Phytochemistry 179 (2020) 112496

Contents lists available at ScienceDirect

Phytochemistry

journal homepage: www.elsevier.com/locate/phytochem

Induction of promising antibacterial prenylated isoflavonoids from different subclasses by sequential elicitation of soybean

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ARTICLE INFO

Keywords:
Soybean (*Glycine max* (L.) Merrill)
Leguminosae
Elicitation
Antimicrobial
H_2O_2
Silver nitrate
Rhizopus spp.
Bacillus subtilis
Prenylated isoflavones
Phaseol
Glyceollins

ABSTRACT

Elicited soybean (*Glycine max* (L.) Merrill, Leguminosae) seedlings can produce prenylated isoflavonoids from different subclasses, namely pterocarpans (glyceollins), isoflavones and coumestans. These prenylated isoflavonoids serve as defence compounds and can possess antimicrobial activity. Recently, we showed that priming with reactive oxygen species (ROS) specifically stimulated the production of glyceollins in *Rhizopus* spp.-elicited soybean seedlings (ROS + *R*). In this study, we achieved diversification of the inducible subclasses of prenylated isoflavonoids in soybean, by additional stimulation of two prenylated isoflavones and one prenylated coumestan. This was achieved by using a combination of the relatively long-lived ROS representative, H_2O_2, with $AgNO_3$ prior to microbial elicitation. Microbial elicitation was performed with a live preparation of either a phytopathogenic fungus, *Rhizopus* spp. or a symbiotic bacterium, *Bacillus subtilis*. *B. subtilis* induced 30% more prenylated isoflavones than *Rhizopus* spp. in (H_2O_2 + $AgNO_3$)-treated seedlings, without significantly compromising the total levels of glyceollins, compared to (ROS + *R*)-treated seedlings. The most abundant prenylated isoflavone induced was 6-prenyl daidzein, which constituted 60% of the total isoflavones. The prenylated coumestan, phaseol, was also induced in the (H_2O_2 + $AgNO_3$)-treated and microbially elicited seedlings. Based on previously developed quantitative structure-activity relationship (QSAR) models, 6-prenyl daidzein and phaseol were predicted to be promising antibacterials. Overall, we show that treatment with H_2O_2 and $AgNO_3$ prior to microbial elicitation leads to the production of promising antibacterial isoflavonoids from different subclasses. Extracts rich in prenylated isoflavonoids may potentially be applied as natural antimicrobial agents.

1. Introduction

The increasing demand for novel, natural antimicrobials for food preservation (Hintz et al., 2015) and for combating drug-resistant pathogens (Subramani et al., 2017) has triggered research to find methods for efficient production of structurally diverse, yet chemically-related compounds. Stressed soybeans (*Glycine max* (L.) Merrill, Leguminosae) produce different subclasses of prenylated isoflavonoids as part of their defence metabolism, from their non-prenylated biosynthetic precursors, daidzein and genistein (Fig. 1). Prenylated pterocarpans (glyceollins) and prenylated isoflavones are the most important subclasses, but prenylated coumestans have been also found in stressed soybeans (Caballero et al., 1986; Simons et al., 2011b; Yuk et al., 2011).

Glyceollins as such, are mainly known for their antifungal properties (Lee et al., 2010), whereas they serve as absolute precursors of the powerful antibacterials, dehydroglyceollins (Araya-Cloutier et al., 2018). Some dehydroglyceollins, for example, were shown to be active against the Gram-positive bacterium, *Listeria monocytogenes,* with minimum inhibitory concentrations (MICs) of 15 μg/mL (47 μM), while for their precursors, MICs higher than 50 μg/mL were found. In contrast, the prenylated isoflavones, wighteone and isowighteone were found to be very potent against both *L. monocytogenes* and *Escherichia coli* (MICs 10–25 μg/mL, 30–74 μM) (Araya-Cloutier et al., 2018). So far, the antimicrobial activity of prenylated coumestans is not well documented. Prenylated coumestans have been reported as moderately or no potent against Gram-positives (Eerdunbayaer et al., 2014; Tanaka et al., 2002), but potent against Gram-negatives (Khatune et al., 2004).

