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ORIGINAL ARTICLE

Effect of elicitors on xanthone accumulation and biomass production in hairy root cultures of *Gentiana dinarica*

Dijana Krstić-Milošević[1] · Teodora Janković[2] · Branka Uzelac[1] · Dragan Vinterhalter[1] · Branka Vinterhalter[1]

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Abstract The effect of abiotic (salicylic acid, jasmonic acid and methyl jasmonate) and biotic (chitosan and yeast extract) elicitors on the growth and xanthone accumulation in two hairy root clones of *Gentiana dinarica* Beck. was studied. The obtained results showed that clone 3 was more responsive to elicitor treatment than clone D. The production of dominant xanthone norswertianin-1-*O*-primeveroside was not significantly affected by either of the abiotic elicitor tested but was stimulated with chitosan treatment. The highest concentrations of all elicitors strongly increased the content of xanthone aglycone norswertianin, but simultaneously reducing the production of its glycoside norswertianin-1-*O*-primeveroside. The most efficient in enhancing norswertianin production was a 7-day treatment with salicylic acid (200 µM) and chitosan (50 mg l^{-1}), which yielded a 7.7- and a 24-fold increase in norswertianin content, respectively. In addition, treatment with biotic elicitors caused the occurrence of new xanthone compounds that were not detected in other samples. Free radical scavenging activity of xanthones was carried out by DPPH assay, and norswertianin showed the strongest activity.

Keywords Abiotic and biotic elicitation · DPPH radical scavenging · *Gentiana dinarica* · Norswertianin

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✉ Dijana Krstić-Milošević
dijana@ibiss.bg.ac.rs

[1] Institute for Biological Research "Siniša Stanković", University of Belgrade, Bulevar despota Stefana 142, Belgrade 11060, Serbia

[2] Institute for Medicinal Plant Research "Dr Josif Pančić", Tadeuša Košćuška 1, 11000 Belgrade, Serbia

Abbreviations

ASA	Ascorbic acid
CH	Chitosan
DPPH	1,1-Diphenyl-2-picrylhydrazyl
DW	Dry weight
JA	Jasmonic acid
MeJA	Methyl jasmonate
NOR	Norswertianin
NOR-1-*O*-PRIM	Norswertianin-1-*O*-primeveroside
SA	Salicylic acid
YE	Yeast extract

Introduction

Gentiana species have a long history of use as herbal bitter preparations in the treatment of digestive disorders, showing beneficial effects in gall and liver diseases (Wichtl 1994). The major active principles of *Gentiana* species are held to be bitter tasting secoiridoid glycosides, but the presence of flavone-*C*-glucosides and xanthones has been reported as well (Hostettmann-Kaldas et al. 1981). Xanthones have a wide range of biological and pharmacological properties with different health-promoting effects mainly based on their antioxidant activity (Pinto et al. 2005; Singh et al. 2012). These polyphenolic compounds play multiple roles in plant protection against stress and pathogen attack, attracting and repelling insects, and participate in plant development (Bhattacharya et al. 2010). In addition, it has been determined that numerous plant extracts and products regularly used as chemotherapeutic agents contain xanthones as active constituents (Negi et al. 2013).

Hairy root cultures obtained by infection of plants with *Agrobacterium rhizogenes* provide a rapid and sustainable way to increase the production of secondary metabolites

(Georgiev et al. 2007). One of the most efficient strategies to increase the productivity of bioactive metabolites is the use of different elicitors that trigger secondary metabolite biosynthesis by activation of the specific genes (Zhao et al. 2005; Baenas et al. 2014). Depending on their origin, elicitors can be classified as biotic or abiotic (Weathers et al. 2010). Biotic elicitors—chitosan, cellulose, alginate and others of biological origin often result from fungal, bacterial, viral or herbivore infections. Complex biological preparations like yeast extract and cell-wall components have also been used as biotic elicitors (Angelova et al. 2006). Abiotic elicitors are chemicals such as inorganic salts or metal ions, and physical factors such as UV irradiation, saline stress, osmotic stress, drought, etc. (Radman et al. 2003; Dornenburg and Knorr 1995). Plant growth regulators, such as salicylic acid and jasmonates, may also be considered as abiotic elicitors (Baenas et al. 2014).

In recent years, there have been a number of studies on enhanced production of secondary metabolites by addition of elicitors to the hairy root cultures (Zhou et al. 2011). Among medicinal plants, there are many reports for elicitor induced effects in *Hypericum* species (Conceição et al. 2006; Gadzovska et al. 2007; Franklin et al. 2008; Tocci et al. 2010, 2011; Coste et al. 2011). However, literature data regarding elicitation of *Gentiana* tissue cultures is very scarce. Zhao et al. (2013) have studied the effect of methyl jasmonate and salicylic acid on the accumulation of oleanolic acid in *Gentiana straminea*, but there are no reports on the influence of elicitors on the xanthone production, one of the most important secondary metabolites in *Gentiana* species.

