

In vitro production of flavonoids in cultures of *Gypsophila glomerata*

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Abstract

Effects of increased concentration of calcium chloride on growth and production of flavonoids in newly established shoot and callus *Gypsophila glomerata* cultures were studied. The highest impact of CaCl₂ on the growth index was determined in callus cultures (GI = 0.92), while in shoot cultures calcium treatment reduced the amount of biomass (GI = 0.38). Total flavonoids in shoot cultures grown on MS medium and MS medium supplemented with double amount of CaCl₂ were 0.36 mg/g d. w. In both callus cultures, 2 mg/g d. w. total flavonoids were quantified. Shoots and callus grown on non-modified media accumulated 0.02 mg/g d. w. quercetin derivatives. Unlike these, both shoots and callus grown on calcium-enriched media accumulated 0.03 and 0.05 mg/g d. w. of isorhamnetin derivatives. *In vitro* shoot cultures grown on MS medium enriched in twice the amount of CaCl₂ accumulated the highest amount of saponarin (0.138 mg/mg d. w.).

Keywords

Gypsophila glomerata, in vitro cultures, total flavonoid content, isorhamnetin, quercetin, saponarin

Introduction

The genus *Gypsophila* L. (Caryophyllaceae) comprises about 150 species of annual to perennial herbaceous plants distributed in the temperate regions of Asia, Europe, Africa, the Pacific Islands, and Australia. The plants are found in calcium-rich places that are high in gypsum, hence the name of the genus. In the Bulgarian flora, the genus *Gypsophila* is represented by six species: *G. paniculata* L., *G. muralis* L., *G. glomerata* Pall. ex M. Bieb., *G. trichotoma* Wend., *G. tekyra* Stef. and *G. petraea* (Baumg.) Reichenb (Valev 1966). *Gypsophila* species are known for their industrial, decorative, and medical applications. Some species are widely used in traditional medicine, such as expectorants, diuretics, contraceptives, for the treatment of hepatitis, gastritis, and bronchitis (Frechet et al. 1991; Jia et al. 2002; Yücekutlu and Bildacı 2008; Yao et al. 2010).

Representatives of the genus *Gypsophila* accumulate different groups of biologically active substances such as triterpene saponins, flavonoids, sterols, and volatile substances. Species of the genus *Gypsophila* have been relatively poorly studied in terms of flavonoids. Till 2014, a total of nineteen C-glycosylflavonoids of apigenin and luteolin, as well as a single flavonol glycoside with aglycone quercetin, were identified in the underground parts of the different *Gypsophila* species (Krasteva et al. 2008). Saponarin is one of the components of the flavonoid fraction in the genus *Gypsophila*. Some studies have shown its presence in *in vitro* grown cultures of *G. altissima*, *G. elegans*, and *G. trichotoma* (Zdraveva et al. 2015). The main flavonoids observed in *G. glomerata* with the highest purity and after HPLC analysis are orientin, homoorientin, vitexin, isovitexin, kaempferol-3-O-glycoside (astragalol) (Böttger et al. 2010), apigenin, quercetin, luteolin (Zheleva-Dimitrova et al. 2018).

The data on the biological activity of the compounds of the genus *Gypsophila* are due to the content of C-glycosylflavones, as well as saponarin. Zhang et al. 2011 investigated the antioxidant activity of flavonoids isolated from *G. elegans*. The effect of isoorientin-2"-O- α -L-arabinopyranosyl on alcohol-induced liver fibrosis in male Wistar rats was reported (Huang et al. 2012). The flavonoid had a positive effect and reduces liver damage, which is why it could be used not only to treat liver fibrosis but even cirrhosis. The hepatoprotective potential of saponarin isolated from *G. trichotoma* was studied using freshly isolated rat hepatocytes. Its effect was evaluated *in vitro/in vivo* under the action of paracetamol-induced liver damage. In freshly isolated hepatocytes, paracetamol (100 μ mol) significantly reduced cell viability. However, pre-treatment with saponarin (60–0.006 μ g/ml) significantly improved paracetamol-induced hepatotoxicity. The beneficial effect of saponarin was also observed *in vivo* in rats. Possible defense mechanisms may reduce the formation of free oxygen radicals by increasing the activity of antioxidant enzyme systems and the amount of endogenous antioxidant GSH. However, additional studies should be performed to evaluate the appropriate dosage for this phytochemical in case of liver damage in humans (Simeonova et al. 2013). *Gypsophila* could be considered a potential source of natural enzyme inhibitors for the treatment of diabetes. This is due to the antilucosidase effect of the flavonoid saponarin. This effect was confirmed after a study of 3 species of *Gypsophila* – *G. glomerata*, *G. trichotoma*, *G. perfoliata* by (Zheleva-Dimitrova et al. 2018).

