Targeted metabolomic profiling reveals interspecific variation in the genus Hypericum in response to biotic elicitors

Miroslava Bálintová, Katarína Bruňáková, Linda Petijová, Eva Čellárová*

Department of Genetics, Institute of Biology and Ecology, Faculty of Science, Pavol Jozef Šafárik University in Košice, Mínskova 23, 04001, Košice, Slovakia

ARTICLE INFO

Keywords: Biotic stress Endophytes PCA Anthraquinones Flavonoids

ABSTRACT

Shoot cultures of eight Hypericum species belonging to the sections Hypericum, Oligostema, Ascyreia and Webbia were evaluated for their phytochemical profiles by high-performance liquid chromatography. In total, 17 secondary metabolites assigned to the groups of anthraquinones, phloroglucinols, hydroxycinnamic acids and flavonoids were detected. Furthermore, the elicitation potential of 18 biotic factors derived from saccharides, endophytic fungi and Agrobacterium rhizogenes was examined and statistically analysed with the paired two-sample t-test and principal component analysis. The production of naphthodianthrones and emodin was predominantly stimulated by elicitors derived from Fusarium oxysporum and Trichoderma crassum, while Piriformospora indica promoted the phloroglucinols production. Among flavonoids, the aglycone amentoflavone was readily increased by several elicitors up to 15.7-fold in H. humifusum treated by potato-dextrose broth. However, the chlorogenic acid proved to be the most susceptible metabolite to elicitation, when 31.7-times increase was detected in H. maralatum shoots upon D-glucose treatment. In spite of several biotic factors have been tested, no metabolite was commonly induced in all Hypericum spp. as a response to elicitor treatments.

1. Introduction

The genus Hypericum comprises more than 500 species that are categorised into 36 taxonomical sections based on morphological and molecular characteristics (Robson, 2016). The representatives of the most primitive sections Campylogenus, Ascyreia and Rascyna possess basal character states and other sections are derived from them (Robson, 1977). Among apomorphic characters belong glandular structures, namely translucent cavities and especially dark glands that are present in members of the advanced taxonomic sections. The dark glands evolved firstly in reproductive parts and later on in vegetative parts in species belonging to core Hypericum (Nürk et al., 2013).

Along with H. perforatum as the most studied and well known species, several other representatives of the genus Hypericum gain attention as a new source of valuable bioactive compounds (Napoli et al., 2018). Phytochemical investigations on the species from various sections (Kitanov, 2001; Crockett et al., 2005; Porzel et al., 2014; Zorzetto et al., 2015; Girak et al., 2016; Kucharíková et al., 2016a, 2016b; Nigutová et al., 2017; Yuk et al., 2017; Bertoli et al., 2018; Tocci et al., 2018) have revealed that these species are valuable sources of naphthodianthrones, phloroglucinols, phenolic acids, flavonoids, xanthones and several volatile constituents. Hypericins as photoactive compounds are studied for their ability of preferential accumulation in neoplastic tissues and production of reactive oxygen species upon light illumination with usage in photodiagnosis and photodynamic therapy (Jendšelovská et al., 2016). Emodin and its chemically derived analogues are able to inhibit proliferation of cancer cells and activate caspase cascade pathway (Wang W et al., 2012). The antidepressant activity of Hypericum extract is carried by phloroglucinols and flavonoids. While hyperforin blocks reuptake of the neurotransmitters by synaptic membranes (Jat, 2013), flavonoids inhibit monoamine oxidase A activity (Butterweck et al., 2000).

Taking into consideration, that the content of secondary metabolites significantly varies in wild-harvested and greenhouse grown plants (Kirakosyan et al., 2003), the production of bioactive compounds free from environmental influence can be achieved by alternative biotechnological approaches. Nowadays, the elicitation is one of the leading biotechnological tools to improve yield of secondary metabolites in in vitro systems. In the genus Hypericum, the elicitation potential of several abiotic and biotic factors has been evaluated (Shakya et al., 2017). Since desired secondary compounds of the genus are usually accumulated in aerial organs and are involved in defence responses to pathogen attacks, greater emphasis is on biotic elicitation. To date, effects of elicitors based on saccharides (Kirakosyan et al., 2006; Tocci...
et al., 2010, 2011; Brasili et al., 2014; Gadzovska-Simic et al., 2014, 2015a; Valletta et al., 2016), phytohormones (Liu et al., 2007; Pavlík et al., 2007; Coste et al., 2011), bacteria (Franklin et al., 2009; Tusevski et al., 2015) or pathogenic fungi (Sivrench and Gibson, 2002; Xu et al., 2005; Conceição et al., 2006; Gadzovska-Simic et al., 2012, 2015b; Azez and Ibrahim, 2013) have been evaluated, but no remarkable enhancement of secondary metabolites has been achieved so far.

The aim of this work was to evaluate the effects of 18 biotic elicitors derived from D-glucose, soluble starch, potato-dextrose broth, chitosan, Thielavia subthermophila, Fusarium oxysporum, Trichoderma crassum, Piriformospora indica, Agrobacterium rhizogenes and nutrient broth no. 2 on secondary metabolism of eight Hypericum species. Based on the phytochemical profiling targeted on 23 metabolites including the major classes of phenolic compounds – the anthraquinones, phloroglucinols and flavonoids, we have further focused on: i) evaluation of the changes in metabolite profiles induced by biotic elicitors, ii) identification of the metabolites that are commonly induced upon the elicitor treatment, and iii) assessment of the treatment mostly affecting the biosynthesis of valuable secondary metabolites. We hypothesise that biotic treatments will induce complex metabolite alterations distinguishable from inherent interspecific variability between Hypericum spp.

2. Material and methods

2.1. Plant material and culture conditions

In vitro grown plants derived from stock cultures of 8 Hypericum species (H. perforatum L., H. maculatum Grantz, H. tetrapertetum Fr., H. erectum Thunb., H. humifusum L., H. monogynum L., H. kouytchense H. Lév., H. canariense L.) were used in this study. The untreated (control) and experimental shoots were cultivated in liquid MS media (Duchefa Biochemie, Netherlands) containing mineral salts according to Murashige and Skoog (1962) with Gamborg’s B5 vitamins (Gamborg et al., 1968), 30 g l\(^{-1}\) sucrose (CentralChem, Slovakia) and 2 mg l\(^{-1}\) glycine with pH adjusted to 5.65 before autoclaving. Each plant growth vessel contained 10 shoots growing in the 20 ml of liquid MS medium supported by glass beads with the diameter of 5 mm. The cultures were grown at 23 ± 2 °C temperature under 16/8 h photoperiod at 90 µmol m\(^{-2}\) s\(^{-1}\) artificial irradiance for 28 days.

2.2. Elicitation treatment

2.2.1. D-glucose & polysaccharides

The shoots were elicited with rich potato-dextrose broth (PDB) commonly used for fungal cultivation. The volume of 1 ml of sterilised PDB (BD, France; composition per litre: 4 g starch, 20 g dextrose) was supplemented into MS media. Additionally, 1 ml of 4 g l\(^{-1}\) soluble starch (Sigma Aldrich, Germany) or 1 ml of 20 g l\(^{-1}\) D-glucose (CentralChem, Slovakia) were added into the MS media to distinguish between the components of PDB which may have elicitation potential. Besides, the shoots were elicited using sterilised chitosan (Sigma Aldrich, Iceland) dissolved in 1% acetic acid with pH adjusted to 5.6 before autoclaving. The experiment started by addition of chitosan to final concentration of 10 mg l\(^{-1}\), 1 mg l\(^{-1}\) or 0.01 mg l\(^{-1}\) media.