We have recently reported *A. rhizogenes*-mediated genetic transformation of *Gentiana dinarica* and establishment of hairy root cultures (Vinterhalter et al. 2015). In this study, we investigated the effects of biotic elicitors chitosan and yeast extract, as well as abiotic elicitors (salicylic acid, jasmonic acid and methyl jasmonate) on the growth and xanthone accumulation in two hairy root clones of *G. dinarica*. Given that xanthones are an important group of plant defense antioxidants, we tested their scavenging activities against stable DPPH free radicals.

Materials and methods

Hairy root cultures

Hairy root cultures of *G. dinarica* were obtained after transformation with *A. rhizogenes* strains A4M70GUS and 15834/PI (Vinterhalter et al. 2015). According to the results obtained in that study, clones 3 (15834/PI) and D (A4M70GUS) were determined as superior, with the highest dry weight and total phenolics content. These two clones were selected for investigation of elicitor treatment. Hairy roots (400 mg fresh weight) were cultured in 100 ml Erlenmeyer flasks with 40 ml of basal liquid medium containing half-strength inorganic Murashige and Skoog (1962) salts and 2% sucrose (pH 5.8). Cultures were mounted on an orbital shaker (85 rpm) in a growth chamber maintained at 25 ± 2 °C and low 2 µmol m^{-2} s^{-1} irradiance under a 16-h photoperiod. Hairy roots were subcultured every 5 weeks.

Elicitor preparation and application

Jasmonic acid (JA, Duchefa), methyl jasmonate (MeJA, Duchefa) and salicylic acid (SA, Duchefa) 10 mM stock solutions were prepared in 50% (v/v) ethanol and then filter-sterilized using 0.22 µm filter. The stock solution of chitosan (20 mg ml^{-1}) was prepared by dissolving 500 mg of crab shell chitosan (CH, Sigma Chemical Company, USA) in 1 ml of glacial acetic acid by adding it drop wise at 60 °C in ultrasonic bath for 30 min. The final volume was made up to 250 ml by adding distilled water. The pH of the solution was adjusted to 5.8 with NaOH before autoclaving at 114 °C for 25 min. Yeast extract (YE, Torlak, Serbia) was added to the culture media prior to autoclaving.

After 28 days of cultivation in the basal medium, hairy roots were transferred to the medium supplemented with elicitors at the following final concentrations: JA (50, 100, 200 µM), MeJA (50, 100, 200 µM), SA (50, 100, 200 µM), CH (5, 10, 20, 50 mg l^{-1}), and YE (1, 2, 5 g l^{-1}). Basal medium (40 ml) without elicitors was added to the control hairy roots. In the treatments with SA, JA and MeJA control hairy roots were grown in the basal medium which contained 1 ml of ethanol. Hairy roots were harvested after treatment with elicitors at day 3 and day 7. Growth index [(final fresh weight − initial fresh weight)/initial fresh weight], dry weight of roots, and accumulation of xanthones were determined for the harvested roots.

Xanthone extraction and HPLC conditions

After elicitor treatment, hairy roots were air-dried, ground and extracted as described previously (Vinterhalter et al. 2015). Briefly, ground dried samples (500 mg) were extracted with 10 ml of methanol in ultrasonic bath for 20 min. After sonication, extraction was continued by maceration for 48 h in the dark at room temperature. The extracts were filtered into 10 ml volumetric flask and the volume was adjusted with methanol. Prior to HPLC analysis, hairy root extracts were filtered through a 0.45 µm membrane filter. Chromatographic analysis was carried out on Agilent series 1100 HPLC instrument, with a DAD detector, on a reverse phase Zorbax SB-C18 (Agilent) analytical column (150 mm × 4.6 mm i.d., 5 µm particle size). The mobile phase consisted of solvent A (1%, v/v solution

of orthophosphoric acid in water) and solvent B (acetonitrile) using the gradient elution as follows: 98−90% A 0−5 min, 90% A 5–10 min, 90−85% A 10–13 min, 85% A 13–15 min, 85−70% A 15–20 min, 70−40% A 20–24 min, 40−0% A 24–28 min. The injection volume was 5 µl. Detection wavelengths were set at 260 and 320 nm, and the flow rate was 1 ml min^{-1}. The isolation, identification and characterization of xanthones norswertianin-1-*O*-primeveroside (NOR-1-*O*-PRIM), norswertianin-1-*O*-glucoside, norswertianin-8-*O*-primeveroside and gentioside were reported in our previous paper (Krstić et al. 2004). Acid hydrolysis of NOR-1-*O*-PRIM with 2N HCl yielded aglycone norswertianin (NOR).

Quantification was performed using calibration curve with external standards. All experiments were repeated at least two times. The results are presented as milligrams per gram of dry weight.

