


Elicitation as a tool to improve the profiles of high-value secondary metabolites and pharmacological properties of *Hypericum perforatum*

Preeti Shakya^a, Gregory Marslin^b, Karthik Siram^c, Ludger Beerhues^d and Gregory Franklin^a 

^aDepartment of Integrative Plant Biology, Institute of Plant Genetics of the Polish Academy of Sciences, Poznań, Wielkopolska, Poland,

^bChinese-German Joint Laboratory for Natural Product Research, Qinling-Bashan Mountains Bioresources Comprehensive Development C.I.C., College of Biological Science and Engineering, Shaanxi University of Technology, Hanzhong, Shaanxi, China, ^cDepartment of Pharmaceutics, PSG College of Pharmacy, Coimbatore, Tamil Nadu, India and ^dInstitute of Pharmaceutical Biology, Technische Universität Braunschweig, Braunschweig, Germany

Keywords

elicitation; *Hypericum perforatum*; *in vitro* cultures; nanoparticles; plant secondary metabolism

Correspondence

Gregory Franklin, Department of Integrative Plant Biology, Institute of Plant Genetics of the Polish Academy of Sciences, Strzeszyńska 34, 60-479 Poznań, Poland. E-mail: fgre@igr.poznan.pl

Received October 17, 2016

Accepted April 10, 2017

doi: 10.1111/jphp.12743

Abstract

Objectives In this review, we aim at updating the available information on the improvement of the *Hypericum perforatum* L. (*Hypericaceae*) phytochemical profile and pharmacological properties *via* elicitation.

Key findings *Hypericum perforatum* seedlings, shoots, roots, calli and cell suspension cultures were treated with diverse elicitors to induce the formation of secondary metabolites. The extracts of the elicitor-treated plant material containing naphthodianthrones, phloroglucinols, xanthonones, flavonoids and other new compounds were quantitatively analysed and tested for their bioactivities. While hypericins were mainly produced in *H. perforatum* cultures containing dark nodules, namely shoots and seedlings, other classes of compounds such as xanthonones, phloroglucinols and flavonoids were formed in all types of cultures. The extracts obtained from elicitor-treated samples generally possessed better bioactivities compared to the extract of control biomass.

Summary Although elicitation is an excellent tool for the production of valuable secondary metabolites in *H. perforatum* cell and tissue cultures, its exploitation is still in its infancy mainly due to the lack of reproducibility and difficulties in scaling up biomass production.

Introduction

Hypericum perforatum L. (*Hypericaceae*) known as St John's wort is an important medicinal plant. Extracts, infusions and decoctions have been used in the treatment of various ailments since ancient times. A number of pharmacological studies and clinical trials have shown that *H. perforatum* extracts possess an astounding array of pharmacological properties including antidepressant, anti-inflammatory, antiviral, anticancer and antibacterial activities. In particular, *H. perforatum* extracts are as efficient as their drug counterparts like fluoxetine (Prozac), sertraline (Zoloft) and other leading antidepressant drugs in the treatment of depression. Nowadays, it is a widely used medicinal plant for the treatment of mild and moderate depression,^[1] prescribed for this indication in some EU countries, while sold in the United States as herbal supplement over the counter.^[2]

In addition to its efficacy in the treatment of neurological disorders, studies suggest that this herb may be useful in

treating cancer, inflammation-related disorders and bacterial and viral diseases.^[3–7] These medicinal properties are related to the composition of the secondary metabolites present in the extract, particularly hypericins, hyperforins, flavonoids, xanthonones and other valuable compounds.^[8] Phytochemical characterization of *H. perforatum* revealed the presence of various classes of compounds, including naphthodianthrones (hypericin and pseudohypericin), prenylated acylphloroglucinols (hyperforin and adhyperforin), flavonoids (quercetin, hyperoside, rutin, catechin and isoquercitrin), biflavones (amentoflavone, biapigenin), phenolic compounds (chlorogenic acid, tannic acid and caffeic acid), xanthonones (1,3,6,7-tetrahydroxyxanthone) and essential oil rich in sesquiterpenes^[9–11] (Figure 1).

Currently, *H. perforatum* is one of the top-selling herbal medicines worldwide. Because of its reputed medicinal values, this species is included in the Pharmacopoeias of several countries including Europe and USA. According to the U.S. and European Pharmacopoeias, the crude drug consists of the dried flowering tops or the aerial parts of the

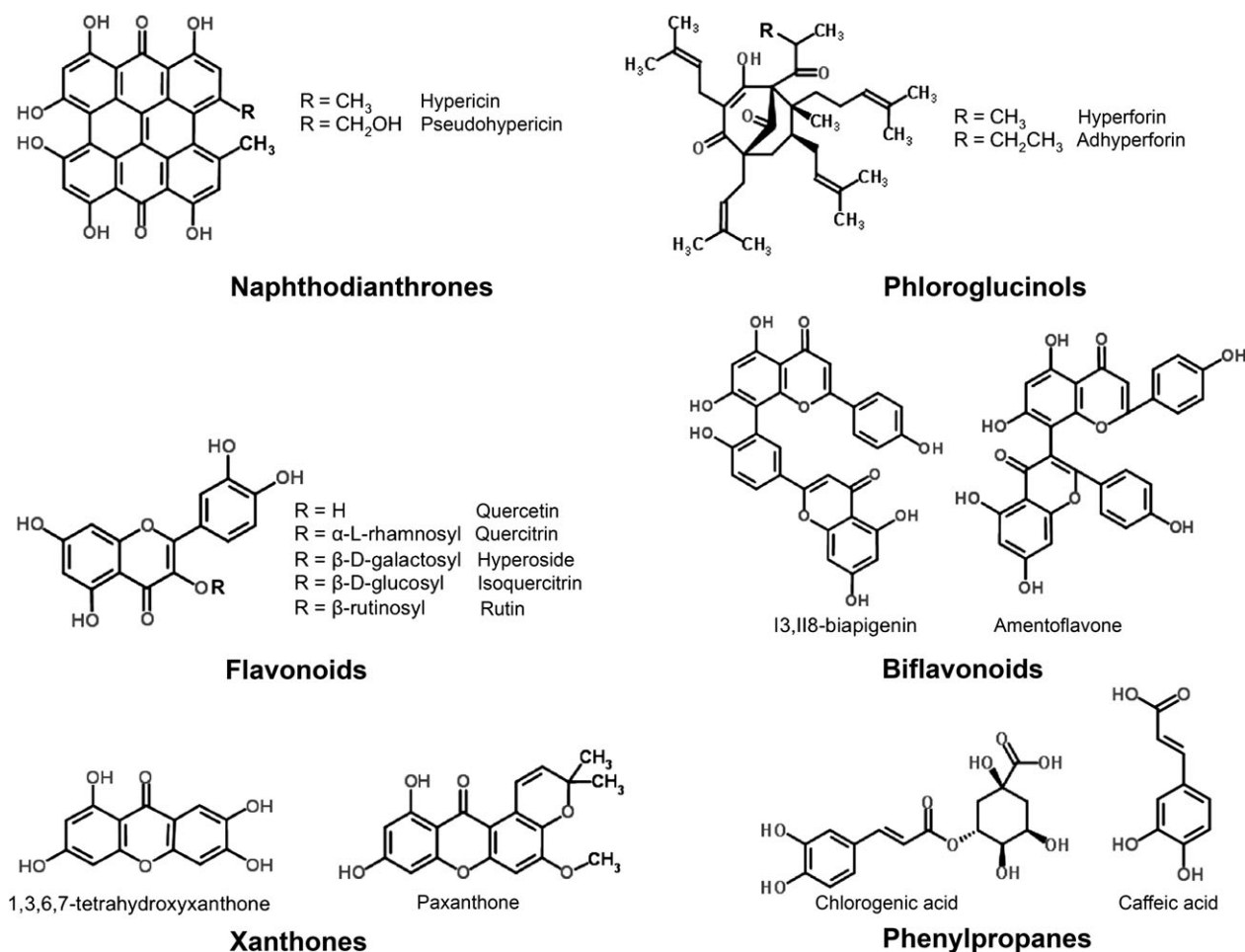


Figure 1 Major classes of secondary metabolites found in *Hypericum perforatum*.

