Article

The Production of Isoflavones by *Genista tinctoria* L. In Vitro Culture after Two Vanadium Compounds Application. Possible Transport Mechanism of Secondary Metabolites through Plasma Membrane

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Abstract: Family *Fabaceae* traditionally serves as food and herbal remedies source. Several plants are already used for menopausal symptoms treatment based on a presence of typical secondary metabolites, isoflavones. Beside soybean and clovers, isoflavones could be produced by other plants or *in vitro* cultures. This type of production can be further enhanced by elicitation that stimulates metabolites biosynthesis via stress reaction. Vanadium compounds have been already described as potential elicitors and the aim of this study was to determine an impact of NH_4VO_3 and $VOSO_4$ solutions on isoflavones production in *Genista tinctoria* L. cell cultures. The significant increase of isoflavones content such as genistin, genistein or formononetin was measured in a nutrient medium or dry mass after NH_4VO_3 treatment for 24 or 48 hours. The possible transport mechanism of isoflavones was also evaluated. An incubation with different transport inhibitors prior elicitation took effect on isoflavones content in the medium. However, there was non-ended result for particular metabolites such as genistein and daidzein, where ABC or alternatively MATE proteins can participate. Possible elicitation by some inhibitors was also discussed as result of their pleiotropic effect. Despite this outcome, the determining of transport mechanism is important step for identification of specific transporter.

Keywords: heavy metals; dyer´s greenweed; elicitation; exudation

1. Introduction

Genista tinctoria (dyer's greenweed) is a bush from the *Fabaceae* family and traditional utilization in the dying industry provided its species name [1]. This plant was also applied in various forms for body detoxification in folk medicine of the western Mediterranean and Turkey [2]. *G. tinctoria* can be also classified as a poisonous plant with regard to quinolizidine alkaloids occurrence [3]. However, their content can be reduced in *in vitro* cultures [4].

G. tinctoria, as a member of legume family, is also a source of various isoflavones. This flavonoid subgroup is typical for different position of phenyl group as a result of isoflavone synthase activity [5,6]. Flavones and isoflavones may be produced as protection against various factors that are classified as either abiotic or biotic stress [7]. Simple isoflavones such as genistein or daidzein can be metabolized further into more complex compounds called pterocarpans. These metabolites (pisatin, medicarpin) have also a significant function in the plant defence [8].

Abiotic chemical stress is often caused by the presence of various substances in soil that occur naturally or as a consequence of human activity [9]. This is also true in the case of heavy metals such as vanadium that, in excess, can have negative effects on plant growth. The level of vanadium in soil

is about 150 μg/g on average, but in the vicinity of a former South African mine, vanadium levels were found to range from 1570 to 3600 μg/g. Increased amounts were also detected in leaf samples from nearby graminoids. The presence of vanadium negatively affects the overall growth of plants [10]. Vanadium toxicity is brought about by its negative effect on various ATPases, protein kinases, ribonucleases, phosphatases and production of reactive oxygen species (ROS) [11]. The EC_{50} of vanadium in solution ranged from 0.8 to 15 mg/L when vanadium toxicity was monitored in soil [12].

Intentional production of certain secondary metabolites (as a consequence of reaction to stress) is referred to as elicitation, and the substance that triggers this process is known as an elicitor [13]. Various types of vanadium compounds were already tested for production of other secondary metabolites in different *in vitro* plant cultures [14-16]. Vanadium was also discussed as a potential elicitor for production phenolic metabolites in a suspension culture of *Vigna radiata* [17]. Orthovanadate showed an increase in both levels of glucoside isoflavones and phenylalanine ammonia-lyase (PAL) enzyme activity. Orthovanadate also affected PAL activity in *Petunia hybrida*, where an increased level of lignin was recorded [18].

PAL is key enzyme for flavonoid biosynthesis, which takes place in the cytosol membrane of the endoplasmic reticulum (ER) [19]. Nevertheless, the formation of metabolites can have negative effects on certain processes occurring within cells, so they are stored inside vacuoles or exported out of the cells [20]. A number of mechanisms contribute to this movement, including a suitable form of transportation of substances within cells and plant body. ATP-binding cassette (ABC) transporters are usually part of the membranes, enabling transport of substances while ATP is hydrolysed. Another way includes multidrug and toxin extrusion (MATE) proteins. The exchange of substrates by these transporters is dependent on the proton/membrane gradient that is maintained by various ion pumps. Some substances are then transported within vesicles that are buded off from various cellular structures (ER, Golgi apparatus) and subsequently merge with the target membrane [21].

The aim of this research was to determine effect of two vanadium compounds on isoflavone production and exudation in a suspension culture of *G. tinctoria* as potential source of these metabolites. Isoflavones content was examined both in the dry matter (DM) and in the nutrient medium (NM). Subsequently, several different inhibitors of transport mechanism were applied to clarify a process of secondary metabolites release into the medium.

2. Results

2.1. The Effect of Vanadium Compounds

The effects of vanadium compounds on the production and exudation of five isoflavones were monitored in a *G. tinctoria* suspension culture. The observed metabolites were aglycone genistein, its 7-*O*-glucoside genistin and 4′-*O*-methylgenistein (biochanin A), as well as aglycone daidzein and 4′-*O*-methyldaidzein (formononetin). The effects of NH_4VO_3 and $VOSO_4$ were monitored at two concentrations (1 and 10 μM) and two cultivation intervals (24 and 48 h).

- NH_4VO_3 (1 μM, Figure 1A) showed that this compound has the potential to increase isoflavone biosynthesis in *G. tinctoria* suspension culture with the possible release of these metabolites into the NM. This compound significantly increased the content of genistin, formononetin and potentially genistein in the NM after 24 h. Significantly higher genistein amount was found in the DM only, but daidzein level was also increased. The amount of biochanin A was not traceable in DM and NH_4VO_3 (1 μM) took no effect on the release of this isoflavone.
- NH_4VO_3 (10 μM, Figure 1B) also caused a significant increase of genistin content, but not formononetin, in the NM after 24 h. In contrast, the content of genistein was significantly lower. After 48 h, the concentration of genistin in both NM and DM was increased. The values of remaining isoflavones were insignificant, although daidzein content was little higher in the both type of samples. NH_4VO_3 (10 μM) did not provide evidence for affecting isoflavone content with the exception of a significant increase in genistin and a decrease in genistein amount.in the DM after 48 h.

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- $VOSO_4$ (1 µM, Figure 1C), as the second tested vanadium compound, affect only formononetin content in the NM that was significantly increased after 48 h of cultivation. Other isoflavones had insignificant change in their levels in comparison with the control medium or DM.
- In a similar way, $VOSO_4$ (10 µM, Figure 1D) took a significant effect on formononetin again, but its concentration was lower after 24 h. There was a non-significant reduction in genistein content in the DM after 24 h. This result was similar to genistein value after $VOSO_4$ (1 µM) application (Figure 1C). Although some results were insignificant, general conclusions suggest that NH_4VO_3 has a stronger impact on the production and release of certain isoflavones (genistin).

