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Gentianella lutescens subsp. *carpatica* J. Holub.: Shoot Propagation *in vitro* and Effect of Sucrose and Elicitors on Xanthones Production

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Abstract: *In vitro* shoot culture of endangered medicinal plant *Gentianella lutescens* was established from epicotyl explants cultured on MS basal medium with 0.2 mg l^{-1} 6-benzylaminopurine (BA) and evaluated for xanthones content for the first time. Five shoot lines were obtained and no significant variations in multiplication rate, shoot elongation and xanthones profile were found among them. The highest rooting rate (33.3%) was achieved in shoots treated for 2 days with 5 mg l^{-1} indole-3-butyric acid (IBA) followed by cultivation in liquid PGR-free ½ MS medium for 60 days. HPLC analysis revealed the lower content of xanthones – mangiferin, bellidifolin, demethylbellidifolin, demethylbellidifolin-8-*O*-glucoside and bellidifolin-8-*O*-glucoside in *in vitro* cultured shoots compared to wild growing plants. The increasing concentration of sucrose, sorbitol and abiotic elicitors salicylic acid (SA), jasmonic acid (JA) and methyl jasmonate (MeJA) influenced the shoot growth and xanthone production. Sucrose and sorbitol applied at the highest concentration of 233.6 mM increased dry matter percentage 2-fold while SA at 100 μM promoted shoot growth. The increased sucrose concentration enhanced accumulation of xanthones in shoot cultures 2-3-fold compared to the control shoots. Elicitors at 100-300 μM increased accumulation of mangiferin, demethylbellidifolin-8-*O*-glucoside, and bellidifolin-8-*O*-glucoside almost equally, while MeJA at the highest concentration of 500 μM enhanced 7-fold amount of aglycones demethylbellidifolin and belidifolin compared to the control. The obtained results facilitate conservation of *G. lutescens* and pave the way for further research on large-scale shoot propagation and production of pharmacologically active xanthones.

Keywords: shoot culture; secondary metabolites; HPLC; bellidifolin; sorbitol

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1. Introduction

The genus *Gentianella* Moench (Gentianeceae) encompasses about 250 species growing mainly in temperate or mountain habitats in Europe, South America, New Zealand, and Australia [1]. In Europe, the genus *Gentianella* consists of 22 species with a numerous of subspecies and taxa that are distributed in the Alps, Carpathians, in the Tatra mountains, as well as in the mountains of the Balkan peninsula [2]. In Serbia, the genus *Gentianella* was represented by six species: *G. austriaca*, *G. bulgarica*, *G. axilaris*, *G. ciliata*, *G. praecox*, and *G. crispata* [3].

Apart their ornamental value, *Gentianella* species are well-known in traditional medicines since ancient times. In South America they have been used as traditional remedy for the treatment of digestive and liver problems [4, 5]. *Gentianella amarella* is known in

traditional medicine of Mongolia to cure headache, hepatitis, fever and gallbladder disorders [6]. Like other species of the Gentianaceae family, *Gentianella* plants are characterized by the universal occurrence of 3 main groups of secondary metabolites such as iridoids, flavonoids, and xanthones [7].

Naturally occurring xanthones have been attracting attention for a long time due to their specific bioactivities and occupy an important position in the pharmacology and chemistry of natural products. Xanthone compounds typical for *Gentianella* species belong to the bellidifolin type of xanthones that mostly occur in the form of *O*-glycosides. They are responsible for wide range of therapeutic properties attributed to *Gentianella* plants. The xanthones bellidifolin and demethylbellidifolin, the principal constituents of many gentianellas, have been reported to show cardioprotective effects, antioxidant, antimicrobial, and antidiabetic activity as well as displayed significant potential to inhibit acetylcholinesterase and monoamino oxidase activity [8]. Bellidifolin exhibited a variety of pharmacological activities including the most prominent hypoglycemic [9] and neuroprotective activities [10]. These findings support the potential use of xanthone compounds as new drugs in treating aging-related neurodegenerative disorders [11] and also as useful candidates in therapy of type 2 diabetes [9].

Although *Gentianella* species have been intensively investigated for the last few decades, many species have not yet been studied due to low availability of plant material. A large number of them are rare and endemic or grow in inaccessible localities while the others became endangered by excessive harvesting or adverse environmental conditions. Such an unfavorable situation is with endemic species *G. lutescens* subsp. *carpatica* which we discovered in Serbia for the first time during field research on the mountain Povlen (Fig. 1). Survey of literature indicated that this species has not been phytochemically investigated so far and very scarce data can be found about it.

Figure 1. *Gentianella lutescens* subsp. *carpatica* in a natural habitat in the mountain Povlen, (locality Razbojište), Serbia.

It is biennial 3-40 cm tall plant, simple or branched above, forming a racemose inflorescence panicle-like, umbrella-shaped with reddish to violet flowers. *Gentianella lutescence* subsp. *carpatica* was located in eastern and central Europe and northern part of Balkan peninsula, mainly in the mountains with Austria, Bulgaria, Czech, Germany, Yugoslavia, and Poland ecotypic variants [12]. This species was reported as relatively common in Chech Republic before 1950, however nowadays it is considered as critically threatened plant survived only on a few sites [13]. The fact that the extant populations are small, often less than ten individuals, signify *G. lutescence* as a critically endangered plant

species that was included in both the European and World red list according to International Union for Conservation of Nature's (IUCN) Red List [14].

The demand for the medicinal plant species has increasing globally due to the resurgence of interest in herbal medicine making heavy pressure on wild medicinal plant populations because of over-harvesting. In order to meet the growing demand of medicinal plants, it becomes important to conserve these species either by way of domestication and cultivation or by other ex-situ and in situ conservation measures for their sustainable exploitation [15]. Advances in plant biotechnology and tissue culture opened up a new area for plant diversity conservation and evaluation. Direct application of biotechnological tools like in vitro culture and cryopreservation proved to be valuable means for large-scale propagation, storage, reintroduction as well as secondary metabolites production of endangered medicinal plant species [16, 17].

Differently from numerous *Gentiana* species that were established in vitro for the last two decades, *in vitro* culture studies were carried out in just a few species of the genus *Gentianella* including *G. austriaca* [18], *G. bulgarica* [19, 20], *Gentianella albifpra* [21] and *G. bicolor* [22].

The tissue culture has been shown as suitable tool for overcoming the deficit of plant material in rare and endangered medicinal species [23]. However, the frequently lower content of secondary metabolites of interest in tissue cultured plants compared to natural plants, may limit the applicability of tissue culture [17]. Since the secondary metabolites are involved in protection response of plants to adverse environment conditions, their production during plant cell or organ culture can give rise to increase by inducing diverse environment stresses [24, 25]. For instance, the formation of phenyl amides and dramatic increasing of polyamines in bean and tobacco [26] and the enhanced accumulation of anthocyanin [27] have been stimulated by various environmental stresses including light conditions, wounding, drought, sugar and nutrient deficiency etc.

Exposure to high sucrose or sorbitol content resulted in cellular dehydration, which causes osmotic stress. This stress can trigger accumulation of some defending secondary metabolites [28]. Eliciting cultures with chemical compounds has also been widely used to enhance the secondary metabolites production during plant cell or organ culture by inducing plant defense responses. The most frequently used compounds with positive elicitation effects on numerous secondary metabolites of interest such as oleanolic acid rosmarinic acid, ginsenosides, anthocyanins, hypericin, kinsenoside and sesquiterpene lactones were jasmonic acid (JA), methyl jasmonate (MeJA) and salicylic acid (SA) [29-34]. The flavonoid production of *H. perforatum* cell cultures was significantly promoted by 100 μM MeJA [35]. MeJA has also been demonstrated to increase xanthone production in *H. perforatum* cell suspension cultures [36] and in combination with sucrose showed remarkable stimulating effects on hypericin and hyperforin production [37]. Positive effect of elicitors on production of xanthone aglycones in hairy roots of *G. dinarica* was reported by Krstić-Milošević et al. (2017) [38] where application of biotic elicitors strongly increased production of aglycone norswertianin while simultaneously reducing the production of its glycoside norswertianin-1-*O*-primeveroside.