plant. In order to meet the ever-increasing demands of the pharmaceutical industry and to obtain quality biomass, *H. perforatum* is cultivated in many countries. Plants growing in the field conditions are generally exposed to biotic and abiotic challenges, which can affect the phytochemical composition. For example, *H. perforatum* plants obtained from different geographical regions, seasons, soil conditions significantly differ in their phytochemical profile.^[12–14] As the pharmacological potential of *H. perforatum* extracts is mainly determined by its phytochemical composition and ratios between important compounds, such changes may affect the treatment efficacy of the extracts.^[15,16] *In vitro* cultures grown under controlled conditions can overcome these issues and have been emerged as an attractive alternative to field cultivation.^[17,18]

Today, enhancing the contents of important bioactive molecules and producing novel compounds are major aspects of *H. perforatum* biotechnology. Developments in plant cell culture systems and molecular biology offered

many ways to improve the production of compounds such as cell line selection, cell immobilization, permeabilization, precursor feeding, product secretion, biotransformation, metabolic engineering, bioreactor engineering, synthetic biology and elicitation.^[19–21] Among all, elicitation emerges as an attractive strategy for enhancement of secondary metabolite production in plant species like *H. perforatum*, in which the application of metabolic engineering or synthetic biology tools remains difficult due to the lack of proficient transformation methods and genetic information about biosynthetic pathways.^[22] Hence, production of secondary metabolites *via* elicitation using various *in vitro* culture systems is of great interest in *H. perforatum* research.

Significant amounts of data on the manipulation of *H. perforatum* secondary metabolism *via* elicitation have been accumulated in the literature in the recent years. However, a consolidated account or a critical analysis of these published data is not available to the best of our knowledge. Here, we holistically review the available

information on the elicitation of *H. perforatum* secondary metabolism, envisage the potential application of nanoparticles as elicitors and discuss how elicitation-mediated improvement of the *H. perforatum* secondary metabolite profile may be exploited for drug discovery.

The basis of elicitation

Plants have to defend themselves against any threats posed by their environment, such as pathogen attack, herbivory, drought, salinity, exposure to UV radiation. Plants perceive such danger signals through their receptors and sensors and activate defence responses to counteract against these stresses.^[23–26] The responses include secondary metabolism.^[27] For instance, plants recognize pathogen-derived elicitors through receptors bound to the plasma membrane and activate, as a defence response, the production of low-molecular-weight antimicrobial compounds. While these phytoalexins are synthesized by and accumulated in plants only after exposure to pathogenic microbes, phytoanticipins are either pre-existing or synthesized after the microbial attack solely from pre-existing constituents of the plant.^[28] In addition to their role in plant defence response, these compounds often possess important pharmacological properties.^[29,30] The ability of plants to counteract with biotic and abiotic stresses *via* mobilizing their secondary metabolism is the central dogma of elicitation.

An elicitor can be an environmental factor or a signal molecule that activates a signal-transduction cascade, which mediates the expression of genes related to the biosynthesis of secondary metabolites in the biotechnological point of view. Elicitors are mainly classified into three categories based on their origin, namely biological, chemical and physical triggers. The biological elicitors are mainly components of microbial cell walls (chitin, chitosan and glucans) and carbohydrates such as poly- and oligosaccharides derived from plant cell walls (pectin, pectic acid, and cellulose). Poly- and oligosaccharides are the most studied signalling molecules for elicitation pathways because these compounds can effectively induce similar plant defence responses to pathogen invasion.^[31] Upon elicitation, a series of metabolic changes are systematically initiated throughout the plant to activate the plant's innate immune system^[32,33] and also to prime the plant for stress challenge.^[34] Furthermore, the plant defence signalling compounds such as salicylic acid (SA), jasmonic acid (JA), methyl jasmonate (MeJA) and nitric oxide which mediate the defence response can also serve as elicitors and their ability to induce secondary metabolism is well-documented.^[8,35–39] Inorganic agents like heavy metals, metal ions and metal oxides can act as chemical elicitors of plant secondary metabolism.^[40–42]

Physical components like cold shock, UV, ozone, osmotic and water stress also induce enzymatic activity and secondary metabolism.^[43]

The mechanism of elicitation is vastly complex because of thousands of intertwined events. Additionally, all these events fluctuate with the origin, specificity and concentration of elicitors, stage of the growth cycle and nutritional uptake of plants, physiochemical environment of the interaction etc. Although it is difficult to propose a universal model for the elicitation mechanism, calcium flux, reactive oxygen species (ROS) burst and mitogen-activated protein kinase (MAPK) phosphorylation are the initial events triggered in most of the elicitor–plant cell interactions.^[44] Later events like activation of signalling pathways and activation of transcription factors leading to the induction of plant secondary metabolism are also well-documented.^[45–47]

Signal recognition is mediated by receptors and elicitor-binding sites present on the plasma membrane in response to elicitors, which activate the next cascade of events like ion fluxes, Ca²⁺ burst, cytoplasmic acidification, ROS burst, NADPH oxidase activation, G-protein activation and MAPK phosphorylation.^[31] The initial plant response is the exchange of ions, for instance K⁺/Cl[−] effluxes and Ca²⁺/H⁺ influxes, in response to elicitors. Ca²⁺ influx is considered as the most important event because of its diverse involvement in physiological and cellular processes.^[48,49] Ca²⁺ signals are elaborated through conformational changes in various Ca²⁺-binding proteins such as calmodulin, calmodulin-like proteins, calcium-dependent kinases (CDPKs) and phospholipases as well as through secondary messengers like inositol 1,4,5- triphosphate (IP₃) and diacylglycerol (DAG).^[50–52] Ca²⁺/Calmodulin-mediated pathways are involved in many physiological responses of plants to stimuli. CDPKs have diverse roles in downstream signalling cascades and protein phosphorylation to coordinate cellular processes like regulation of the oxidative burst, hormonal signalling and gene expression.^[53] ROS generation is another important phenomenon in plant defence response, as is the effect of NADPH oxidase and other oxidases in plant cells, and even Ca²⁺ spiking is also responsible for ROS generation.^[49,54,55] Studies have shown the linked role of G-proteins in stimulating ion channels, phospholipase A, phospholipase C and phospholipase D, ROS generation and cell death in plants.^[50,56,57] Activated G-protein can stimulate the level of cAMP, IP₃ and DAG, which triggers the target kinases PKA and PKC. These induced protein kinases cause phosphorylation of MAPKs, which results in gene expression leading to enzymatic reactions, which in turn reprogram the pathway of secondary metabolite production^[58] (Figure 2).

