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Research article

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

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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. maculatum* 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 *Campylosporus, Ascyreia* and *Roscyna* possess basal character states and all 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; Cirak 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 source 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 (Jendželovská 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., 2000; Tocci

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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 (Sirvent and Gibson, 2002; Xu et al., 2005; Conceição et al., 2006; Gadzovska-Simic et al., 2012, 2015b; Azeez 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* Crantz, *H. tetrapterum* 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 Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, accession number DSM-21024. *Piriformospora indica* was kindly provided by Dr. Ahmad Mazen (Sohag 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 Kusari 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′-TCCTCCGCTTATTGATA TGC-3′) and ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) primers (White et al., 1990). The elicitors were prepared in accordance with Yu et al. (2001) and Wang Y 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 Cultures Collection. 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′-AATGCGTCTCTCTCGTGCAT-3′ and 5′-AAACCGACCACTAACGCGAT-3′) were selected according to Komarovská et al. (2009) and primers for the amplification of *rolC* gene (5′-AAATGCGAAGTAGGCGCTCCG-3′ and 5′-TACGTCGACTGCCCGAC GATGATG-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 2×10^9 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 Bruňáková and Čellárová (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 Kinetex 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, quercitrin and amentoflavone (Extrasynthese, 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 subsistent standards. The peaks for hypericin, pseudohypericin and their protoforms 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 $p \leq 0.05$.

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 hydroxycinnamic 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 quercetin. 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-L-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.

	H. perforatum	*H. maculatum*	*H. tetrapterum*	*H. erectum*	*H. humifusum*	*H. monogynum*	*H. kouytchense*	*H. canariense*
Emodin	0.058 ± 0.021	0.011 ± 0.003	0.046 ± 0.004	0.045 ± 0.006	0.294 ± 0.047	nd	nd	nd
Pseudohypericin	1.102 ± 0.368	0.531 ± 0.118	1.280 ± 0.346	0.492 ± 0.069	3.386 ± 0.448	nd	nd	nd
Hypericin	0.179 ± 0.089	0.043 ± 0.014	0.452 ± 0.163	0.272 ± 0.043	0.812 ± 0.251	nd	nd	nd
Phloroglucinol (RT = 8.8 min)	nd	nd	nd	nd	6.647 ± 1.763	nd	nd	4.487 ± 2.272
Phloroglucinol (RT = 9.2 min)	nd	nd	nd	nd	nd	nd	nd	4.966 ± 0.897
Hyperforin (RT = 9.9 min)	27.552 ± 4.664	nd	nd	nd	nd	1.679 ± 0.250	3.605 ± 0.876	nd
Phloroglucinol (RT = 10.1 min)	nd	nd	nd	nd	nd	nd	5.963 ± 1.986	nd
Chlorogenic acid	0.366 ± 0.263	0.152 ± 0.078	3.726 ± 0.694	9.185 ± 0.833	9.751 ± 4.166	1.557 ± 0.893	0.977 ± 0.605	4.200 ± 0.647
Naringenin	0.095 ± 0.077	nd	nd	nd	nd	nd	nd	nd
Amentoflavone	nd	nd	0.122 ± 0.045	0.020 ± 0.031	0.351 ± 0.399	nd	nd	nd
Quercetin	nd	nd	nd	0.037 ± 0.057	nd	nd	nd	nd
Rutin	0.969 ± 0.329	nd	nd	0.265 ± 0.028	nd	0.506 ± 0.180	0.421 ± 0.067	0.228 ± 0.037
Hyperoside	0.507 ± 0.218	0.597 ± 0.235	0.332 ± 0.071	1.540 ± 0.077	1.052 ± 0.261	0.450 ± 0.046	1.443 ± 0.107	nd
Isoquercetin	0.264 ± 0.090	0.188 ± 0.070	0.205 ± 0.045	0.862 ± 0.112	0.545 ± 0.081	0.107 ± 0.034	0.331 ± 0.073	0.066 ± 0.011
Quercitrin	0.722 ± 0.333	0.057 ± 0.019	0.381 ± 0.082	0.100 ± 0.033	0.554 ± 0.103	2.620 ± 0.162	1.019 ± 0.222	0.072 ± 0.005
Kaempferol-3-O-glucoside	0.050 ± 0.033	nd	0.075 ± 0.018	0.261 ± 0.010	nd	0.067 ± 0.032	0.371 ± 0.098	nd
Catechin	0.116 ± 0.027	0.135 ± 0.061	0.297 ± 0.185	1.694 ± 0.254	0.161 ± 0.040	0.206 ± 0.085	0.271 ± 0.143	1.886 ± 0.295