The amounts of defence metabolites in plants are relatively low and dependent on plant's physiological and developmental stage (Abbasi

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https://doi.org/10.1016/j.phytochem.2020.112496
Received 4 June 2020; Received in revised form 27 July 2020; Accepted 16 August 2020
Available online 15 October 2020

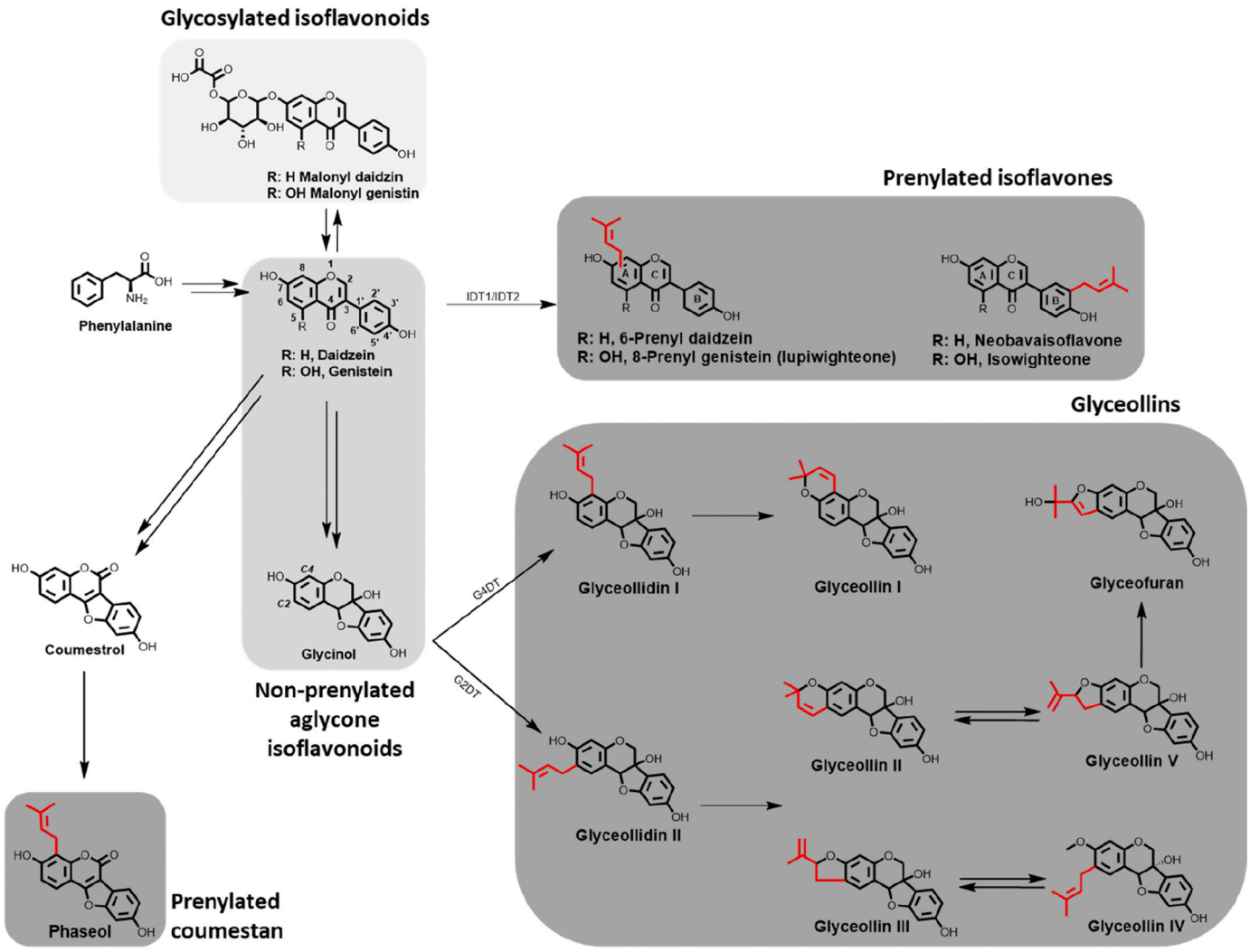

Fig. 1. Simplified biosynthetic pathway of the main prenylated isoflavonoids and their corresponding subclasses encountered in stressed soybeans (*Glycine max*). The prenyl group in its different configurations is highlighted in red. The different prenyltransferases involved in the biosynthesis of the two main subclasses of prenylated isoflavonoids (ie. glyceollins and prenylated isoflavones) are demonstrated. Both possible prenylation positions (*C2* and *C4*) on the glycinol backbone for the synthesis of glyceollins are also depicted. Based on Suzuki et al., 2006, Yoneyama et al., 2016 and Dewick et al., 1970. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