In recent decades, studies have also been conducted on the conditions under which different species of *Gypsophila* can be cultured in *in vitro* cultures. Thus, the focus of the present study was to establish *in vitro* cultures of *G. glomerata* and to determine their flavonoid content.

Materials and methods

Plant material

G. glomerata seeds were obtained from the Madara plateau, Shumen, Bulgaria in August 2018. The seeds were sterilized under standard procedure (Ionkova et al. 2010) and after 30 days of cultivation at a constant temperature of 25 °C on DoH medium (Zdraveva et al. 2017), they were germinating. Shoots were established from seedlings on a standard solid MS culture medium (Murashige and Skoog 1962) and a modified MS medium supplemented with a double amount of CaCl₂ (0.88 g/L). Every four weeks shoot explants were transferred to a fresh medium. The amorphous callus tissue was formed when shoots were cultivated on G48 medium (Zdraveva et al. 2017) and G48 medium supplemented with a double amount of CaCl₂ (0.88 g/L). Calli were transferred every three weeks on a fresh medium. All the cultures were kept under sterile conditions in a light regimen (2000 lx).

Analysis of saponarin

For saponarin analysis, 200 mg of each *in vitro* culture were extracted individually twice with 80% MeOH (2 \times 4 mL) on a boiling water bath under reflux. The extracts were filtered through a pleated filter paper, transferred in a volumetric flask and the volume was adjusted to 10.0 mL with 80% MeOH. For total flavonoid determination, as well as for the differentiated flavonol content, a previously described method was used (Shkondrov et al. 2017).

An HPLC system Young Lin 9100 (Hogye-dong, Anyang, Korea), consisting of YL 9101 vacuum degasser, YL 9110 quaternary pump, YL 9131 column compartment, YL 9160 PDA detector, and 7725i manual injector was used. Clarity software (v. 2) was employed to perform calibration and to calculate results. Analysis of saponarin was performed on a pre-packed RP-C₁₈ column Luna (100Å, 250 \times 4.6 mm, 5 μ m, Phenomenex, USA), coupled with Security Guard ODS cartridge. The column temperature was 35 °C. Solvents were H₂O + 0.1% H₃PO₄ (A) and MeCN (B), filtered (0.45 μ m, PVDF) prior to use. The gradient program for saponarin determination was: initial 10% B; from 5 to 25 min 10% \rightarrow 100% B; from 25 to 28 min maintained at 100% B; from 28 to 30 min back to 10% B; all segments linear, with flow rate 1 mL/min. Separations were monitored at 330 nm. An aliquot (10 μ L) of each sample was injected and each injection was done in triplicate. Separations to determine total flavonoids and differentiated flavonol content were performed on a Purospher STAR column (RP C18, 4.6 \times 125 mm, 5 μ m, Merck, Darmstadt, Germany). The binary solvent system consisted of solvents A (H₂O + 0.1% H₃PO₄) and B (MeOH). The flow rate was 1.2 mL/min. The temperature was set at 35 °C. The used gradient program was performed as follows: initial 40% B; from 1 to 20 min 40% to 55% B, linear; from 20 to 21 min 55% to 100% B, linear; from 21 to 25 min 100% B. The injected volume was 10 μ L. All chromatograms were recorded at 370 nm. The results were expressed as mean \pm SD. Saponarin, rutin, isorhamnetin, and quercetin CRS, as well as acetonitrile, methanol, and *o*-phosphoric acid, were purchased from Sigma Aldrich (St. Louis, MO, USA). Rutin, isorhamnetin, and quercetin were treated as reported before (Shkondrov et al. 2017). For calibration purposes, saponarin CRS was diluted in MeOH to obtain a stock solution (1 mg/mL), which was serially diluted to obtain the standard solutions.

Results and discussion

In vitro cultivation

In vitro shoots and callus cultures of *G. glomerata* were successfully established. The highest growth index was determined in callus cultures, grown on G48 medium, supplemented with double amount of CaCl₂ (GI = 0.92), while the amount of biomass of the calli, grown on G48 medium was 0.83. The lowest growth index (GI = 0.38)

was detected in shoots, grown on MS medium supplemented with double amount of CaCl_2 . The amount of biomass of the shoot cultures, grown on MS medium was 0.53 (Suppl. material 1: Table S1 and Fig. 1).