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

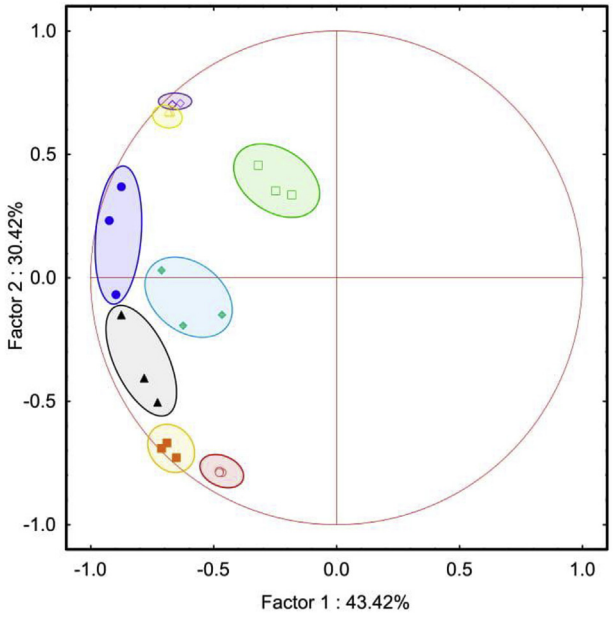

Fig. 1. The scores plot obtained from PCA of eight untreated *Hypericum* species depict the projection of the variables in 2-D space for *H. perforatum* (empty red circles), *H. maculatum* (empty green squares), *H. tetrapterum* (empty purple diamonds), *H. erectum* (empty yellow triangles), *H. humifusum* (full dark blue circles), *H. monogynum* (full light blue diamonds), *H. kouytchense* (full orange squares) and *H. canariense* (full black triangles). 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.)

each cluster could be further divided into subgroups representing control plants or plants after exposure to biotic factors. The subdivision of species clusters into smaller units (subgroups) was further confirmed by the HCA (data not shown).

Elicitors: Glc: D-glucose; **starch**: soluble starch; **PDB**: potato dextrose broth; **10 CH**: 10 mg l^{-1} chitosan; **1 CH**: 1 mg l^{-1} chitosan; **0.01 CH**: 0.01 mg l^{-1} chitosan; **TS**: sterilised hyphae of *T. subthermophila*; **TS + CH**: TS elicitor combined with chitosan; **FO**: sterilised hyphae of *F.oxysporum*; **FO + CH**: FO elicitor combined with chitosan; **TC**: sterilised hyphae of *T. crassum*; **TC + CH**: TC elicitor combined with chitosan; **PI**: sterilised hyphae of *P. indica*; **PI + CH**: PI elicitor combined with chitosan; **ArA4neg**: *A. rhizogenes* A4 lacking pRi; **ArATCCneg**: *A. rhizogenes* ATCC 15834 lacking pRi; **ArATCCpos**: *A. rhizogenes* ATCC 15834 carrying pRi; **NB**: nutrient broth.

Metabolites: k-3-O-glc: kaempferol-3-O-glucoside; **chlor. acid**: chlorogenic acid; **phlorogluc.**: phloroglucinols; **amentofl.**: amentoflavone; **isoquer.**: isoquercetin; **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. tetrapterum* 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. kouytchense* 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. kouytchense* 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. tetrapterum* 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

Table 2
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 $p \leq 0.05$.