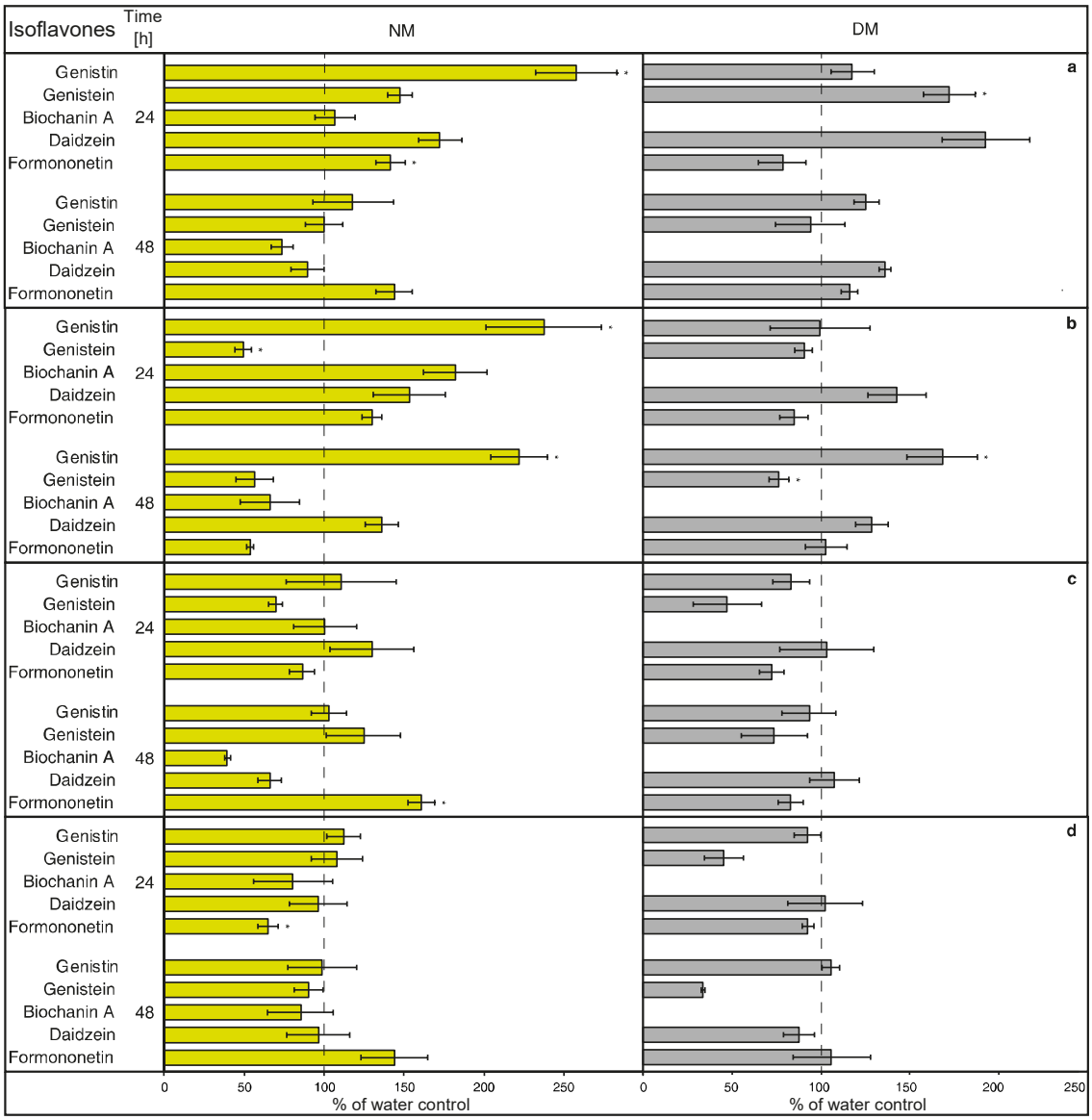

Figure 1. Effects of elicitors on isoflavone content in nutrient medium (NM) and dry matter (DM) after 24 or 48 h. *Genista tinctoria* cell culture was treated by 1 µM (**a**) or 10 µM (**b**) of NH_4VO_3 and 1 µM (**c**) or 10 µM (**d**) of $VOSO_4$. Bars indicate isoflavone levels in treated samples recalculated to a relative 100% isoflavone content in water control samples. Data are mean of three repeats ± SE; (*) represent significant difference ($P < 0.05$) between tested and water control samples within Tukey´s test.

Therefore, NH_4VO_3 (1 µM) was subsequently chosen for study *G. tinctoria* transport mechanisms. This solution significantly increased content of two isoflavones (genistin, formononetin) in the NM after 24 h. The concentration of genistein and daidzein may also be positively affected; the increase of the former aglycone amount was clearly visible in DM.

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2.2. The Effects of Transport Mechanism Inhibitors

In order to identify the possible transport mechanism of the five isoflavones mentioned above, adapted method for *Silybum marianum* was used [22]. Cell cultures were treated with several compounds that inhibited specific transport processes in various species.

- NH_4Cl (Figure 2) is ranked between protonophores [23]. It can disrupt the proton gradient and inhibit transport of certain substances, such as nicotine (Shoji et al. 2009). The concentration of isoflavones from *G. tinctoria* was mostly reduced in NM, while significant results were measured for genistein (1 mM, 10 mM) and daidzein (10 mM) in comparison with NH_4VO_3 treated samples. Whereas formononetin content was strongly decreased in DM, results of remaining isoflavones were not conclusive against elicited samples. However, this inhibitor (10 mM) increased genistin and genistein presence in DM; this difference was only evident in the glycoside against water control.

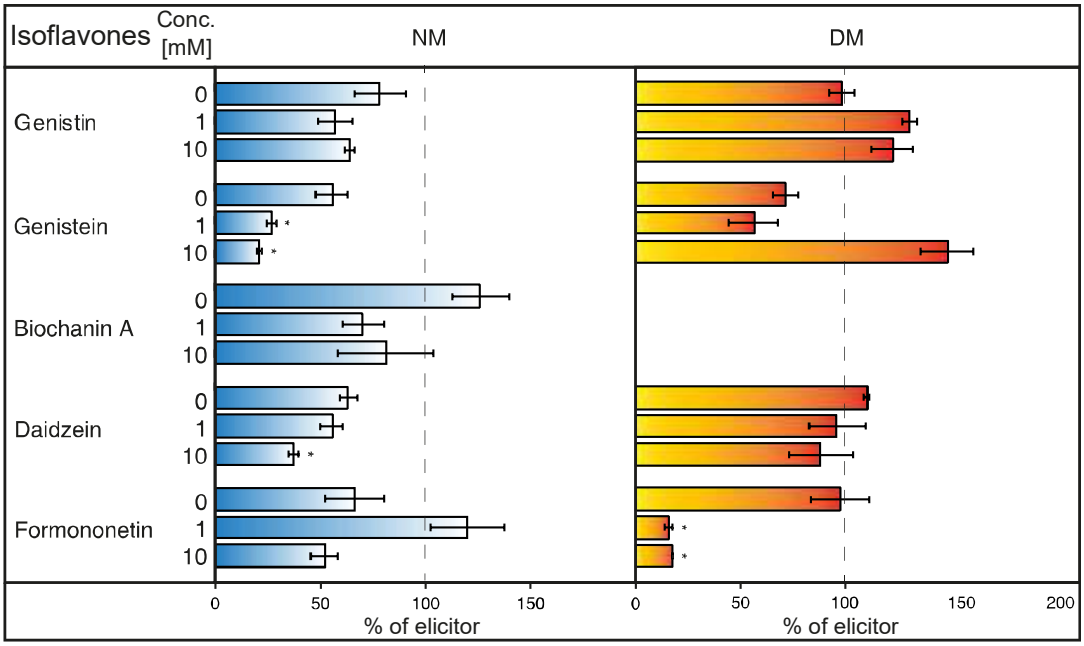

Figure 2. Effects of NH4Cl on isoflavone content in nutrient medium (NM) and dry matter (DM) of *Genista tinctoria* cell culture. Zero concentration of inhibitor represents water control. All bar values were recalculated to a relative 100% isoflavone content in samples with NH_4VO_3 (1 μM) after 24 h. Data are mean of three repeats ± SE; (*) represent significant difference ($P < 0.05$) between elicited and inhibited or water control samples within Tukey´s test.

