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RESEARCH NOTE

In vitro culture type and elicitation affects secoiridoid and xanthone LC–ESI–TOF MS profile and production in *Centaurium erythraea*

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Abstract *Centaurium erythraea* is medicinal plant with high content of secoiridoid glycosides and xanthones with gastroprotective, hepatoprotective and antioxidative properties. In vitro culture based *C. erythraea* production would offer sustainable and economically feasible alternative to wild plant harvest provided that comparable productivity could be achieved in artificially propagated plants. To identify most productive *C. erythraea* system the secoiridoid glycoside and xanthone production and profiles using liquid chromatography-mass spectrometry were assessed in in vitro shoot, callus and cell suspension culture-derived material in comparison with wild *C. erythraea* plants. In vitro shoot culture was the most productive system for *C. erythraea* secoiridoid production (total yield 86 mg g^{-1} dw) and cell culture was the most productive system for phenolic compound production (19 mg GAE g^{-1} dw). The effect of elicitors yeast extract, methyljasmonate (MeJA) and chitosan on secondary metabolite yield indicated that elicitation with MeJA and chitosan significantly decreased yield of sweroside and eustomin, respectively. Significant quantitative differences of major secoiridoids and xanthones indicated that further methodological improvements for increasing yield of major secondary metabolites of in vitro origin need to be explored for in vitro cultivation to become a feasible alternative to *C. erythraea* wild plant harvest.

Keywords *Centaurium erythraea* · LC–ESI–TOF MS · Secoiridoids and xanthones · In vitro shoot culture · Callus culture · Cell suspension culture

Introduction

Centaurium erythraea Rafn. is a *Gentiana* family medicinal plant, traditionally used for treatment of gastrointestinal disorders, while its pharmacological potential is mainly associated with gastroprotective and hepatoprotective properties. In addition, it also exhibits antioxidative, antibacterial, antifungal, anti-diabetic and diuretic activity (reviewed by European Medicines Agency 2016). Pharmacological properties have mainly been attributed to high content of secoiridoid glycosides, iridoids and xanthones (Andrade et al. 1998; Aberham et al. 2011; Piatczak et al. 2011; Stefkov et al. 2014; Šiler 2012; Šiler et al. 2014).

Overharvesting, low ecological competitiveness and unfavorable habitat management adversely affects wild *C. erythraea* populations in certain European regions (Šiler et al. 2012), necessitating artificial propagation techniques (Piatczak et al. 2005a, b; Subotić et al. 2009). Successful in vitro propagation has been achieved by applying callus (Barešová et al. 1988), cell (Beerhues and Berger 1994), shoot (Piatczak et al. 2005a, b), leaf (Filipović et al. 2015; Simonović et al. 2015) and root (Subotić et al. 2009) cultures. However, the ability of different types of in vitro culture types to support production of major secoiridoids

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and xanthones in quantities comparable to wild plants is not known. Significant differences in chemical composition between wild, cultivated and in vitro material have been observed in many plant species including related *C. pulchellum* (Krstić et al. 2003). In vitro based production of secoiridoids in comparison to wild plants have previously been studied in shoot cultures only (Piatczak et al. 2005a, b), whereas the other studies of cell suspension and callus cultures have reported xanthone, but not secoiridoid production (Meravy 1987). Thus, integrated information regarding production of both secoiridoids and xanthones in response to different in vitro culture types is missing. Elicitation is successfully applied for enhanced production of secondary metabolites in many species (Murthy et al. 2014). Elicitation with methyljasmonate (MeJA) in related *Gentianaceae* species *Swertia mussotii* resulted in overexpression of geraniol 10-hydroxylase gene and concomitant accumulation of swartiamarin (Wang et al. 2010), while chitosan elicited xanthone accumulation in *Hypericum perforatum* root cultures (Valletta et al. 2016).

The objective of this study was to use LC–ESI–TOF MS (liquid chromatography electrospray ionization time of flight mass spectrometry) to compare amount of secoiridoid and xanthone metabolites, total phenolic content (TPC) and antiradical activity (ARA) in ethanol-aqueous extracts from three types of *C. erythraea* in vitro cultures and wild plants. In addition, the effect of elicitation with MeJA, yeast extract (YE) and chitosan was assessed in in vitro shoot cultures of *C. erythraea* for the first time.