Treatment	*H. perforatum*	*H. maculatum*	*H. tetrapterum*	*H. erectum*	*H. humifusum*	*H. monogynum*	*H. kouytchense*	*H. canariense*
Glc	1.5 k-3-O-glc	31.7 chlor. acid	1.7 catechin	1.3 catechin	3.6 catechin	3 k-3-O-glc	2.3 phlorogluc.	2.3 phlorogluc.
starch	2.7 k-3-O-glc	3.9 chlor. acid	1.7 catechin	1.3 rutin	4.2 amentofl.	3 k-3-O-glc	1.9 phlorogluc.	2 phlorogluc.
PDB	1.7 k-3-O-glc	3 chlor. acid	6.2 amentofl.	1.3 hypericins	15.7 amentofl.	1.8 chlor. acid	3.7 chlor. acid	3 quercitrin
10 CH	7.3 chlor. acid	7.8 chlor. acid	5.5 amentofl.	2.6 quercitrin	5.3 amentofl.	9.3 chlor. acid	14.9 chlor. acid	5.1 quercitrin
1 CH	2.4 chlor. acid	2 isoquer.	1.9 amentof.	1.5 isoquer.	2.6 amentofl.	6.5 isoquer.	1.8 chlor. acid	2.2 chlor. acid
0.01 CH	4.7 chlor. acid	1.4 chlor. acid	2.6 amentofl.	1.4 quercitrin	1.4 isoquer.	5.9 isoquer.	1.5 quercitrin	3.9 isoquer.
TS	3.7 chlor. acid	2.7 chlor. acid	13.1 amentofl.	1.8 quercitrin	5 amentofl.	11.5 chlor. acid	11.4 chlor. acid	1.9 chlor. acid
TS + CH	3.1 chlor. acid	4.7 chlor. acid	3.1 amentofl.	non-sig.	3.9 amentofl.	2.4 chlor. acid	3.2 chlor. acid	2 chlor. acid
FO	3.7 k-3-O-glc	7.3 chlor. acid	3.2 amentofl.	non-sig.	1.8 amentofl.	2.7 k-3-O-glc	1.9 k-3-O-glc	1.7 rutin
FO + CH	4.7 k-3-O-glc	7.4 chlor. acid	1.5 emodin	non-sig.	4.2 amentofl.	non-sig.	1.7 k-3-O-glc	1.5 catechin
TC	4.1 k-3-O-glc	8.4 chlor. acid	2.5 amentofl.	non-sig.	2.1 amentofl.	1.2 rutin	non-sig.	1.4 quercitrin
TC + CH	1.3 hypericins	1.6 chlor. acid	1.2 amentofl.	non-sig.	1.8 amentofl.	2.8 k-3-O-glc	1.6 phlorogluc.	non-sig.
PI	1.5 emodin	non-sig.	3 amentofl.	non-sig.	non-sig.	non-sig.	1.4 rutin	1.2 phlorogluc.
PI + CH	1.6 k-3-O-glc	3.2 chlor. acid	4.7 isoquer.	non-sig.	2.2 amentofl.	13 isoquer.	5.9 isoquer.	1.8 catechin
ArA4neg	7.2 chlor. acid	8.7 chlor. acid	10.7 amentofl.	2.1 quercitrin	2.9 amentofl.	9.3 chlor. acid	7.5 chlor. acid	2.7 chlor. acid
ArATCCneg	4 chlor. acid	3.6 chlor. acid	7.9 amentofl.	1.7 quercitrin	4.3 amentofl.	7.7 chlor. acid	8.2 chlor.acid	1.9 chlor. acid
ArATCCpos	2.3 k-3-O-glc	1.5 hyperoside	1.3 k-3-O-glc	non-sig.	1.4 quercitrin	1.8 chlor. acid	2.1 chlor. acid	2.5 rutin
NB	4.9 chlor. acid	11.5 chlor.acid	13.3 amentofl.	2 quercitrin	5.6 amentofl.	9.4 chlor. acid	7.9 chlor. acid	3.3 rutin

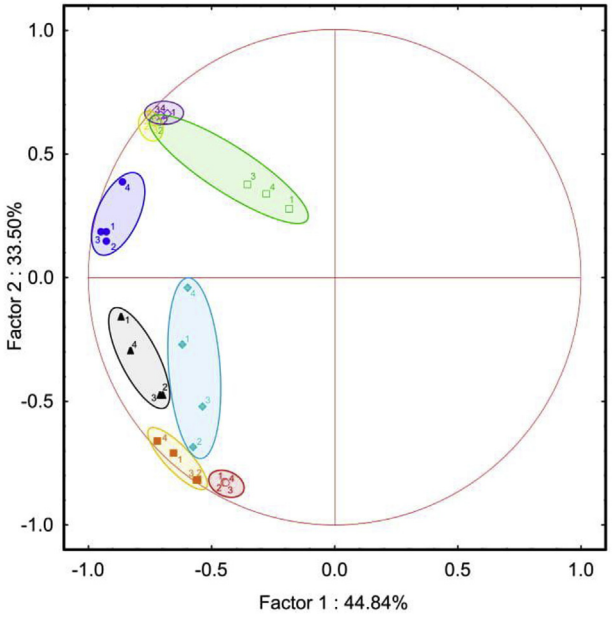

Fig. 2. The PCA plot derived from chemometric data of control *Hypericum* shoots (1) and shoots treated with D-glucose (2), soluble starch (3) and potato-dextrose broth (4). Each species is depicted as follows: *H. perforatum* (empty red circles), *H. maculatum* (empty green squares), *H. tetrapterum* (empty purple 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). 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.)