Thus, in view of medicinal and conservation importance of *G. lutescens*, the aim of the current work was to evaluate in vitro grown shoot culture of *G. lutescens* as an alternative, sustainable and stable source of xanthones. In order to increase biomass and xanthones content in *G. lutescens* shoots, the effect of sucrose, sorbitol and elicitors JA, MeJA, and SA, on shoot growth and xanthones production was investigated.

2. Results and discussion

2.1. HPLC analysis of secondary metabolites of wild grown G. lutescens plants

The chemical profile of methanol extract of aerial parts of wild-growing *G. lutescens* plants analyzed by HPLC-DAD technique is presented in Fig. 2. Similarly as in other *Gentianella* species, three groups of secondary metabolites such as secoiridoids, flavone-*C*-

glucosides and xanthones were detected. The chromatographic analysis identified the presence of swertiamarin and gentiopicrin (peaks 1 and 2, respectively), as the most common secoiridoid compounds which appeared to be present in all species of the Gentianaceae [39].

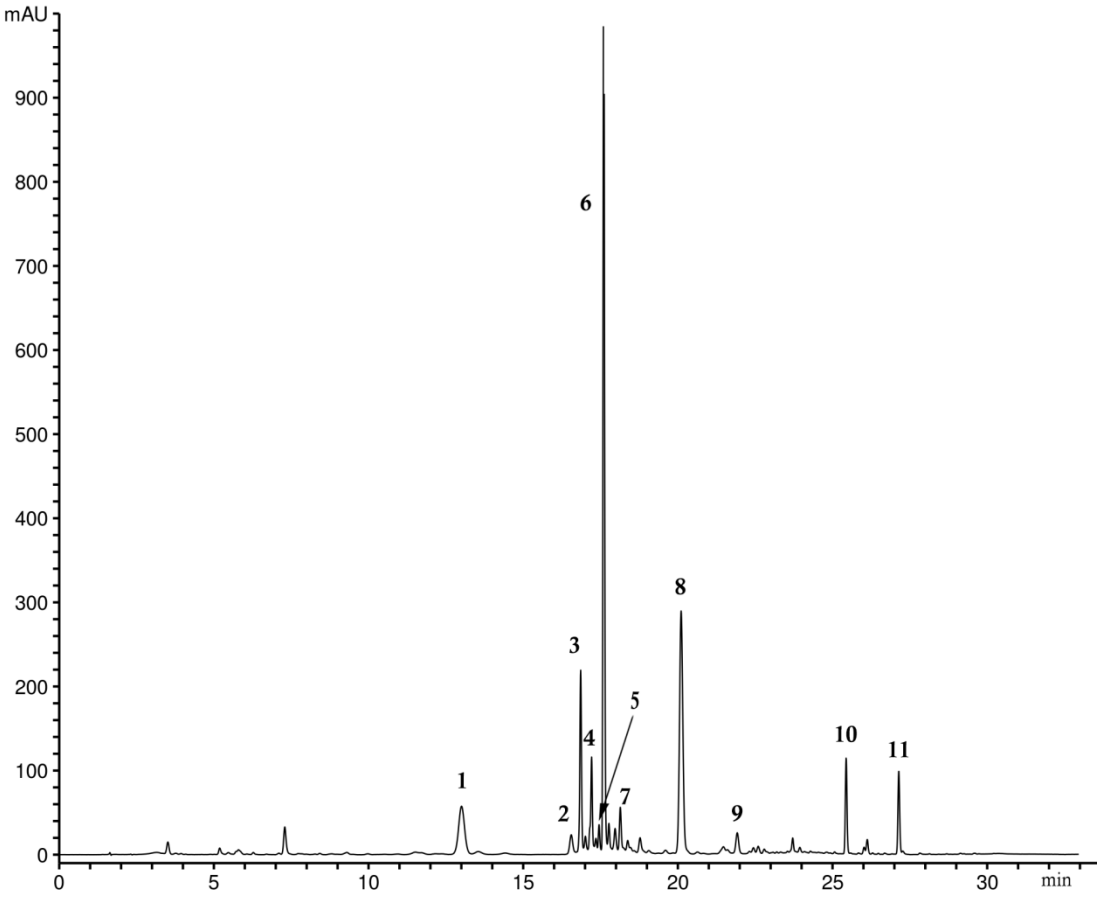


Figure 2. HPLC profile (λ=260 nm) of methanol extract of G. lutescens. Peaks: 1- gentiopicrin, 2- swertiamarin, 3- mangiferin, 4- campestroside, 5- isooorientin, 6- demethylbellidifolin-8-*O*-glucoside, 7- swertisin, 8- bellidifolin-8-*O*-glucoside, 9- veratriloside, 10- demethylbellidifolin, 11- bellidifolin.

The peaks 5 and 7 were identified as isoorientin and swertisin, the most represented **C**-glucoflavones in the *Gentianella* species. The seven remaining peaks presented in chromatogram were detected as xanthone compounds. The precise identification of each xanthone was confirmed by HPLC co-injection method using reference xanthone standards isolated previously in our laboratory [40]. The two dominant peaks (Fig. 2) belong to demethylbellidifolin-8-*O*-glucoside (6) and bellidifolin-8-*O*-glucoside (8), xanthones with 1,3,5,8-oxidation pattern characteristic for *Gentianella* species. HPLC also revealed the presence of a tetrahydroxanthone glucoside named campestroside (4), a partially saturated analogue of demethylbellidifolin-8-*O*-glucoside. Xanthone with such structure need special attention since it is rare in the nature and its occurrence is of particular chemotaxonomic and biogenetic significance [40]. Compound 9 was identified as xanthone-*O*-glucoside veratriloside, and this compound was reported to be the first 1,3,4,7-oxygenated xanthone isolated from the genus *Gentianella* [20,40]. The peak 3 belongs to *C*-glucoxanthone mangiferin, one of the well-known naturally occurring xanthone, widespread among angiosperms. The occurrence of mangiferin together with flavone-*C*-glucosides isoorientin and swertisin is most common and typical for *Gentianella* species. The peaks

detected at the end of the chromatogram were identified as xanthone aglycons demethylbellidifolin (10) and bellidifolin (11). Fig. 3 shows chemical structures of the secoiridoid and xanthone compounds identified in *G. lutescens*.

Considering that xanthones are becoming increasingly important compounds that possess a broad spectrum of biological and pharmacological activities, our study on secondary metabolites from *G. lutescens* cultured *in vitro* will be focused on the chemical analysis of five dominant xanthones which include mangiferin, demethylbellidifolin-8-O-glucoside (DMB-8-O-glc), bellidifolin-8-O-glucoside (bell-8-O-glc), and aglycons demethylbellidifolin (DMB) and bellidifolin.

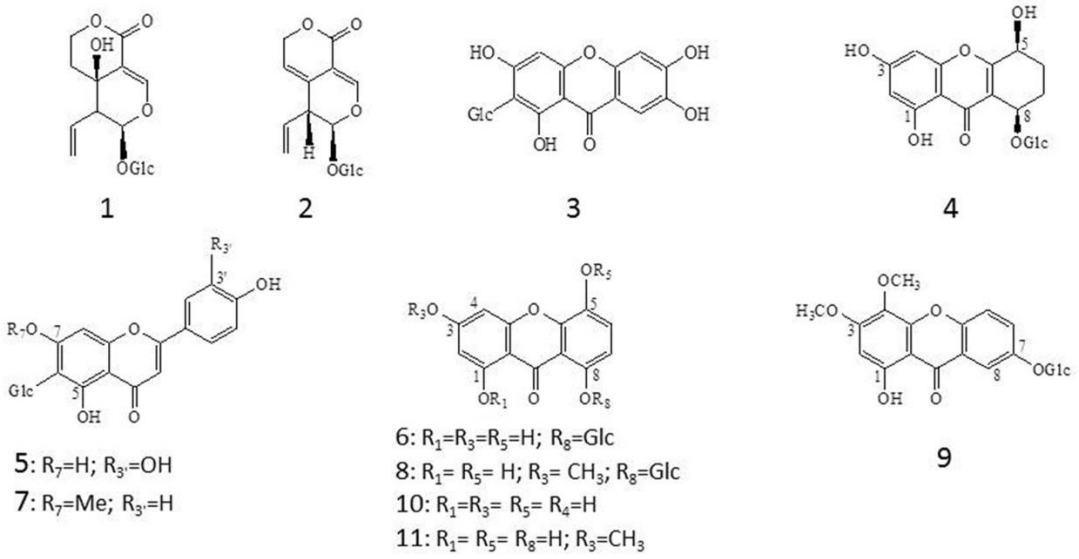


Figure 3. Chemical structures of secoiridoid and xanthone compounds identified in *G. lutescens*. 1- gentiopicrin, 2- swertiamarin, 3- mangiferin, 4- campestroside, 5- isooorientin, 6- demethylbellidifolin-8-O-glucoside, 7- swertisin, 8- bellidifolin-8-O-glucoside, 9- veratriloside, 10- demethylbellidifolin, 11- bellidifolin.