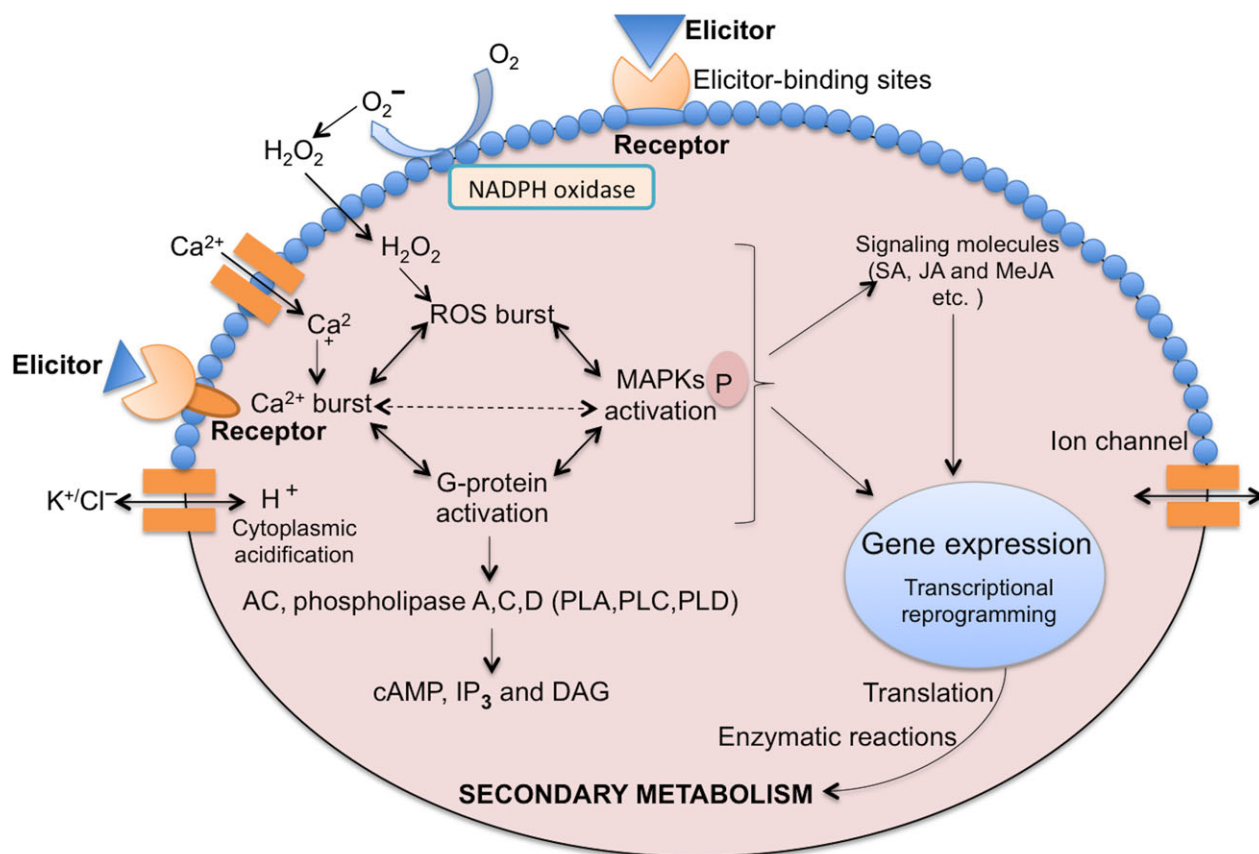


Figure 2 The molecular mechanism of elicitation: Recognition of elicitors by plasma membrane-bound receptors results in ion fluxes, Ca^{2+} burst, cytoplasmic acidification, ROS burst, NADPH oxidase activation, G-protein activation and mitogen-activated protein kinase phosphorylation. It also activates downstream signalling pathway messengers like salicylic acid, jasmonic acid and methyl jasmonate. Messengers activate transcription factors and gene expression, which lead to reprogramming secondary metabolism. [Colour figure can be viewed at wileyonlinelibrary.com]

Manipulation of *Hypericum perforatum* Secondary Metabolism Via Elicitation

In general, the type of culture rather than the type of elicitor defines the compounds induced in *H. perforatum* upon elicitation. Hypericins are the most frequently elicitor-induced compounds in seedlings and shoot cultures, probably due to the presence of hypericin nodules. On the other hand, cell suspensions, calli and root cultures mostly form flavonoids and xanthenes.

In addition to the type of culture, several other factors affect the success of elicitation, which include the type of elicitor, concentration, incubation conditions and duration of elicitor treatment. Various biotic and abiotic elicitors tested for the manipulation of *H. perforatum* secondary metabolism are categorized in Figure 3. The important compounds induced by these elicitors in various types of *H. perforatum* cultures such as seedling, shoot, root, callus and cell suspension are listed in Table 1.

Biotic elicitation

Fungi and extracts from fungal mycelia

In *H. perforatum* shoot cultures, yeast extract did not show any stimulatory effect on either hypericin or pseudohypericin production.^[59] The arbuscular mycorrhizal fungi (AMF) species *Rhizophagus intraradices* enhanced *H. perforatum* seedling growth and hypericin and pseudohypericin production, when supplied to the soil either alone or as mixture with AMF, namely *Funneliformis constrictum*, *F. geosporum* and *F. mosseae*.^[60]

Hypericum perforatum cell suspension cultures treated with *Colletotrichum gloeosporioides* cell wall extract showed a significant increase in xanthone accumulation.^[35] Interestingly, this xanthone accumulation was increased 12-fold when the cultures were primed with MeJA or SA, before adding the fungal extract. *Aspergillus niger*, *Fusarium oxysporum* and yeast extracts increased the accumulation of phenolic compounds and flavonoids in addition to the

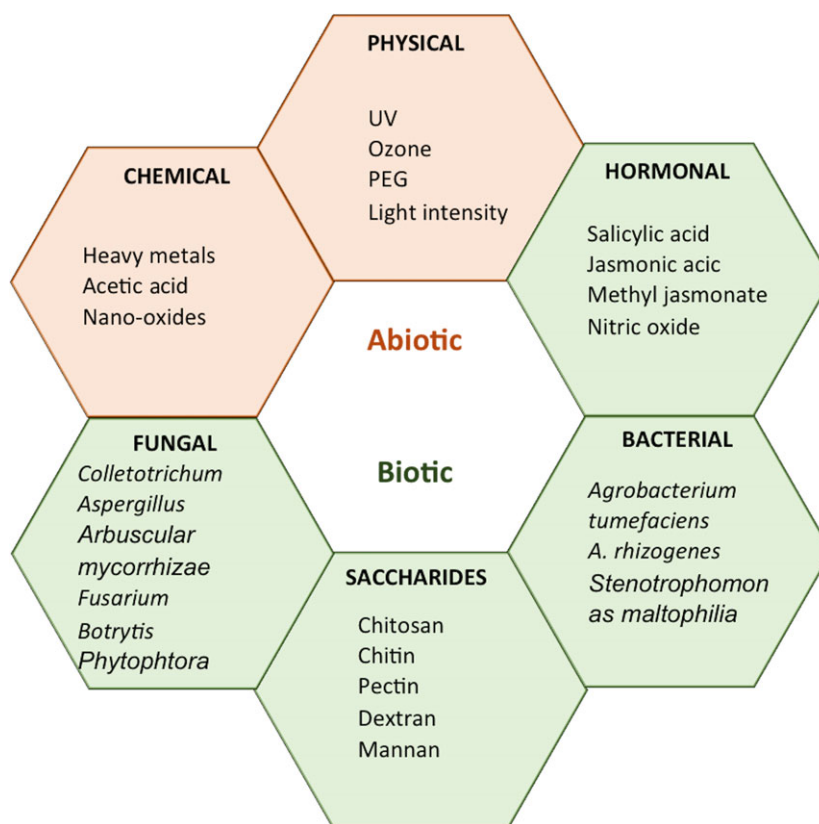


Figure 3 Elicitors of biotic and abiotic origin tested for the induction of secondary metabolites in *Hypericum perforatum*. [Colour figure can be viewed at wileyonlinelibrary.com]

production of new constituents such as *p*-hydroxybenzoic acid in the suspended cells of *H. triquetrifolium*.^[61] On the other hand, mycelium extract from *Aspergillus flavus* only stimulated the level of anthocyanins in *H. perforatum* cell suspensions.^[62]

Interestingly, cell wall extracts from *F. oxysporum*, *Phomaexigua* and *Botrytis cinerea* showed rapid stimulation of naphthodianthrone (hypericin and pseudohypericin) in addition to flavonoids and anthocyanins in cell suspension cultures of *H. perforatum*.^[63] *A. niger* cell walls induced hypericin biosynthesis in *H. perforatum* cell suspension cultures.^[36]

Polysaccharides

Oligosaccharides and polysaccharides, respectively, from fungal and plant cell walls are the most studied signalling molecules in elicitation pathways.^[31] In *H. perforatum* shoot cultures, mannan, β -1,3-glucan, pectin and yeast extract were studied for the production of naphthodianthrone.^[59] Although mannan stimulated the production of pseudohypericin and hypericin substantially, β -1,3-glucan and pectin showed a weak effect on pseudohypericin production but had no effect on hypericin production.

While pectin and dextran enhanced the content of pseudohypericin and hypericin, chitin did not show any stimulatory effect in *H. perforatum* shoot cultures.^[64] Mannan and pectin were tested for the biosynthesis of hypericins in *H. adenotrichum* seedlings grown *in vitro*.^[65] Although both elicitors stimulated the biosynthesis of pseudohypericin and hypericin, the stimulatory potential of mannan was lower than that of pectin.