2.2.2. Endophytic fungi

In this work, the sterilised hyphae derived from axenic cultures of 4 different fungi were used for elicitation. Thielavia subthermophila was purchased from Leibnitz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, accession number DSM-21024. Piriformospora indica was kindly provided by Dr. Ahmad Mazen (Soah University; Egypt). Fusarium oxysporum and Trichoderma crassum were isolated from H. perforatum grown in vitro and subsequently adapted to outdoor conditions according to the method described by Rusari et al. (2008). The fungal DNA was isolated by E.Z.N.A. Fungal DNA Mini Kit (Omega Bio-tek, USA) according to the manufacturer’s protocol. The identification of all fungi was assessed by sequencing of ITS (internal transcribed spacer) region using ITS4 (5′-TCTTCCGCTATTGATA TGC-3′) and ITS5 (5′-GGAATTAAGTCGTTAACAAGG-3′) primers (White et al., 1990). The elicitors were prepared in accordance with Yu et al. (2001) and Wang et al. (2012) with some modifications. The fungi were cultivated in PDB on orbital shaker Biosan OS-10 (Latvia) at 130 rpm and 28 °C in the darkness for 3 (P. indica), 4 (T. crassum), 5 (T. subthermophila) or 6 weeks (F. oxysporum) depending on growth rate of the fungal isolate. Afterwards, the mycelia were filtered, grounded with mortar and pestle, and diluted 10-times with distilled water. The mixture was then sterilised and 1 ml was added to each shoot culture for treatment. In the co-culture experiments, chitosan (0.01 mg l\(^{-1}\)) was added to cultures after 14 days of pre-treatment with elicitors derived from fungi and cultivated for next 14 days.

2.2.3. Agrobacterium rhizogenes & nutrient broth no. 2

The agropine wild-type strains of Agrobacterium rhizogenes, ATCC 15834 and A4, used in this study were obtained from American Type Culture Collections. The single cell colonies were established on nutrient agar no. 2 (Imuna Pharm, Slovakia). The presence or absence of Ri plasmid was determined by the colony PCR (polymerase chain reaction) amplification of virC and rolC genes. The primers used for the amplification of virC gene (5′-AAATGGTCTTCCTCGTGAT-3′ and 5′-AACCGGACACTAAACCGGAT-3′) were selected according to Komarovská et al. (2009) and primers for the amplification of rolC gene (5′-AAATGGCAAGTAGGCGCTCG-3′ and 5′-TACGTGACCTGGCGGAT-3′) were chosen according to Di Guardo et al. (2003). The single cell colonies were cultivated overnight in nutrient broth no. 2 (Imuna Pharm, Slovakia) and afterwards the optical density at 600 nm was evaluated. When the liquid cultures reached the final concentration of 2 × 10^6 cells ml\(^{-1}\), they were inactivated by sterilisation in the autoclave for 30 min. 1 ml of resultant suspension or sterilised cell-free nutrient broth no. 2 (composition per litre: 5 g beef extract, 11 g peptone for microbiology, 6 g nutrient base, 3 g sodium chloride) were added to MS media containing Hypericum plants at the beginning of experiment.

2.3. Determination of secondary metabolite content

The content of secondary metabolites was analysed by high-performance liquid chromatography (HPLC) using the Agilent 1260 HPLC system (Agilent Technologies, USA) equipped with DAD (diode array detector) and UV–Vis (ultraviolet–visible) lamp.

The content of anthraquinones and phloroglucinols was assessed according to protocol proposed by Tolonen et al. (2003) and further modified as described by Brífáková and Cellárová (2016). Each sample consisted of 50 mg of air dried shoots homogenised at TissueLyser II Qiagen (Germany). The powdered material was extracted with 1.5 ml of the mixture consisting of HPLC grade methanol (Sigma Aldrich, Germany): Uvasol ethanol (Merck, Germany): HPLC grade acetone (Sigma Aldrich, United Kingdom) (1/1/1, v/v/v) for 30 min in ultrasonic bath PS04000A Ultrasonic Compact Cleaner 4L Powersonic (Slovakia) at 25 °C followed by centrifugation in centrifuge U-32R Boeco (Germany) at 14,000 rpm and 20 °C for 20 min. The resultant supernatant was separated by Agilent Poroshell 120 EC-C18 3.0 × 50 mm 2.7 µm column (Agilent Technologies, USA) heated to 40 °C with the injection volume of 10 µl. The mobile phase consisted of gradient of Solvanal acetonitril (ACN; CentralChem, Slovakia) mixed from phase A, 10% ACN with pH adjusted to 2.7 by trifluoroacetic acid (Sigma Aldrich, USA), and from phase B, 100% ACN, applied at flow rate of 1.3 ml min\(^{-1}\). The starting ratio of phases was 80:20 (A:B) gradually changing to 20:80 in 8.5 min and to 0:100 in 9.5 min, thereafter returning to 80:20 in 16.7 min and held at this composition for 3.3 min giving the total analysis time of 20 min per sample.

The content of phenolic acids (chlorogenic, caffeic, ferulic, cinnamic and gallic acid) and flavonoids (naringenin, apigenin, amentoflavone,
kaempferol, kaempferol-3-O-glucoside, catechin, quercetin, rutin, hyperoside, quercitrin and isoquercetin) was assessed as follows: 50 mg of dried and homogenised plant material was extracted by 1.5 ml of 70% HPLC grade methanol and thereafter separated by Kinex C18 100 Å 150 × 4.6 mm 5 μm column (Phenomenex, USA) heated to 30 °C. The mobile phase was mixed from phase A, 5% ACN (pH 2.7), and phase B, 80% ACN (pH 2.7), applied at flow rate of 0.9 ml min⁻¹. The gradient started at 100:0 (A:B) changing through 70:30 in 25 min to 0:100 in 30 min and held for 5 min followed by returning to 100:0 in 40 min.

The identification of the analysed substances was ascertained by comparing the absorption spectra and retention times of each peak with those of the respective standards of hypericin (AppliChem, Germany), emodin, ferulic acid, cinnamic acid (Sigma Aldrich, China), hyperforin, apigenin, quercetin and naringenin (Sigma Aldrich, USA), caffeic acid, (+) – catechin (Sigma Aldrich, Germany), chlorogenic acid, gallic acid (Sigma Aldrich, Switzerland), kaempferol-3-O-glucoside (Roth, Germany), kaempferol, rutin, hyperoside, isoquercetin, quercetin and amentoflavone (Extrasythene, France). The amount of investigated phytochemicals was determined according to the calibration curves of the reference standards. The emodin was analysed at 440 nm, (+) – catechin and naringenin were identified at 229 nm, and phenolic acids and flavonoids were detected at 254 nm according to their sub-sistent standards. The peaks for hypericin, pseudohypericin and their protomers were detected at 590 nm and quantified according to hypericin calibration curve. Similarly, the phloroglucinols were analysed at 270 nm and calculated according to hyperforin standard. The content of all secondary metabolites in this manuscript is denoted in mg g⁻¹ DW (dry weight).