2.2 *In vitro* shoot propagation of *G. lutescens*

Immature seeds of *G. lutescens* (Fig. 4a) were germinated for 10 days at the rate of 5%, and non-contaminated seeds were found. Seedling developed from each seed was designated as unique seedling line. For shoot culture initiation the epicotyl explants of five seedling genotypes (lines) were individually transferred onto BM+0.2 mg l^{-1} 6-benzylaminopurine (BA) where they started to elongate and regenerate shoots during the first few days of culture. Maximum response of the explants to produce new shoots was observed after 35 days of culture on shoot induction medium (Fig. 4b). The results indicated the genotype of the individual seedling had not considerable effect on shoot proliferation response since multiplication index of about 3 was recorded in all five lines but significantly influenced elongation of the main shoot that ranged from 14.49 mm in line 1 to 23.05 mm in line 3 (Table 1).

Figure 4. *In vitro* propagation of *G. lutescens*. (a) Open pod with immature seeds, (b) Shoot multiplication on BM+0.2 mg l^{-1} BA, (c) Root elongation in PGR-free ½ MS liquid medium after treatment of shoots with 5.0 mg l^{-1} IBA for 2 days.

Table 1. Shoot induction and multiplication and the length of the main shoot in *G. lutescens*. Epicotyl explants of five seedling lines were cultivated on BM+0.2 mg l^{-1} BA for 35 days. Values represent the means ± SE from 2-3 experiments with 25-40 samples per line (n=75-80). Data were analyzed by one-way ANOVA analysis. Within each column means followed by different letters are significantly different according to Fisher's LSD test at p≤0.05. Multiplication index: main shoot + axillary buds.

Line (BA 0.2 mg l^{-1})	**No. of explants**	**Multiplication index ± SE**	**Lenght of main shoot (mm) ± SE**	
line 1	80	3.03 ± 0.23 ab	14.49 ± 0.65 a	
line 2	77	3.31 ± 0.19 ab	17.40 ± 0.92 ab	
line 3	80	3.53 ± 0.23 b	23.05 ± 1.67 c	
line 4	75	2.76 ± 0.25 a	17.19 ± 1.22 ab	
line 5	80	3.41 ± 0.26 ab	19.39 ± 1.07 b	
ANOVA source of variation	**Df**	**Mean Square**	***F*- Ratio**	***p*-Value**
Multiplication index	4	7.48476	1.73	0.1428
Lenght of main shoot	4	804.293	7.67	**0.0000**

Elongated shoots (≥15 mm) sporadically formed flower bud. The shoots with intense blue-violet flowers developed normally on the cytokinin containing medium. With the aim to enhance shoot proliferation, the individual shoots of the line 5 were transferred onto BM with increasing concentrations of BA (0-2.0 mg l^{-1}). According to ANOVA, shoot multiplication and shoot length were significantly affected by concentration of BA (Table 2). The higher BA concentration (above 0.5 mg l^{-1}) not only contributed to the enhancement of shoot multiplication rate but also increased percent of low quality vitrified shoots (Table 2). Moreover the length of the main shoot was significantly decreased with BA concentration increasing. Since the highest multiplication rate close to 4 was achieved on BM+0.2 mg l^{-1} BA along with minimum reduction of the main shoot length compared to cytokinin-free medium, and reasonable percent of vitrified shoots (7.75%) this medium formulation was selected as optimal for shoot culture multiplication and maintenance. In

cultures maintained on BM+0.2 mg l^{-1} BA more than one year, the number of shoots with spontaneously developed flowering buds gradually decreased in time and became rareness in those maintained more than 5 years. Unexpected relatively satisfactory multiplication rate, (2.89) that was achieved on cytokinin-free medium when compared to BA containing media, can be explained by prolonged effect of BA from shoot induction medium.

Table 2. Effect of increasing concentrations (0-2.0 mg l^{-1}) of BA on shoot multiplication, the length of the main shoot and vitrification incidence of G. lutescens line 5. Values represent the means ± SE from 3-4 experiments with 25-40 samples per treatment (n=80-129). Data were analyzed by one-way ANOVA analysis. Within each column means followed by different letters are significantly different according to Fisher's LSD test at p≤0.05. Results were scored after 35 days. Multiplication index - main shoot + axillary buds.

BA (**mg l^{-1}**)	**No. of expl.**	**Multiplication index ± SE**	**Lenght of main shoot (mm)** ± SE	**Vitrification**	
				No.	**%**
0	85	2.89±0.16 a	15.02±0.66	1	0.8
0.05	100	3.16±0.17 a	15.85±0.50 e	1	1.0
0.1	128	3.20±0.14 a	13.66±0.46 cd	7	5.47
0.2	90	3.92±0.25 b	15.48±0.64 cd	10	7.75
0.5	129	3.22±0.17 a	13.09±0.48 bc	24	18.6
1.0	128	2.88±0.15a	12.09±0.38 b	20	15.6
2.0	80	2.94±0.20 a	10.46±0.47 a	14	17.5
ANOVA source of variation	**Df**	**Mean Square**	***F*- Ratio**	***p*-Value**	
Multiplication index	6	12.2028	3.72	**0.0012**	
Lenght of main shoot	6	351.866	12.96	**0.0000**	

To our knowledge, this is the first report on shoot culture establishment of endemic *G. lutescens*. In general, *Gentianella* species were so far diminutively investigated compared to their closed relatives belonging to the genus *Gentiana*. Thus Huo and Zheng (2002) [21]was firstly reported shoot regeneration from calli of *G. albifpra* cultured on medium with 3.0 mg l^{-1} 2,4-D + 1.0 mg l^{-1} KIN. Later on *in vitro* propagation was achieved from epicotyls of sterile germinated mature seeds of *G. austriaca* [18], and *G. bulgarica* [19] which are endemic in central part of Balkan Peninsula. Incorporation of cytokinin promoted shoot explant proliferation in *Gentianella* species and BA was found superior for new shoot formation. BA was also effective for inducing shoot proliferation in previously mentioned *Gentianella* species, *G. austriaca* where BA at 0.2-0.5 mg l−1 (depending on species) with the addition 0.1 mg l−1 NAA was applied. Most recently Solorzano et al. (2014) [22] reported shoot regeneration from leaf-explant-derived calli of *G.bicolor* on medium containing combination of KIN and 2,4-D. However shoot tips, epicotyl and nodal segments are generally preferred explants for multiplication of most of the plant species due to the presence of pre-existing meristems. They can be easily developed into shoots that ensure clonal fidelity [41]. Genetic fidelity and true-to-type regenerated plants are very important

for both the germplasm maintenance for plant conservation purpose and mass shoot propagation that ensures continuous supply of genetic uniform plant material for large scale secondary metabolite production [42,43]. Cytokinin was also a common requirement for in vitro flowering, occurred in G. lutescens shoot cultures during multiplication phase. Shoot cultures of *G. austriaca* [18] and *G. bulgarica* [19] also displayed precocious in vitro flowering which significantly affected shoot multiplication due to decay of the main shoot after flowering. However, this phenomenon sporadically occurred in *G. lutescens* and did not threat multiplication at the higher rate compared to related *G. austriaca* and *G. bulgarica* [18,19]. In vitro flowering could be a valuable tool to release new cultivars more rapidly [44].