Treatment with chitin, pectin and dextran improved production of phenylpropanoids (phenolics, flavanols, anthocyanins) in cell suspension cultures.^[66] Brasil et al.^[67,68] studied the effect of chitosan treatment in *H. perforatum* adventitious root cultures. Chitosan increased the synthesis of epicatechin, xanthones and isoprenoids^[68] and a new xanthone, brasilixanthone B, was identified in treated root cultures.^[67]

Bacteria

Elicitation of *H. perforatum* shoot cultures with a combination of saccharose and inactivated *Agrobacterium tumefaciens* promoted the hypericin and hyperforin production.^[69] Treatment of *H. perforatum* seedlings with *Stenotrophomonas maltophilia* increased the hypericin and pseudohypericin

Table 1 Various types of *Hypericum perforatum* cultures and elicitors used in the induction of secondary metabolites

Culture type	Elicitor	Compounds elicited	Reference	
Cell suspension	Zinc nano-oxide	Hypericin and hyperforin	[41]	
	Iron nano-oxide	Hypericin and hyperforin	[41]	
	Chitin	Phenolics, flavonols, flavanols, anthocyanins, hypericin and pseudohypericin	[66]	
	Pectin	Phenolics, flavonols, flavanols, anthocyanins, hypericin and pseudohypericin	[66]	
	Dextran	Phenolics, flavonols, flavanols, anthocyanins, hypericin and pseudohypericin	[66]	
	<i>Phytophthora cinnamoni</i> fungal cell wall extract	None	[39]	
	<i>Colletotrichum gloeosporioides</i> mycelium extract	Xanthenes and flavonoids	[35]	
	<i>Aspergillus flavus</i> mycelium extract	Phenolic compounds, flavanols, flavonols, anthocyanins and hypericins	[62]	
	<i>Fusarium oxysporum</i>	Phenolics, flavonoids, anthocyanins, hypericin and pseudohypericin	[63]	
	<i>Phoma exigua</i>	Phenolics, flavonoids, anthocyanins, hypericin and pseudohypericin	[63]	
	<i>Botrytis cinerea</i>	Phenolics, flavonoids, anthocyanins, hypericin and pseudohypericin	[63]	
	JA	Hypericins	[39]	
	JA	Phenolic compounds, flavanols, flavonols, hypericin and pseudohypericin	[38]	
	MeJA	Flavones	[35]	
	MeJA	Flavonoids	[8]	
	SA	Xanthenes	[85]	
	SA	None	[35]	
	SA	None	[39]	
	SA	Hypericins, pseudohypericins	[86]	
	SA	Flavonoids	[8]	
	MeJA + <i>C. gloeosporioides</i>	Xanthenes	[35]	
	SA + <i>C. gloeosporioides</i>	Xanthenes	[35]	
	<i>Agrobacterium tumefaciens</i>	Xanthenes	[71]	
<i>A. tumefaciens</i>	Lignin and flavanoids	[72]		
<i>A. tumefaciens</i>	Phenolics, flavonols, flavanols and xanthenes	[73]		
<i>A. rhizogenes</i>	Phenolics, flavonols, flavanols and xanthenes	[73]		
Callus	SA	Hypericins, pseudohypericins	[86]	
Root	MeJA	Hypericin	[84]	
	IBA	Hypericin	[84]	
	Acetic acid	Xanthenes	[91]	
	Chitosan	Xanthenes	[68]	
	Chitosan	Xanthenes	[68]	
Shoot	SA	Hypericins and pseudohypericins	[86]	
	Mannan	Pseudohypericin and hypericin	[59]	
	β -1,3-glucan	Pseudohypericin	[59]	
	Pectin	Pseudohypericin	[59]	
	Yeast extract	None	[59]	
	Chitin	None	[64]	
	Pectin	Hypericin and pseudohypericin	[64]	
	Dextran	Hypericin and pseudohypericin	[64]	
	Saccharose	Hypericin and hyperforin	[69]	
	Saccharose + PEG	Hypericin and hyperforin	[69]	
	Saccharose + MeJA	Hypericin and hyperforin	[69]	
	Saccharose + <i>A. tumefaciens</i>	Hypericin and hyperforin	[69]	
	<i>Stenotrophomonas maltophilia</i> culture filtrate	Pseudohypericin	[70]	
	Seedling	Chromium	Protopseudohypericin, hypericin and pseudohypericin	[40]
		Nickel	None	[12]
<i>Rhizophagus intraradices</i>		Hypericin and pseudohypericin	[60]	
<i>S. maltophilia</i>		Hypericin and pseudohypericin	[70]	

SA, salicylic acid; JA, jasmonic acid; MeJA, methyl jasmonate; PEG, polyethylene glycol.

contents.^[70] These authors also reported that treating *H. perforatum* shoot cultures with a *S. maltophilia* culture filtrate induced only pseudohypericin production.^[70]

Analysis of the methanolic extract of cell suspension cultures treated with *A. tumefaciens* revealed a 12-fold increase in the total xanthone concentration and the emergence of

many new xanthenes.^[71] In addition, the contents of lignin and flavonoids (quercetin, quercetrin etc.) were also significantly increased in the cell wall fraction of phenolics after elicitation with *A. tumefaciens*.^[72] Similarly, improvement of phenolic, flavonol, flavanol and xanthone concentrations in response to *A. tumefaciens* and *A. rhizogenes* treatment was observed in cell suspension cultures.^[73] However, *A. rhizogenes* was less effective in the induction of secondary metabolism compared to *A. tumefaciens*.^[73] Induction of *H. perforatum* plant secondary metabolism by *Agrobacterium* might be attributed to components such as cold-shock protein, flagellin, peptidoglycan and elongation factor-Tu.^[74–80]

Signalling compounds and plant growth regulators

Plant defence signalling compounds such as SA, MeJA, JA have been shown to modulate *H. perforatum* secondary metabolism. When treated with an analogue of MeJA, 2,3-dihydroxypropyl jasmonate, hypericins and hyperforin production was increased in *H. perforatum* and *H. sampsonii* shoot cultures.^[81] Different combinations of plant growth regulators and signalling compounds (JA and SA) enhanced the accumulation of hypericins, pseudohypericin and hyperforin in shoot cultures of *H. hirsutum* and *H. maculatum*.^[82] The supplementation of plant growth regulators in culture medium variously affected naphthodianthrone accumulation in shoot cultures.^[83] This study reported that the presence of IBA actually decreased the concentration of hypericin and pseudohypericin although the naphthodianthrone production in plantlets was not influenced by IAA supplementation.

The MeJA concentration showed a negative correlation with biomass production and a positive correlation with hypericin production in adventitious root cultures grown in balloon-type airlift bioreactors, in which an optimum hypericin content of 1.61 mg/g DW was achieved at 350 μ M MeJA.^[84]

The effect of signalling compounds on the induction of secondary metabolism in cell suspension cultures of *H. perforatum* is divergently reported between groups. Conceicao *et al.*^[35] reported that MeJA-treated *H. perforatum* cell suspension cultures accumulated new flavonoids (flavones), whereas SA was unable to induce any change of secondary metabolism. Elicitation of cell cultures with 100 μ M MeJA on day 15 resulted in 2.7 times higher flavonoid production (280 mg/l) compared to control cultures.^[8] Another study reported that JA treatment significantly increased phenolic compounds, flavanols and flavonols, concomitantly reducing the anthocyanin content.^[38] A twofold increase in the xanthone, cadensin G and paxanthone contents was found in cell suspension cultures of *H. perforatum* following elicitation with SA.^[85]

Walker *et al.*^[39] found that JA dramatically enhanced hypericin production in cell cultures incubated in the dark compared to cultures grown under photoperiodic conditions, although SA treatment could not induce hypericin production in cell suspensions. Contrarily, SA remarkably influenced the production of hypericin and pseudohypericin in *H. perforatum* cell suspension cultures as per the report of Gadzovska-Simic *et al.*^[86]