- Gramicidin (Figure 3) is an antibiotic formed by *Bacillus brevis* that acts as a selective ionophore for cations. Gramicidin disrupted processes associated with potassium ions [24] or nicotine transport in a similar way as NH_4Cl [25]. The levels of genistein and daidzein significantly decreased in the NM after the application of this inhibitor. Gramicidin, however, did not affect the presence of these aglycones in DM, as well as other isoflavones. The content of genistin and biochanin A was higher in NM in comparison with water control, while the result of the second compound was significant.

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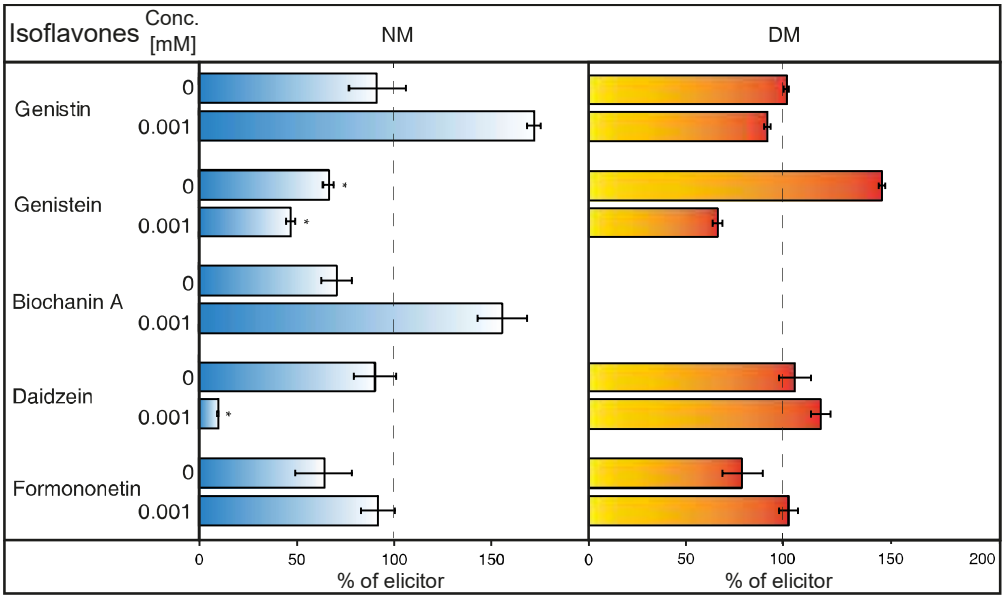

Figure 3. Effects of gramicidin on isoflavone content in nutrient medium (NM) and dry matter (DM) of *Genista tinctoria* cell culture. Zero concentration of inhibitor represents water control. All bar values were recalculated to a relative 100% isoflavone content in samples with NH_4VO_3 (1 μM) after 24 h. Data are mean of three repeats ± SE; (*) represent significant difference ($P < 0.05$) between elicited and inhibited or water control samples within Tukey´s test.

- Brefeldin A (Figure 4) is a macrolide lactone produced by specific ascomycetes [26]. This inhibitor suppresses the guanine nucleotide exchange factor involved in vesicular transport of substances [27]. It also dissolves the Golgi apparatus, which contribute on various molecules movement [28]. Brefeldin A adversely affected some isoflavones content in the NM; significant decrease: genistein, daidzein (2.5 and 5 μM) and formononetin (5 μM). Reduced concentration of these metabolites in the NM insignificant after treatment in other cases. On the other hand, brefeldin A caused a significant increase in the levels of genistein (5 μM) and biochanin A (2.5 and 5 μM) in the NM.

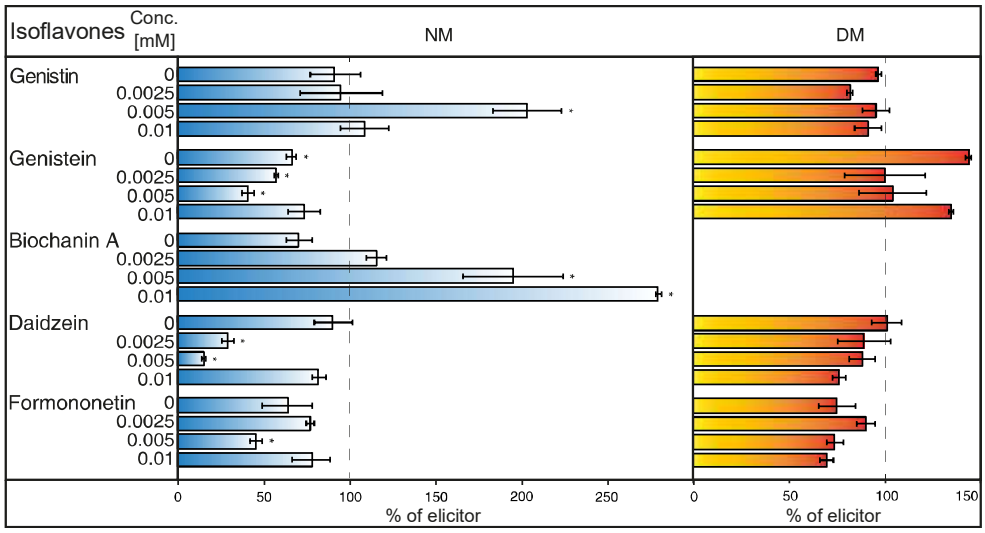

Figure 4. Effects of brefeldin A on isoflavone content in nutrient medium (NM) and dry matter (DM) of *Genista tinctoria* cell culture. Zero concentration of inhibitor represents water control. All bar values were recalculated to a relative 100% isoflavone content in samples with NH_4VO_3 (1 μM) after 24 h. Data are mean of three repeats ± SE; (*) represent significant difference ($P < 0.05$) between elicited and inhibited or water control samples within Tukey´s test.

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- Na_3VO_4 (sodium orthovanadate; Figure 5) is known and used as a plasma membrane (PM) H^+-ATPase inhibitor. Application of this substance to cell culture of *Eschscholtzia californica* caused gradual alkalisation of surrounding media and lower rate of excretion of benzophenanthridine alkaloids [29]. In addition, the inhibition caused by Na_3VO_4 was discussed for the ABC transporter in *Salmonella typhimurium* [30] and the plants [31]. Na_3VO_4 affected isoflavone content according to used concentration. Application of Na_3VO_4 (1 mM) reduced strongly the content of genistein and daidzein in the NM, while genistein was also negatively affected by the less concentrated solution. The content of isoflavones was usually lower in comparison with the elicited samples except daidzein in DM. However, no result of this aglycone was significant.