G. lutescens appeared recalcitrant for rooting. The attempts of rooting of shoots on solid ½ MS and WPM media containing IBA, NAA (1-naphthaleneacetic acid) or IAA (indole-3-acetic acid) at 0.1, 0.2, 0.5, 1.0, and 2.0 mg l^{-1} were not successful (data not shown). A maximum of 10% shoots cultured on media containing lower concentrations of IBA and IAA formed low number of root primordia, while at higher concentrations root primordia were absent but larger black calli were formed instead. Subculturing of shoots with developing root primordia onto solid ½ MS auxin free medium with or without 1% of active charcoal did not favorize elongation of the root primordia too. Rooting of shoots on solid ½ MS media containing IBA or NAA (0.5-2.0 mg l^{-1}) with addition GA3 (gibberelic acid) 0.1 mg l^{-1} did not stimulate root primordia elongation too, but shoots with root primordia was increased from 10% to 26% on IBA 1.0 mg l^{-1} + GA3 0.1 mg l^{-1}. The attempt of shoots rooting using short treatments with high IBA concentration (50.0 mg l^{-1}) for 4h, 8h, 20h and 24h following by transfer into liquid PGR-free ½ MS medium was also unsuccessful since the low number of non-elongated root primordia were formed but instead the higher percent of callus and vitrified shoots were observed too (data not shown).

Accordingly, in order to define advanced rooting conditions, the micropropagated shoots of *G. lutescens* were primarily cultured on solid ½ MS containing increasing concentrations (0.2-5.0 mg l^{-1}) of IBA for different time period followed by transfer into liquid PGR-free ½ MS where they have been cultured at different duration of time (Table 3). Despite the number of combinations were performed, the highest rooting percentage (33%), the number of shoots with induced root primordia (23.3 and 16.7), the number of roots per rooted shoots (5.4 and 3.5) and root elongation (11.2 and 7.7 mm) were achieved when shoots were cultured on solid ½ MS+5.0 mg l^{-1} for 2 and 4 days, respectively following by culture in liquid plant growth regulator free ½ MS for 61days (Table 3; Fig. 4c. Apart satisfactory frequency of rooting, these procedures required minimal rooting time lasting 63 or 65 days compared to other combination examined (Table 3). These results pointed out *G. lutescens* as highly recalcitrant species regarding rooting potential that should be further improved in order to obtain enough healthy and functional plants for successful acclimatization and *ex situ* and *in situ* conservation. The other *Gentianella* species also displayed restricted rooting potential. The highest rooting percentage was obtained in *G. austriaca* where 47.3% of shoots formed roots on solid MS medium with 4.92 μM (1.0 mg l^{-1}) IBA [18]. However, only 1-2% shoots of *G. bulgarica* spontaneously rooted on plant growth regulator free medium [19].

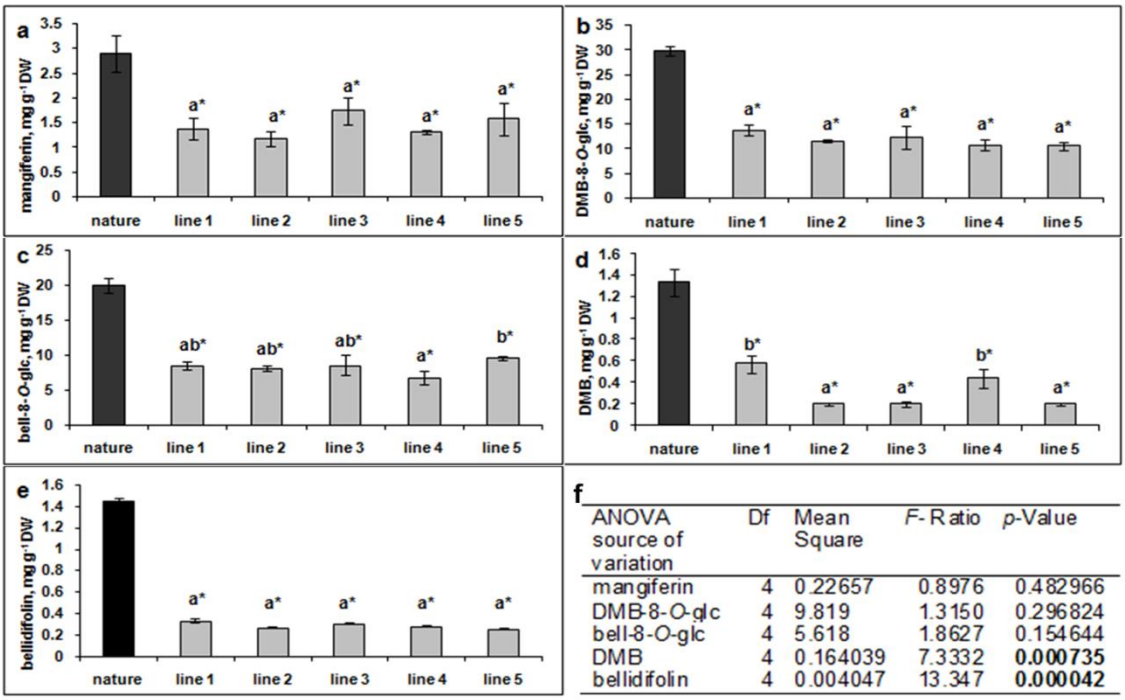
Table 3. Effect of pretreatments with IBA on rooting of *G. lutensces* line 5 shoots. After IBA tretment the shoots were cultivated on PGR-free liquid MS ½ medium with 2% sucrose and fresh medium was added every 7 days. Experiment was repeated 2 times with 15-26 shoots per treatment (n= 30-52). Within the each group of experiments (distinct colour) means followed by different letters in the column are significantly different according to Fisher's LSD test at $p \leq 0.05$. SE – Standard error.

IBA (mg l^{-1})	Auxin treatment + PGR-free liquid medium = duration of experiment (day)	Explants No.	Shoot with root primordia (%)	Rooting (%)	Roots per rooted explant ± SE	Length of the longest root (mm) ± SE
0.2	50 + 55 = 105	40	12.5	17.5	2.57 ± 0.84 a	7.29 ± 1.49 a
0.5	50 + 55 = 105	52	13.5	7.7	2.25 ± 0.48 a	8.75 ± 2.56 a
1.0	50 + 55 = 105	50	16.0	14.0	3.43 ± 0.95 a	5.86 ± 2.41 a
2.0	50 + 55 = 105	50	6.0	6.0	2.33 ± 0.33 a	10.51 ± 7.51 a
1.0	14 + 66 = 80	34	8.8	0	-	-
1.0	21 + 59 = 80	37	8.1	3.3	2.0 ± 0	2.0 ± 0
1.0	28 + 52 = 80	45	13.3	0	-	-
2.0	14 + 66 = 80	30	16.7	6.7	4.5 ± 0.5 a	14.0 ± 2.0 a
2.0	21 + 59 = 80	30	16.7	13.3	5.25 ± 1.1 a	13.75 ± 4.27 a
2.0	28 + 52 = 80	33	6.1	0	-	-
5.0	1 + 61 = 62	30	13.3	30.0	2.89 ± 0.5 a	12.4 ± 2.02 a
5.0	2 + 61 = 63	30	23.3	33.3	5.4 ± 1.1 b	11.2 ± 2.6 a
5.0	4 + 61 = 65	30	16.7	33.3	3.5 ± 0.6 ab	7.7 ± 1.23 a
5.0	6 + 59 = 65	30	10.0	16.7	2.4 ± 1.2 a	7.6 ± 1.21 a

2.3 HPLC analysis of secondary metabolites of G. lutescens cultured in vitro

HPLC analysis of methanol extracts obtained from five shoot lines of *G. lutescens* showed no significant differences between them in either qualitative or in quantitative xanthone composition. It can be noticed that all 5 lines produced a lower content of xanthones compared to the plant material collected from nature (Fig. 5). It was not surprising since the relatively low number of tissue cultured plants outpassed wild plants in the amounts of the secondary metabolites [45].