Materials and methods

In vitro shoot culture was established from seeds of *C. erythraea* harvested in 2014 on the bank of river Ogre, Ogresgals municipality, Latvia. Seeds were surface sterilized using commercial bleach containing 5% sodium hypochlorite and germinated on ½ MS (Murashige and Skoog 1962) basal medium (agar 7.8 g L^{-1}, sucrose 30 g L^{-1}, no growth regulators, pH 6.5 adjusted before autoclaving at 121 °C for 20 min) in growth room with a 16 h photoperiod at 23 °C. After 1 week plantlets were transferred to a fresh full strength MS medium and in vitro shoots were cultivated as for germination. For LC–ESI–TOF MS, TPC and ARA analysis 6 week old in vitro grown plants were used.

Callus cultures were established from leaf fragments of 4 weeks old in vitro grown plants using MS basal medium supplemented with 1.5 mg L^{-1} 1-naphthaleneacetic acid (NAA), 1 mg L^{-1} 6-benzylaminopurine (BAP). Callus culture was subcultured every month and sampled for phytochemical analyses 14 days after subculturing.

Cell suspension cultures were derived from fourth passage of 2 weeks old calluses by suspending 2 g of calli in 20 ml of liquid MS basal medium supplemented with 1.5 mg L^{-1} NAA, 1 mg L^{-1} BAP. Cultures were maintained in 100 ml conical flasks in darkness at 25 °C on rotary shaker at 120 rpm and subcultured every 10 days. After two subcultures cells for phytochemical analyses were collected after 9 days by vacuum filtration.

Leaves from wild *C. erythraea* (identity confirmed by PhD Agnese Priede, president of Botanical society of Latvia) were collected during flowering in Tukums district, Latvia.

For elicitation experiments 4 week old in vitro *C. erythraea* plants were transferred to MS basal medium containing corresponding elicitor and no growth regulators. Elicitation details are provided in the Online resource 2. Leaves of elicitor treated plants were collected for phytochemical analyses after 14 days.

Additional materials and methods are described in Online resource 1.

Results and discussion

In vitro cultures for production of plant derived compounds are increasingly gaining industrial application, because of availability on demand, potential for technological process standardization, independence of seasonality, purity of the product and low environmental impact (Steingroewer et al. 2013). Secoiridoid glycosides and xanthones have been reported as characteristic secondary metabolites in *C. erythraea* (Aberham et al. 2011; Piatczak et al. 2011; Stefkov et al. 2014). In this study amount of secoiridoid glycosides and xanthones was assessed for the first time in *C. erythraea* in vitro shoot, cell suspension and callus cultures in comparison with wild plants. Comparison of the high resolution LC–ESI–TOF MS mass spectra with external standards and previously published data identified the five major peaks as secoiridoid glycosides, sweroside (Swer), swertiamarin (Swam) and gentiopicroside (Gent), and xanthones, eustomin (Eust) and demethyleustomin (Dmeust) (Online resource 2, Supplementary Table 1, Supplementary Fig. 1). LC–ESI–TOF MS profiles of cultures varied considerably among cultures and wild plant derived material (Online resource 2, Supplementary Fig. 2).

C. erythraea in vitro shoot culture extracts contained significantly lower amounts of Swam and Gent compared to wild plants, while presence of Swer was detected only in in vitro shoot culture and Eust was detected in all three in vitro cultures, but not in wild plants. Moreover, statistically significant differences between in vitro cultures

Table 1 LC–ESI–TOF MS-based comparison of major secondary metabolite amount in in vitro culture-derived *C. erythraea* plant material and wild plants

	Secondary metabolite amount (mg g^{-1} dw)				
	Swer	Swam	Gent	Eust	Dmeust
Wild plants	n.d.	220.05 ± 2.31	24.39 ± 0.53	n.d.	0.43 ± 0.03
In vitro shoots	1.93 ± 0.09	76.71 ± 4.40***[a,b]	5.96 ± 0.3***[a,b]	1.80 ± 0.06[a,b]	0.62 ± 0.04***[a]
Cell suspens.	n.d.	19.75 ± 0.10***[a,c]	0.93± 0.06***[a,c]	1.54 ± 0.04[a,c]	0.15 ± 0.01***[c]
Callus	n.d.	17.92 ± 0.06***[b,c]	1.68 ± 0.09***[b,c]	2.65 ± 0.06[b,c]	0.71 ± 0.06***[c]