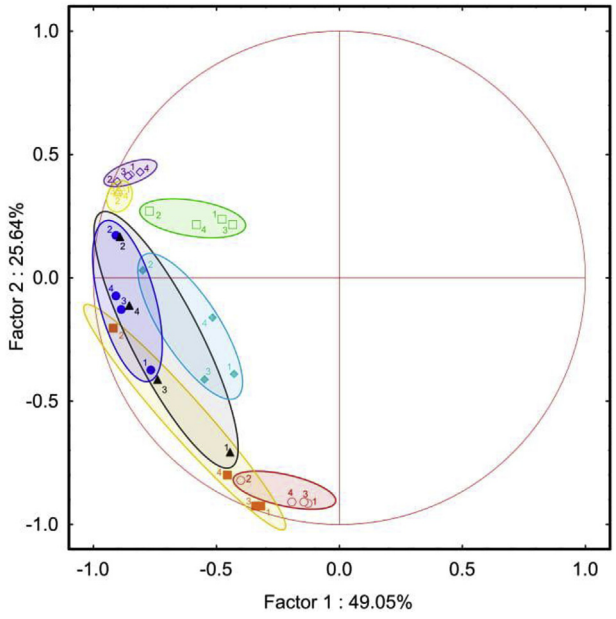

Fig. 3. The PCA plot derived from chemometric data of control *Hypericum* shoots (1) and shoots treated with chitosan in a final concentration up to 10 mg l^{-1} (2), 1 mg l^{-1} (3) and 0.01 mg l^{-1} (4). Each species is depicted as follows: *H. perforatum* (empty red circles), *H. maculatum* (empty 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). 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 quercitrin 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

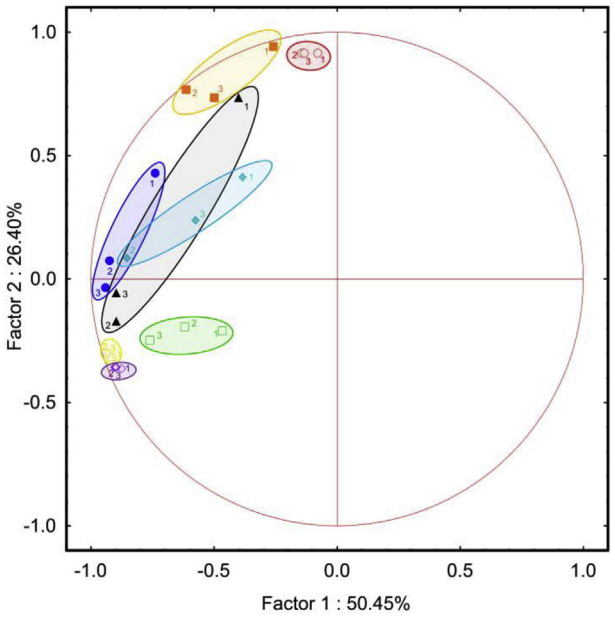

Fig. 4. The PCA plot derived from chemometric data of control *Hypericum* shoots (1) and shoots treated with *Thielavia subthermophila* sterilised hyphae (2) and *T. subthermophila* sterilised hyphae combined with 0.01 mg l^{-1} chitosan (3). Each species is depicted as follows: *H. perforatum* (empty 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). 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.)