DPPH radical scavenging activity

The DPPH (1,1-diphenyl-2-picrylhydrazyl) method, originally published by Blois (1958) and modified by Brand-Williams et al. (1995), is a simple and rapid method for determining the antiradical activity of plant extracts and chemical compounds by using DPPH as stable radical. The reaction mixture (1 ml) contained 500 µl of daily prepared DPPH solution (250 µM), 400 µl of Tris–HCl buffer pH 7.4 (100 mM) and 100 µl of methanol solutions of the tested xanthones at various concentrations (20, 30, 40, 60 and 80 µM). After thorough mixing, the solutions were kept in the dark at room temperature for 20 min. Thereafter, the absorbance was measured at 517 nm. All tests were performed in triplicate, with Trolox and ascorbic acid as a positive control. The percentage of inhibition of DPPH radical by xanthones was calculated according to equation:

$$\%\ \text{Inhibition (DPPH radical scavenging activity)} = \left[\left(A_{blank} - A_{test}\right) / A_{blank}\right] \times 100$$

where A_{blank} is the absorbance of solution without test sample (antioxidant), and A_{test} is the absorbance of test solution.

Statistical analysis

All results were reported as mean ± standard error (SE) of 3–4 independent experiments performed in triplicate. All percentage data were subjected to angular transformation ($\arcsin\sqrt{X}$) and SE number data to square root transformation prior to analysis. After analysis, data were subjected to inverse transformation for presentation. Significant differences between means of each treatment after 3 and 7 days separately within each clone, were determined using Fisher's least significant difference (LSD) test at $P \leq 0.05$ using the StatGraphics Plus software package for Windows 2.1 (Statistical Graphics Corp.,Rockville, MD, USA).

Results

Effect of abiotic elicitors on growth parameters and xanthone production

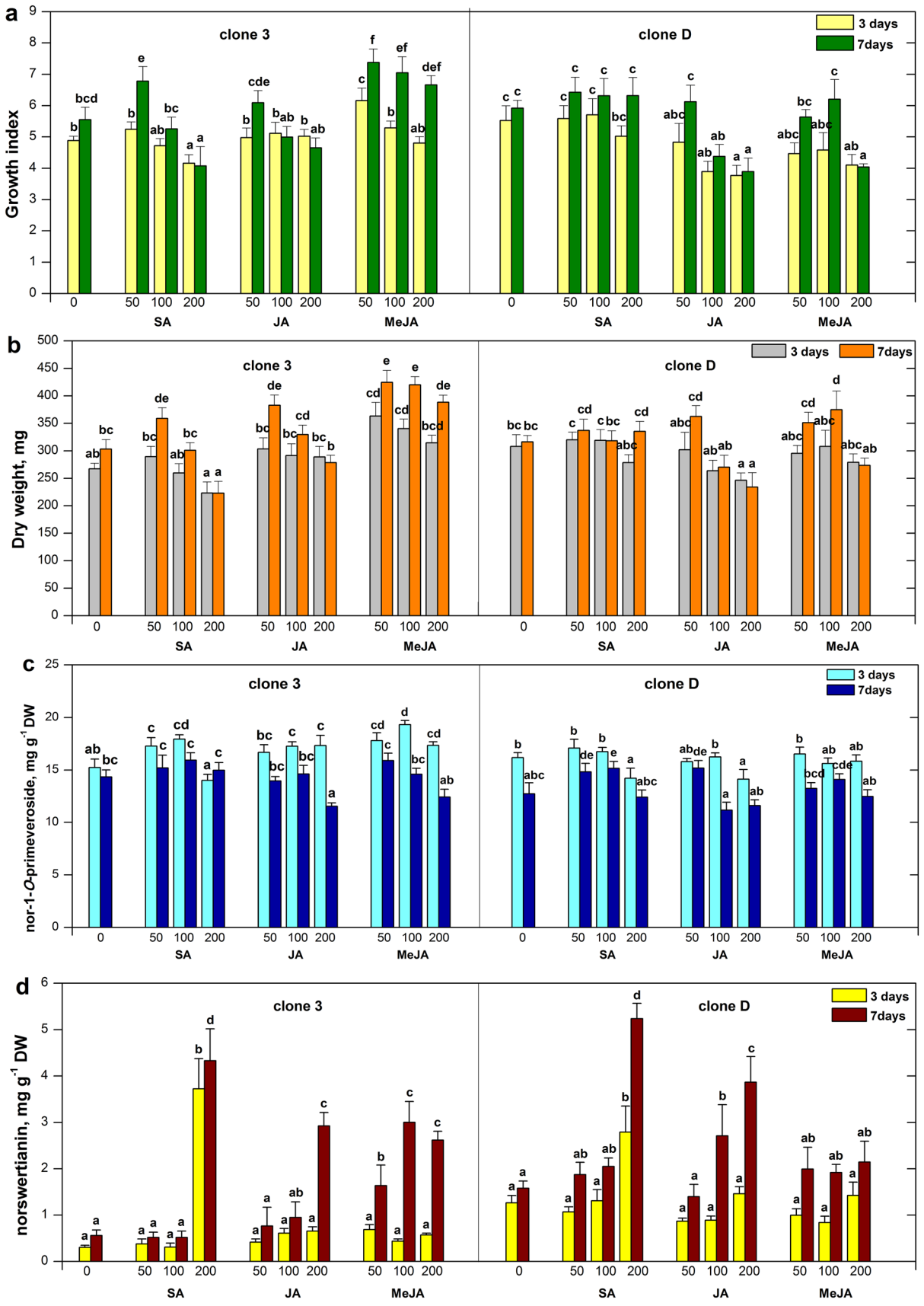
The effects of abiotic elicitation (SA, JA and MeJA at different concentrations) on root growth and biomass production depended on the genotype of hairy root clones of *G. dinarica* and duration of elicitor treatment (Fig. 1a, b). Three-day elicitor treatment had no stimulating effect on the root growth and biomass production of either clone, compared to the control, with exception of MeJA at 50 µM in clone 3. Seven-day treatment stimulated root growth of clone 3 at lowest elicitor concentrations. Either elicitor at the highest concentration (200 µM) decreased root growth and induced necrosis in both clones.