Swer Sweroside, *Swam* Swertiamarin, *Gent* Gentiopicroside, *Eust* Eustomin, *Dmeust* Demethyleustomin. *n.d.* compound could not be determined

*** Statistically significant difference between wild plants and in vitro cultures (single factor ANOVA, $p < 0.001$)

[a] Statistically significant difference between in vitro shoot and cell suspension culture (single factor ANOVA, $p < 0.05$)

[b] Statistically significant difference between in vitro shoot and callus culture (single factor ANOVA, $p < 0.01$)

[c] Statistically significant difference between cell suspension and callus culture (single factor ANOVA, $p < 0.05$)

were found for amount of Swam, Gent, Eust and Dmeust (Table 1). Similarly, considerably lower yield of Swam, but significantly higher content of Swer was found in in vitro shoot culture compared to wild (Piatczak et al. 2005a, b) or greenhouse grown *C. erythraea* plants (Radović et al. 2013) suggesting that conditions of in vitro shoot cultivation might be favorable for production of Swer.

Although Swam and Gent were detectable in cell suspension and callus cultures, amount of compounds was considerably lower than in in vitro shoots and wild plants (Table 1). Lower content of target secondary metabolites in cell cultures has been reported, e.g., for *Hypericum perforatum* (Gadzovska et al. 2013). In vitro shoot and callus culture-derived material contained significantly higher amount of Eust and Dmeust in comparison to wild plants (Table 1) suggesting that in vitro culture conditions favor xanthone production. Increased production of xanthones in in vitro cultures compared to wild plants has been reported, e.g., in *C. pulchellum* (Krstić et al. 2003).

High xanthone and flavonoid content resulted in significant anti-radical activity in *C. erythraea* extracts (Sefi et al. 2011; Šiler et al. 2014). This study provides the first assessment of TPC and ARA in different *C. erythraea* in vitro cultures with cell suspension culture exhibiting similar amount of TPC and significantly higher ARA than wild plants, even though in vitro shoot and callus cultures contained significantly lower TPC (Fig. 1).

Elicitation is used to improve yield of phytochemicals in in vitro cultures (Murthy et al. 2014). We assessed amount of secoiridoids and xanthones in *C. erythraea* in vitro shoot culture treated with chitosan, YE and MeJA. Fresh weight, plant rosette size and leaf number in the same sample before and after elicitation was not affected (data not shown). Phytochemical analysis of elicited *C. erythraea*

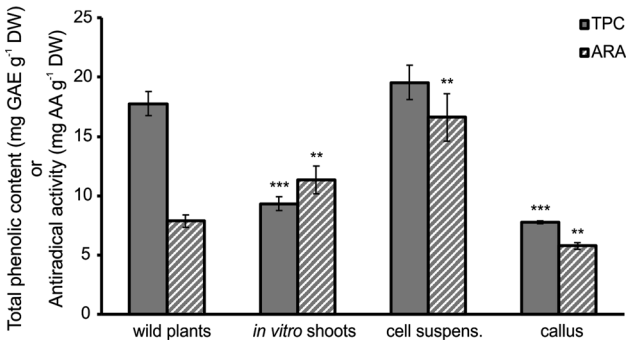

Fig. 1 Total phenolic content (TPC) and anti-radical activity (ARA) of aqueous-alcoholic extracts of different in vitro culture-derived and wild *Centaurium erythraea*. Data represent mean values of three biological replicates from three independent repetitions. *GAE* gallic acid equivalents, *AA* ascorbic acid equivalents. Statistically significant differences (single factor ANOVA) from wild plants is indicated by asterisks, $**p < 0.01$; $***p < 0.001$

shoot cultures revealed altered content and proportions of secoiridoids and xanthones (Table 2, Online resource 2, Supplementary Fig. 3). PCA clearly distinguished samples suggesting that different elicitors can trigger distinct changes in phytochemical profiles (Fig. 2). YE significantly reduced the amount of Swam, while chitosan reduced Eust (Table 2). Most pronounced effect was observed with MeJA, where amount of Swer was reduced in a dose dependent manner (Table 2). TPC and ARA were not significantly affected by elicitation (data not shown). MeJA (100 µM) and YE (2–16 g L^{-1}) can significantly enhance xanthone production in *C. erythraea* cell suspension cultures (Beerhues and Berger 1995). MeJA elicitation of secoiridoid production has not been reported in *C. erythraea*; however, elicitation of *Swertia mussotii* seedlings with 50 µM MeJA doubled the amount of Swam (Wang et al. 2010).