and Graham, 2001; Aisyah et al., 2015; Dixon, 2001; Oksman-Caldentey and Inzé, 2004) as well as on environmental factors (Isah, 2019; Yang et al., 2018). Plant elicitation, ie. the stimulation of biosynthesis of these metabolites upon the addition of small amounts of elicitors (Radman et al., 2003), is one of the most practically feasible techniques for the induction of these molecules (Namdeo, 2007). Elicitation is often intertwined with priming, as the agents that can induce elicitation can act also act as primers (Mauch-Mani et al., 2017; Namdeo, 2007). Priming refers to a physiological state that a plant acquires in response to warning signals (eg. microbially-derived, physical and chemical stimuli) in its environment (Mauch-Mani et al., 2017; Pastor et al., 2013). Contrary to elicitation, priming involves short (and sometimes repetitive) (Baenas et al., 2016; Singh et al., 2014) exposure to the stimuli, preserves the fitness of the plant and involves no or minimal induction of defensive genes (Slaughter et al., 2012). After priming, plant's defence mechanisms are more effectively induced upon subsequent attack (Mauch-Mani et al., 2017; Pastor et al., 2013).

Different classes of agents, such as biotic, including live microorganisms or fragments thereof, and abiotic, including metal ions and endogenous signalling molecules, can stimulate defence responses in different ways (Farrell et al., 2017; Yoshikawa, 1978). Regarding microbial stress, fungal elicitation is the most employed strategy to stimulate production of prenylated isoflavonoids in legumes (Aisyah et al., 2013; Feng et al., 2007; Simons et al., 2011a; Sobolev et al., 2008). On the contrary, reports on the efficiency of bacterial elicitation are often contradicting (Dakora and Phillips, 1996; Hynes et al., 1994; Mañero et al., 2012; Ramos-Solano et al., 2010). The most relevant and systematic study on soybean elicitation with plant-growth promoting (PGPR) bacteria was reported by Ramos-Solano et al. (2010). There, it was suggested that *Bacillus* spp. cause mobilization of non-prenylated aglycone isoflavones from soybeans' roots to shoots where they may play a role in defence. This effect did not compromise soybeans' growth. However, no subsequent analysis on prenylated isoflavonoids was performed.

Plants might respond differently to microbial stresses due to variability in pathogen recognition (Berenbaum, 1995; Ferrari, 2010) and discrepancies have been observed even between different hosts of the same plant species (Kalli et al., 2020). Therefore, alternatives to microbial stress are sought for more controllable and reliable inductions (Poulev et al., 2003). Exogenous application of chemicals is readily used as an abiotic stimulator of plants' secondary metabolism (Ghosh et al., 2020; Namdeo, 2007; Thakur et al., 2019). Farrell et al. (2017), for

example, reported specific biosynthesis of glyceollin I in soybean seeds by 1 mM silver nitrate ($AgNO_3$) but not with the same concentration of $CuCl_2$ (Farrell et al., 2017). Later, 1 mM $AgNO_3$ was shown to upregulate prenyltransferases involved in the production of both glyceollins and prenylated isoflavones in soybean (Sukumaran et al., 2018). Furthermore, endogenous signalling molecules are also exploited to chemically stress plants. Reactive oxygen species (ROS) generated through Fenton's reaction (H_2O_2 + Fe (II)), have been exogenously applied alone (Degousee et al., 1994) or as a primer prior to fungus elicitation to stimulate glyceollin biosynthesis (Kalli et al., 2020).