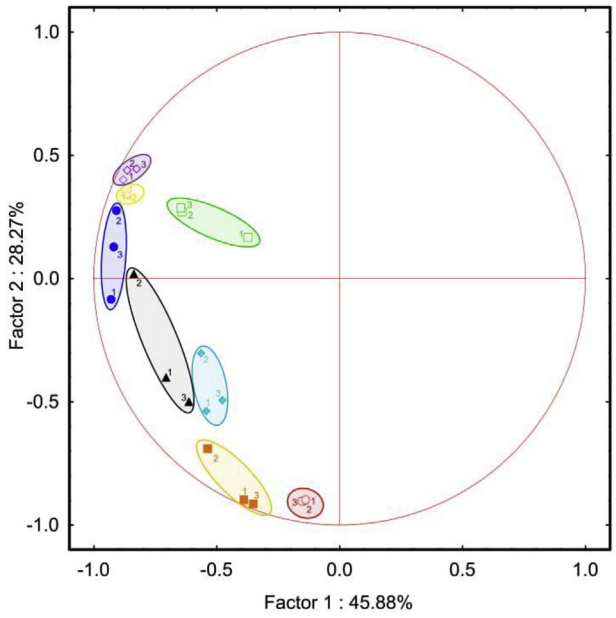

Fig. 5. The PCA plot derived from chemometric data of control *Hypericum* shoots (1) and shoots treated with *Fusarium oxysporum* sterilised hyphae (2) and *F. oxysporum* sterilised hyphae combined with 0.01 mg l^{-1} chitosan (3). Each species is depicted as follows: *H. perforatum* (empty red circles), *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). 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.)

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. tetrapterum* 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. tetrapterum* (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 an exception of three species belonging to the section *Hypericum* (*H. perforatum, H. erectum* and *H. tetrapterum*), 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. tetrapterum* 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

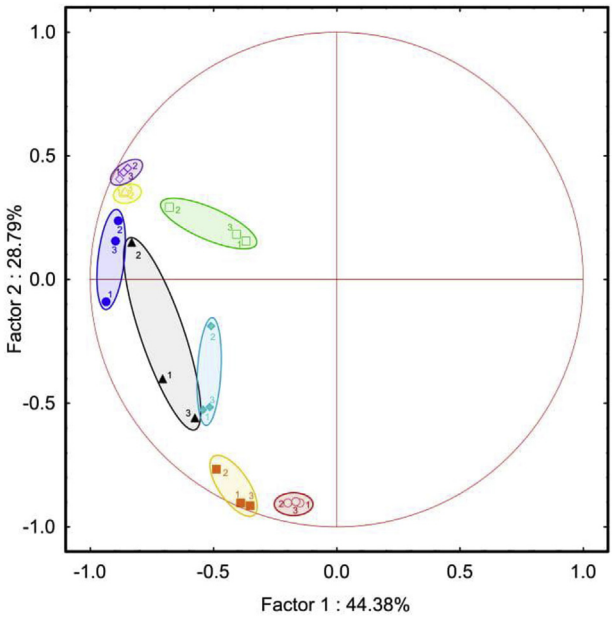

Fig. 6. The PCA plot derived from chemometric data of control *Hypericum* shoots (1) and shoots treated with *Trichoderma crassum* sterilised hyphae (2) and *T. crassum* sterilised hyphae combined with 0.01 mg l^{-1} chitosan (3). Each species is depicted as follows: *H. perforatum* (empty red circles), *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). 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.)

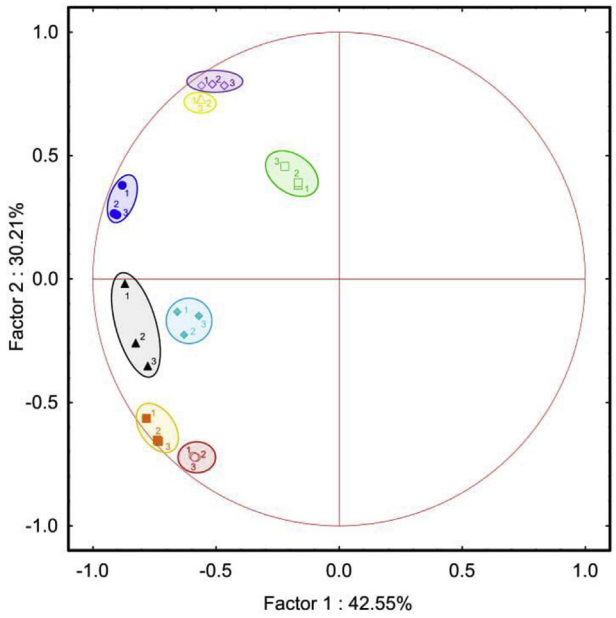

Fig. 7. The PCA plot derived from chemometric data of control *Hypericum* shoots (1) and shoots treated with *Piriformospora indica* sterilised hyphae (2) and *P. indica* sterilised hyphae combined with 0.01 mg l^{-1} chitosan (3). Each species is depicted as follows: *H. perforatum* (empty red circles), *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). 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. 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 contents 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 naphthodianthrones, 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,