According to ANOVA, cytokinin BA necessary for shoot multiplication of *G. lutescens*, did not has a significant effect on the production of xanthones (Supplementary material, Fig. S1). However, a different effect of BA was reported in *G. bulgarica* shoots where the xanthones production was strongly affected by BA. Naimely, it has been shown that the content of xanthones linearly increased with increasing BA concentration in the medium [19]. Stimulatory effect of BA on the secondary metabolite production has been shown in shoot cultures of *G. austriaca* [18] and *Gentiana asclepiadea* [46] too.



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ANOVA source of variation	Df	Mean Square	*F*-Ratio	*p*-Value
mangiferin	4	0.22657	0.8976	0.482966
DMB-8-*O*-glc	4	9.819	1.3150	0.296824
bell-8-*O*-glc	4	5.618	1.8627	0.154644
DMB	4	0.164039	7.3332	**0.000735**
bellidifolin	4	0.004047	13.347	**0.000042**

Figure 5. The content of xanthones mangiferin (a), demethylbellidifolin-8-*O*-glucoside (DMB-8-*O*-glc) (b), bellidifolin-8-*O*-glucoside (bell-8-*O*-glc) (c), demethylbellidifolin (DMB) (d) bellidifolinin (e) in five shoot lines of *G. lutescens* cultured on BM+0.2 BA mg l^{-1} for 35 days. Wild *G. lutescens* herb grown in nature was used as a control. Values are the means ± SE of four to six biological replicates (n=4-6). Data of xanthones content in shoot lines were analyzed by one-way ANOVA (f). The values followed by different letters were significantly different according to Fisher's LSD test at $p \leq 0.05$; Asterisk (*) indicate a significant difference of values between *in vitro* shoot lines and wild plants samples according to Student's *t*-test at $p \leq 0.05$.

2.4 The effect of sucrose and sorbitol on shoot growth and xanthone production

According to ANOVA, increasing concentration of sucrose in the growth medium significantly affected shoot growth, dry matter, and flowering (Supplemntary material, Table S1). Thus, additional sucrose from 58.4 mM to 233.6 mM gradually decreased shoot fresh weight and growth index (Fig. 6 a,c). At the same time dry weight of the shoots was not significantly affected while dry matter percentage even significantly increased with sucrose concentration increasing (Fig. 6 b,d).

In plant tissue culture, sucrose serves as a carbon and energy source necessary for cell division and differentiation [47] and for regulation of osmotic potential [48]. In the present study, the highest shoot fresh weight (1600 mg) and the growth index (3.2) were recorded in control *G. lutescens* shoots grown at the lowest 58.4 mM sucrose. According to Grattapaglia and Machado (1998) [49], sugar concentrations lower or higher than 58.42 mM can cause chlorosis or explant deterioration, respectively, in in vitro cultures. The increased concentration of sucrose lead to the osmotic stress due to enhanced osmotic potential in the growth medium that inhibited shoots water and nutrients uptake from the medium [50], affecting shoots fresh weight and the growth index more significantly than dry weight. Osmotic potential may interfere with nutrient abortion by the cells, which is essential to growth and cell division in the aerial parts [51]. This can partly explain the observations of reduced *G. lutescens* shoots fresh weights with sucrose concentrations increasing above 58.4 mM (Fig. 6d). Increment of flowering (Fig. 7) from 0 to 1.4 flowers per Erlenmeyer flask with increasing sucrose concentration from 58.4 in control to 175.2 mM, respectively and then gradually decreased at 175.2 mM and 233.6 mM sucrose (1.2 and 0.4 flowers per Erlenmeyer flask, respectively) (data not shown) could be also stress related phenomenon [52].

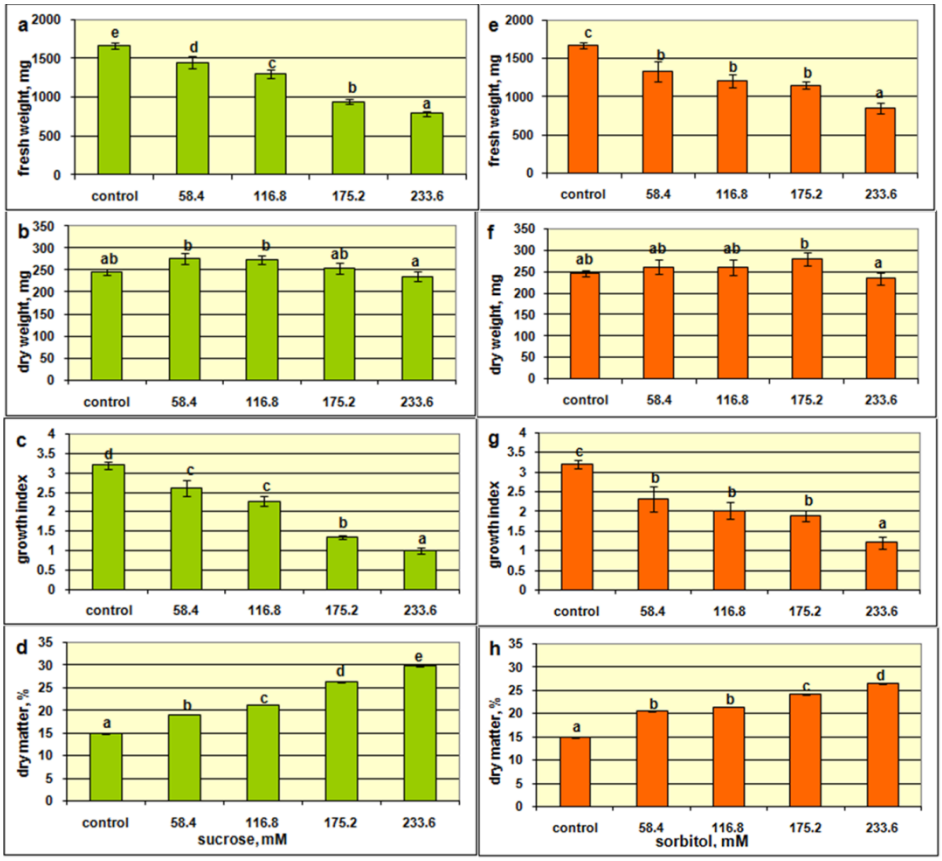

Figure 6. The effect of increasing concentrations (58.4, 161.8, 175.2, and 233.6 mM) of sucrose (a-d) and sorbitol (e-h,) on the growth of *G. lutescens* shoot cultures (line 5) after 35 days of culture. The increasing concentrations of sucrose or sorbitol were added into control medium (BM+0.2 mg l^{-1} BA containing 58.4 mM sucrose). Values are the means ± SE of eight to ten biological replicates (n=8-10). Data were analyzed by one-way ANOVA. Values followed by different letters are significantly different according to Fisher's LSD test at $p \leq 0.05$.

Additionally, the higher sucrose concentrations at 175.2 and 233.6 mM also caused colour change of *G. lutescens* shoots from green to yellow-browning along with stunted growth (Fig. 7). High sugar concentrations (45 and 60 g l^{-1}) was found to inhibit the growth of aerial plant parts [53], with a reduction of photosynthetic pigments content [54] compared to plants grown on medium without sucrose as was verified on *B. zebrina* shoots [55]. The reduction in chlorophyll content in *in vitro* plants may reduce photosynthetic ability by decreasing light absorption [56].

On the other hand, although the increased sucrose concentration in the medium above 58.4 mM was not optimal for *G. lutescens* shoot fresh weight and growth index, continuing increasing of dry matter percentage *G. lutescens* shoots over all higher ranges of sucrose tested was indicative (Fig. 6). A positive correlation between sucrose in the medium and dry matter content in *in vitro* plants has been reported previously. Increased availability of sugars in heterotrophic systems has been shown to increase cellulose synthesis, which was correlated to increase in dry weight [57]. Besides, sucrose cleavage in the medium results in glucose and fructose production. It may accelerate cell division and consequently increase the explant weight and volume [58].