Abiotic elicitation

Physical factors

A few physical factors such as exposure to high light intensity, ozone, UV and osmotic stress have been tested for the induction of secondary metabolism in *H. perforatum*. The hyperforin, pseudohypericin and hypericin concentrations were altered in *H. perforatum* plants after UV-B exposure during the vegetative growth.^[87] However, the level and length of UV-B radiation negatively affected the hypericin concentration in leaves, although the flavonoid and tannin contents increased with enhanced levels of UV-B exposure.^[88] Treatment with polyethylene glycol (PEG) increased the hypericin and pseudohypericin concentrations in cultured *H. adenotrichum* seedlings. However, this increase was dependent on the concentration of PEG and the treatment period, 10 g/l PEG for a 15-day treatment being optimal.^[89] On the other hand, synthesis of hypericin and hyperforin in *H. perforatum* shoot cultures was not increased in the presence of 10 and 15 g/l PEG, although a lower PEG concentration stimulated the production of those compounds.^[69] Sucrose as an osmotic agent was shown to stimulate hypericins in cultured seedlings of *H. adenotrichum* with increasing concentrations until 45 g/l, followed by decline.^[89] Correspondingly, the presence of saccharose (10–30 g/l) influenced the production of hypericin and hyperforin in *H. perforatum* shoot cultures and a combination of PEG (1.25–5 g/l) and saccharose (10–30 g/l) synergistically increased the production of the above compounds.^[69]

Hypericum perforatum cell suspension cultures accumulated anthocyanins and flavonoids with concomitant reduction of xanthenes upon increasing the irradiance of cultures from 20 μ mol/s per m² to 60 μ mol/s per m².^[90] Exposure to ozone stimulated hypericin synthesis in *H. perforatum* cell suspension cultures, with the cell cultures at the exponential phase being more responsive than the ones at the lag and stationary phases.^[91]

Chemical

Chromium treatment induced the production of protopseudohypericin, hypericin and pseudohypericin in

H. perforatum seedlings, whereas nickel treatment did not show any stimulatory effect.^[40] In *H. perforatum* root cultures, acetic acid enhanced xanthone production.^[91]

Treatment of *H. perforatum* cell suspension cultures with 100 ppb zinc and iron nano-oxides promoted hypericin and hyperforin production, while higher concentrations of these nanomaterials negatively affected the production of these compounds.^[41]

Pharmacological properties of *Hypericum perforatum* after elicitation

Although a number of studies report the elicitor-induced modulation of the phytochemical profile of *H. perforatum* *in vitro* cultures, only a few of them actually tested the bioactivity of the treated cultures. The methanolic extract of *H. perforatum* cell suspension cultures challenged with *A. tumefaciens* was studied for various bioactivities. This extract showed a higher antioxidant potential, a greater capacity to prevent synaptosomal lipid peroxidation^[71] and improved antimicrobial activity against several human pathogenic bacteria.^[92] It also offered human HepG2 cells more protection against t-BOOH-induced oxidative damage, compared to control extract.^[93] Tocci et al.^[94] have shown that extracts of root tissue elicited with chitosan, O-carboxymethyl chitosan and its derivatives showed antifungal activity against human fungal pathogens such as *Candida* spp., *Cryptococcus neoformans* and dermatophytes.

Future Perspectives

Potential application of nanotechnology

In addition to several well-known biotic and abiotic elicitors discussed above, nanoparticles are currently emerging as new class of elicitors. A few studies on the induction of secondary metabolites in response to nanoparticle treatment provide insights in the exploitation of these submicron size particles as elicitors of secondary metabolites. For instance, the artemisinin content was increased in a hairy root culture of *Artemisia annua* upon treatment with core-shell silver nanoparticles.^[95] In *Calendula officinalis*, the anthocyanin and flavonoid contents were decreased, whereas carotenoid and saponin contents were increased in response to silver nanoparticles, as revealed by classical colorimetric assays.^[96] In addition, multiwalled carbon nanotubes stimulated the biosynthesis of secondary metabolites and the antioxidant capacity in the medicinal plant *Satureja khuzestanica* grown *in vitro*.^[97] In the case of *H. perforatum*, one study has reported that zinc and iron nano-oxides stimulate hypericin and hyperforin production in cell suspension cultures.^[41]

In addition to the use of metallic nanoparticles as elicitors, signalling compounds such as JA, SA and MeJA can be encapsulated in biodegradable polymers as nanoparticles and can be exploited for sustained release of the signalling molecules into the culture medium, which might be useful in sustained production of secondary metabolites without affecting the growth of cultures. The possibility of using metallic nanoparticles as trappers of secondary metabolites from plants has been demonstrated in *A. thaliana*.^[98] The authors reported that anatase TiO₂ nanoparticles (smaller than 20 nm) enter plant cells, conjugate enediol- and catechol group-rich flavonoids *in situ* and exit plant cells as flavonoid-nanoparticle conjugates. The compound adsorption capacities of nanoparticles may be further improved by functionalization. For instance, the adsorption capacity of SiO₂ nanoparticles towards quercetin could be improved by TiO₂ functionalization compared to non-functionalized and decyl group-functionalized SiO₂ nanoparticles due to possible binding of quercetin to the metal oxide.^[99] This adsorption capacity increased linearly with surface coverage of TiO₂ emphasizing the correlation between the functional surface and quercetin adsorption. This phenomenon may provide us an attractive opportunity to establish nanotrapping strategies for a wide variety of secondary metabolites in the near future.

Elicitation of *Hypericum perforatum* secondary metabolism: a road to drug discovery

Although manipulation of *H. perforatum* secondary metabolism *via* elicitation has been reported in numerous studies, very few authors have actually tested the pharmacological properties of induced compounds and extracts from treated cells.^[92–94] To promote the discovery of novel drugs, extracts from elicitor-treated cells containing new compounds should be analysed and screened for a variety of bioactivities. Bioactivity-guided fractionation may be used. The possible road to drug discovery after elicitation is summarized in the following Figure 4.

In spite of the exciting possibility of inducing diverse classes of bioactive compounds in *H. perforatum* cell and tissue cultures *via* elicitation, industrial exploitation of elicitation-based changes in secondary metabolism and pharmacological properties is still in its infancy. The production of significant quantities of aseptic biomass for elicitation is the major concern. In this context, small-scale bioreactors of *in vitro* cultures for the production of active compounds have been reported.^[18] Recently, adventitious root cultures in large-scale bioreactors for the production of *H. perforatum* phytochemicals have been developed.^[84,100] The choice of the correct culture vessels and the determination of exogenous signals needed for *in vitro* production of biomass and