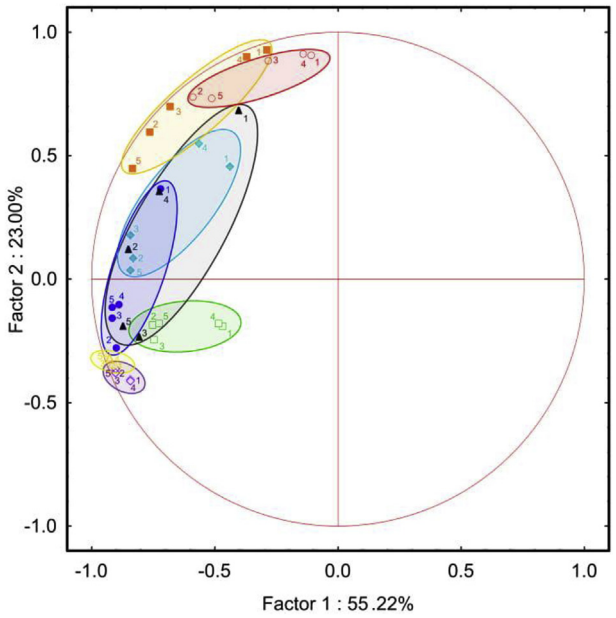

Fig. 8. The PCA plot derived from chemometric data of control *Hypericum* shoots (1) and shoots treated with *Agrobacterium rhizogenes* A4 lacking pRi (2), *A. rhizogenes* ATCC 15834 lacking pRi (3), *A. rhizogenes* ATCC 15834 carrying pRi (4) and cell-free nutrient broth no. 2 (5). Each species is depicted 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.)

but it could be accounted to the presence of flowers carrying dark glands under given culture conditions. Anthraquinone emodin was exclusively presented in hypericin-producing species, which is in concordance with our previous results (Nigutová et al., 2017), but more sensitive analytical method revealed the presence of emodin also in hypericin-lacking species like *H. monogynum* and *H. kouytchense* (Kucharíková et al., 2016b).

The representatives of the genus *Hypericum* produce different types of acylphloroglucinols which share a common acylated 1,3,5-trihydroxybenzene core (Verotta, 2002; Ishida et al., 2010; Porzel et al., 2014; Kucharíková et al., 2016a). We detected four different phloroglucinols that have the same absorbance spectrum as hyperforin, but differed in retention times. Phloroglucinols accounted for the highest abundance among all metabolites, in line with previous findings reported by Porzel et al. (2014). None of the four targeted phloroglucinols were present in *H. maculatum, H. erectum* and *H. tetrapterum*.

From five studied phenolic acids, we detected chlorogenic acid in all species similar to Kucharíková et al. (2016b). Tocci et al., (2018) studied metabolite profile of Andean *Hypericum* species. They detected chlorogenic acid only in *H. carinosum*, but its content exceeded that of other phenolic acids several folds (Tocci et al., 2018). It seems that we were able to detect only the most abundant phenolic acid, while other hydroxycinnamic acids might be hidden below detection limit of our method.

Flavonoids represent the most prevalent group of phenolic compounds in the genus *Hypericum*. In this work we studied metabolites belonging to five main subgroups of flavonoids. Despite the fact that flavanones are intermediates in the biosynthesis of most other flavonoid groups, including flavones and flavonols, their presence in the *Hypericum* 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 (Saddiqe 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 Rusalepp 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 Rusalepp 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 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 flavonols. 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; Rusalepp 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 evokes 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 flavonoids 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., 2014). 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