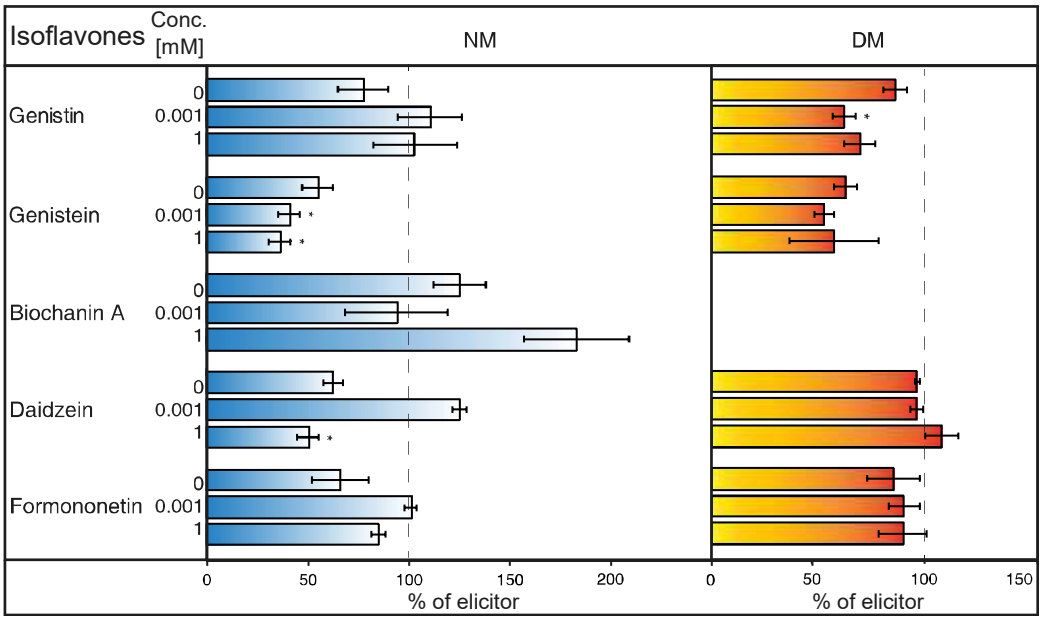

Figure 5. Effects of Na_3VO_4 on isoflavone content in nutrient medium (NM) and dry matter (DM) of *Genista tinctoria* cell culture. Zero concentration of inhibitor represents water control. All bar values were recalculated to a relative 100% isoflavone content in samples with NH_4VO_3 (1 μM) after 24 h. Data are mean of three repeats ± SE; (*) represent significant difference ($P < 0.05$) between elicited and inhibited or water control samples within Tukey´s test.

- Verapamil (Figure 6) inhibits the activity of calcium transport channels, but suppressing effect on ABC proteins, multidrug resistance protein 1 subfamily (MDR1), has been also observed. This effect was studied as a way to overcome the tolerance of some drugs caused by P-glycoprotein [32] and alkaloid berberine was accumulated within the *Thalictrum minus* cells after this inhibitor application, [33]. Verapamil caused insignificant decrease in any studied isoflavone level in the NM, while a small reduction for all compounds except daidzein was found. This particular aglycone had a higher concentration than a water control (unmarked in figures).

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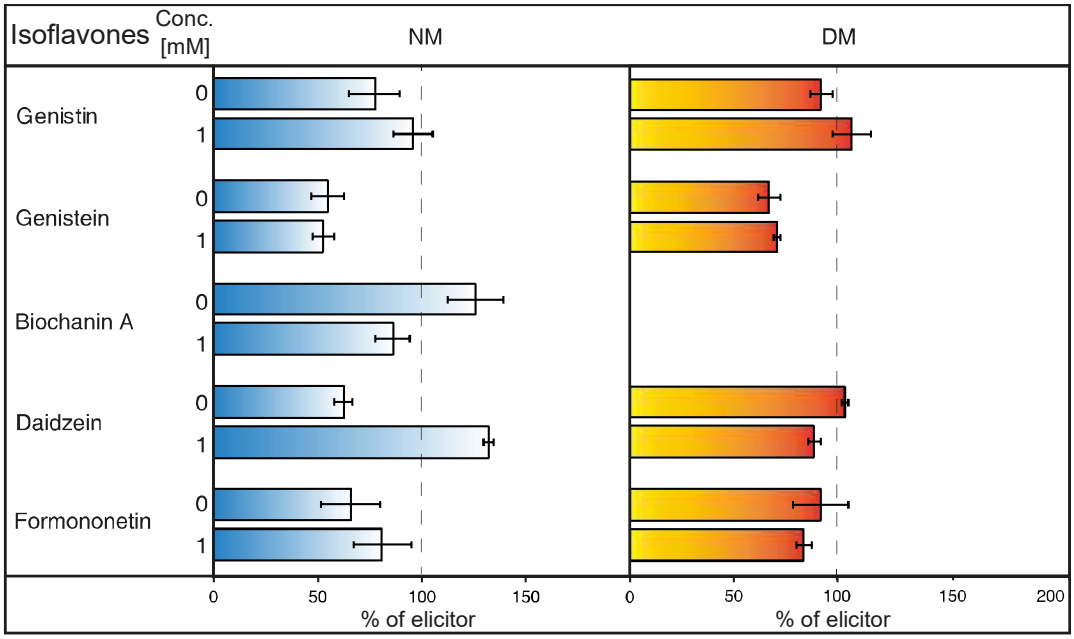

Figure 6. Effects of verapamil on isoflavone content in nutrient medium (NM) and dry matter (DM) of *Genista tinctoria* cell culture. Zero concentration of inhibitor represents water control. All bar values were recalculated to a relative 100% isoflavone content in samples with NH_4VO_3 (1 μM) after 24 h. Data are mean of three repeats ± SE; (*) represent significant difference ($P < 0.05$) between elicited and inhibited or water control samples within Tukey´s test.

- Probenecid (Figure 7) primarily affects the excretion of uric acid in kidneys. This drug can take effect on the ABC transporters, multidrug resistance-associated protein 1 and 2 subfamily (MRP1 and MRP2), and inhibits the transfer of organic anions [23]. After probenecid treatment, a statistically significant reduction of genistein (0.5 mM) and daidzein (0.5 and 1 mM) was measured in the NM; their content in the DM has not changed significantly. On the other hand, this inhibitor also positively affect concentration of genistin (1 mM) and biochanin A in a medium compared to water control samples.