2.4. Statistical analysis

The experimental data were evaluated by three statistical approaches. The paired two-sample t-test performed with the SPSS 16.0 statistical software program (Statistical Package for the Social Sciences version 16.0; IBM Corporation, USA) was used to assess differences in content of metabolites in the control and experimentally influenced shoots. The principal component analysis (PCA), performed with the STATISTICA version 7.0 package software (StatSoft, USA), was exploited to reveal interspecific variability under given experimental conditions. The principal components (PCs) represent the axes, which are the orthogonal projections for the values representing the highest possible variability, in this case at least 70%. The hierarchical cluster analysis (HCA; STATISTICA 7) with complete-linkage clustering method was used to assess intraspecific variability of chemometric data caused by elicitation treatment. All statistical tests were considered as significant at p ≤ 0.05.

3. Results

3.1. Metabolic profiles of the untreated Hypericum spp.

The metabolic analysis using HPLC involved the quantification of 23 targeted compounds belonging to four subgroups of phenolics – anthraquinones, phloroglucinols, phenolic acids and flavonoids present in eight Hypericum species involved in the study. According to Robson’s classification (Robson, 1977 onwards), these species belong to four taxonomical sections, namely Hypericum (H. perforatum, H. maculatum, H. tetrapterum, H. erectum), Oligostema (H. humifusum), Ascyreia (H. kouytchense, H. monogynum) and Webbia (H. canariense).

Among the studied species, H. perforatum, H. maculatum, H. tetrapterum and H. erectum from the section Hypericum and H. humifusum (section Oligostema) accumulated anthraquinones represented by emodin and naphthodianthrones, namely hypericin, pseudohypericin and their protoforms. The maximum content of naphthodianthrones was detected in H. humifusum reaching 4.3 mg g⁻¹ DW (Table 1). Regardless the species, pseudohypericin was the prevalent anthraquinone whose content exceeded that of hypericin 2- to 12-times.

Four phloroglucinol derivatives with identical absorption spectra as hyperforin but distinct retention times were detected. Hyperforin and phloroglucinol derivatives were identified in the representatives of all sections. H. perforatum, H. humifusum, H. kouytchense, H. monogynum and H. canariense differed in the spectrum and quantity of these metabolites. Reaching above 27 mg g⁻¹ DW in H. perforatum, hyperforin was concurrently the major phloroglucinol and phenolic compound quantified in the shoot cultures of all species involved in the study (Table 1).

Among phenolic acids, the potential of Hypericum species to accumulate hydroxybenzoic acids was evaluated. Although chlorogenic acid was present in all species, cinnamic, caffeic, ferulic and gallic acids were not detected. The content of chlorogenic acid ranged from 0.15 mg g⁻¹ DW in H. maculatum to 9 mg g⁻¹ DW in H. erectum and H. humifusum shoot cultures (Table 1).

The flavonoid composition of the eight species revealed variation in the spectrum and abundance of metabolites belonging to several subgroups of flavonoids, namely the flavanones (naringenin), flavones (apigenin) and biflavones (amentoflavone), flavonols (quercetin, kaempferol and theirs glycosides) and flavan-3-ols (catechin). The flavanone naringenin was detected only in H. perforatum in spite of its position as precursor in biosynthetic pathway leading to apigenin, kaempferol and quercitin. The flavone apigenin was not detected, but its biflavone amentoflavone was present in H. tetrapterum, H. erectum and in the highest amount in H. humifusum. Similarly, the presence of flavonol kaempferol can only be predicted by detection of kaempferol-3-O-glucoside in the shoots of H. perforatum, H. tetrapterum, H. erectum, H. monogynum and H. kouytchense. Despite the aglycone quercetin was solely found in H. erectum of the section Hypericum, its glycosidic forms, rutin (quercetin-3-rutinoside), hyperoside (quercetin-3-D-galactoside), isoquercetin (quercetin-3-glucoside) and quercitrin (quercetin-3-4-rhamnoside), were detected in almost all species. The flavanol catechin was present in all species with the highest content in H. erectum and H. canariense (Table 1).

Interspecific differences in the metabolic profiles of the untreated plants were evaluated by the hierarchical cluster analysis (HCA) and the principal component analysis (PCA). The first two principal components (PCs), PC1 and PC2, accounted for 73.84% of the total variability and therefore were used to visualise the interspecific variability of the untreated plants (Fig. 1). The 17 chemotaxonomy markers (Table 1) allowed grouping of almost all species to separate clusters, besides H. tetrapterum and H. erectum, both from section Hypericum, which formed an overlapping cluster. The slight differences in metabolite fingerprints of H. maculatum, H. humifusum, H. monogynum and H. canariense shoots resulted in formation of less compact clusters in comparison to the rest of species.

3.2. Changes in the metabolic profiles of Hypericum spp. induced by elicitor treatment

The elicitation potential of several biotic factors, including glucose, starch, chitosan, elicitors derived from fungi and Agrobacterium rhizogenes, were evaluated after co-culture with in vitro shoot cultures of Hypericum species. In response to elicitation treatments, the paired two-sample t-tests revealed numerous significant changes in the content of 17 metabolites belonging to anthraquinones, phloroglucinols, phenolic acids and flavonoids (Table 2). Therefore the PCA was applied to distinguish between the natural variability among the studied Hypericum species in the metabolite profiles of the elicitor-untreated plants and the metabolite alterations caused by the elicitors applied to a particular species. The first two principal components (PCs) had the greatest eigenvalues and explained at least 70% of total variability of each treatment. Containing the chemically relevant variability, PC1 and PC2 were used for projection of the treatments to factor-planes. While an individual species was represented by a single cluster at PCA projection,
Table 1
Phenolic compounds [mg g⁻¹ DW] in the untreated Hypericum species.

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>H. perforatum</th>
<th>H. maculatum</th>
<th>H. tetrapertum</th>
<th>H. erectum</th>
<th>H. humifusum</th>
<th>H. monogynum</th>
<th>H. kouytkense</th>
<th>H. canariense</th>
</tr>
</thead>
<tbody>
<tr>
<td>Emodin</td>
<td>0.058 ± 0.021</td>
<td>0.011 ± 0.003</td>
<td>0.046 ± 0.004</td>
<td>0.045 ± 0.006</td>
<td>0.294 ± 0.047</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Pseudohypericin</td>
<td>1.102 ± 0.368</td>
<td>0.531 ± 0.118</td>
<td>1.280 ± 0.346</td>
<td>0.492 ± 0.069</td>
<td>3.386 ± 0.448</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Hypericin</td>
<td>0.179 ± 0.089</td>
<td>0.043 ± 0.014</td>
<td>0.452 ± 0.163</td>
<td>0.272 ± 0.043</td>
<td>0.812 ± 0.251</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Phloroglucinol</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Phloroglucinol (RT = 9.2 min)</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>6.647 ± 1.763</td>
<td>nd</td>
<td>nd</td>
<td>4.487 ± 2.272</td>
<td></td>
</tr>
<tr>
<td>Hyperforin</td>
<td>27.552 ± 4.664</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>1.679 ± 0.250</td>
<td>3.605 ± 0.876</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Phloroglucinol (RT = 10.1 min)</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>5.963 ± 1.986</td>
<td>nd</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chlorogenic acid</td>
<td>0.366 ± 0.263</td>
<td>0.152 ± 0.078</td>
<td>3.726 ± 0.694</td>
<td>9.185 ± 0.833</td>
<td>9.751 ± 4.166</td>
<td>1.557 ± 0.893</td>
<td>0.977 ± 0.605</td>
<td>4.200 ± 0.647</td>
</tr>
<tr>
<td>Rutin</td>
<td>0.905 ± 0.077</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Amentoflavone</td>
<td>nd</td>
<td>nd</td>
<td>0.122 ± 0.045</td>
<td>0.020 ± 0.031</td>
<td>0.351 ± 0.399</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Quecetin</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>0.037 ± 0.057</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Quercetin</td>
<td>0.507 ± 0.218</td>
<td>0.352 ± 0.071</td>
<td>0.552 ± 0.071</td>
<td>0.754 ± 0.261</td>
<td>0.450 ± 0.046</td>
<td>0.107 ± 0.034</td>
<td>0.331 ± 0.073</td>
<td>0.66 ± 0.011</td>
</tr>
<tr>
<td>Kaempferol-3-O-glucoside</td>
<td>0.050 ± 0.033</td>
<td>nd</td>
<td>0.075 ± 0.018</td>
<td>0.261 ± 0.010</td>
<td>0.067 ± 0.032</td>
<td>0.371 ± 0.098</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Catechin</td>
<td>0.116 ± 0.027</td>
<td>0.135 ± 0.061</td>
<td>0.297 ± 0.185</td>
<td>1.694 ± 0.254</td>
<td>0.161 ± 0.040</td>
<td>0.206 ± 0.085</td>
<td>0.271 ± 0.143</td>
<td>1.886 ± 0.295</td>
</tr>
</tbody>
</table>