Table 2 Effect of elicitation on secondary metabolite amount in *C. erythraea* in vitro shoot cultures

	Secondary metabolite amount (mg g dw^{-1})				
	Swer	Swam	Gent	Eust	Dmeust
Control	5.62 ± 1.87	43.39 ± 5.01	7.82 ± 3.50	1.39 ± 1.00	1.12 ± 0.48
Chit 10 mg L^{-1}	5.62 ± 1.54	49.68 ± 6.80	6.32 ± 2.43	0.90 ± 0.37*	1.00 ± 0.12
Chit 20 mg L^{-1}	8.05 ± 2.00	62.01 ± 6.66*	11.48 ± 4.25	1.89 ± 1.41	1.88 ± 1.04
Chit 50 mg L^{-1}	4.60 ± 0.39	46.29 ± 2.41	5.82 ± 0.94	0.65 ± 0.11**	0.74 ± 0.10
YE 1500 mg L^{-1}	5.19 ± 0.20	41.95 ± 3.91	7.25 ± 1.00	0.57 ± 0.24	0.89 ± 0.10
YE 3000 mg L^{-1}	5.60 ± 0.57	35.36 ± 2.98*	6.85 ± 1.47	0.38 ± 0.12	0.63 ± 0.04
YE 4500 mg L^{-1}	4.22 ± 2.66	30.16 ± 3.13**	5.97 ± 1.67	0.54 ± 0.15	0.66 ± 0.08
MeJA 11 mg L^{-1}	1.81 ± 0.12***	55.44 ± 8.08	7.02 ± 1.73	0.70 ± 0.38	0.63 ± 0.04
MeJA 22 mg L^{-1}	0.57 ± 0.11***	73.84 ± 31.57	7.93 ± 3.04	0.68 ± 0.38	0.73 ± 0.07
MeJA 45 mg L^{-1}	0.39 ± 0.07***	49.27 ± 3.85	6.20 ± 0.11	1.15 ± 0.41	0.77 ± 0.26

Chit shrimp shell chitosan, *YE* purified yeast extract, *MeJA* methyl jasmonate

Statistically significant differences from control samples are indicated (single factor ANOVA), * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

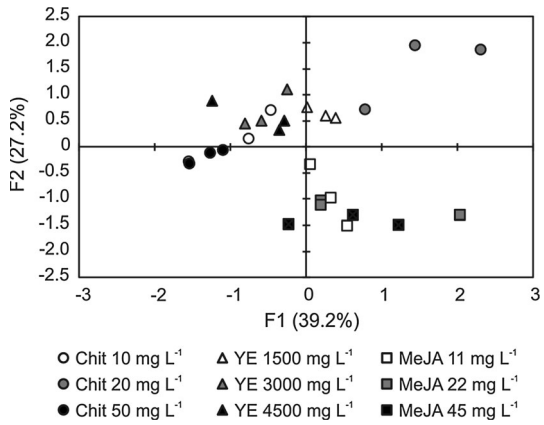

Fig. 2 Principal component analysis of secondary metabolite profiles from *Centaurium erythraea* in vitro shoot cultures elicited with different elicitors at indicated concentrations. *Chit* chitosan, *YE* yeast extract, *MeJA* methyl jasmonate

In conclusion, the use of LC–ESI–TOF MS for comparison of five major secondary metabolites in three different *C. erythraea* in vitro culture types with wild plants provided novel data on advantages of different culture types for production of secoiridoids and xanthones. Shoot culture was more appropriate for secoiridoid production due to higher Swam and Swer content, whereas cell culture could provide a solution for production of phenolics due to high xanthone yield and considerable ARA and TPC. Secoiridoid yield was compound-specific and depended on in vitro system applied; therefore, to match secoiridoid content in wild plants development of in vitro production system will require additional solutions, such as elicitation.

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