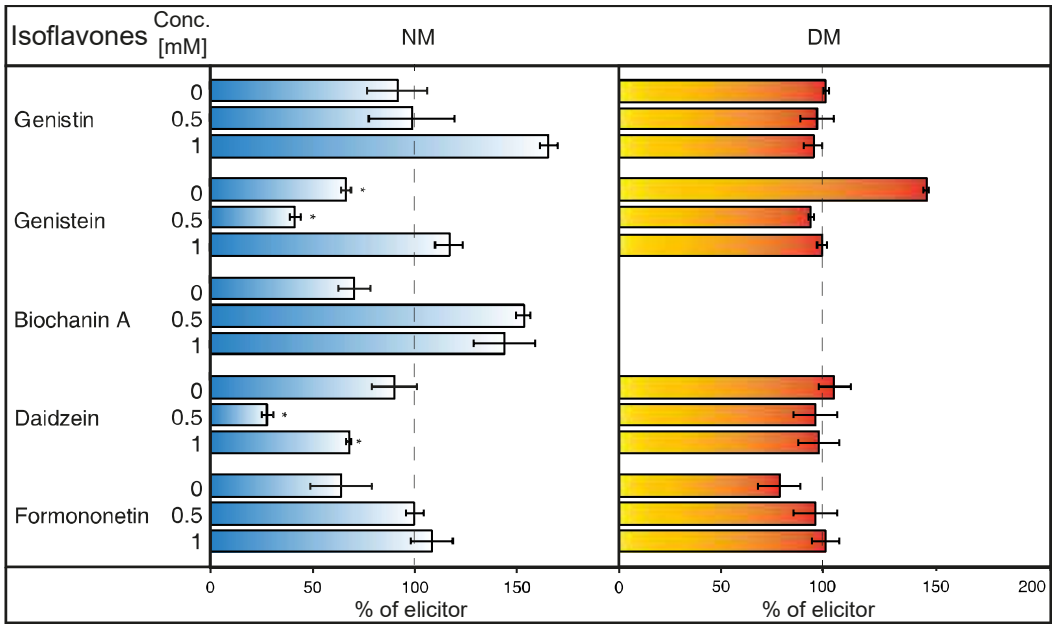

Figure 7. Effects of probenecid on isoflavone content in nutrient medium (NM) and dry matter (DM) of *Genista tinctoria* cell culture. Zero concentration of inhibitor represents water control. All bar values were recalculated to a relative 100% isoflavone content in samples with NH_4VO_3 (1 μM) after

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24 h. Data are mean of three repeats ± SE; (*) represent significant difference (P < 0.05) between elicited and inhibited or water control samples within Tukey´s test.

- Glibenclamide (Figure 8) is a drug that acts primarily on ABC proteins in pancreatic B-cells. This drug inhibited the activity of MRP1 in the lung tumour [34] or AtMRP5 in *Arabidopsis thaliana* [35]. No isoflavone had significantly lower content in the NM after glibenclamide application. Nevertheless, there was some reduction of isoflavones (genistein, biochanin A, formononetin). On the contrary, glibenclamide positively affected daidzein content in the NM after administration of both concentrations of inhibitor, resulting in the opposite effect compared to probenecid. The same effect was found for genistein (0.5 mM) in spite of water control.

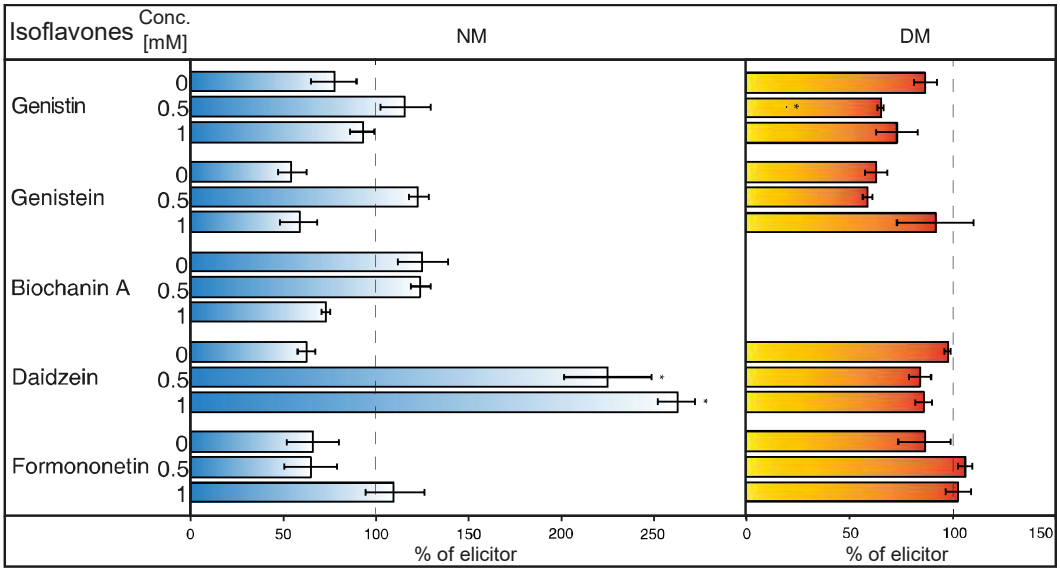

Figure 8. Effects of glibenclamide on isoflavone content in nutrient medium (NM) and dry matter (DM) of *Genista tinctoria* cell culture. Zero concentration of inhibitor represents water control. All bar values were recalculated to a relative 100% isoflavone content in samples with NH_4VO_3 (1 μM) after 24 h. Data are mean of three repeats ± SE; (*) represent significant difference (P < 0.05) between elicited and inhibited or water control samples within Tukey´s test.

3. Discussion

3.1 Impact of vanadium compounds

Vanadium compounds were tested as potential elicitors for different plants and their products in past It was stated by various authors that $VOSO_4$ increased the production of ajmalicine and catharanthine in *Catharanthus roseus* [14], nicotine in *Nicotiana tabacum*, cv. Virginiana, but not in cv. Burley [16], as well as ginsenosides in *Panax ginseng* [15]. Some authors also discussed suppressing effect of this salt on plant growth [16] and the concentration of vanadium solutions was usually higher in these studies too. The application of $VOSO_4$ (6 mg/L) caused a loss of weight and calcium content in hydroponically grown *Phaseolus vulgaris* (*Fabaceae*) [36] and vanadium was mainly concentrated in the reduced roots [37]. With regard to this research, the cell culture of *G. tinctoria* was treated with less concentrated solutions of elicitors.

Vanadium and other heavy metals can have direct adverse effect on plants, when these elements bind to cell sites instead of natural ions. They can also harm tissues indirectly through self-oxidation or Fenton reaction by production of free radicals by [38]. H_2O_2 and eventually a hydroxyl radical can be formed by activity of certain NAD(P)H dependent enzymes that are able to make reduction of V^{+V} too [39].

Plant defence reaction against stress develops as a result of signal cascade triggering. The signal is carried by various messenger molecules from receptors and the final step is protective mechanism

activation [9]. The perception of the metal could be mediated by proteins in PM such as receptor-like protein kinases, which react on other types of stress too [40]. As described for human cells [11], vanadium can get into cytoplasm through anionic channel (V^{+V}) or pass across PM (V^{+IV}). Its compounds can create ROS inside cells then, which can also react with appropriate receptors. With regard to an inhibition of ATPases of PM by orthovanadate, subsequent change of membrane potential was proposed as activation signal for flavonoid biosynthesis pathway in *Arachis hypogaea* [41]. Higher isoflavone production and exudation was also discussed in another *Fabaceae* plant, *Cicer arietinum*, as a result of different pH values [42]. These changes leading to the flavonoids and related molecules (phenolic acids, lignin) biosynthesis that is dependent on activity of above mentioned PAL [17,43]. Heavy metals, such as cadmium and lead, also increased mRNA of this enzyme and isoflavones production in *Lupinus luteus* [44].