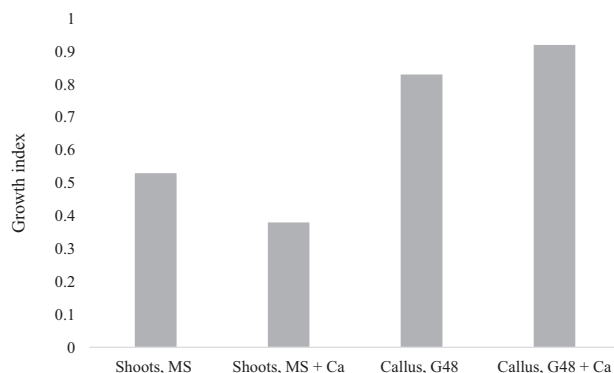


Figure 1. Growth index of *in vitro* cultures of *G. glomerata*.

Analysis of total flavonoids

Total flavonoids were detected in the obtained *in vitro* cultures by HPLC-UV. Shoots grown on MS and MS supplemented with an additional amount of CaCl_2 had a similar quantity of total flavonoids – 0.36 mg/mg d. w. and alternatively, calli both had nearly 2 mg/ mg d. w. The results are presented in Fig. 2 and Table 1 and expressed as flavonoid equivalents.

Callus cultures both produced total flavonoids, expressed as rutin in triple amount than the shoots.

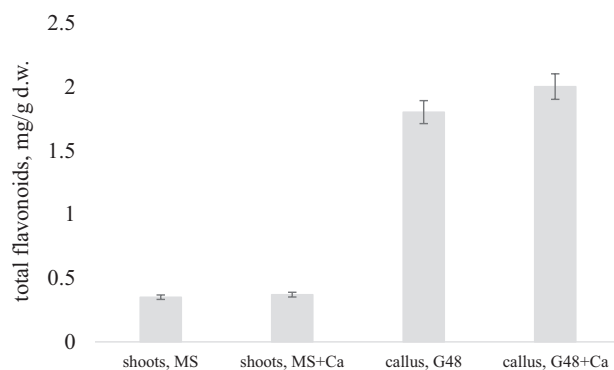


Figure 2. Total flavonoids in the cultures.

Table 1. Content of total flavonoids.

Culture	Total flavonoids, mg/g d. w.
Shoots, MS	0.351
Shoots, MS + Ca^{2+}	0.370
Callus, G48	1.801
Callus, G48 + Ca^{2+}	2.002

Analysis of quercetin and isorhamnetin derivatives

It was previously established that differences in flavonol content may occur amongst different *in vitro* cultures (Zdraveva et al. 2017). Both shoots and calli grown on me-

dia supplemented with Ca had a higher amount of isorhamnetin derivatives – 0.03 and 0.05 mg/g d. w. (Fig. 2 and Table 2).

Table 2. Content of isorhamnetin and quercetin derivatives.

Culture	Quercetin derivatives, mg/g dw	Isorhamnetin derivatives, mg/g dw
Shoots, MS	0.020	0.005
Shoots, MS + Ca^{2+}	0.010	0.030
Callus, G48	0.020	0.020
Callus, G48 + Ca^{2+}	0.010	0.050

This finding is rather different from the quercetin glycoside content – both non-supplemented with an additional amount of CaCl_2 shoots and calli had 0.02 mg/g d. w. quercetin derivatives. Although there is a slight chemical difference between quercetin and isorhamnetin, i.e. one methyl group, the quantity difference between cultures of these two flavonols was significant.

Quantitation of saponarin

The mean AUC of each calibration solution was used to construct the calibration curve. The equation was: $y = 19217.086x$ ($r^2 = 0.9955$). The validation of the method concerning saponarin was performed according to the approved ICH standards (ICH Guideline 2002). Reagent specificity was tested by a blank – no peaks were corresponding to retention time (t_R) of saponarin in the chromatogram obtained. Six saponarin-containing solutions were analyzed by three injections each. The standard deviation (SD) in absorption units (AU) and the relative SD (%) were found to be $\pm 1.0\%$. The limit of detection (LOD) was $0.0011 \text{ mg}\cdot\text{mL}^{-1}$ ($y_{\text{LOD}} = y_b + 3S_b$). The limit of quantification was $0.005 \text{ mg}\cdot\text{mL}^{-1}$ ($y_{\text{LOQ}} = y_b + 10S_b$). Linearity in the concentration range 0.005–0.9 mg/mL was studied; the correspondence between the peak area measured in AUC and concentrations in $\text{mg}\cdot\text{mL}^{-1}$ was proportional in the ranges with $r^2 > 0.99$. Identification of saponarin in the samples was performed based on retention time (t_R) compared to a standard saponarin solution, injected under the same conditions.