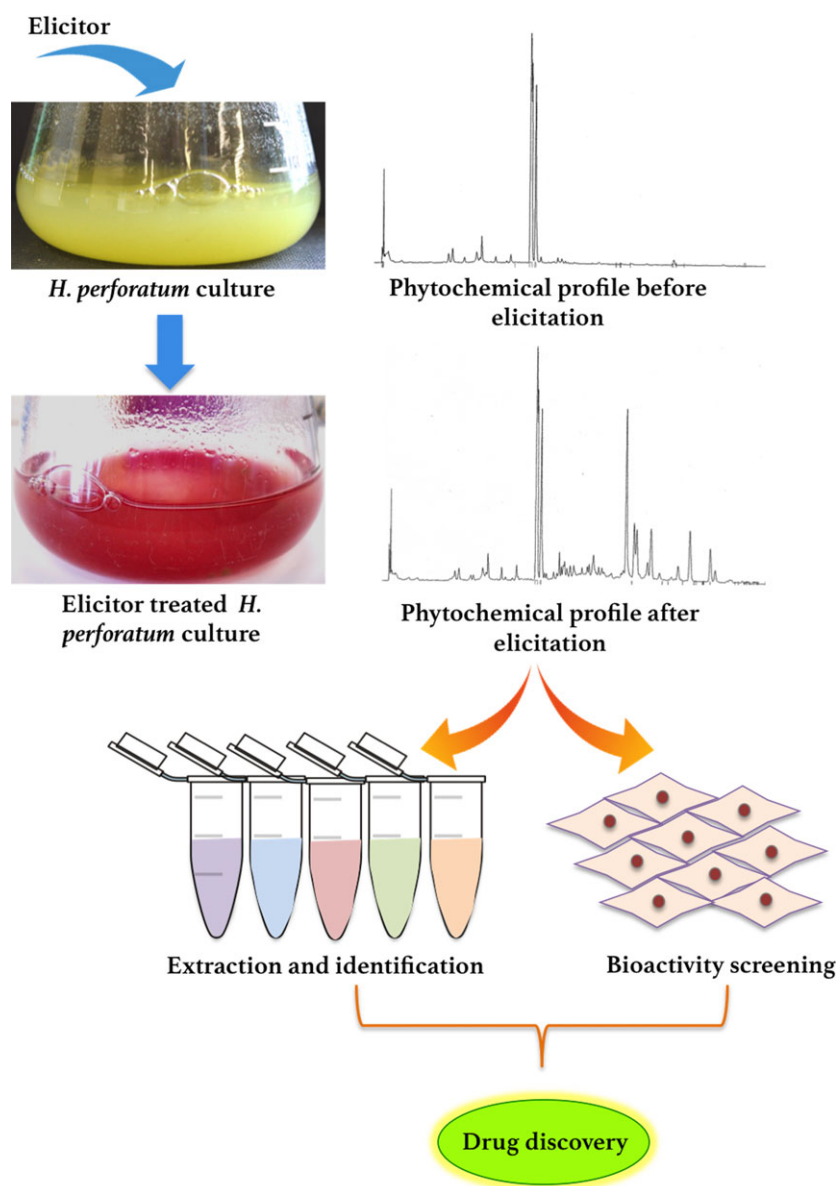


Figure 4 Scheme of possible exploitation of elicitation-mediated changes in the *Hypericum perforatum* secondary metabolite profile for drug discovery via bioactivity-guided fractionation. [Colour figure can be viewed at wileyonlinelibrary.com]

optimization of elicitation measures are essential factors for the further progress in this area.

Conclusion

Based on our analysis of the literature, it is evident that seedlings and *in vitro* cultures of *H. perforatum* are efficient systems to produce a wide variety of bioactive secondary metabolites via elicitation. The paradoxical results obtained between groups in terms of phytochemical profile, namely hypericin production after elicitation, argue for the complexity of culture maintenance, elicitation and analytical

methods. For example, under normal conditions, hypericin is produced only in the presence of dark hypericin nodules. Several studies have confirmed a clear positive correlation between the presence of dark cell clusters and hypericin accumulation irrespective of the tissue, genotype, species etc.^[101–104] Roots of *H. perforatum* plants lack these clusters, where no traces of hypericin were found.^[105] In spite of the absence of these nodules in roots and cell suspension cultures of *H. perforatum*, few groups have actually reported the formation of hypericins in these cultures after elicitation.^[38,39,41,63,66,84,86] Further investigation on the ability of *H. perforatum* cell and tissue cultures without

these dark cell clusters to produce hypericin might offer novel clues on the hypericin biosynthetic pathway.

As the secondary metabolite elicitation potential varies between the types of cultures, elicitors, treatment conditions and other parameters, further research is needed to optimize the best and reproducible protocols. In this context, understanding the metabolic pathways leading to the production of specific secondary metabolites and their regulation is imperative. However, with the exception of the flavonoid pathway, complex biosynthetic pathways specifically evolved in *Hypericum* spp. are far from being understood. The lack of relevant information on the enzymes and genes involved as well as the transcription factors and master switches controlling these pathways is the major obstacle for developing efficient strategies for elicitation of *H. perforatum* secondary metabolism. Comparative transcriptomic, metabolomic and proteomic studies carried out

on both control and elicitor-treated cultures are expected to offer important clues.

Declarations

Acknowledgements

This work has received funding from the National Science Center, Poland (2016/21/B/NZ9/01980). PS and GF are supported by European Union's 7th Framework Programme for research, technological development and demonstration under grant agreement no 621321 and co-financed by funds allocated for education through project no W26/7.PR/2015 [GA 3413/7.PR/2015/2] for the years 2015–2019.

References

- Oliveira AI et al. Neuroprotective activity of *Hypericum perforatum* and its major components. *Front Plant Sci* 2016; 7: 1004.
- Shelton RC. St John's wort (*Hypericum perforatum*) in major depression. *J Clin Psychiatry* 2009; 70 (Suppl 5): 23–27.
- Franchi GG et al. Composition and antioxidant activity in vitro of different St. John's wort (*Hypericum perforatum* L.) extracts. *J Med Plants Res* 2011; 5: 4349–4353.
- Klemow KM et al. 11 Medical Attributes of St. John's Wort (*Hypericum perforatum*). Lester Packer, PhD. 2011: 211.
- Silva BA et al. Phytochemical and antioxidant characterization of *Hypericum perforatum* alcoholic extracts. *Food Chem* 2005; 90: 157–167.
- Silva BA et al. St. John's wort (*Hypericum perforatum*) extracts and isolated phenolic compounds are effective antioxidants in several in vitro models of oxidative stress. *Food Chem* 2008; 110: 611–619.
- Olivo M et al. New frontier in hypericin-mediated diagnosis of cancer with current optical technologies. *Ann Biomed Eng* 2012; 40: 460–473.
- Wang J et al. Enhanced production of flavonoids by methyl jasmonate elicitation in cell suspension culture of *Hypericum perforatum*. *Bioresour Bioprocess* 2015; 2: 1–9.
- Tatsis EC et al. Identification of the major constituents of *Hypericum perforatum* by LC/SPE/NMR and/or LC/MS. *Phytochemistry* 2007; 68: 383–393.
- Guedes AP et al. *Hypericum* sp.: essential oil composition and biological activities. *Phytochem Rev* 2012; 11: 127–152.
- Bruni R, Sacchetti G. Factors affecting polyphenol biosynthesis in wild and field grown St. John's wort (*Hypericum perforatum* L. Hypericaceae/Guttiferae). *Molecules* 2009; 14: 682–725.
- Murch SJ et al. Nickel contamination affects growth and secondary metabolite composition of St. John's wort (*Hypericum perforatum* L.). *Environ Exp Bot* 2003; 49: 251–257.
- Bruni R et al. Herbal drug quality and phytochemical composition of *Hypericum perforatum* L. affected by ash yellows phytoplasma infection. *J Agric Food Chem* 2005; 53: 964–968.
- Southwell IA, Bourke CA. Seasonal variation in hypericin content of *Hypericum perforatum* L. (St. John's Wort). *Phytochemistry* 2001; 56: 437–441.
- Marrelli M et al. *Hypericum perforatum*: influences of the habitat on chemical composition, photo-induced cytotoxicity, and antiradical activity. *Pharm Biol* 2014; 52: 909–918.
- Božin B et al. Impact of origin and biological source on chemical composition, anticholinesterase and antioxidant properties of some St. John's wort species (*Hypericum* spp., Hypericaceae) from the Central Balkans. *Molecules* 2013; 18: 11733–11750.
- Murch SJ, Saxena PK. St. John's wort (*Hypericum perforatum* L.): challenges and strategies for production of chemically-consistent plants. *Can J Plant Sci* 2006; 86: 765–771.
- Zobayed SMA et al. In vitro production and chemical characterization of St. John's wort (*Hypericum perforatum* L. cv 'New Stem'). *Plant Sci* 2004; 166: 333–340.
- Rao SR, Ravishankar G. Plant cell cultures: chemical factories of secondary metabolites. *Biotechnol Adv* 2002; 20: 101–153.
- Wilson SA, Roberts SC. Recent advances towards development and commercialization of plant cell culture processes for the synthesis of biomolecules. *Plant Biotechnol J* 2012; 10: 249–268.
- Trosset J-Y, Carbonell P. Synthetic biology for pharmaceutical drug discovery. *Drug Des Devel Ther* 2015; 9: 6285.
- Hou W et al. A perspective on *Hypericum perforatum* genetic transformation. *Front Plant Sci* 2016; 7: 879.