The production of dominant xanthone NOR-1-*O*-PRIM after 3-day treatment was stimulated in clone 3 and unaffected in clone D. In both clones NOR-1-*O*-PRIM production was generally higher after 3-day elicitation compared to 7-day treatment with each elicitor (Fig. 1c). The production of aglycone NOR strongly increased in both clones after 7-day treatment with SA and JA (2.4- to 7.8-fold increase compared to non-elicited controls, respectively). The highest increase in NOR accumulation was recorded for 200 µM SA in both clones (Fig. 1d).

Effect of biotic elicitors on growth parameters and xanthone production

When YE was applied for elicitation, only a 7-day treatment improved root growth and biomass production in both clones of *G. dinarica* (Fig. 2a, b). The effect of YE was more pronounced in clone 3, where the highest growth index (11.56) was achieved. CH elicitation was effective only in clone D, at lower CH concentrations (growth index of 9.30). The highest elicitor concentrations resulted in necrosis, accompanied by the browning of the culture medium.

Changes in the xanthone content in hairy roots after biotic elicitation are shown in Fig. 2c, d. YE did not stimulate the accumulation of NOR-1-*O*-PRIM in either clone. CH stimulated NOR-1-*O*-PRIM production only in clone 3, when applied at lower doses (Fig. 2c). The level of xanthone aglycone NOR was not affected or only slightly enhanced upon treatment with lower concentrations of YE and CH. However, hairy roots elicited with the highest elicitor concentrations showed a significant increase in


a
Growth index
clone 3
clone D
3 days
7days
SA
JA
MeJA
b
Dry weight, mg
c
nor-1-O-primeveroside, mg g-1 DW
d
norswertianin, mg g-1 DW

◂**Fig. 1** Effect of abiotic elicitors SA, JA and MeJA (μM) on growth parameters and xanthone production of *G. dinarica* hairy root clones: **a** growth index, **b** dry weight, **c** norswertianin-1-*O*-primeveroside content, **d** norswertianin content. Data represent mean values (±SE) of three replicates. Values denoted by the *same letter* are not significantly different according to the Fisher's (LSD) test at $P \leq 0.05$ following ANOVA multifactorial analysis

NOR accumulation (Fig. 2d). In clone 3, an 8- and 24-fold increase in NOR content was observed after 3-day treatment with YE and CH, respectively. Similar stimulatory effects of YE and CH were recorded in clone D, where maximum level of NOR was 16- and 15-fold higher than in controls, respectively.

Chromatograms of methanol extracts of hairy root clones treated with the highest concentrations of YE and CH revealed that, apart from a significant increase of norswertianin content, two additional peaks appeared which were not detected in the control roots. One of these peaks was identified as isogentisin (peak 6) by comparison with the external standard, and the second peak was identified as xanthone (peak X) also based on its characteristic UV spectra (Fig. 3).

DPPH radical scavenging activity

In order to evaluate antioxidant potential of xanthones from hairy roots of *G. dinarica*, we tested their scavenging activities against stable DPPH free radicals in comparison with Trolox and ascorbic acid (Fig. 4). Results obtained in our study showed that radical scavenging activity decreased in the following order: norswertianin > norswertianin-1-*O*-primeveroside > norswertianin-1-*O*-glucoside > norswertianin-8-*O*-primeveroside. Aglycone norswertianin exhibited the highest scavenging activity that follows that of ascorbic acid.

Discussion

In the present work, we studied the influence of different biotic and abiotic elicitors on the growth parameters and xanthone accumulation in hairy roots of *G. dinarica*.