Data are the mean ± standard deviation (SD) of three biological replicates; nd – not detected.

Metabolites: k-3-O-glc: kaempferol-3-O-glucoside; chlor. acid: chlorogenic acid; phlorogluc.: phloroglucinols; amentofl.: amento-flavone; isoquer.: isoqueretin; non-sig.: non-significant increase.

3.2.1. D-glucose & polysaccharides

The stimulatory effects of saccharides on the biosynthesis of secondary compounds were studied by addition of monosaccharide D-glucose (elicitor Glc) and its polymer - soluble starch (elicitor starch) into the culture media. Furthermore, combined effect of Glc and starch elicitors was determined by co-culture of shoots of Hypericum spp. in MS media with potato-dextrose broth (elicitor PDB).

The PCA (Fig. 2) showed the compact clusters of H. perforatum, H. tetrapertum and H. erectum suggesting that the effects of saccharides on the accumulation of secondary compounds were not cumulative or caused no significant metabolite alterations. On the other hand, H. monogynum cluster was clearly divided into four subgroups referring to each treatment and control. H. kouytkense and H. canariense formed two subgroups representing the metabolite profiles of starch and Glc treated plants detached from those of PDB treated and control plants. While the metabolite profiles of Glc treated plants created a clearly isolated subgroup in H. maculatum cluster, the PDB subgroup was the most separated one in H. humifusum.

Although significant changes in metabolite content were detected in Hypericum plants treated with all elicitors derived from saccharides, the PDB seems to be, in comparison to both the Glc and starch, more effective. While saccharide elicitors had no effect on anthraquinones production, the Glc elicitor stimulated the phloroglucinols accumulation in H. monogynum, H. kouytkense and H. canariense. Among the flavonoids, the content of flavonol glycosides was elevated in all species regardless of the elicitor used; on the other hand the production of aglycone amentoflavone raised 15.7-times in H. humifusum shoots after PDB treatment. The accumulation of chlorogenic acid was stimulated 31.7-fold in H. maculatum shoots treated with Glc elicitor (Supp. Table 1 part A-C).

Chitosan is a linear polysaccharide produced by deacetylation of chitin, which is a structural component of fungal cell walls and insect and crustacean exoskeletons. In this work the chitosan in a final concentration up to 10 mg l⁻¹ (elicitor 10 CH), 1 mg l⁻¹ (1 CH) or 0.01 mg l⁻¹ (0.01 CH) was used.

As shown by PCA (Fig. 3), the changes in the metabolite content of H. erectum and H. tetrapertum were minimal as both species formed tightly connected clusters. The shoots of H. perforatum and H. maculatum treated by elicitor 10 CH were separated from subgroup consisted of control, 1 CH and 0.01 CH plants. The clusters representing the

Fig. 1. The scores plot obtained from PCA of eight untreated Hypericum species depict the projection of the variables in 2-D space for H. perforatum (empty red circles), H. maculatum (empty green squares), H. tetrapertum (empty purple diamonds), H. erectum (empty yellow triangles), H. humifusum (full dark blue circles), H. monogynum (full light blue diamonds), H. kouytkense (full orange squares) and H. canariense (full black triangles). Three points representing the metabolite profile of each species are clustered. Each point accounts for two biological replicates. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Fig. 2. 2-D scores plot obtained from PCA of Hypericum species. The two subgroups representing the metabolite profile of each species are clustered. Each point accounts for two biological replicates and each treatment and control. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Fig. 3. The scores plot obtained from PCA of eight Hypericum species. The two subgroups representing the metabolite profile of each species are clustered. Each point accounts for two biological replicates and each treatment and control. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
in all species with the highest up to 15-times increase in susceptible metabolite to 10 CH elicitation as its content was elevated in cultures treated by 10 CH elicitor. The chlorogenic acid was the most prominent increases of metabolite content [x-fold] for each treatment. The significance of differences was assessed by pair two-sample t-test at p ≤ 0.05.