The results from the quantitative analysis of saponarin are presented in Table 3.

Table 3. Saponarin content in the samples.

Culture	Saponarin, mg/mg dw
Shoots, MS	0.042
Shoots, MS + Ca^{2+}	0.138
Callus, G48	not detected
Callus, G48 + Ca^{2+}	not detected

There were no peaks with retention time, similar to that of saponarin, in the chromatograms from callus grown on G48 medium without and with the addition of calcium. This means that saponarin was not produced in callus. The data from the analysis (Fig. 4) showed that CaCl_2 in the nutrient medium had a positive effect on saponarin accumulation in *in vitro* shoots.

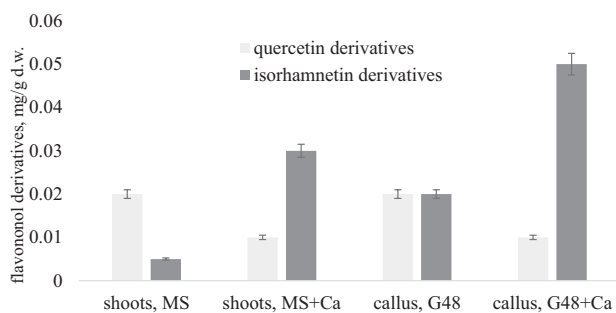


Figure 3. Differentiated content of flavonols.

The quantity of saponarin determined in samples obtained from shoots cultured on MS was 0.042 mg/mg d. w. After analysis of the chromatogram of the extract obtained from *in vitro* shoot culture, cultivated on MS supplemented with double amount of CaCl_2 , the content of saponarin (0.138 mg/mg dw) was higher compared to shoots on MS. This was probably due to the calcium content in the nutrient medium, which is an essential element in many biosynthetic pathways in halophyte-plants, like *Gypsophila*.

Conclusion

For the first time, we investigated the flavonoid content in *in vitro* cultures from *G. glomerata*.

The ability to manipulate flavonoid biosynthesis in plant species is rapidly gaining importance as new economically important uses these secondary metabolites emerged, incl. food quality and nutraceuticals. Culture productivity is critical to the practical application of cell culture technology to produce flavonoids. This study demonstrates that *in vitro* cultures of *G. glomerata* can produce important phenolic compounds such as quercetin and isorhamnetin

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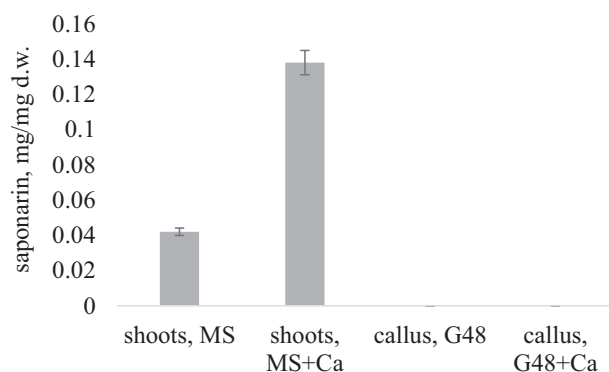


Figure 4. Saponarin content in the *in vitro* cultures.

derivatives, as well as saponarin. It is considered that calcium chloride treatment had a significant impact on the vitality of *in vitro* cultures, regulating both growth rates as well as secondary metabolism. Callus cultures produced total flavonoids, expressed as rutin in amounts triple than the shoots. Cell-culture-derived *G. glomerata* flavonoids can be more easily separated in an intact polymeric form than flavonoids within complex plant tissues. Our results show that *G. glomerata* can serve as an alternative way of production of flavonoids, particularly of C-glycosyl flavones, which is quite rare. In addition, differences in flavonoid content of the cultures established will serve not only as a basis for culturing media optimization but also as a leading point for *in vitro* cultivation of calciphylic taxa.

Acknowledgements

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Supplementary material 1

Table S1. Growth index of *in vitro* cultures of *Gypsophila glomerata*

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Data type: docx. file

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