However, the effects of $VOSO_4$ were dependent on the plant genotype, the monitored tissue and the concentration of applied solution [16]. This would explain various values of isoflavones content between samples with individual vanadium compounds (Figures 1A, 1B). The effects of different compounds might vary even at identical concentrations [45]. In a suspension culture of *P. ginseng*, solutions of NH_4VO_3 and $NaVO_3$ (50 μM) caused the significant increase of ginsenosides in DM, but$VOSO_4$ had only effectivity 65 % of $NaVO_3$. These secondary metabolite content in the medium was not measured in their study, while differences in isoflavone levels were determined here afterNH_4VO_3 and $VOSO_4$ treatment (Figures 1A, 1C).

Beside the character of elicitor and its quantity, resulting improvement of secondary metabolites production is dependent on a number of other factors such as growing conditions and culture type [46]. With regard to genistin (Figure 1B), some elicitors in summary of secondary metabolites production research also caused a higher quantity of substances in both cells and the medium of certain cultures [47]. In a case of other plants, their metabolites were exported out, but the dry matter content remained unchanged too.

The production of isoflavones in *in vitro* cultures of *G. tinctoria* was also compared to that of wild growing plants [48]. Upon establishing suitable conditions, the *in vitro* cultures produced more secondary metabolites. The age of the culture is also one factor for secondary metabolites biosynthesis and young calluses were used in the above-mentioned study. On the other hand, cell cultures of *G. tinctoria* treated with vanadium compounds or used in subsequent transport experiments were subcultures from older calluses.

3.2 Transport of isoflavones across membranes

In the research of the secondary metabolite transport mechanism are used isolated PM [49] or tonoplast vesicles [24] along with a known amount of a tested metabolite. These experiments are based on the metabolite uptake in the presence of some molecule with potential to affect transport process. In contrary, similar study was conducted [22], but metabolites were produced directly by the plant cell. Moreover, to bring new light to inhibition effect evaluation, the isoflavone content in the NM of *G. tinctoria* was determined together with DM.

Natural substances may be transported by different ways such as above-mentioned ABC transporters, MATE proteins [31,50] and vesicular transport [51]. Various mechanisms were identified with regard to the selected plant, examined membrane (plasma or vacuolar) or the selected metabolite.

In the case of some samples, where inhibitors produced by microorganisms (*Bacillus brevis*, *Eupenicillium brefeldianum*) were used (gramicidin, brefeldin A), a noticeable increase of genistin and particularly biochanin A in the NM was recorded (Figures 4, 5) despite inhibitors divergences. This could be explained by isoflavones classification between phytoalexins. They play a significant role in case of the plant's exposure to abiotic or biotic stress and this group included a number of different compounds, such as pisatin from *Pisum sativum* or medicarpin from *Medicago truncatula* [52]. These metabolites have a vital part in the interaction between the plant and the microbial pathogen and used inhibitors could act as biotic elicitors.

The *Fabaceae* family is known for its symbiosis with nitrification bacteria and nodules created on the roots as the result. Aside from a protective function, the ability of isoflavones and other flavonoids to attract nitrification bacteria is under consideration. The participation of ABC transporters on the isoflavones transport through PM were found in the roots of *Glycine max*, although some used ABC inhibitors did not affect this process. However, possible uptake could be dependent on another subgroup, classified as ABCG or pleiotropic drug resistance (PDR) proteins [49]. It was determined in *G. tinctoria* that Na_3VO_4 (1 mM) solution also caused a strong decrease of genistein and daidzein levels in NM (Figure 5). Moreover, substances such as verapamil suppressing the activity of multidrug resistance protein (ABCB) or probenecid, active against MRP (ABCC), may not have a noticeable impact in *G. max*, as in case of *G. tinctoria* (Figure 6). Probenecid (0.5 mM) in the NM only decreased the content of genistein and daidzein; the amount of other isoflavones was not affected (Figure 7).

Another outcome was discussed in a study, where flavanone naringenin was applied to the root cap of *A. thaliana* [53]. The movement of this metabolite was mostly inhibited in plant samples treated with glibenclamide or glutathione in various redox states. Glutathione, specifically glutathione S transferase, tends to be associated with the transport dependent on MRP proteins [54]. In spite of mentioned experiment with *G. max*, Na_3VO_4 did not significantly suppress the movement of naringenin too. Moreover, the treatment of cell suspension of *G. tinctoria* with the glibenclamide brought insignificant decrease of some isoflavones level (Figure 8) and involvement of MRP subgroup could be excluded.

The participation of PDR transporters was discussed in the case of *M. truncatula* hairy-root culture, where MtABCG10 was identified. To clarify this protein importance, its gene was silenced and the amount of medicarpin precursors such as daidzein, formononetin and vestitonin decreased then. The plant culture was also more susceptible to the fungal pathogen *Fusarium oxysporum*, indicating the role of these substances as phytoalexins [55]. Other PDR transporter, AtABCG29, was identified in *A. thaliana* that is responsible for the transmission of monolignol p-coumarylalcohol through PM [56]. This metabolite production is also PAL dependent and it is a component of lignin. It was discussed that transmission of these phenolic aglycones primarily took place on PM, while their glycosides were transported more through tonoplast [57]. The authors referred that various forms of molecules require different transporters for their transmission and structure contributes to the placement of metabolites.

However, transport of the individual isoflavones may be affected by the presence of other similar metabolites. The uptake of genistein into *G. max* PM vesicles was decreased in the presence of other aglycones, particularly daidzein, formononetin and biochanin A. Therefore, same type of transporter was hypothesised for these molecules. With regard to glycosides, only genistin had more noticeable effect on uptake inhibition in comparison with 7-*O*-glucosides of other flavonoids [49]. In a case of *G. tinctoria,* an export of several isoflavones was observed at the same time; their possible mutual effect on transport cannot be excluded. Abundance of metabolite inside cells caused by the inhibitors could cause further transformation of individual substances (conjugation, methylation, acylation) [58]). Moreover, other isoflavonoids are present in *G. tinctoria* that were not measured. It could explain some large differences between the measured results then.

On the other hand, studies focusing on a tonoplast transport of flavonoids brought more examples of MATE proteins and proton pumps participation within this membrane. The uptake of glycoside saponarin in *Hordeum vulgare* was disrupted by the inhibitors of the proton gradient, but ABC proteins was affected in *A. thaliana* then [31]. Saponarin is not natural metabolite of *A. thaliana*; the participation of a transporter of different mechanism was discussed with regard to its character as remover of toxic substances. In *M. truncatula*, more proteins were identified later, such as MtMATE1 that contributed to the transport of flavan-3-ols [50] or MtMATE2 transporting some acylated flavonoids [59]. The vacuolar uptake of daidzin in this plant was 0more sensitive to inhibitors of the ABC proteins, but the specific transporter was not identified here [50]. One of the first identified ABC transporters on the tonoplast were ZmMRP3 for anthocyanins in *Zea mays* [60] and VvABCC1 in *Vitis vinifera* [61] then.

Despite tonoplast, participation of MATE proteins on flavonoid transport across PM has not been reported yet. In the case of *Vaccinium corymbosum*, several VcMATEs were identified as possible transporters due to their similarity with some known transporters from this family [62]. Therefore, decreased genistein and daidzein content in the NM could indicate MATE protein involving (Figure 2). The gramicidin decreased the content of genistein and daidzein too, but level of these isoflavones did not changed in DM (Figure 3).