In this study, a combination of H_2O_2 and $AgNO_3$ was selected to treat soybean seedlings prior to microbial elicitation to stimulate the production of prenylated isoflavonoids from different subclasses, including the more antibacterial prenylated isoflavones. H_2O_2, the freely diffusible and relatively long-lived representative of ROS, is expected to regulate the fast entry of the plant into the primed state (Graham and Graham, 1999). $AgNO_3$ is expected to specifically target the biosynthesis of prenylated isoflavonoids (Farrell et al., 2017; Sukumaran et al., 2018). Ag (I) does not participate in Fenton's reaction (Nishimoto et al., 2018), as is known for Fe (II) (Degousee et al., 1994; Kalli et al., 2020), thus H_2O_2 and $AgNO_3$ should act independently. As microbial elicitors, a phytopathogenic, live fungus preparation (a mixture of *Rhizopus oligosporus and Rhizopus oryzae*) (Ghosh and Ray, 2011; Partida-Martinez et al., 2007) or a live bacterial preparation of a symbiotic bacterium (*Bacillus subtilis*) (Nagórska et al., 2007) were used. We hypothesized that the symbiont will affect less the fitness of soybeans seedlings, thereby allowing more extensive secondary metabolism. Last, the effect of soybeans' developmental stage on the induction of prenylated isoflavonoids was also investigated, by applying the treatments in 2d- and 4d-old soybean seedlings. The treatments and the sequence by which they were applied to the seedlings are depicted in Fig. 2.

2. Results and discussion

2.1. Chromatographic profile and annotation of isoflavonoids in (H_2O_2 + $AgNO_3$)-treated soybean seedlings

The RP-UHPLC-PDA chromatograms of ethanol extracts of (H_2O_2 + $AgNO_3$)-treated (and subsequently elicited) soybean seedlings are shown in Fig. 3 and compared to the recently proposed ROS + *R*, i.e. (H_2O_2 + Fe (II)), which is used as a benchmark in this study. Treatment with (H_2O_2+ $AgNO_3$) stimulated the production of **12** and **13** which were tentatively annotated as neobavaisoflavone (3′-prenyldaidzein) and 6-prenyl daidzein (Araya-Cloutier et al., 2017), respectively. When (H_2O_2 + $AgNO_3$)-treated seedlings were subsequently elicited with *Rhizopus* spp. *or Bacillus subtilis*, the levels of prenylated isoflavones increased significantly. In addition to **12** and **13**, they also substantially

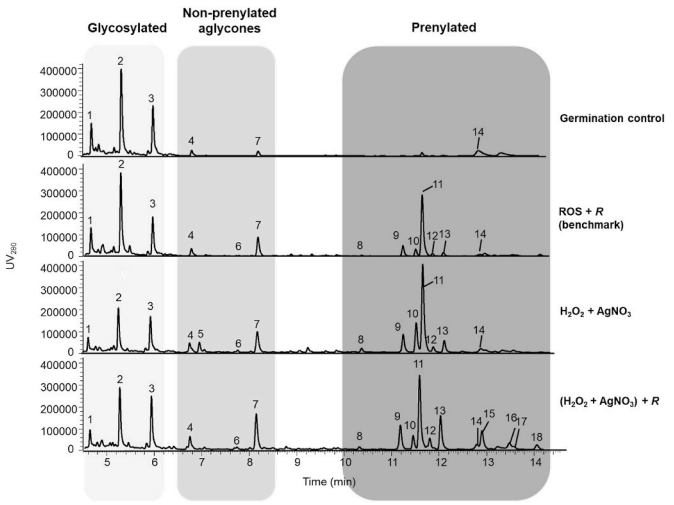

Fig. 3. RP-UHPLC-PDA (280 nm) profiles of 96% (v/v) EtOH extracts of germinated (without any treatment application), ROS-primed and subsequently *Rhizopus* spp. (*R*)-elicited (Kalli et al., 2020), (H_2O_2 + $AgNO_3$)-treated and (H_2O_2 + $AgNO_3$)-treated and subsequently *R*-elicited soybean seedlings. Extracts correspond to 7d-old seedlings, where treatments (if any) were applied on the 4th day of germination. Peak numbers refer to compounds in Table S1.

accumulated phaseol (4-prenylcoumestrol) (Caballero et al., 1986; Simons et al., 2011b) (**15**), lupiwighteone (8-prenyl genistein) (**16**), isowighteone (3′-prenyl genistein) (Araya-Cloutier et al., 2017) (**17**) and the *C*2-glyceollin, glyceollin IV (**18**) (Van De Schans et al., 2016). Interestingly, compounds **12**–**17** were weakly or not consistently induced over time in ROS + *R* (Kalli et al., 2020).