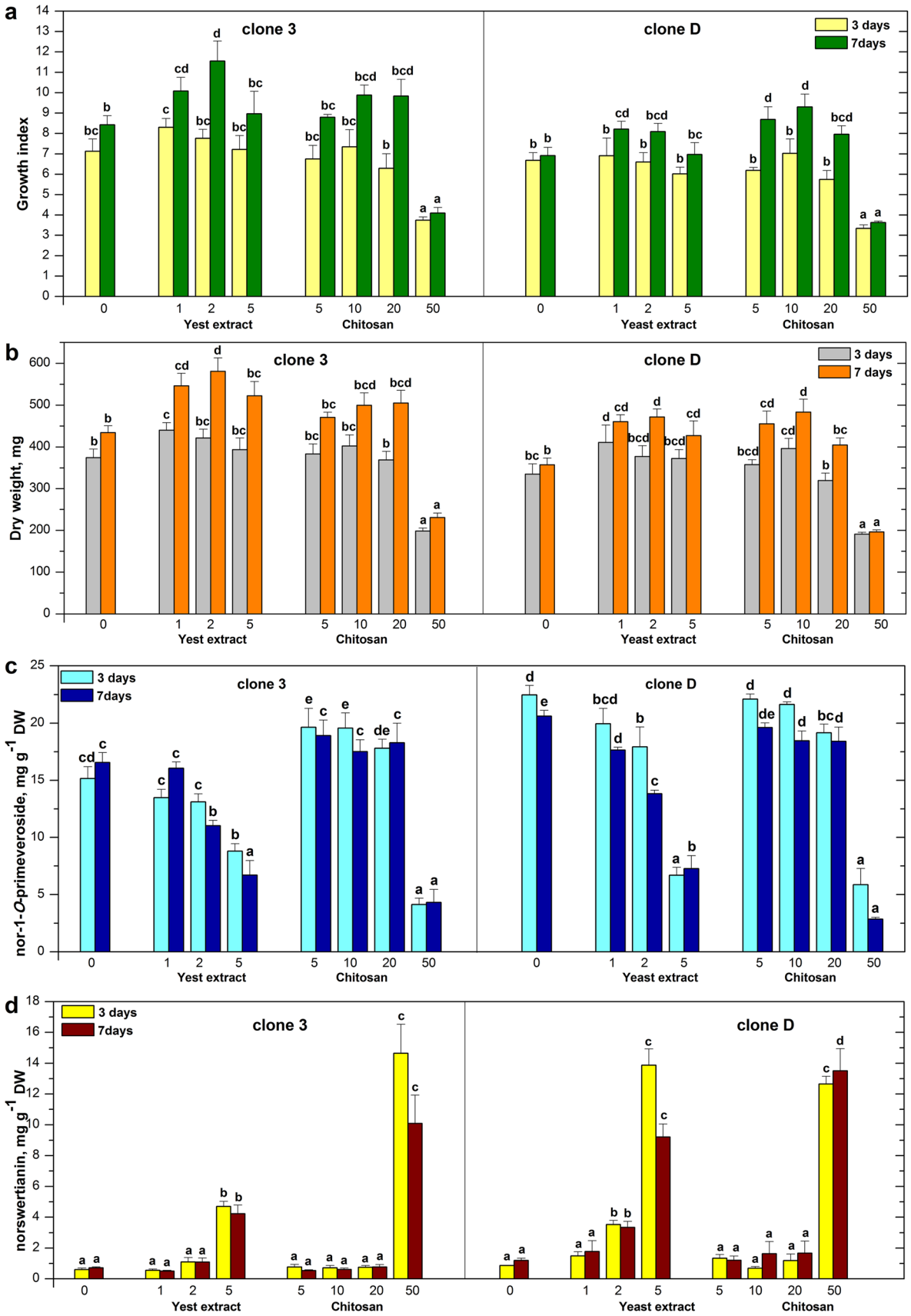
Effect of elicitors on growth parameters

SA and jasmonates had similar effects on the growth and biomass production in both examined clones. SA, JA or MeJA applied for 7 days showed slight stimulatory effect on the growth parameters, while 3-day treatments were apparently too short in duration providing no significant effect compared to the non-treated transformed roots. Our findings concerning the effects of SA and MeJA on root growth are similar to those previously reported for hairy root cultures of *Salvia castanea* (Li et al. 2016) and *Tropaeolum majus* (Wielanek and Urbanek 2006). Insignificant effect of SA on the biomass production was also observed in *Hypericum hirsutum* and *Hypericum maculatum* shoot cultures, while JA inhibited shoot growth in both species (Coste et al. 2011). Treatment with SA as well as JA negatively affected the growth of *Hypericum perforatum* cell suspension cultures (Gadzovska et al. 2007, 2013) and *H. perforatum* plantlets (Sirvent and Gibson 2002). Study on *Eleutherococcus koreanum* adventitious roots subjected to MeJA and SA elicitation for 7 days decreased biomass accumulation in a concentration dependent manner (Lee et al. 2015).

Biotic elicitors YE and CH improved root growth and biomass production in both clones of *G. dinarica*, and higher growth index was gained when elicitors were applied at lower concentrations. Similar positive effects of biotic elicitation were reported by Bayraktar et al. (2016), where YE and CH enhanced biomass production of micropropagated *Stevia rebaudiana*. Increased biomass accumulation of elicited culture compared to that of the control has been also observed in hairy root cultures of *Tropaeolum majus* (Wielanek and Urbanek 2006) and *Salvia castanea* (Li et al. 2016) treated with YE.