### Table 2

<table>
<thead>
<tr>
<th>Treatment</th>
<th>H. perforatum</th>
<th>H. maculatum</th>
<th>H. tetrapterum</th>
<th>H. erectum</th>
<th>H. humifusum</th>
<th>H. monogynum</th>
<th>H. kouytchense</th>
<th>H. canariense</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glc</td>
<td>1.5 k-3-O-glc</td>
<td>31.7 chlor. acid</td>
<td>1.7 catechin</td>
<td>1.3 catechin</td>
<td>3.6 catechin</td>
<td>3 k-3-O-glc</td>
<td>2.3 phlorogluc.</td>
<td>2.3 phlorogluc.</td>
</tr>
<tr>
<td>starch</td>
<td>2.7 k-3-O-glc</td>
<td>3.9 chlor. acid</td>
<td>1.7 catechin</td>
<td>1.3 rutin</td>
<td>4.2 amentof.</td>
<td>3 k-3-O-glc</td>
<td>1.9 phlorogluc.</td>
<td>2 phlorogluc.</td>
</tr>
<tr>
<td>PDB</td>
<td>1.7 k-3-O-glc</td>
<td>3 chlor. acid</td>
<td>6.2 amentofl.</td>
<td>1.3 hypericins</td>
<td>15.7 amentofl.</td>
<td>1.8 chlor. acid</td>
<td>3.7 chlor. acid</td>
<td>3 quercitrin</td>
</tr>
<tr>
<td>10 CH</td>
<td>7.3 chlor. acid</td>
<td>7.8 chlor. acid</td>
<td>5.5 amentofl.</td>
<td>2.6 quercitrin</td>
<td>5.3 amentofl.</td>
<td>9.3 chlor. acid</td>
<td>14.9 chlor. acid</td>
<td>5.1 quercitrin</td>
</tr>
<tr>
<td>0.01 CH</td>
<td>4.7 chlor. acid</td>
<td>1.4 chlor. acid</td>
<td>2.6 amentofl.</td>
<td>1.4 quercitrin</td>
<td>1.4 quercitrin</td>
<td>5.9 isoorqu.</td>
<td>1.5 quercitrin</td>
<td>3.9 isoorqu.</td>
</tr>
<tr>
<td>TS</td>
<td>3.7 chlor. acid</td>
<td>2.7 chlor. acid</td>
<td>13.1 amentofl.</td>
<td>1.8 quercitrin</td>
<td>5 amentofl.</td>
<td>11.5 chlor. acid</td>
<td>11.4 chlor. acid</td>
<td>1.9 chlor. acid</td>
</tr>
<tr>
<td>TS + CH</td>
<td>3.1 chlor. acid</td>
<td>4.7 chlor. acid</td>
<td>3.1 amentofl.</td>
<td>non-sig.</td>
<td>3.9 amentofl.</td>
<td>2.4 chlor. acid</td>
<td>3.2 chlor. acid</td>
<td>2 chlor. acid</td>
</tr>
<tr>
<td>FO</td>
<td>3.7 k-3-O-glc</td>
<td>7.3 chlor. acid</td>
<td>3.2 amentofl.</td>
<td>non-sig.</td>
<td>1.8 amentofl.</td>
<td>2.7 k-3-O-glc</td>
<td>1.9 k-3-O-glc</td>
<td>1.7 rutin</td>
</tr>
<tr>
<td>FO + CH</td>
<td>4.7 k-3-O-glc</td>
<td>7.4 chlor. acid</td>
<td>1.5 emodin</td>
<td>non-sig.</td>
<td>4.2 amentofl.</td>
<td>1.7 k-3-O-glc</td>
<td>1.5 catechin</td>
<td></td>
</tr>
<tr>
<td>TC</td>
<td>4.1 k-3-O-glc</td>
<td>8.4 chlor. acid</td>
<td>2.5 amentofl.</td>
<td>non-sig.</td>
<td>2.1 amentofl.</td>
<td>1.2 rutin</td>
<td>non-sig.</td>
<td>1.4 quercitrin</td>
</tr>
<tr>
<td>TC + CH</td>
<td>1.3 hypericins</td>
<td>1.6 chlor. acid</td>
<td>1.2 amentofl.</td>
<td>non-sig.</td>
<td>1.8 amentofl.</td>
<td>2.8 k-3-O-glc</td>
<td>1.6 phlorogluc.</td>
<td>non-sig.</td>
</tr>
<tr>
<td>PI</td>
<td>1.5 emodin</td>
<td>non-sig.</td>
<td>3 amentofl.</td>
<td>non-sig.</td>
<td>1.8 amentofl.</td>
<td>1.4 rutin</td>
<td>non-sig.</td>
<td>1.2 phlorogluc.</td>
</tr>
<tr>
<td>PI + CH</td>
<td>1.6 k-3-O-glc</td>
<td>3.2 chlor. acid</td>
<td>4.7 isoorqu.</td>
<td>non-sig.</td>
<td>2.2 amentofl.</td>
<td>13 isoorqu.</td>
<td>5.9 isoorqu.</td>
<td>1.8 catechin</td>
</tr>
<tr>
<td>Ara4neg</td>
<td>7.2 chlor. acid</td>
<td>8.7 chlor. acid</td>
<td>10.7 amentofl.</td>
<td>2.1 quercitrin</td>
<td>2.9 amentofl.</td>
<td>9.3 chlor. acid</td>
<td>7.5 chlor. acid</td>
<td>2.7 chlor. acid</td>
</tr>
<tr>
<td>AraATCCneg</td>
<td>4 chlor. acid</td>
<td>3.6 chlor. acid</td>
<td>7.9 amentofl.</td>
<td>1.7 quercitrin</td>
<td>4.3 amentofl.</td>
<td>7.7 chlor. acid</td>
<td>8.2 chlor.acid</td>
<td>1.9 chlor. acid</td>
</tr>
<tr>
<td>AraATCCpos</td>
<td>2.3 k-3-O-glc</td>
<td>1.5 hyperoside</td>
<td>1.3 k-3-O-glc</td>
<td>non-sig.</td>
<td>1.4 quercitrin</td>
<td>1.8 chlor. acid</td>
<td>2.1 chlor. acid</td>
<td>2.5 rutin</td>
</tr>
<tr>
<td>NB</td>
<td>4.9 chlor. acid</td>
<td>11.5 chlor.acid</td>
<td>13.3 amentofl.</td>
<td>2 quercitrin</td>
<td>5.6 amentofl.</td>
<td>9.4 chlor. acid</td>
<td>7.9 chlor. acid</td>
<td>3.3 rutin</td>
</tr>
</tbody>
</table>

**Fig. 2.** The PCA plot derived from chemometric data of control *Hypericum* shoots (1) and shoots treated with D-glucose (2), soluble starch (3) and potato-dextrose broth (4). Each species is depicted as follows: *H. perforatum* (empty red circles), *H. maculatum* (empty green squares), *H. tetrapterum* (empty purple squares) and *H. erectum* (empty yellow triangles). Four points representing the metabolite profile of each species are clustered. Each point accounts for two biological replicates. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

species *H. monogynum*, *H. kouytchense* and *H. humifusum* formed three subgroups, from which the 10 CH subgroup was the most remote from the control. The most profound changes in the metabolite profiles evoked by chitosan were evident in *H. canariense* cluster that was segregated into three well separated subgroups representing each treatment.

The greatest number of metabolite alterations was observed in shoot cultures treated by 10 CH elicitor. The chlorogenic acid was the most susceptible metabolite to 10 CH elicitation as its content was elevated in all species with the highest up to 15-times increase in *H. kouytchense*. Among flavonoid aglycones, the amentoflavone accumulation was elevated 5-times in *H. tetrapterum* and *H. humifusum*. The kaempferol and quercetin glycosides productions were positively affected by 10 CH elicitation in almost all species by a 3-fold increment in average, while the 5.1-times increase of quercetin content was detected in *H. canariense* (Supp. Table 1 part D-F).

**3.2.2. Endophytic fungi**

The elicitors established from hyphae of *Thielavia subthermophila* (elicitor TS), *Fusarium oxysporum* (elicitor FO), *Trichoderma crassum* (elicitor TC) and *Piriformospora indica* (elicitor PI) were used to evaluate the potential to stimulate biosynthesis of profiling bioactive compounds. Furthermore, the combined effect of pre-treatment with fungal
elicitors followed by the addition of 0.01 mg l−1 chitosan to media was assessed (elicitors TS + CH, FO + CH, TC + CH, PI + CH).

The PCA plot obtained from the data of shoots treated by T. subthermophila (Fig. 4) revealed the compact clusters of H. perforatum, H. tetramerum and H. erectum suggesting that neither TS nor TS + CH caused alterations in metabolite fingerprints of these Hypericum representatives. On the other side, H. humifusum, H. kouytchense and H. canariense shoots treated by TS or TS + CH formed connected subgroups different from the control plants. The division of TS and TS + CH subgroup was visible in H. maculatum and even more apparently in H. monogynum shoots.

T. subthermophila elicitors induced almost the same amount of significant alterations in the metabolome of all species regardless the phenolic compounds. While TS slightly increased the production of anthraquinones and phloroglucinols, both the TS and TS + CH elicitors stimulated the accumulation of flavonol glycosides. The most noticeable positive effects were observed by TS elicitor, which stimulated the biosynthesis of chlorogenic acid 11.4-times in H. monogynum and H. kouytchense shoots and increased the content of amentoflavone 13.0-times in H. tetramerum (Supp. Table 1 part G-H).

The PCA of the data from Hypericum plants co-cultured with elicitors derived from F. oxysporum (Fig. 5) revealed significant changes in metabolite fingerprints in almost all species, with the exception of three species belonging to the section Hypericum (H. perforatum, H. erectum and H. tetramerum), which formed compact clusters. The clusters of H. maculatum, H. monogynum, H. kouytchense and H. canariense were divided into two well separated subgroups; FO clustered with FO + CH in H. maculatum, or formed individual subgroups departed from the controls and FO + CH-treated plants in H. monogynum, H. kouytchense and H. canariense. The cluster of H. humifusum was separated into three subgroups referring to control, FO and FO + CH treatment.