In the vesicles prepared from the xylem of various woody plants (*Populus sieboldii*, *Pinus densiflora*), the transport of coniferin (coniferyl alcohol glucoside) particularly occurred on the tonoplast and possibly on the ER membrane [63]. The uptake of this metabolite was dependent on the presence of MgATP, while its aglycone was minimally transported under the same conditions. Unlike the transport in *A. thaliana*, this process was inhibited by bafilomycin A1 in hybrid poplar tree (*Populus sieboldii* × *Populus grandidentata*) that affected the proton gradient through vacuolar ATPase.

In the above mentioned experiments, Na_3VO_4 was primarily applied as the inhibitor of ABC proteins. However, older studies used this compound against other enzymes with ATPase activity. In animals, vanadate affected some pumps, such as the (Na^+/K^+)-ATPase [64] and (H^+/K^+)-ATPase. The inhibition of ATPase was subsequently also shown on the PM in *Beta vulgaris* and *Z. mays* [65]. Aside from orthovanadate, vanadyl sulphate was able to inhibit (Na^+/K^+)-ATPase too [66], as was sodium metavanadate [64]. The activity of the electrogenic ATPase was disrupted by ammonium metavanadate on the peribacteriod membrane in *G. max* then [67]. The inhibition effect of metavanadate and vanadyl on these enzymes was not studied in plant cultures such as *P. ginseng* and *N. tabacum* during elicitation.

In contrary, the effect of orthovanadate as H^+-ATPase inhibitor on PM that could act as the elicitor by this mechanism was also discussed [42]. A treatment of *C. arietinum* with vanadate (50 μM, 100 μM) caused an increased production and release of pterocarpans (maackiain, medicarpin) and isoflavones (biochanin A, formononetin) from the roots into the medium. The authors proposed a hypothesis that the change of pH of the external environment to more alkaline, as the result of protein pumps inhibition had a positive effect on the elicitation. There was no reduction of secondary metabolites in the medium with increased concentrations of vanadate. The application of NH_4VO_3 and $VOSO_4$ could cause elicitation in *G. tinctoria* cell culture without affecting the release of isoflavones, while their potential effect on ATPase may not have manifested at the concentrations used.

Another aspect of flavonoids movement, particularly anthocyanin, was clarified by describing of transport vesicles in line with occurrence of specific bodies in plant cells [68]. The cyanoplasts and anthocyanoplasts had high level of these secondary metabolites. It was stated that vesicles contain precursors of proanthocyanidins, which could have been formed by splitting from ER, where the metabolites were initially transported from the cytosol membrane. Protein GFS9 (green fluorescent seed) present in the Golgi apparatus of *A. thaliana* was discussed as one of the vesicular transport factors for vacuolar uptake of flavonoids. Mutants with the *gfs9* gene had a different colour in comparison with wild-type plants and were equated to mutant *tt9* [69].

In a hairy-root culture of *Ophiorrhiza pumila*, an application of brefeldin A caused a release of alkaloid camptothecin into the extracellular space. According to [51], this metabolite is also formed on ER and stored in the vacuole with potential passive transport across PM. Brefeldin A suppressed camptothecin uptake into vacuole and subsequently increased metabolite in the medium.

Therefore, the disruption of isoflavones exudation by vesicles would manifest as their increased content in the DM. The possibility of flavonoids passive transport was not extensively researched [70]. However, participation of vesicular transport was reported in culture of *Silybum marianum* [22], where brefeldin A caused a decrease of flavonoids in the medium. The application of brefeldin A could have affected the NM concentration of genistein, daidzein and formononetin (Figure 4) within *G. tinctoria* cell culture. Overall, it was not possible to definitively determine, whether the studied isoflavones are transported by vesicles.

4. Materials and Methods

4.1 In Vitro Culture Preparation

The seeds for the plant material cultivation were provided by the Agricultural Faculty of the Mendel University in Brno (CZ). Manipulation of plant material and all experiments took place in a disinfected box with a laminar flow. The seedlings of *G. tinctoria* were cultivated on a Schenk and Hildebrandt medium [71] with agar under stable conditions (24° C, 16-h light and 8-h dark regime) under ColorLux Plus bulb (NARVA Lichtquellen GmbH, Brand-Erbisdorf, Germany) with irradiance of 81.51 μW m^{-2}.

A callus cultures were prepared by cutting the stem of seedlings to smaller parts that were placed onto paper bridges immersed in a Schenk and Hildebrandt medium. The medium contained 2,4-D (2.2621 μM; SERVA Electrophoresis GmbH, Heidelberg, Germany) and kinetin (0.4646 μM; SERVA Electrophoresis GmbH, Heidelberg, Germany) as growth hormones that support the formation and development of unorganized tissue. The formed callus cultures were subsequently cultivated in closed Erlenmeyer flasks (250 mL) under same conditions and subcultured every 4 weeks until the tissue became homogenous and sufficiently grown.

A suspension culture was then prepared from this tissue by transferring the plant inoculum (approximately 5 g FW) into a liquid Schenk and Hildebrandt medium with same concentration of used growth hormones. The callus mass was carefully disrupted to smaller cell clusters that was cultivated in Erlenmeyer flasks under same conditions again. An aeration and prevention of cell sedimentation was achieved by placing the flasks into a Multi-flask shaker (115 rotations per min; VKS 75, Edmund Buhler GmbH, Hechingen, Germany). The subculturing of the suspension culture took place every 14 days, when approximately 15 mL of thick suspension was transferred into 20 mL of fresh Schenk and Hildebrandt medium.

4.2 Vanadium Treatment

The effects of two vanadium compounds were tested in 3-day old suspension culture of *G. tinctoria* This culture were treated with 1 mL of ammonium metavanadate (NH_4VO_3; Lachema, Brno, CZ) or vanadyl sulfate ($VOSO_4$; Honeywell Riedel de Haën, Seelze, Germany) and the concentration of the elicitors was 1 and 10 μM. At the same time, cultures with only 1 mL of distilled water were prepared as a control group.

Flasks with the tested sample suspensions were returned to the shaker and cultivated under the same conditions for 24 and 48 h. Individual samples were later removed and separated at normal pressure through a filter paper. The separated NM was immediately used for extraction of isoflavones or this liquid samples were frozen and kept refrigerated no more than one week. Cellular sediment on the filtration paper was carefully rinsed and dried at laboratory temperature. Samples of DM were stored in a dark, dry place until their extraction.

4.3 Transport Mechanism Inhibitors Treatment

The result of elicitation with vanadium compounds indicated that the NH_4VO_3 (1 μM) solution caused an increase of certain isoflavones level in the NM after 24 h. Several compounds affecting various transport mechanisms were applied as possible inhibitors of these metabolites exudation then. A 3-day-old suspension cultures were used again and 30 μL of the inhibitors NH_4Cl (PENTA, Prague, CZ), gramicidin, brefeldin A, Na_3VO_4, verapamil (Sigma-Aldrich®, Schnelldorf, Germany), probenecid (MSD, Prague, CZ) and glibenclamide (Roche, Basel, Swiss) from the stock solution prepared according to [22] was added.

The cultures were incubated with the inhibitors for 1 h and once again with 1 mL of NH_4VO_3 (1 μM) and cultivated for 24 h in accordance with the above conditions for the suspension culture. At the same time, samples containing only 1 mL of the elicitor or 1 mL of distilled water without inhibitors (water control) were prepared by the same method. All samples were filtrated after 24 h and processed as before (4.2). The solvents had statistically insignificant effect on the level of isoflavones in the DM or the NM.