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 *H. maculatum* and *H. tetrapterum* 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 *H. perforatum*, while isoquercetin was stimulated in *H. monogynum* shoots. To date, the effects of chitosan were evaluated in cell suspension and root cultures of *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-dihydroxyxanthone, while the amount of quercitrin 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 *F. oxysporum* and *T. crassum* isolates obtained from *H. perforatum* plants adapted to outdoor conditions, but both fungi are recognised endophytes of other plant species and were previously used for elicitation experiments (Moreno et al., 2009; Samarina et al., 2017; Warhade and Badere, 2018). Additionally, we used elicitors derived from endophytic fungus *T. subthermophila* originally isolated from *H. perforatum* grown in India (Kusari et al., 2008) and from a plant growth promoting endophyte *P. indica* (Varma et al., 1999). Besides *F. oxysporum* originated elicitors, the elicitation potential of other fungi to stimulate secondary metabolism of *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 *T. subthermophila, F. oxysporum* and *T. crassum* treatments, *P. indica* treated plants formed clusters similar to control plants. The PCA revealed that *H. perforatum, H. tetrapterum* and *H. erectum* were the least susceptible species to fungal elicitation, while *H. canariense* shoots responded most sharply to elicitation stimuli regardless 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 *H. maculatum*, while TS evoked its 11.4-times increase in *H. monogynum* and *H. kouytchense*. The amount of amentoflavone was higher in shoots treated by chitosan free fungal elicitors with the exception of FO + CH treated *H. humifusum*. Among flavonol glycosides, kaempferol-3-O-glucoside was strongly stimulated by FO + CH in *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 isoquercetin production in *H. monogynum* shoots. The elicitation potential of *F. oxysporum* was previously studied by Gadzovska-Simic et al. (2015b) alongside elicitors derived from pathogenic fungi *Botrytis cinerea* and *Phoma exigua*. Among them, *F. oxysporum* showed the strongest stimulation of phenylpropanoids and naphthodianthrones production in *H. perforatum* cell suspensions (Gadzovska-Simic et al., 2015b). The application of *F. oxysporum* elicitors to *H. triquetrifolium* cells promoted production of rutin, hyperoside and quercitrin, but inhibited the phenolic acids biosynthesis (Azeez and Ibrahim, 2013). Additionally, elicitors derived from *Aspergillus flavus* stimulated production of anthocyanins, while hypericin and flavonoid production decreased or remained unchanged (Gadzovska-Simic et al., 2012). On the other side, *A. niger* caused 4.2-fold increase of hypericin production in *H. perforatum* cell suspensions (Xu et al., 2005) and boosted flavonoid production in *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 *H. perforatum* plants treated by *Colletotrichum gloeosporioides* (Sirvent and Gibson, 2002), *Phytophthora capsici* and *Diploceras hypericinum* (Çirak et al., 2005); while *Nomuraea riley* stimulated production of chlorogenic acid, hyperoside and isoquercetin in *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 (Çirak 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 *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, agropine strains *A. rhizogenes* A4 and *A. rhizogenes* ATCC 15834 cultivated in nutrient broth no. 2 were used. Before elicitation experiments the presence of Ri plasmid was confirmed in *A. rhizogenes* ATCC 15834 strain (ArATCCpos), while *A. rhizogenes* A4 (ArA4neg) and *A. rhizogenes* ATCC 15834 (ArATCCneg) lines lacked pRi. 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 *H. tetrapterum* shoots and the amount of phloroglucinols increased in *H. monogynum*. Similarly, the inhibition of hypericin synthesis in *H. perforatum* shoots after *A. tumefaciens* treatment was observed by Pavlík et al. (2007). The production of chlorogenic acid and amentoflavone 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 pRi evoke less serious plant defence responses than *Agrobacterium* missing pRi or even cell free nutrient broth. Recently, Tusevski et al. (2015) applied elicitors prepared from inactivated *A. rhizogenes* A4 and *A. tumefaciens* B6S3 to *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 amentoflavone 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 *Hypericum* extracts.

5. Conclusions

Among 23 studied compounds belonging to anthraquinones, phloroglucinols, hydroxycinnamic acids and flavonoids, only 17 were present in the shoots of *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 after treatments, but total increase was species-dependent. Among flavonoids, amentoflavone was elicitable up to 15.7-fold in *H. humifusum* treated by PDB, while quercetin glycosides were usually elevated up to 5-times in comparison to control plants. However, the production of naphthodianthrones and phloroglucinols was enhanced only slightly. It seems that *Hypericum* plants are able to accumulate hypericins to certain level derived from morphology of leaves and present dark glands (Kimáková et al., 2018), which cannot be overcome by exogenous factors. Despite several tested elicitors, no common response in *Hypericum* spp. exposed to biotic stress was detected.

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. **Katarína Bruňáková:** Formal analysis, Writing – original draft. **Linda Petijová:** Formal analysis. **Eva Čellárová:** Conceptualization, Formal analysis, Project administration, Writing – review & editing.

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Appendix A. Supplementary data

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

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