The combination of FO + CH induced more significant changes in the metabolite contents compared to FO treatment. Reaching 7.3-fold increase, the chlorogenic acid was the most positively influenced metabolite regardless the presence of chitosan in F. oxysporum elicitors. Similarly, the production of naphthodianthrones and emodin was elevated 1.5-fold in H. humifusum after both, the FO and FO + CH treatments. While FO evoked a higher number of increases in the metabolite content, the FO + CH induced higher enhancement in particular metabolites, e. g. a 4.2-times increase of amentoflavone in H. humifusum and 4.7-times increase of kaempferol-3-O-glucoside in H. perforatum (Supp. Table 1 part I-J).

The PCA plot of T. crassum treated Hypericum plants (Fig. 6) revealed a similar pattern of species-dependent metabolite alterations as seen in plants cultured in the presence of elicitors derived from T. subthermophila and F. oxysporum. H. perforatum, H. erectum and H. tetramerum formed the tightly packed clusters with a perfect grouping of control and treated plants. In the cluster of H. humifusum, the TC and TC + CH elicited shoots created a subgroup separated from the controls. The subgroups of TC treated plants of H. maculatum, H. monogynum and H. kouytchense differed from the control and TC + CH plants. The greatest effect of T. crassum on metabolite content was apparent in the cluster of H. canariense, where different treatments were discriminated into three independent subgroups.

In comparison to TC, the TC + CH accounted for more alterations, which could be attributed to a higher number of negative changes in metabolite contents. Similarly to the effects of other fungal elicitors, the level of anthraquinones reached a maximum of 1.6-fold increase in H. humifusum plants treated with T. crassum elicitors when compared to control. From among the other phenolic compounds, the chlorogenic acid was the most susceptible metabolite to elicitation reaching 8.4-fold increase after TC treatment (Supp. Table 1 part K-L).

The metabolite data obtained from Hypericum plants cultivated in the presence of elicitors derived from P. indica subjected to the PCA
that treated plants formed subgroups resembling the controls suggesting served in ttered into a subgroup with ArATCCneg and NB treated plants as ob-
hyphae of prominent enhancements were observed under PI + CH treatment. The Hypericum shoots treated with common response to these elicitors.
the other species showed more or less compact clusters suggesting no increase of phloroglucinols in H. humifusum, H. kouytchense seen in
H. canariense cluster was divided into two subgroups based on the meta-
plasmid. The most significant influence on the metabolite content was recorded under the effect of NB elicitor, e. g. the 11.5-times increase of the chlorogenic acid in shoots of H. maculatum or 13.3-times increase of the amentoflavone in H. tetrapterum (Supp. Table 1 part O-R).

(Fig. 7) revealed the most compact species-dependent clustering. The H. canariense cluster was divided into two subgroups based on the metabolite profiles referring to both the control and treated plants. While H. canariense was the most susceptible species to the P. indica treatments, the other species showed more or less compact clusters suggesting no common response to these elicitors.

Despite a similar number of changes in metabolite content of Hypericum shoots treated with P. indica-derived elicitors, majority of prominent enhancements were observed under PI + CH treatment. The hyphae of P. indica combined with chitosan resulted in 2.3-times increase of phloroglucinols in H. kouytchense. The PI + CH treatment also resulted in 13-times higher content of quercetin glucoside, isoquercetin, in H. monogynum (Supp. Table 1 part M-N).

3.2.3. Agrobacterium rhizogenes & nutrient broth no. 2

Two different agropine strains of A. rhizogenes, A4 and ATCC 15834, were used in this study. Based on the presence of Ri plasmid (pRi) determined by amplification of virC and rolC genes, the A. rhizogenes ATCC 15834 strain containing pRi (elicitor ArATCCpos) and two plasmid missing lines of the strains of A4 (elicitor ArA4neg) and ATCC 15834 (elicitor ArATCCneg), were used. In addition to A. rhizogenes derived elicitors, the nutrient broth no. 2 (elicitor NB) used for cultivation of bacteria, was applied to Hypericum species.

The PCA of Agrobacterium treated plants (Fig. 8) revealed the overlapping clusters belonging to H. tetrapterum and H. erectum shoots. The ArA4neg treated plants created either independent subgroups as seen in H. humifusum, H. kouytchense and H. canariense, or were clustered into a subgroup with ArATCCneg and NB treated plants as observed in H. maculatum and H. monogynum. In H. perforatum, H. maculatum, H. monogynum, H. kouytchense and H. canariense, the ArATCCpos treated plants formed subgroups resembling the controls suggesting that Hypericum shoots were less susceptible to the treatment with the elicitor, which was derived from A. rhizogenes line containing Ri plasmid.

Among four tested elicitors derived from strains of A. rhizogenes or nutrient broth, the ArATCCpos caused fewer significant changes in metabolite content in comparison to ArATCCneg, ArA4neg and NB elicitors. The most significant influence on the metabolite content was recorded under the effect of NB elicitor, e. g. the 11.5-times increase of the chlorogenic acid in shoots of H. maculatum or 13.3-times increase of the amentoflavone in H. tetrapterum (Supp. Table 1 part O-R).

4. Discussion

The representatives of the genus Hypericum produced a plethora of bioactive compounds. While H. perforatum is used world-wide due to balanced composition of secondary metabolites, like naphthodian-thrones, phloroglucinols, flavonoids or melatonin, other species of the genus possess similar or even superior pharmacological activity (Napoli et al., 2018). In the present work, eight Hypericum species were used, from which H. perforatum, H. maculatum, H. tetrapterum and H. humifusum are native to Slovak republic; H. canariense is endemic to Canary Islands; and H. erectum, H. kouytchense and H. monogynum originate in China, Siberia and Japan (Petijová et al., 2014).

Naphthodianthrones are unique compounds of the genus Hypericum that are produced by representatives of phylogenetically younger sections. While H. kouytchense and H. monogynum from primitive section Ascyreia (Crockett and Robson, 2011) and H. canariense from monotypic section Webbia did not produce hypericin and its derivatives, species belonging to clade core Hypericum (Nürk et al., 2013), H. perforatum, H. maculatum, H. erectum, H. tetrapterum and H. humifusum, accumulated naphthodianthrones. Similar pattern of distribution among sections was reported by Kitanov (2001). H. humifusum (Oligostema) accumulated the highest amount of naphthodianthrones from among all studied species,
Averages of all metabolites were expressed as concentration per gram dry weight (g DW). As a result of our PCA analysis, the variability of metabolite profiles among Hypericum species was successfully evaluated, with each species forming a distinct cluster. The main separation was based on the presence and amounts of flavonoids, with the exception of H. kouytchense, which did not accumulate hyperoside. The PCA analysis revealed that the presence of flavonoids in the shoots of Hypericum species was directly correlated with the presence of hyperoside. As a result, we concluded that the presence of flavonoids in the shoots of Hypericum species is a potential taxonomic marker specific to H. maculatum subsp. maculatum and is missing in other subspecies (Kladar et al., 2015) or is present in a minute amount and eludes the detection limit of some methods. Besides, H. tetrapterum and H. humifusum shoots did not contain rutin as found out by Nigutová et al. (2017) or Kucharíková et al. (2016b). H. canariense was the only species that did not accumulate hyperoside. Although its content was comparable to the amount of quercitrin similarly to our previous results (Nigutová et al., 2017), Zorzetto et al. (2015) were unable to detect any quercitrin in flowering aerial parts of H. canariense.