4.4 Extracts Preparation and HPLC Analysis

Genista tinctoria extracts and HPLC analysis was done in line with methodology of the Department of Pharmacognosy. The specific volume of the nutrient media was reduced in an Laborota 4010 Rotary Vacuum Evaporator (Heidolph Instruments, Schwabach, Germany) and the evaporated residue was dissolved in 10 mL of methanol 80 %. This solution was placed into a measuring flask (10 mL) and filled up to the mark by the same solvent. The content of the flask was transferred by a syringe through a 0.45 μm microfilter (VWR International, Radnor PA, USA) into a vial for HPLC analysis.

Dried sediment from the suspension culture was carefully crushed into powder in a mortar, and 0.30 to 0.50 g of DM was placed in a laboratory boiling flask then. The dry cells were extracted by 10 mL of methanol 80 % on water bath (75 °C) under Liebig cooler for 10 min.

The mixture was cooled and filtered through cotton; the liquid component was transferred into a measuring flask (20 mL). DM from the cotton was placed back into the boiling flask, and the extraction process was repeated under the same conditions. The liquid part of the second extraction was mixed with the first and the resulting solution was also placed in to a vial for HPLC analysis using a 0.45 μm microfilter. The isoflavones content in the NM and DM was established using HPLC. Individual isoflavone standards (Sigma-Aldrich, Schnelldorf, Germany; Figure 9), the chromatography set (JASCO International, Tokyo, Japan) and analysis assay follow used methodology [72].

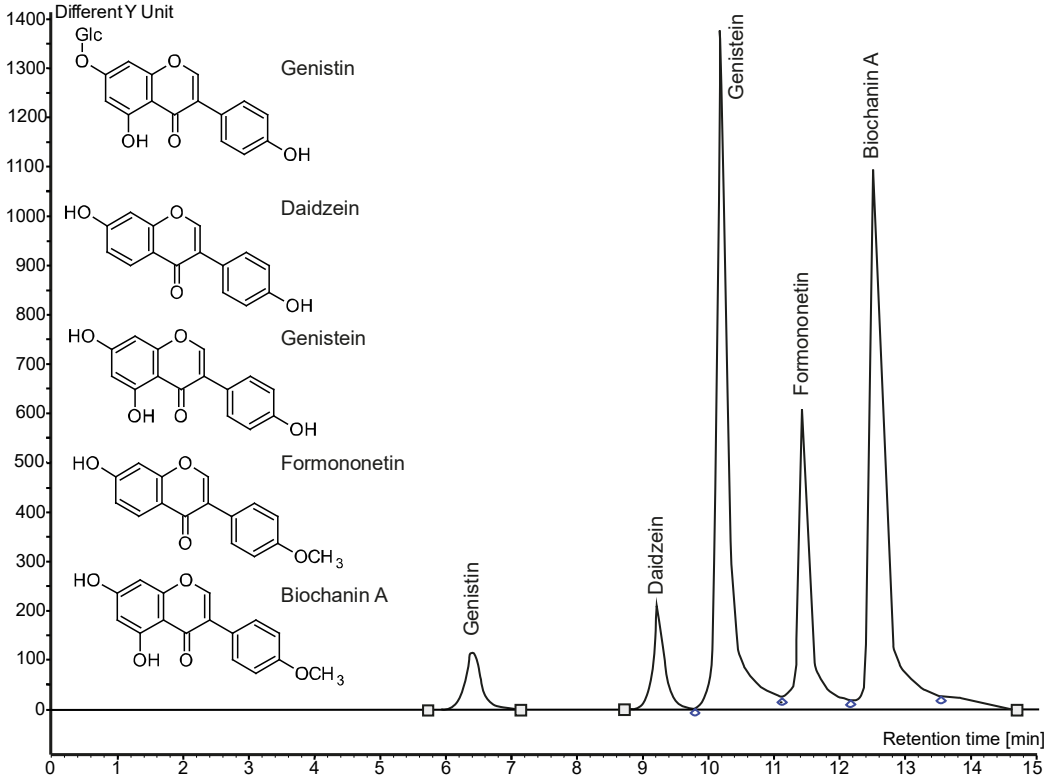

Figure 9. HPLC chromatogram of monitored isoflavones standards and their chemical formula.

4.5 Statistical Analysis

The values of isoflavones content in control samples in case of Figure 1 and elicited samples in case of Figures 2 – 8 were taken as 100 % and they are given in tables (Supplemental Information, Table S1 and S2). The results of other samples were related to this value. Two subsequent subcultures of suspension culture were used for potential transport mechanism determination. Each tested group was only compared with isoflavone content in corresponding subculture. A mixed-model procedure, with a repeated statement for each parameter, was used to analyse the data set. Data from each measurement was tested separately. Tukey´s test ($P < 0.05$) was used to

determine significant differences. All statistical tests presented in this study were performed using a Statistica 13 (StatSoft Inc., Tulsa, USA) software package.

5. Conclusions

In summary, this study presented ammonium metavanadate as a potential elicitor of several isoflavones in *G. tinctoria*, because higher production and release of these metabolites has been found. Beside other well-known plants, this one also represents possible alternative source of isoflavones or model for their further research. The activity of important biosynthesis enzymes or stress signal molecules like as jasmonic acid was not examined in vanadium treated *G. tinctoria* suspension culture in contrast to previous studies [45]. The exact clarification of elicitor mechanism in this cell culture could therefore be considered as challenge for future research. Monitoring the release of isoflavones had less evident results after treatment with transport inhibitors; genistein or daidzein transport could be affected by more types of inhibitors, similarly to mentioned daidzin in *M. truncatula* [50]. There is also possibility that specific inhibitor would show more significant result confirming hypothetical participation of protein from ABCG subfamily. Naturally, plant secondary metabolites transporters are not identical to proteins inhibited by used drugs. However, other plant model systems explained a variability of inhibitors efficacy too and other different compounds can be tested. Unlike *G. max*, *C. arietinum* or *M. truncatula*, there is no described genome of *G. tinctoria* yet and the determination of specific transporter protein could be more difficult. Therefore, further experiments dedicated to transport mechanism are needed such as the determination of isoflavones movement in the intact plant or across the tonoplast.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Table S1: The content of isoflavones in nutrient medium (mg/100 mL) and dry matter (mg/g) of *Genista tinctoria* after application of distilled water for 24 or 48 h., Table S2: The content of isoflavones in nutrient medium (mg/100 mL) and dry matter (mg/g) of *Genista tinctoria* after application of NH_4VO_3 (10 μM) alone for 24 hours. The values were used for a comparison with specific inhibitor in one passage of cell culture.

Author Contributions: Conceptualization, L.T. and J.M[2].; Methodology, J.K., L.T. and J.M[2].; Software, M.S.; Formal Analysis, M.S.; Investigation, J.K.; Data Curation, M.S. and H.H.; Writing-Original Draft Preparation, J.K.; Writing-Review & Editing, M.S., J.K. and J.M[1].; Visualization, M.S. and J.K.; Supervision, V.H. and L.T.

Funding: This research was funded by [the Ministry of Education, Youth and Sports of the Czech Republic] grant number [SVV 260 416] and grant number [S grant of MSMT CR].

Acknowledgments: We would like to thank Miroslava Karlova, Adela Kohoutkova and Marketa Simunkova, who provided the technical background.

Conflicts of Interest: The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

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Sample Availability: Samples of the compounds are available from the authors.