Effect of elicitors on xanthone production

It is well known that SA, JA and its derivate are involved in systematic signaling pathways for plant defense response to the pathogens or herbivores (Conrath et al. 2002). Treatment of *G. dinarica* hairy roots with SA, JA and MeJA affected the content of two main xanthones in a different manner. In clone 3, all tested elicitors increased the content of NOR-1-*O*-PRIM, whereas in clone D the amount of this xanthone was highest at low concentrations of each elicitor, and then decreased at the higher concentrations. It was also noticed that short-term (3 day) treatment had a more pronounced effect than 7 day treatment. On the contrary, hairy roots of both clones treated with higher concentrations of all elicitors yielded the highest content of aglycone NOR, and this was highly expressed in 7 day treatment. It has been previously reported that the concentration of elicitor and treatment duration are the factors that strongly influence the intensity of the response (Vasconsuelo and Boland 2007). In addition, each elicitor exhibited different effect. In our study, SA exerted the highest stimulatory effect on NOR production in both hairy roots clones, while MeJA was more effective in NOR-1-*O*-PRIM accumulation in clone 3. The stimulatory effect of SA on the tropane alkaloids production from *Brugmansia candida* hairy roots was reported by Pitta-Alvarez et al. (2000). Coste et al. (2011) showed different influence of JA and SA on secondary metabolite production in *H. hirsutum* and *H. maculatum*.


a
clone 3
clone D
3 days
7days
Growth index
Yest extract
Yeast extract
Chitosan
b
Dry weight, mg
c
nor-1-O-primeveroside, mg g-1 DW
d
norswertianin, mg g-1 DW

◂**Fig. 2** Effect of biotic elicitors, yeast extract (g l^{-1}) and chitosan (mg l^{-1}) on the growth parameters and xanthone production of *G. dinarica* hairy root clones: **a** growth index, **b** dry weight, **c** norswertianin-1-*O*-primeveroside content, **d** norswertianin content. Data represent mean values (± SE) of four replicates. Values denoted by the *same letter* are not significantly different according to the Fisher's (LSD) test at $P \leq 0.05$ following ANOVA multifactorial analysis

Addition of JA stimulated accumulation of hypericin and pseudohypericin in *H. hirsutum* and *H. maculatum* shoot cultures, but negatively affected hyperforin biosynthesis in *H. hirsutum*. However, in both *Hypericum* species SA significantly increased hypericin and hyperforin production. The application of MeJA significantly enhanced the accumulation of oleanolic acid in *G. straminea* (Zhao et al. 2013). Beerhues and Berger (1995) showed that treatment with MeJA improved xanthone accumulation in cell cultures of *Centaurium erythraea*. In *H. perforatum* shoots Pavlik et al. (2007) reported that MeJA in combination with sucrose showed remarkable stimulating effects on hypericin and hyperforin production. Also, MeJA has been demonstrated to increase xanthone production in *H. perforatum* cell suspension cultures (Conceição et al. 2006).

Chitosan was more efficient elicitor for the enhancement of major xanthones content in hairy roots than yeast extract. The stimulating effect of CH and YE on the isoflavonoid production in *Pueraria candollei* hairy roots has been reported previously (Udomsuk et al. 2011). Cell and root cultures of *H. perforatum* subsp. *angustifolium* elicited with CH accumulate increased amount of xanthones (Tocci et al. 2010, 2011). Sivanandhan et al. (2012) reported that CH enhanced withanolides production in adventitious root cultures of *Withania somnifera*, while addition of YE elevated accumulation of tropane alkaloids in hairy root cultures of *B. candida* (Pitta-Alvarez et al. 2000) and rosmarinic acid content in cell suspension cultures of *Ocimum sanctum* (Lukmanul Hakkim et al. 2011). In the recent study of Bayraktar et al. (2016), YE and CH treatments enhanced the stevioside production in *S. rebaudiana* cultured in vitro.

Furthermore, phytochemical profile of hairy roots treated with YE and CH differed in comparison with untreated roots. HPLC chromatogram revealed the appearance of additional xanthone compounds which were not detected in other samples. Accumulation of de novo synthesized xanthone compounds was also observed in *H. perforatum* cells elicited with *Colletotrichum gloeosporioides* (Conceição et al. 2006) or *Agrobacterium tumefaciens* (Franklin et al. 2009). Franklin et al. (2009) attributed a 10-fold increase in antimicrobial activity observed in these cells to both newly synthesized xanthones and an increase in the amount of total xanthones. There are several studies that reported antimicrobial activity of xanthones found in *G. dinarica* hairy roots. Antibacterial and antifungal activity of norswertianin has been described by Fotie and Bohle (2006). One of de novo synthetized compounds identified in hairy roots of *G. dinarica* treated with biotic elicitors, isogentisin, has been reported to exhibit antimicrobial activity against *Mycobacterium bovis* (Menković et al. 1999), *Micrococus luteus, Escherichia coli* and *Pseudomonas aeruginosa* (Šavikin et al. 2009).

