicates evaluated. (Below) Quantitative determination of COS-induced production of H_2O_2. Bars represent SE (n = 3).

increased the content of terpenoids in rice leaves.[26] In sweet basil, applications of chitosan solutions (0.01−1%) promoted the total amounts of phenolics and terpenoids, where the most effective concentration for the production of phenolics and terpenoids was 0.1 and 0.5%.[19] However, a 0.5% COS solution was reported to have no effect on the content of terpenoids in tomato.[18] These reports suggest that the ability of COS to induce secondary metabolites varies in different plant systems. The response may vary from plant to plant and depend upon many factors, such as the concentration of COS, growth stage, timing of elicitor, and contact time of elicitation.

In this study, 12 polyphenols (4 phenolic acids and 8 flavonoids) and 17 VOC were identified and quantified in Greek oregano MeOH and DCM extracts, respectively (Table 1 and Figure 3). The content of polyphenols and VOC corresponds well to the findings in earlier work with Greek oregano.[2] Following COS treatment, no marked effect on VOC, consisting primarily of mono- and sesquiterpenes, was seen (Table 2). However, the concentration of total flavonoids was upregulated by 50 and 200 ppm COS treatment, and the concentration of total polyphenols was upregulated by 50 and 200 ppm COS (Table 3). The total phenolic acids and flavonoids showed the same tendency, with a declining concentration with an increasing COS concentration, suggesting that there was some coherence between COS regulation of polyphenols. Some authors suggested that the upregulation of

Table 4. Height Growth Rate (%) and Secondary Metabolites (mg/g of FW) Production of Greek Oregano Plants Affected by Different Applications of H_2O_2 Concentrations[a]

		terpenes (mg/g of FW)					polyphenols (mg/g of FW)							
							phenolic acids			flavonoids				
treatment	height growth rate (%)	carvacrol	γ-terpinene	*p*-cymene	thymol	total terpenes	rosmarinic acid	lithospermic acid B	total	Ap-6,8-digluc	Lu-7-glu	Lut-7,4′-diglu	total	total polyphenols
1 mM H_2O_2	5.8 ± 1.1 a	8.6 ± 0.8 a	1.0 ± 0.08 a	0.3 ± 0.05 a	0.025 ± 0.005 a	11.0 ± 0.9 a	2.8 ± 0.5 a	2.3 ± 0.4 a	5.4 ± 0.9 a	1.2 ± 1.0 a	2.3 ± 1.0 a	2.0 ± 0.5 a	7.2 ± 1.4 a	12.6 ± 1.9 a
10 mM H_2O_2	2.5 ± 0.2 b	7.1 ± 0.3 ab	1.1 ± 0.09 a	0.32 ± 0.04 a	0.025 ± 0.001 a	9.5 ± 0.4 a	3.1 ± 0.2 a	1.8 ± 0.6 a	5.4 ± 0.5 a	1.1 ± 0.5 a	1.6 ± 0.5 a	2.6 ± 0.5 a	6.9 ± 0.7 a	12.3 ± 0.7 a
control	2.8 ± 0.8 b	7.0 ± 0.4 b	0.9 ± 0.06 a	0.33 ± 0.03 a	0.025 ± 0.002 a	9.2 ± 0.5 a	2.9 ± 0.9 a	1.7 ± 0.4 a	4.9 ± 1.3 a	0.8 ± 0.2 a	2.5 ± 0.2 a	2.0 ± 0.2 a	6.8 ± 0.5 a	11.7 ± 1.6 a

[a]Data are presented as averages (n = 4) 1 week after treatment, with ±SE. Different letters within a column (between treatments) indicate a significant difference at $p < 0.05$ with the LSD test. Ap-6,8-digluc, apigenin 6,8-di-*C*-glucoside (vicenin-2); Lu-7-glu, luteolin 7-*O*-glucuronide; Lu-7,4′-diglu, luteolin 7,4′-di-*O*-glucuronide.

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polyphenols may result from the effect of COS on the enzymes, which are involved in the biosynthesis of polyphenols, such as phenylalanine ammonia lyase (PAL) and chalcone synthase (CHS). For example, in *Vitis vinifera* cell suspensions, chitosan promoted the polyphenol levels and both the gene transcript and protein levels of some polyphenol-synthase-related enzymes (PAL and CHS).[27] In our study, the low concentrations of COS (50 and 200 ppm) were more effective in inducing polyphenols than the high concentrations (1000 ppm), and this is in accordance with former results that PAL enzyme activity was upregulated by 50 ppm COS in both oilseed rape and tobacco plants.[28]

In Greek oregano, we have only seen a positive effect of COS on polyphenols and not terpenes. The terpene content may be more difficult to enhance. For instance, following nitrogen application (40, 80, and 120 kg/ha), the number of stems and branches and the DM yield of Greek oregano were significantly increased, but there was no effect on the essential oil content.[11] In another experiment, foliar Ca^{2+} and Mg^{2+} treatments promoted plant height and DM yield and decreased the number of days required for oregano plants to flower. However, these treatments also did not affect the concentration of secondary metabolites in oregano.[29] Azizi et al.[30] reported that high nitrogen levels decreased the content of essential oils[30] but that water deficiency at flowering increased secondary metabolite content; however, this treatment reduced the production of DM. Therefore, in comparison to previous research in oregano plants, the COS treatments evaluated here seem to provide both a positive effect on plant growth rate and the concentration of some secondary metabolites. Application of COS should be investigated further in both a controlled growth environment to reduce the plant−plant variability and a combination with other cultivation treatments.

Function of H_2O_2. H_2O_2 is a significant secondary signal messenger, which plays an important role in many plant activities, including plant growth and secondary metabolite production.[21,22] In the present experiment in Greek oregano, COS induced H_2O_2 production in leaves shortly after application. This result is consistent with previous findings, where H_2O_2 production (generated in tobacco and oilseed rape epidermis) was rapidly upregulated following COS treatment.[31] There was an effect of the low concentrations (50 and 200 ppm) of COS on both H_2O_2 and the production of polyphenols. This effect declined at higher concentrations of COS (500 ppm). These results suggest a relationship between COS-induced H_2O_2 and accumulation of polyphenols. The high concentration (1000 ppm) COS treatment rapidly induced H_2O_2 accumulation, but there was no effect on the production of secondary metabolites. This may reflect a stress-induced upregulation of H_2O_2 at such high concentrations of COS, and plant cell death may have resulted.[32,33]

This study in Greek oregano showed that a low concentration of H_2O_2 has the potential to induce the accumulation of both terpenes and polyphenols and revealed a potential role of H_2O_2 in COS-regulated oregano secondary metabolite production. Furthermore, there was a significant growth promotion effect of low concentrations of H_2O_2 (1 mM) on oregano, which was consistent with the result that 200 and 500 ppm COS treatments activated weaker H_2O_2 accumulation but higher oregano growth. The treatment with different concentrations of COS induced distinct H_2O_2 generation patterns, and oregano responded differently to the different COS concentrations, suggesting that different COS concentrations had a differentiated function on the oregano plants and that H_2O_2 is likely to be involved in these processes. Our results may be important for the production of high-quality herbs and medicinal plants and certainly give rise to questions and motivation for new and exciting experiments with plant elicitors.

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ABBREVIATIONS USED

CHS, chalcone synthase; COS, chitosan oligosaccharides; DCM, dichloromethane; H_2DCF-DA, 2′,7′-dichloroluorescin diacetate; LC−PDA−MS, liquid chromatography−photodiode array−mass spectrometry; PAL, phenylalanine ammonia lyase; VOC, volatile organic compounds

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