Since numerous studies indicated sorbitol was the best carbon source for plant multiplication in many species [59,60] as well as to distinguishe nutritive effect of enhanced sucrose in the growth medium from its osmotic effect the influence of increased sorbitol concentrations was evaluated at the *G. lutescens* shoot cultures.

Results of the present study revealed analogical effect of increasing concentrations of sorbitol (58.4 mM – 233. 6 mM) with that of sucrose on *G. lutescens* shoot fresh weight, dry weight, and growth index (Fig. 6). Moreover, the effect of enhanced sorbitol on increased dry matter percentage (Fig. 6h) at almost identical range compared to that of sucrose (Fig. 6d) was observed. These findings strongly confirmed osmotic effect of sucrose on the *G. lutescens* and appointed 58.4 mM sucrose as optimal for shoots growth *in vitro*.

Figure 7. The effect of sucrose at increasing concentrations (58.4, 161.8, 175.2, and 233.6 mM) on the growth and flowering of shoot cultures of *G. lutescens* after 35 days of culture. The increasing concentrations of sucrose were added into control medium (BM+0.2 mg l^{-1} BA containing 58.4 mM sucrose). Arrows indicate floral bud development. Right – shoot with normally developed flower regenerated on the medium with 175.2 mM sucrose.

Regarding the effect of sucrose and sorbitol on the xanthone contents in *G. lutescens* shoots, the obtained results showed that increased sucrose concentration stimulated the production of xanthones, whereas the effect of sorbitol was weak or even apsent (Fig. 8., Supplementary material, Table S2). Namely, the level of either xanthone analysed significantly increased with increasing sucrose in the medium and the highest content of xanthones was recorded at the higher sucrose concentration applied. Thus, the content of mangiferin at 233.6 mM of sucrose was more than 3-fold higher compared to shoots cultured on the control medium containing 58.4 mM of sucrose. The highest sucrose concentration enhanced the production of xanthone glucosides DMB-8-*O*-glc and bell-8-*O*-glc for 2.8- and 1.8-times, respectively. The amount of aglycon bellidifolin increased 2.8-times, while the accumulation of DMB raised more than 4 times compared to the control.

The results presented in Fig. 8 (a-e) showed that the xanthone content linearly increased with increasing sucrose concentration up to 116.8 mM. With additional increase in sucrose at 175.2 and 233.6 mM, the content of each xanthone reaches its highest level. On the other hand, the content of xanthones in shoots cultured with sorbitol was mainly at the level recorded in the control shoots. Compared to the control, only the production of DMB-8-*O*-glc, DMB, and bellidifolin was slightly higher in shoots cultured with the lowest sorbitol concentration (Fig 8 g-j).

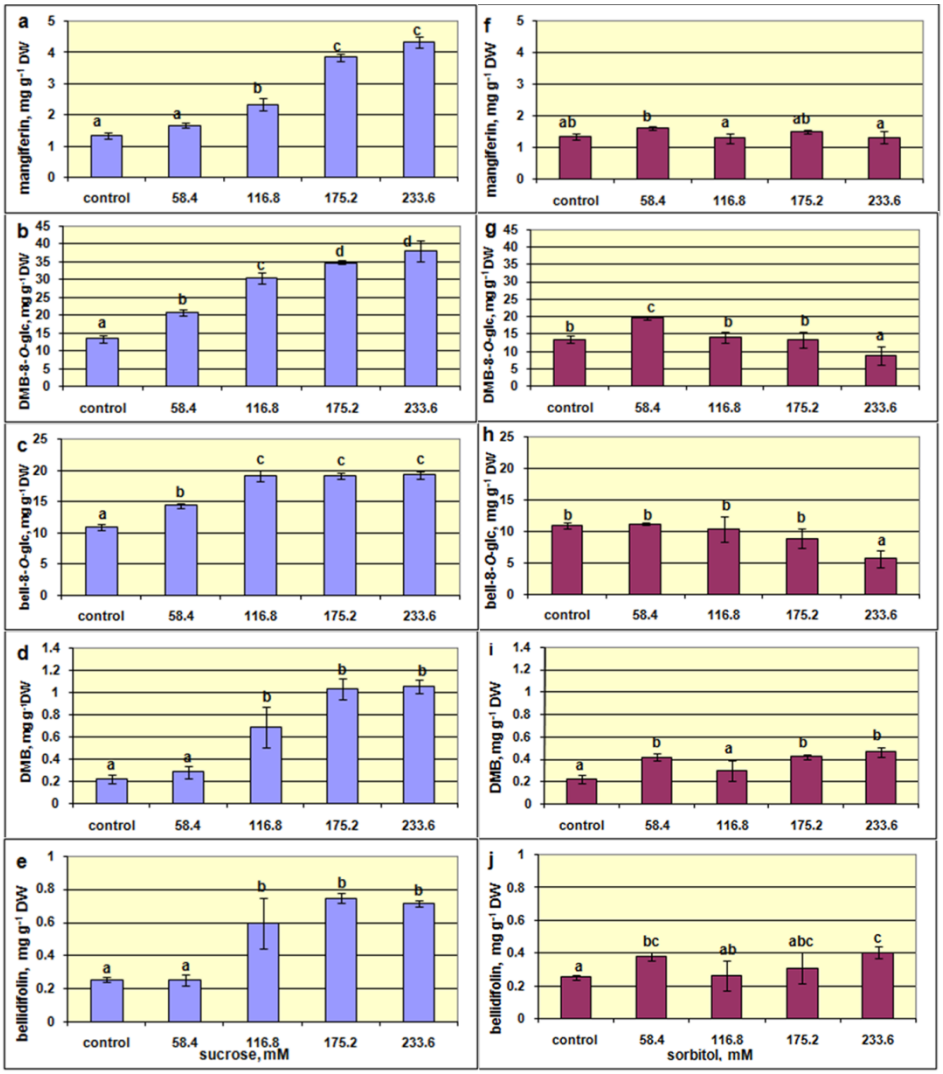

Figure 8. The effect of increasing concentrations (58.4, 161.8, 175.2, and 233.6 mM) of sucrose (a-e) and sorbitol (f-j) on the content of xanthones mangiferin, demethylbellidifolin-8-*O*-glucoside (DMB-8-*O*-glc), bellidifolin-8-*O*-glucoside (bell-8-*O*-glc), demethylbellidifolin (DMB) and bellidifolin in shoots cultures of *G. lutescens* after 35 days of culture. The increasing concentrations of sucrose or sorbitol were added into control medium (BM+0.2 mg l^{-1} BA containing 58.4 mM sucrose). Values are the means ± SE of four to eleven biological replicates (n=4-11). Values denoted by the same letter are not significantly different according to the Fisher's LSD test at $p \leq 0.05$ following one-way ANOVA.

These results indicate that enhanced accumulation of xanthones in the shoots caused by increased concentration of sucrose might be a consequence of the nutritional effect of sucrose. Numerous studies have shown that higher content of sucrose in the medium stimulated the production of useful secondary metabolites in plant cell cultures. Sucrose is considered to be one of the key sugars in plant life. In addition to its primary building role, sucrose is also an energy supplier for production of plant biomass. It is also involved in growth, development, storage, signaling, plant stress responses and various metabolic processes [61]. The abundance of sucrose in the culturing medium quite certainly affects and alters metabolic activities in the shoots of *G. lutescens*. The accretion of available source of carbon and energy directs metabolic pathways to formation more complex compounds with a large carbon skeleton such as xanthones. Further, xanthones may undergo hydroxylation reactions to give xanthone glycosides. As explained in our previous study on the root cultures of *Gentiana dinarica*, higher metabolic activities, due to the increase of

available carbohydrates sources as biochemical substrates, may direct xanthone biosynthesis to the enhanced production of xanthone glycosides [28].

The similar positive effect of increased sucrose in the medium on the xanthone content has been reported in *Centaurium erythraea* cultured *in vitro* [62]. The higher sucrose concentration was more favorable for accumulation of phenols, flavonoids, chlorogenic acid, and total hypericin in adventitious root cultures of *Hypericum perforatum* [63]. The roots of *Gentiana dinarica* produced higher content of xanthones when cultured on medium with more sucrose [28].