Our results revealed that all elicitors at the highest concentrations reduced the production of glycoside NOR-1-*O*-PRIM while simultaneously strongly increased aglycone NOR accumulation. This implies that the elicitor treatment affected specifically some metabolic steps in the xanthone biosynthesis. As reported by Beerhues and Berger (1995), hexaoxygenated-xanthone primeveroside is formed by the addition of sugar residue to the hexaoxygenated xanthone aglycone in cell cultures of *Centaurium erythraea* and *C. littorale*. In our study, the addition of elicitor might have caused the suppression of glycosylation, the late step of the constitutive pathway, which led to the increased accumulation of norswertianin aglycone.

Antiradical activity of xanthones from hairy roots of *G. dinarica*

Reactive oxygen species (ROS) play an important role as signaling molecules in the regulation of responses to biotic and abiotic stresses in plants (Baxter et al. 2014). However, increased accumulation of ROS in response to stress stimuli might be detrimental for plant cells and the presence of free radical scavenging compounds is a decisive factor for protecting cells from oxidative damage. It has been reported that xanthones showed great capacity for preventing lipid peroxidation and decreasing ROS production (Pinto et al. 2005; Zheng et al. 2014). Our results confirmed findings of Franklin et al. (2009) that xanthones have dual function in plant cells, and act both as phytoalexins to impair the pathogen growth and as antioxidants to protect the cells from oxidative damage. Free radical scavenging activity of polyphenolic compounds, including xanthones, is related to the hydroxylation of phenolic rings. As discussed by Rice-Evans et al. (1995), polyphenols with *o*-dihydroxyl structure in the B ring could scavenge free radicals effectively. Our finding that NOR-1-*O*-PRIM and NOR-1-*O*-glucoside were stronger DPPH radical scavengers than NOR-8-*O*-PRIM, due to the presence of *ortho*-dihydroxy groups in B ring of 1-*O*-glycosides, is in accordance with previous report that the presence of *ortho*-trihydroxy group in the B ring of flavonoids contributes to the strong radical scavenging activity of the compounds (Nanjo et al. 1996). Moreover, it has been shown that glycosylation of flavonoids reduces their activity when compared to the corresponding aglycones (Shahidi and Wanasundara 1992). Since