23. Ulm R, Jenkins GI. Q&A: how do plants sense and respond to UV-B radiation? *BMC Biol* 2015; 13: 45.
24. Wu L *et al.* Go in for the kill: how plants deploy effector-triggered immunity to combat pathogens. *Virulence* 2014; 5: 710–721.
25. Wu J, Baldwin IT. New insights into plant responses to the attack from insect herbivores. *Annu Rev Genet* 2010; 44: 1–24.
26. Osakabe Y *et al.* Sensing the environment: key roles of membrane-localized kinases in plant perception and response to abiotic stress. *J Exp Bot* 2013; 64: 445–458.
27. Dixon RA, Paiva NL. Stress-induced phenylpropanoid metabolism. *Plant Cell* 1995; 7: 1085–1097.
28. VanEtten HD *et al.* Two classes of plant antibiotics: phytoalexins versus “phytoanticipins”. *Plant Cell* 1994; 6: 1191.
29. González-Lamothe R *et al.* Plant antimicrobial agents and their effects on plant and human pathogens. *Int J Mol Sci* 2009; 10: 3400–3419.
30. Park S *et al.* Glyceollins, one of the phytoalexins derived from soybeans under fungal stress, enhance insulin sensitivity and exert insulinotropic actions. *J Agric Food Chem* 2010; 58: 1551–1557.
31. Zhao J *et al.* Elicitor signal transduction leading to production of plant secondary metabolites. *Biotechnol Adv* 2005; 23: 283–333.
32. Jones JD, Dangl JL. The plant immune system. *Nature* 2006; 444: 323–329.
33. Van Wees SC *et al.* Plant immune responses triggered by beneficial microbes. *Curr Opin Plant Biol* 2008; 11: 443–448.
34. Conrath U. Molecular aspects of defence priming. *Trends Plant Sci* 2011; 16: 524–531.
35. Conceicao LF *et al.* Induction of phenolic compounds in *Hypericum perforatum* L. cells by *Colletotrichum gloeosporioides* elicitation. *Phytochemistry* 2006; 67: 149–155.
36. Xu M-J *et al.* Nitric oxide mediates the fungal elicitor-induced hypericin production of *Hypericum perforatum* cell suspension cultures through a jasmonic-acid-dependent signal pathway. *Plant Physiol* 2005; 139: 991–998.
37. Sivakumar G, Paek K. Methyl jasmonate induce enhanced production of soluble biophenols in Panax ginseng adventitious roots from commercial scale bioreactors. *Chem Nat Compd* 2005; 41: 669–673.
38. Gadzovska S *et al.* Jasmonic acid elicitation of *Hypericum perforatum* L. cell suspensions and effects on the production of phenylpropanoids and naphthodianthrones. *Plant Cell Tissue Organ Cult* 2007; 89: 1–13.
39. Walker TS *et al.* Jasmonic acid-induced hypericin production in cell suspension cultures of *Hypericum perforatum* L. (St. John’s wort). *Phytochemistry* 2002; 60: 289–293.
40. Tirillini B *et al.* Induction of hypericins in *Hypericum perforatum* in response to chromium. *Fitoterapia* 2006; 77: 164–170.
41. Sharafi E *et al.* Improvement of hypericin and hyperforin production using zinc and iron nano-oxides as elicitors in cell suspension culture of St John’s wort (*Hypericum perforatum* L.). *J Med Plants By-Prod* 2013; 2: 177–184.
42. Ramirez-Estrada K *et al.* Elicitation, an effective strategy for the biotechnological production of bioactive high-added value compounds in plant cell factories. *Molecules* 2016; 21: 182.
43. Ramakrishna A, Ravishankar GA. Influence of abiotic stress signals on secondary metabolites in plants. *Plant Signal Behav* 2011; 6: 1720–1731.
44. Seybold H *et al.* Ca²⁺ signalling in plant immune response: from pattern recognition receptors to Ca²⁺ decoding mechanisms. *New Phytol* 2014; 204: 782–790.
45. Schluttenhofer C, Yuan L. Regulation of specialized metabolism by WRKY transcription factors. *Plant Physiol* 2015; 167: 295–306.
46. Naoumkina MA *et al.* Elicitor-induced transcription factors for metabolic reprogramming of secondary metabolism in *Medicago truncatula*. *BMC Plant Biol* 2008; 8: 132.
47. Maeda K *et al.* DcMYB1 acts as a transcriptional activator of the carrot phenylalanine ammonia-lyase gene (DcPAL1) in response to elicitor treatment, UV-B irradiation and the dilution effect. *Plant Mol Biol* 2005; 59: 739–752.
48. Trewavas AJ, Malhó R. Ca²⁺ signalling in plant cells: the big network!. *Curr Opin Plant Biol* 1998; 1: 428–433.
49. White PJ, Broadley MR. Calcium in plants. *Ann Bot* 2003; 92: 487–511.
50. Meijer HJ, Munnik T. Phospholipid-based signaling in plants. *Annu Rev Plant Biol* 2003; 54: 265–306.
51. Wang X. Phospholipase D in hormonal and stress signaling. *Curr Opin Plant Biol* 2002; 5: 408–414.
52. Bigeard J *et al.* Signaling mechanisms in pattern-triggered immunity (PTI). *Mol Plant* 2015; 8: 521–539.
53. Boudsocq M, Sheen J. CDPKs in immune and stress signaling. *Trends Plant Sci* 2013; 18: 30–40.
54. Bolwell GP, Wojtaszek P. Mechanisms for the generation of reactive oxygen species in plant defence – a broad perspective. *Physiol Mol Plant Pathol* 1997; 51: 347–366.
55. Zhao J *et al.* Elicitor-induced indole alkaloid biosynthesis in *Catharanthus roseus* cell cultures is related to Ca²⁺ influx and the oxidative burst. *Plant Sci* 2001; 161: 423–431.
56. Yang Z. Small GTPases versatile signaling switches in plants. *Plant Cell* 2002; 14(Suppl 1): S375–S388.
57. Roos W *et al.* A redox-dependent, G-protein-coupled phospholipase A of the plasma membrane is involved in the elicitation of alkaloid biosynthesis in *Eschscholtzia californica*. *Biochim Biophys Acta* 1999; 1448: 390–402.
58. Vasconsuelo A, Boland R. Molecular aspects of the early stages of elicitation of secondary metabolites in plants. *Plant Sci* 2007; 172: 861–875.
59. Kirakosyan A *et al.* Stimulation of the production of hypericins by mannan in *Hypericum perforatum* shoot cultures. *Phytochemistry* 2000; 53: 345–348.
60. Zubek S *et al.* Hypericin and pseudo-hypericin concentrations of a