2.5 The effect of elicitors JA, MeJA and SA on shoot growth and xanthone production

In attempts to enhance xanthones production JA, MeJA and SA at increasing concentrations (100-500 μM) were applied in shoot culture of *G. lutescens*. Shoot growth and xanthones amounts were analyzed in shoots grown 7 days on elicitor containing medium following by 7 days on elicitor-free medium. Figure 9a shows that growth index of the shoots significantly increased after SA low concentration (100 μM) treatment and significantly decreased after treatment with MeJA at the highest one (500 μM), whereas shoot growth was not affected by JA at all concentrations (Fig. 9a, Supplementary material, Table S3). Positive effect of low concentration of SA on *G. lutescens* is not surprised. Generally low concentrations of applied SA promote plant growth under unfavorable conditions whereas high SA concentrations inhibit growth while threshold between low and high concentrations depend on plant species. Thus, SA exhibited growth-promoting (50 μM) and growth-inhibiting (250 μM) effect on *Matricaria chamomilla* seedlings [64].

SA, JA and MeJA, were reported to elicit a wide spectrum of phytochemicals in different plant species by inducing the expression of genes for various biosynthetic pathways. Thus MeJA was reported to stimulate rosmarinic acid accumulation in the cell cultures of *Satureja khuzistannica* [29] and anthocyanins in shoot cultures of *Prunus salicina* × *P. Persica* [31], while SA stimulated the production of hypericin and pseudohypericin in the cell cultures of *Hypericum perforatum* [32].

Changes in the xanthone content in shoot culture of *G. lutescens* after elicitation with JA, MeJA and SA are shown in Fig. 9b-f. These compounds contributed almost equally in enhanced accumulation of xanthones magniferin, DMB-8-*O*-glc, and bell-8-*O*-glc, while MeJA was superior for elicitation of xanthone aglycons DMB and bellidifolin (Fig. 9e,f). The content of xanthone glucosides mangiferin, DMB-8-*O*-glc, and bell-8-*O*-glc at all applied concentration of JA was almost 2-fold higher than in control shoots. However, with the increase of MeJA concentration, their production decreased to the level of non-elicited controls. When SA was applied for elicitation, increased concentration did not significantly affect the level of mangiferin, but reduced the content of DMB-8-*O*-glc and bell-8-*O*-glc.

In contrast to xanthone glucosides, the production of aglycones DMB and bellidifolin were not affected or only slightly enhanced upon treatment with lower concentrations of JA and SA. However, shoots elicited with the higher MeJA concentrations showed a significant increase in accumulation of both aglycones. The highest DMB and bellidifolin content (1.14 mg g^{-1} DW and 1.68 mg g^{-1} DW, respectively) was recorded at concentration of 500 μM MeJA which was 7.4 and 7.6 times higher than in the control shoots, respectively.

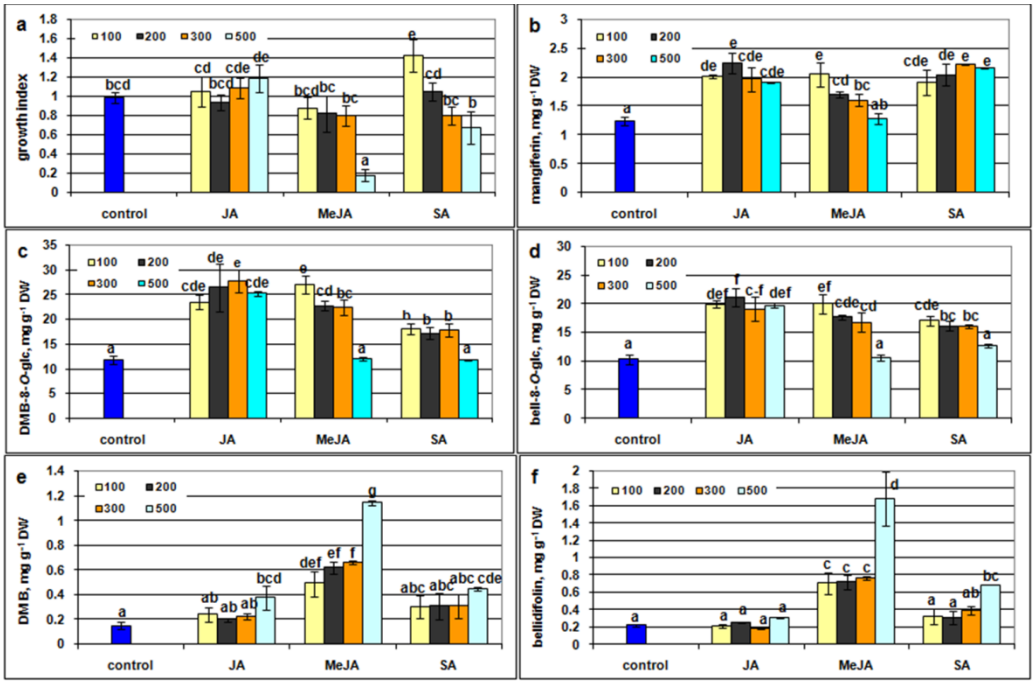

Figure 9. Effect of elicitors jasmonic acid (JA), methyl jasmonat (MeJA) and salicilic acid (SA) on growth index and xanthone production of *G. lutescens* shoot cultures. Growth index (a**)**, mangiferin (b), DMB-8-*O*-glc (c), bell-8-*O*-glc (d), DMB (e), and bellidifolin (f) content. Data represent means ± SE of four to seven biological replicates (n=4-7). 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.

JA has also been reported as an effective elicitor of secondary metabolite production in many plant species. Thus, enhanced production of oleanolic acid was obtained in cell suspension cultures of *Calendula officinalis* by elicitation with 100 μM JA for 72h [65], while supplementation of 50 μM JA on day 12 induced the highest anthraquinone content in cell suspension cultures of *Morinda elliptica* [66].

According to the findings exposed it can be concluded that the effectiveness of elicitation of secondary metabolites depends on several factors including the type of elicitor, the elicitor concentration, the time of exposure to elicitor treatment and the culture conditions [67].

3. Materials and Methods

3.1. Plant material

Gentianella lutescens plants at fruitful stage were collected in their native habitat on the south outskirts of the large mountain field Veliko Košlje at Povlen mountain, locality Razbojište (latitude 440 10' 3.43" north and longitudes 140 37' 33.474" east), the Republic of Serbia. Voucher specimen (Co. 6392113/04) is deposited in the Herbarium at the Natural History Museum, Belgrade.

3.2 In vitro seed germination and shoot culture initiation

Immature fruits harvested from collected plants were disinfected in 100 ml 20% (v/v) commercial bleach solution (4–5% NaOCl) with two drops of liquid detergent (Fairy®) for 25 min and then rinsed 3 times with sterile distilled water.

Immature seeds (1.5 mm) were isolated under stereomicroscope and germinated in Ø90 mm Petri dishes filled with 25 ml of germinating medium consisting of Murashige and Skoog's (MS) mineral salts [68], LS vitamins [69], and 0.64% (w/v) agar (Institute of Virology, Vaccines and SeraTorlak, Belgrade, Serbia). For shoot culture initiation the

epicotyl explants were isolated from five seedlings lines and placed onto shoot induction medium which is basal medium (BM) consisting of MS mineral salts, LS vitamins, 100 mg l^{-1} myo-inositol (Sigma-Aldrich), 58.4 mM sucrose with addition 0.2 mg l^{-1} BA. The medium was gelled with 0.64% (w/v) agar (Institute of Virology, Vaccines and SeraTorlak, Belgrade, Serbia). Individual epicotyl explant of each seedling line was cultured separately in 100-ml wide-neck Erlenmeyer flask with cotton-wool plugs. Obtained shoots were maintained on the same medium (BM + 0.2 mg l^{-1} BA) and subcultured every 5 weeks on fresh medium. For shoot multiplication 8-10 shoots of the same line were cultured in Erlenmeyer flask containing BM with addition of BA at increasing concentrations 0, 0.05, 0.1, 0.2, 0.5, 1.0, and 2.0 mg l^{-1} for 5 weeks and the number of propagated shoots were recorded. Multiplication index was calculated as main shoot + new axillary shoots (length ≥2 mm).