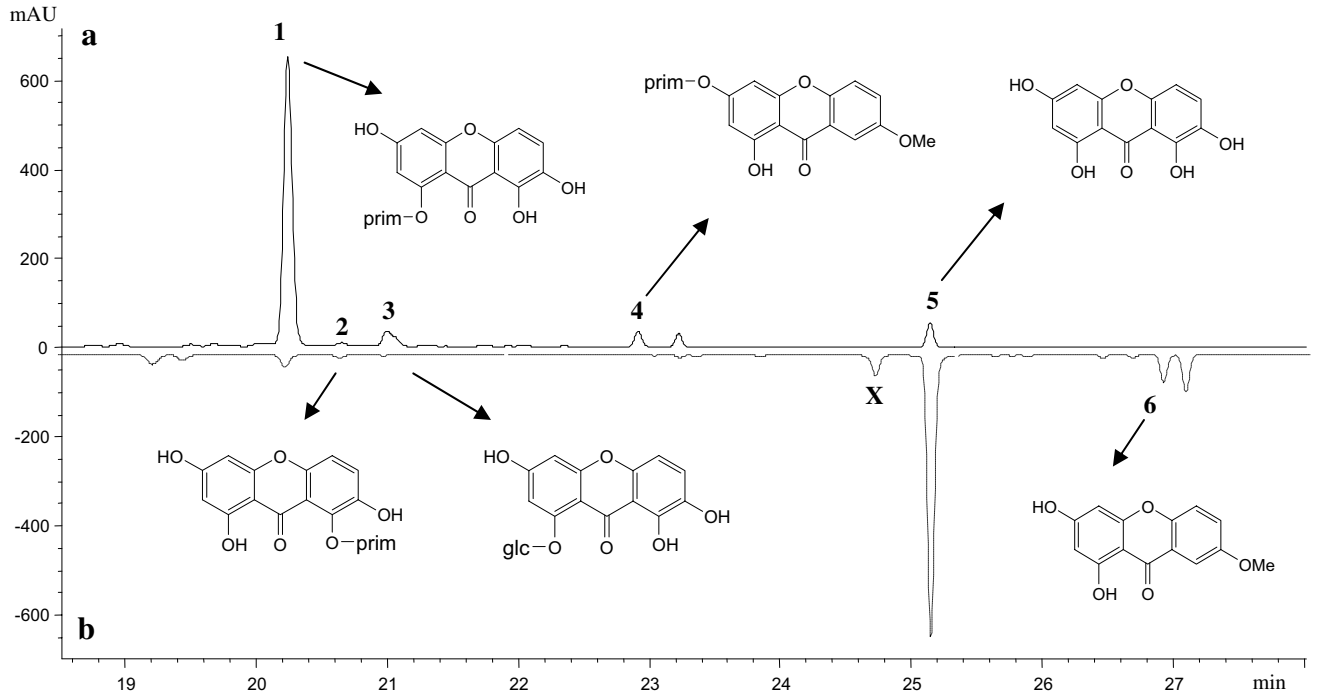


Fig. 3 Comparative HPLC profiles (λ = 260 nm) of *Gentiana dinarica* (clone D) methanolic extracts of **a** control and **b** hairy roots treated with chitosan (50 mg l^{-1}): *1* norswertianin-1-*O*-primeveroside; *2* norswertianin-8-*O*-primeveroside; *3* norswertianin-1-*O*-glucoside; *4* gentioside; *5* norswertianin; *6* isogentisin; (*X*) unidentified xanthone compound

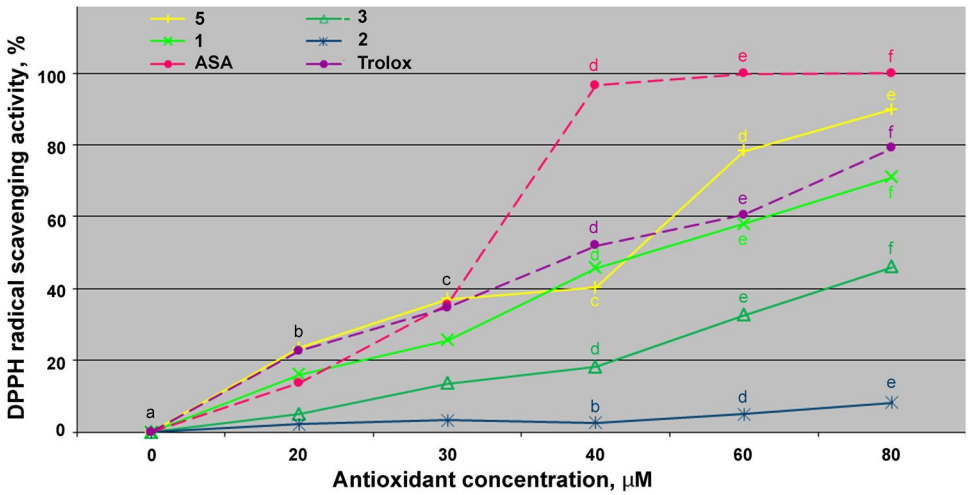


Fig. 4 DPPH radical scavenging activity of xanthones: *1* norswertianin-1-*O*-primeveroside; *2* norswertianin-8-*O*-primeveroside; *3* norswertianin-1-*O*-glucoside; *5* norswertianin compared with ASA (ascorbic acid) and Trolox. Data represent mean values (±SE) of three replicates. Values denoted by the *same letter* are not significantly different according to the Fisher's (LSD) test at $P \leq 0.01$ following ANOVA multifactorial analysis

the addition of elicitors reduced production of NOR-1-*O*-PRIM and other glycosides, it can be concluded that biosynthetic pathway in the presence of elicitors is directed to the accumulation of the xanthones with the highest antioxidant efficacy.

Conclusion

This is the first study on the influence of different elicitors on biomass production and xanthone accumulation in hairy roots of *Gentiana* species. Seven-day elicitor treatment exerted a more pronounced stimulatory effect on root growth and biomass production in both examined clones, with clone 3 exhibiting a greater sensitivity towards elicitor treatment than clone D and biotic elicitors being more efficient in increasing the growth index. The highest concentrations of all elicitors reduced the production of glycoside NOR-1-*O*-PRIM but simultaneously strongly increased aglycone norswertianin content, with biotic elicitors CH and YE being the most effective. Elicitation with CH resulted in the highest amount of

NOR, yielding a 24-fold increase compared to the control. High content of NOR, xanthone with the strongest antioxidant capacity, provided protection from self-oxidative damage and supported cellular ROS homeostasis in elicited roots. Furthermore, the occurrence of new xanthone compounds indicated that biotic elicitors caused changes in xanthone biosynthesis and led to formation of xanthones that could act as protective agents against pathogens. These findings indicated that priming with different elicitors could alter plant secondary metabolism, resulting in the production of "protective compounds" with strong antioxidant and antimicrobial properties. Further studies on elicitor-induced effects in *G. dinarica* hairy roots will allow us to determine the activity of the enzymes involved in xanthone biosynthesis.

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Author contributions BV produced and maintained in vitro cultures and designed and supervised the whole study. DKM and TJ provided plant material, performed phytochemical analysis and wrote the manuscript. DV helped with experimental design and performed statistical analysis of the data. BU contributed to the writing and correction of the manuscript.

Compliance with ethical standards

Conflict of interest The authors have no conflict of interest to declare.

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