The principal component analysis is usually used to assess the relation between metabolites or plants based on chemometric data acquired by analytical methods. In this work, the PCA revealed the formation of separated clusters of untreated plants, even though H. erectum and H. tetrapterum formed overlapping clusters. The main separation of the species was primarily a consequence of the presence and amounts of hypericins, emodin and phloroglucinol derivatives, while more discrete distinction was based on different amounts of chlorogenic acid and flavonoids. In the genus Hypericum, PCA was successfully employed to reveal interspecific differences (Porzel et al., 2014; Zorzetto et al., 2015; Yuk et al., 2017; Bertoli et al., 2018), intraspecific differences between populations (Kladar et al., 2015; Rusallepp et al., 2017), and to distinguish between the metabolite profiles of the plant parts (Cirak et al., 2016) or ontogenetic phases (Bertoli et al., 2011), but this is the first time when the PCA was used to evaluate elicitation potential of biotic stressors. Due to good species-dependent clustering, any further division into subgroups after elicitation reflected the alterations in metabolite compositions evoked by used biotic factors.

Saccharides are natural parts of plants and also microorganisms. While single saccharides are involved in signalling pathways of stress perception, sucrose can evoke osmotic stress that causes dehydration of plants (Zobayed et al., 2003). In this work we tested potential of D-glucose, soluble starch and potato-dextrose broth to boost biosynthesis of valuable secondary compounds. The PCA implied that the response to saccharides stimulation is to some extent species-specific. While naphthodianthrones production was inhibited after all treatments, phloroglucinol and flavonoid contents were elevated. On the other side, previous results indicated that saccharides are suitable elicitors of hypericins production (Kirakosyan et al., 2000; Gadzovska-Simic et al., 2011). The species specificity was the most noticeable in the case of Glc elicitation, when the production of chlorogenic acid increased 31.7-times in H. maculatum shoots, while its total content decreased in other species. Similarly Gadzovska-Simic et al. (2015a) detected increase of total phenolic and flavonoid contents in cell-suspension cultures of H. perforatum treated by pectin, dextran or chitin. When the same elicitors were used in H. perforatum shoot cultures, the content of phenolics and flavonoid compounds in the extracts is rarely evaluated. From among studied species we were able to detect naringenin only in H. perforatum shoots in minute amounts. Although apigenin was not observed in any species, even though it was previously detected in extract from greenhouse grown plants (Saddique et al., 2011; Kladar et al., 2015), we detected amentoflavone in H. erectum, H. tetrapterum and H. humifusum shoots. It should be noted that all the mentioned treated and control species were in flower during each experiment, so we accounted the presence of amentoflavone to generative stage as pointed out by Hölscher et al. (2009), Kladar et al. (2015) or Cirak et al. (2016). Flavonols, especially quercetin and kaempferol glycosides, are the best studied group of flavonoids. Five species accumulated kaempferol-3-O-glucoside up to 0.37 mg g⁻¹ DW in H. kouytchense. Its presence was also reported in H. maculatum and H. perforatum by Rusallepp et al. (2017), though Yuk et al. (2017) did not detect kaempferol-3-O-glucoside in H. erectum. Among the studied species, H. perforatum, H. erectum, H. monogynum and H. kouytchense accumulated all the studied quercetin glycosides – rutin, hyperoside, isoquercetin and quercitrin, as previously noted (Crockett et al., 2005; Porzel et al., 2014). We did not detect rutin in the shoots of H. maculatum alike to Rusallepp et al. (2017), even though several reports stated otherwise (Bagdonaite et al., 2009; Kladar et al., 2015). We incline to explanation provided by Kladar et al. (2015) that rutin is a potential taxonomic marker specific to H. maculatum subsp. maculatum and is missing in other subspecies (Kladar et al., 2015) or is present in a minute amount and eludes the detection limit of some methods. Besides, H. tetrapterum and H. humifusum shoots did not contain rutin as found out by Nigutová et al. (2017) or Kucharíková et al. (2016b). H. canariense was the only species that did not accumulate hyperoside. Although its content was comparable to the amount of quercitrin similarly to our previous results (Nigutová et al., 2017), Zorzetto et al. (2015) were unable to detect any quercitrin in flowering aerial parts of H. canariense.

Fig. 8. The PCA plot derived from chemometric data of control Hypericum shoots (1) and shoots treated with Agrobacterium rhizogenes A4 lacking pRi (2), A. rhizogenes ATCC 15834 lacking pRi (3), A. rhizogenes ATCC 15834 carrying pRi (4) and cell-free nutrient broth no. 2 (5). Each species is depicted as follows: H. perforatum (empty red circles), H. maculatum (empty green squares), H. tetrapterum (empty purple diamonds), H. erectum (empty yellow triangles), H. humifusum (full dark blue circles), H. monogynum (full light blue diamonds), H. kouytchense (full orange squares) and H. canariense (full black triangles). Five points representing the metabolite profile of each species are clustered. Each point accounts for two biological replicates. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
flavonoids decreased (Gadzovska-Simic et al., 2014). It seems that saccharides influence secondary metabolism by both, species and type of explants-dependent ways.

Chitosan is a linear polysaccharide that is known to mimic the effects of pathogenic fungi in stimulating defence responses (Hadwiger and Loschke, 1981). The chitosan was previously applied in final concentration up to 200 mg l\(^{-1}\) (Tocci et al. 2010, 2011; Brasili et al., 2014; Valletta et al., 2016), but the cultures used in the present study were unable to sustain such high concentration similarly to Zubrická et al. (2015). The PCA showed that 10 mg l\(^{-1}\) chitosan caused the most prominent metabolite alterations, even though hypericins and emodin content was stimulated only in 1 CH or 0.01 CH treated \textit{H. maculatum} and \textit{H. tetrapertum} shoots. The potential of chitosan to elicit chlorogenic acid production was dose dependent and 10 CH treatment caused 3-4-fold increase in comparison to 1 CH and 0.01 CH treatments. The total flavonoids content was elevated in all species after elicitation, but type of affected metabolite differ between species and concentration of chitosan; for instance catechin and kaempferol-3-O-glucoside contents were elevated in 10 CH treated \textit{H. perforatum}, while isoqueretin was stimulated in \textit{H. monogynum} shoots. To date, the effects of chitosan were evaluated in cell suspension and root cultures of \textit{H. perforatum}; the addition of chitosan into cell suspension cultures caused increase in total xanthones content (Tocci et al., 2010; Zubrická et al., 2015) and even promoted de novo production of cadensin G and 1,7-dihydroxy-xanthone, while the amount of quercetin and hyperoside decreased (Tocci et al., 2010). On the other hand, the production of epicatechin increased 2-fold in treated root cultures (Brasili et al., 2014), which was accompanied by significant increment in xanthones content (Tocci et al., 2011; Brasili et al., 2014; Valletta et al., 2016).