- valuable medicinal plant *Hypericum perforatum* L. are enhanced by arbuscular mycorrhizal fungi. *Mycorrhiza* 2012; 22: 149–156.
61. Azeez H, Ibrahim K. Effect of biotic elicitors on secondary metabolite production in cell suspensions of *Hypericum triquetrifolium* Turra. *Bull Univ Agric Sci Vet Med Cluj-Napoca Horticult* 2013; 70: 26–33.
 62. Gadzovska-Simic S et al. Secondary metabolite production in *Hypericum perforatum* L. cell suspensions upon elicitation with fungal mycelia from *Aspergillus flavus*. *Arch Biol Sci* 2012; 64: 113–121.
 63. Simic SG et al. Fungal elicitor-mediated enhancement in phenylpropanoid and naphthodianthrone contents of *Hypericum perforatum* L. cell cultures. *Plant Cell Tissue Organ Cult* 2015; 122: 213–226.
 64. Gadzovska Simic S et al. Effects of polysaccharide elicitors on secondary metabolite production and antioxidant response in *Hypericum perforatum* L. shoot cultures. *ScientificWorldJournal* 2014; 2014: 609649.
 65. Yamaner Ö et al. Stimulation of the production of hypericins in in vitro seedlings of *Hypericum adenotrichum* by some biotic elicitors. *Turk J Botany* 2013; 37: 153–159.
 66. Simic SG et al. Polysaccharide elicitors enhance phenylpropanoid and naphthodianthrone production in cell suspension cultures of *Hypericum perforatum*. *Plant Cell Tissue Organ Cult* 2015; 122: 649–663.
 67. Brasili E et al. Metabolic profile and root development of *Hypericum perforatum* L. in vitro roots under stress conditions due to chitosan treatment and culture time. *Front Plant Sci* 2016; 7: 507.
 68. Brasili E et al. A non-targeted metabolomics approach to evaluate the effects of biomass growth and chitosan elicitation on primary and secondary metabolism of *Hypericum perforatum* in vitro roots. *Metabolomics* 2014; 10: 1186–1196.
 69. Pavlik M et al. Hypericin and hyperforin production in St. John's wort in vitro culture: influence of saccharose, polyethylene glycol, methyl jasmonate, and *Agrobacterium tumefaciens*. *J Agric Food Chem* 2007; 55: 6147–6153.
 70. Mañero FJG et al. Elicitation of secondary metabolism in *Hypericum perforatum* by rhizosphere bacteria and derived elicitors in seedlings and shoot cultures. *Pharma Biol* 2012; 50: 1201–1209.
 71. Franklin G et al. Xanthone biosynthesis in *Hypericum perforatum* cells provides antioxidant and antimicrobial protection upon biotic stress. *Phytochemistry* 2009; 70: 60–68.
 72. Singh RK et al. Lignin and flavonoid content increases in *Hypericum perforatum* cell wall after *Agrobacterium tumefaciens* co-cultivation. *Planta Med* 2014; 80: 1388.
 73. Tusevski O et al. *Agrobacterium* enhances xanthone production in *Hypericum perforatum* cell suspensions. *Plant Growth Regul* 2015; 76: 199–210.
 74. Felix G, Boller T. Molecular sensing of bacteria in plants – the highly conserved RNA-binding motif RNP-1 of bacterial cold shock proteins is recognized as an elicitor signal in tobacco. *J Biol Chem* 2003; 278: 6201–6208.
 75. Felix G et al. Plants have a sensitive perception system for the most conserved domain of bacterial flagellin. *Plant J* 1999; 18: 265–276.
 76. Kunze G et al. The N terminus of bacterial elongation factor Tu elicits innate immunity in *Arabidopsis* plants. *Plant Cell* 2004; 16: 3496–3507.
 77. Zipfel C et al. Bacterial disease resistance in *Arabidopsis* through flagellin perception. *Nature* 2004; 428: 764–767.
 78. Zipfel C et al. Perception of the bacterial PAMP EF-Tu by the receptor EFR restricts *Agrobacterium*-mediated transformation. *Cell* 2006; 125: 749–760.
 79. Erbs G et al. Peptidoglycan and muropeptides from pathogens *Agrobacterium* and *Xanthomonas* elicit plant innate immunity: structure and activity. *Chem Biol* 2008; 15: 438–448.
 80. Bauer Z et al. Sensitivity of different ecotypes and mutants of *Arabidopsis thaliana* toward the bacterial elicitor flagellin correlates with the presence of receptor-binding sites. *J Biol Chem* 2001; 276: 45669–45676.
 81. Liu X-N et al. Effects of cytokinins and elicitors on the production of hypericins and hyperforin metabolites in *Hypericum sampsonii* and *Hypericum perforatum*. *Plant Growth Regul* 2007; 53: 207–214.
 82. Coste A et al. Effects of plant growth regulators and elicitors on production of secondary metabolites in shoot cultures of *Hypericum hirsutum* and *Hypericum maculatum*. *Plant Cell Tissue Organ Cult* 2011; 106: 279–288.
 83. Gadzovska S et al. Identification and quantification of hypericin and pseudohypericin in different *Hypericum perforatum* L. in vitro cultures. *Plant Physiol Biochem* 2005; 43: 591–601.
 84. Wu S-Q et al. Several factors affecting hypericin production of *Hypericum perforatum* during adventitious root culture in airlift bioreactors. *Acta Physiol Plant* 2014; 36: 975–981.
 85. Zubrická D et al. Xanthonas from roots, hairy roots and cell suspension cultures of selected *Hypericum* species and their antifungal activity against *Candida albicans*. *Plant Cell Rep* 2015; 34: 1953–1962.
 86. Gadzovska S et al. The influence of salicylic acid elicitation of shoots, callus, and cell suspension cultures on production of naphthodianthrone and phenylpropanoids in *Hypericum perforatum* L. *Plant Cell Tissue Organ Cult* 2013; 113: 25–39.
 87. Brechner ML et al. Effects of UV-B on secondary metabolites of St. John's wort (*Hypericum perforatum* L.) grown in controlled environments. *Photochem Photobiol* 2011; 87: 680–684.
 88. Germ M et al. Flavonoid, tannin and hypericin concentrations in the leaves of St. John's wort (*Hypericum perforatum* L.) are affected by UV-B radiation levels. *Food Chem* 2010; 122: 471–474.
 89. Yamaner O, Erdag B. Effects of sucrose and polyethylene glycol on

- hypericins content in *Hypericum adenotrichum*. *EurAsian J Biosci* 2013; 7: 101–110.
90. Franklin G, Dias A. *Hypericum perforatum* cells accumulate anthocyanins and flavonoids at the expense of xanthone biosynthesis during light adaptation PlantEngine 1 (COST Action FA 1006- Plant Metabolic Engineering for high value products, 1st Annual Conference), 2011 (Nov 17–18) Murcia, Spain 2011.
 91. Valletta A et al. Acetic acid acts as an elicitor exerting a chitosan-like effect on xanthone biosynthesis in *Hypericum perforatum* L. root cultures. *Plant Cell Rep* 2016; 35: 1009–1020.
 92. Singh RK et al. Agrobacterium-elicited *Hypericum perforatum* cell methanolic extract shows antibacterial activity against human pathogens. *Planta Med* 2014; 80: 1424–1425.
 93. Carvalho AC et al. Methanolic extract of *Hypericum perforatum* cells elicited with *Agrobacterium tumefaciens* provides protection against oxidative stress induced in human HepG2 cells. *Ind Crops Prod* 2014; 59: 177–183.
 94. Tocci N et al. Bioassay-guided fractionation of extracts from *Hypericum perforatum* in vitro roots treated with carboxymethylchitosans and determination of antifungal activity against human fungal pathogens. *Plant Physiol Biochem* 2013; 70: 342–347.
 95. Zhang B et al. Stimulation of artemisinin production in *Artemisia annua* hairy roots by Ag-SiO₂ core-shell nanoparticles. *Curr Nanosci* 2013; 9: 363–370.
 96. Ghanati F, Bakhtiarian S. Effect of methyl jasmonate and silver nanoparticles on production of secondary metabolites by *Calendula officinalis* L (Asteraceae). *Trop J Pharm Res* 2014; 13: 1783–1789.
 97. Ghorbanpour M, Hadian J. Multi-walled carbon nanotubes stimulate callus induction, secondary metabolites biosynthesis and antioxidant capacity in medicinal plant *Satureja khuzestanica* grown in vitro. *Carbon* 2015; 94: 749–759.
 98. Kurepa J et al. Direct isolation of flavonoids from plants using ultra-small anatase TiO₂ nanoparticles. *Plant J* 2014; 77: 443–453.
 99. Schlipf DM et al. Flavonoid adsorption and stability on titania-functionalized silica nanoparticles. *Colloids Surf A* 2015; 478: 15–21.
 100. Cui X-H et al. Pilot-scale culture of *Hypericum perforatum* L. adventitious roots in airlift bioreactors for the production of bioactive compounds. *Appl Biochem Biotechnol* 2014; 174: 784–792.
 101. Onelli E et al. Ultrastructural studies on the developing secretory nodules of *Hypericum perforatum*. *Flora Morphol Distrib Funct Ecol Plants* 2002; 197: 92–102.
 102. Piovan A et al. Detection of hypericins in the “red glands” of *Hypericum elodes* by ESI-MS/MS. *Phytochemistry* 2004; 65: 411–414.
 103. Zobayed S et al. Plant–environment interactions: accumulation of hypericin in dark glands of *Hypericum perforatum*. *Ann Bot* 2006; 98: 793–804.
 104. Kornfeld A et al. The production of hypericins in two selected *Hypericum perforatum* shoot cultures is related to differences in black gland structure. *Plant Physiol Biochem* 2007; 45: 24–32.
 105. Gaid M et al. Hyperforin production in *Hypericum perforatum* root cultures. *J Biotechnol* 2016; 222: 47–55.