For rooting, solid ½ MS medium supplemented with 2% sucrose and 0.1-50.0 mg l^{-1} IBA (indole-3-butyric acid, Sigma-Aldrich), 0.1-2.0 mg l^{-1} NAA (1-naphthaleneacetic acid, Sigma-Aldrich) or 0.1-2.0 mg l^{-1} IAA (indole-3-acetic acid, Sigma-Aldrich) with or without GA3 0.1 mg l^{-1} (gibberelic acid, Duchefa) was used. For elongation of induced roots, the shoots were transferred into liquid or solid ½ MS medium with or without 1 g l^{-1} of active charcoal. Individual shoots were transferred in test tubes 20x150 mm with metal holders containing ≈15 ml of liquid medium. Lasting of the individual treatments depended on the concentration of auxin applied. Shoots were treated with high concentration IBA at 50.0 mg l^{-1} by 24 h, while treatments with lower IBA concentration of 5.0 mg l^{-1} lasting 1-8 days and with the lowest IBA at 0.1-2.0 mg l^{-1} 35-50 days. Two replicates with 15-26 shoots per rooting treatment were performed and the percentage of rooted shoots and root features were estimated.

All media pH was adjusted to 5.8 prior to autoclaving for 25 min at 114 °C. Cultures were grown at 25 ± 2 °C in a controlled environment room illuminated with cool-white Phillips fluorescent lamps providing 35–45 μmol m^{-2} s^{-1} under a 16-h (long day) photoperiod.

3.3. Increase in sucrose and sorbitol level

To examine the effect of enhanced osmotic pressure on the shoot growth and xanthone production, 400 mg of *G. lutescens* shoots were cultivated per one Erlenmeyer flask on BM + 0.2 mg l^{-1} BA wherein sucrose or sorbitol at increasing concentrations 58.4, 116.8, 175.2, 233.6 mM were added. Shoot growth was measured after 5 weeks of culture in terms of fresh weight and dry weight of shoots per Erlenmeyer flask. Growth index [(final fresh weight - initial fresh weight)/initial fresh weight], % dry matter [(dry weight/ final fresh weight) x 100], and accumulation of xanthones were determined for the harvested shoots.

3.4 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 shoots (400 mg weight) were isolated from 35-40 days old cultures and grown in the BM+0.2 mg l^{-1} BA supplemented with elicitors at the following final concentrations: 100, 200, 300, 500 μM. The treatment with elicitor lasted 7 days, and then the shoots were cultured on a control medium for another 7 days. Control shoots were cultured on BM+0.2 mg l^{-1} BA without elicitors. At the end of the experiment growth index [(final fresh weight - initial fresh weight)/initial fresh weight] and accumulation of xanthones were determined for the harvested shoots.

3.5 Xanthone extraction and HPLC conditions

Xanthone compounds extraction from dry powered plant material was performed as previously reported by Krstić-Milošević et al. (2020) [70]. Obtained extracts were filtered into volumetric flasks (10 ml) and adjusted to the volume with methanol. Prior to HPLC analysis, extracts were filtered through a nylon syringe filters (Captiva syringe filters, 0.45 mm, 13 mm, Agilent Technologies). Chromatographic analysis was carried out on Agilent series 1100 HPLC instrument, with a diode array detector, on a reverse phase Zorbax SB-C18 (Agilent) analytical column (150 mm x 4.6 mm i.d., 5 μm particle size) thermostated at 25 °C. The mobile phase consisted of two solvents: A (1% v/v solution of orthophosphoric acid in water) and B (acetonitrile) in the following gradient elution: 98-90% A 0–5 min, 90% A 5–13 min, 90-75% A 13–15 min, 75% A 15–18 min, 75-70% A 18–20 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 demethylbellidifolin-8-*O*-glucoside (DMB-8-*O*-glc), bellidifolin-8-*O*-glucoside (bell-8-*O*-glc), demethylbellidifolin (DMB) and bellidifolin were reported in previous study [40]. Mangiferin was purchased from Sigma-Aldrich (Steinheim, Germany). Quantification was performed using standardized calibration curves of xanthones. The content of xanthones in the samples was determined by calculation of peak area and expressed as milligrams per gram of dry weight.

3.6. Statistical analysis

All in vitro culture experiments were repeated at least 2–4 times with 15–40 shoots/explants per treatment. Biochemical analyses were repeated 4-11 times. The data were subjected to standard one-way analysis of variance (ANOVA) except the data related to the effects of elicotors where two-way ANOVA was applied. Percentage data were subjected to angular transformation before statistical analysis, followed by inverse transformation for presentation. Means and standard errors were calculated for numerical parameters and their differences was analyzed by t-test or Fisher's multiple range LSD test at $p \leq 0.05$ using the StatGraphics Plus software package for Windows 2.1 (Statistical Graphics Corp.,Rockville, MD, USA).

4. Conclusions

The present study reports the establishment of an *in vitro* shoot multiplication culture system from immature epicotyl explants of endangered *G. lutescens* as a promising source of important xanthones. The maximum shoot multiplication was obtained on ½ MS medium containing BA at 0.2 mg l^{-1} with 3.92 shoots per explant, 15.48 mm length of main shoot and

7.5% of vitrified shoots only. The sucrose concentration of 58.4 mM provided the highest shoot fresh weight, dry weight and growth index while sucrose and sorbitol applied at the highest concentration of 233.6 mM increased dry matter percentage 2-fold. The best rooting with a frequency 33% was achieved after shoots were initially treated with IBA 5.0 mg l^{-1} 2-4 days following by trasfer on liquid PGR-free ½ MS medium.

HPLC analysis of methanolic extracts of shoots revealed the presence of xanthones such as mangiferin, DMB-8-*O*-glc, bell-8-*O*-glc, DMB and bellidifolin. Increased sucrose in the culture medium of 233.6 mM enhanced production of all xanthones in in vitro propagated shoots for more than 2-fold. Abiotic elicitors JA, MeJA and SA at 100-300 μM increased accumulation of mangiferin, DMB-8-*O*-glc, bell-8-*O*-glc almost equally, while MeJA at the highest concentration of 500 μM enhanced 7-fold amount of aglycones BMD and bellidifolin compared to the control shoots. The results of the present study provides a first approach on the establishment of in vitro shoot culture of *G. lutescens* that can be used for the mass shoot propagation contributing to the conservation of this valuable

medicinal plant. This is also the first report of the composition of pharmacologically active xanhones in *G. lutescens* wild and tissue cultured shoots. Application of increased sucrose concentration and abiotic elicitors JA, MeJA and SA in *in vitro* shoot culture is a promising approach that can facilitate further research on large-scale shoot propagation and production of pharmacologically active xanthones.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1**, Figure S1:** The effect of increasing concentrations of BA (0-2 mg l^{-1}) on the content of xanthones mangiferin (a), demethylbellidifolin-8-*O*-glucoside (DMB-8-*O*-glc) (b), bellidifolin-8-*O*-glucoside (bell-8-*O*-glc) (c), demethylbellidifolin (DMB) (d), and bellidifolin (e) in shoots cultures of *G. lutescens* line 5 after 35 days of cultivation. Values are the means ± SE of six biological replicates (n=6). Data were analysed by one-way ANOVA (f). Values followed by different letters are significantly different according to Fisher's LSD test at $p \leq 0.05$. **Table S1:** The results of nested ANOVA for the effects of sucrose and sorbitol at increasing concentrations on the growth parameters (growth index, fresh and dry weight and % of dry matter) of shoot cultures of *G.lutescens* line 5. The bold values indicate statistically significant differences ($p \leq 0.05$). **Table S2:** The results of nested ANOVA for the effects of sucrose and sorbitol at increasing concentrations on the xanthones production in shoot cultures of *G.lutescens* line 5. The bold values indicate statistically significant differences ($p \leq 0.05$). **Table S3:** The results of nested ANOVA for the effects of elicitor type and concentrations on the shoot growth and xanthones production in shoot cultures of *G.lutescens* line 5. The bold values indicate statistically significant differences ($p \leq 0.05$).

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