Fungal elicitors are usually cell wall fragments derived from pathogenic fungi that induce hypersensitive response in plants. In this work, we tested elicitors prepared from \textit{F. oxysporum} and \textit{T. crassum} isolates obtained from \textit{H. perforatum} grown in India (Kusari et al., 2008) and from a plant growth promoting endophyte \textit{P. indica} (Varma et al., 1999). Besides \textit{F. oxysporum} originated elicitors, the elicitation potential of other fungi to stimulate secondary metabolism of \textit{Hypericum} spp, have not been studied yet. While the PCA projections depicted similar division of species clusters to subgroups depending on metabolite alterations evoked by \textit{T. subterrithophila}, \textit{F. oxysporum} and \textit{T. crassum} treatments, \textit{P. indica} treated plants formed clusters similar to control plants. The PCA revealed that \textit{H. perforatum}, \textit{H. tetrapertum} and \textit{H. erectum} were the least susceptible species to fungal elicitation, while \textit{H. canariense} shoots responded most sharply to elicitation stimuli regardless of the type of fungus and the presence of chitosan. The t-tests further uncovered that the production of naphthodianthrones and emodin was slightly elevated by all fungal elicitors, but the phloroglucinols content increased only after PI or PI + CH treatments. TC and FO elicitors stimulated the chlorogenic acid production only in \textit{H. maculatum}, while TS evoked its 11.4-times increase in \textit{H. monogynum} and \textit{H. kouytchense}. The amount of amentonflavone was higher in shoots treated by chitosan free fungal elicitors with the exception of FO + CH treated \textit{H. humifusum}. Among flavonol glycosides, kaempferol-3-O-glucoside was strongly stimulated by FO + CH in \textit{H. perforatum} cultures. Although the content of all quercetin glycosides increased up to 2-fold after TS or TS + CH treatment, the other fungal elicitors did not showed such consistent elevation across different types of glycosides. Among used elicitors, PI + CH caused the most prominent 13-fold increase of isoorchetin production in \textit{H. monogynum} shoots. The elicitation potential of \textit{F. oxysporum} was previously studied by Gadzovska-Simic et al. (2015b) alongside elicitors derived from pathogenic fungi \textit{Botrytis cinerea} and \textit{Phoma exigua}. Among them, \textit{F. oxysporum} showed the strongest stimulation of phenylpropanoids and naphthodianthrones production in \textit{H. perforatum} cell suspensions (Gadzovska-Simic et al., 2015b). The application of \textit{F. oxysporum} elicitors to \textit{H. triquetrifolium} cells promoted production of rutin, hyperoside and quercetin, but inhibited the phenolic acids biosynthesis (Azeez and Ibrahim, 2013). Additionally, elicitors derived from \textit{Aspergillus flavus} stimulated production of anthocyanins, while hypericin and flavonoid production decreased or remained unchanged (Gadzovska-Simic et al., 2012). On the other side, \textit{A. niger} caused 4.2-fold increase of hypericin production in \textit{H. perforatum} cell suspensions (Xu et al., 2005) and boosted flavonoid production in \textit{H. triquetrifolium} cells (Azeez and Ibrahim, 2013). However, the shoot cultures or greenhouse grown plants are more suitable for hypericin biosynthesis as they have fully developed leaves carrying dark glands. To date, the hypericins production was stimulated in \textit{H. perforatum} plants treated by Colletotrichum gloeosporioides (Sirvent and Gibson, 2002), \textit{Phytophthora capsici} and Diplloceras hypericinum (Círak et al., 2005); while Nomuraecra riley stimulated production of chlorogenic acid, hyperoside and isoorquetin in \textit{H. polyanthemum} plants (Meirelles et al., 2013). Furthermore, it seems that elicitors derived from pathogenic fungi commonly caused the inhibition of biomass production or even necrosis of tissues (Círak et al., 2005; Gadzovska-Simic et al., 2012, 2015b), which was not detected in the cultures used in this study upon elicitation with any of the endophytic fungi.

The pathogenic Gram-negative soil bacteria of the genus \textit{Agrobacterium} cause tumours in plants via horizontal gene transfer. Besides, bacteria are source of cell wall components, like peptidoglycans, and non-structural bacterial metabolites that trigger the process of elicitation. In this work, agroplasm strains \textit{A. rhizogenes} A4 and \textit{A. rhizogenes} ATCC 15834 cultivated in nutrient broth no. 2 were used. Before elicitation experiments the presence of Ri plasmid was confirmed in \textit{A. rhizogenes} ATCC 15834 strain (ArATCCpos), while \textit{A. rhizogenes} A4 (ArA4neg) and \textit{A. rhizogenes} ATCC 15834 (ArATCCneg) lines lacked plRi. The PCA revealed that NB, ArA4neg and ArATCCneg treated plants were detached from control plants. Surprisingly it seems that ArATCCpos evoked less prominent metabolite changes than other elicitors, which was further confirmed by t-tests. The content of hypericins and emodin was elevated only in NB and ArATCCneg treated \textit{H. tetrapertum} shoots and the amount of phloroglucinols increased in \textit{H. monogynum}. Similarly, the inhibition of hypericin synthesis in \textit{H. perforatum} shoots after \textit{A. tumefaciens} treatment was observed by Pavlík et al. (2007). The production of chlorogenic acid and amentonflavone was strongly stimulated by NB, ArA4neg and ArATCCneg elicitors. Additionally, we detected almost no stimulation of flavonol glycosides biosynthesis by ArATCCpos, even though other elicitors caused 2- to 6-fold elevation in some quercetin glycosides. These results indicated that elicitors derived from bacteria carrying plRi evoke less serious plant defence responses than \textit{Agrobacterium} missing plRi or even cell free nutrient broth. Recently, Tusevski et al. (2015) applied elicitors prepared from inactivated \textit{A. rhizogenes} A4 and \textit{A. tumefaciens} B653 to \textit{H. perforatum} cell suspensions. They detected 17-fold higher levels of xanthones compared to control cells. These results are partly in concordance with Franklin et al. (2009), who also detected increase in xanthone production, but flavonoids remained unaltered.

The content of chlorogenic acid and flavonoids, especially amentonflavone and quercetin glycosides, significantly increased upon different treatments (Table 2). These compounds were identified to possess cytotoxic effects under dark conditions (Napoli et al., 2018). Therefore we anticipate that the flavonoids, along with photoactive hypericins, could synergistically contribute to overall antineoplastic effects of \textit{Hypericum} extracts.

5. Conclusions

Among 23 studied compounds belonging to anthraquinones, phloroglucinols, hydroxycinnamic acids and flavonoids, only 17 were present in the shoots of \textit{Hypericum} spp. Each studied species could be
distinguished based on its unique phytochemical pattern, while only chlorogenic acid, isoquercetin, quercitrin and catechin were present in every species. Additionally, the effects of 18 biotic elicitors were evaluated. The content of chlorogenic acid changed the most distinctively in the current study are available from the corresponding author on request. Despite several tested elicitors, no common response in every species. Additionally, the effects of 18 biotic elicitors were evaluated. The content of chlorogenic acid changed the most distinctively.

**Compliance with ethical standards**

**Declaration of interests**

The authors declare that they have no conflict of interest.

**Data availability**

All data generated or analysed during this study are included as mean values in this published article [Supplementary Table 1]. The raw data and or chromatograms generated during and or analysed during the current study are available from the corresponding author on reasonable request.

**CRediT authorship contribution statement**

Miroslava Bálintová: Formal analysis, Writing – original draft. Katarina Brňašká: Formal analysis, Writing – original draft. Linda Petičová: Formal analysis. Eva Čelárová: Conceptualization, Formal analysis, Project administration, Writing – review & editing.

**Acknowledgements**

Funding: This work was supported by the Slovak Research and Development Agency [grant number APVV-14-0154]; and the Scientific Grant Agency of Slovak Republic [grant number VEGA 1/0090/15].

**Appendix A. Supplementary data**

Supplementary data to this article can be found online at https://doi.org/10.1016/j.phyto.2018.12.024.

**References**