All treated seedlings accumulated the three main glyceollin isomers, namely the only *C*4-glyceollin present in the extracts, glyceollin I (**11**) and the *C*2-glyceollins, glyceollin II and III (**9** and **10**). In addition, the *C*2-glyceollins, glyceofuran (**6**) and glyceollidin II (**8**) were also identified (Aisyah et al., 2013). The concomitant production of the newly induced prenylated isoflavones and prenylated coumestans together with glyceollins shows that the latter were not sacrificed at the expense of the former two (Aisyah et al., 2013; Kalli et al., 2020). This finding shows that (H_2O_2 + $AgNO_3$)-treatment prior to microbial elicitation results in diversification in the induced subclasses of prenylated isoflavonoids; from mainly glyceollins as induced by ROS + *R* (Kalli et al., 2020) to the additional induction of prenylated isoflavones and of a prenylated coumestan (Fig. 1).

The major non-prenylated isoflavonoids typically found in soybeans, namely daidzein (**4**) and genistein (**7**) (Suzuki et al., 2006), together with their glycosides, daidzin (**1**), the malonylated glycoside of daidzein

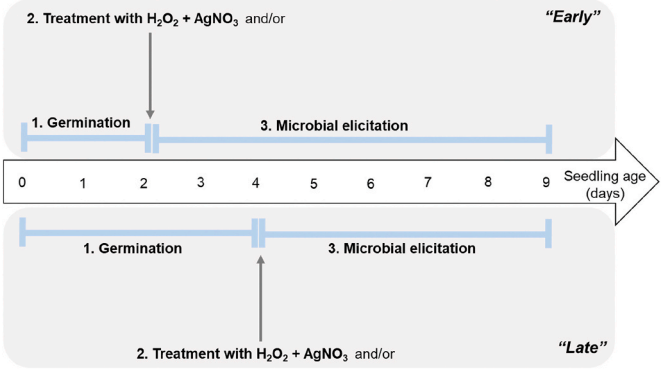

Fig. 2. Timeline of (H_2O_2 + $AgNO_3$)-treatment with or without subsequent microbial elicitation of soybean seedlings. Microbial elicitation was performed with a live preparation of either a phytopathogenic fungus, *Rhizopus* spp. or a symbiotic bacterium, *Bacillus subtilis*. "Early" and "Late" refer to the time point of application of the (H_2O_2 + $AgNO_3$)-treatment to 2d- and 4d-germinated seedlings, respectively.

(6″-O-malonyldaidzin) (**2**) and of genistein (6″-O-malonylgenistin) (**3**), were also identified in the extracts and represented more than 90% of the annotated peaks in the germination control (Fig. 3). Soyasaponin βg (**14**), constitutively present in the soybeans, was not considered further due to its weak antimicrobial potential (Araya-Cloutier et al., 2017). The annotated isoflavonoids in all (H_2O_2 + $AgNO_3$)-treated seedlings can be found in Table S1.

2.2. *Abiotic elicitation with H_2O_2 + $AgNO_3$ alone can circumvent biotic agents in the induction of glyceollins*

The effect of soybeans' developmental stage on the induction of the different subclasses of prenylated isoflavonoids by the (H_2O_2 + $AgNO_3$)-treatments was studied. For this, the treatments were applied to the seedlings at different moments, i.e. after 2 days of germination ("early") and after 4 days of germination ("late") (Fig. 2). "Late" treatment with H_2O_2+ $AgNO_3$ without subsequent elicitation triggered a 1.5-times increase in glyceollin levels compared to "Early" application, with a maximum of 4.5 ± 0.3 μmol/g DW on the 4th day after the treatment (Fig. 4A). This finding further corroborates the fact that older soybean seedlings respond better to elicitation treatments with respect to glyceollin induction than their younger counterparts (Abbasi and Graham, 2001; Aisyah et al., 2015; Kalli et al., 2020). "Late" (H_2O_2+ $AgNO_3$)--treatment without subsequent elicitation performed similarly to "Late"

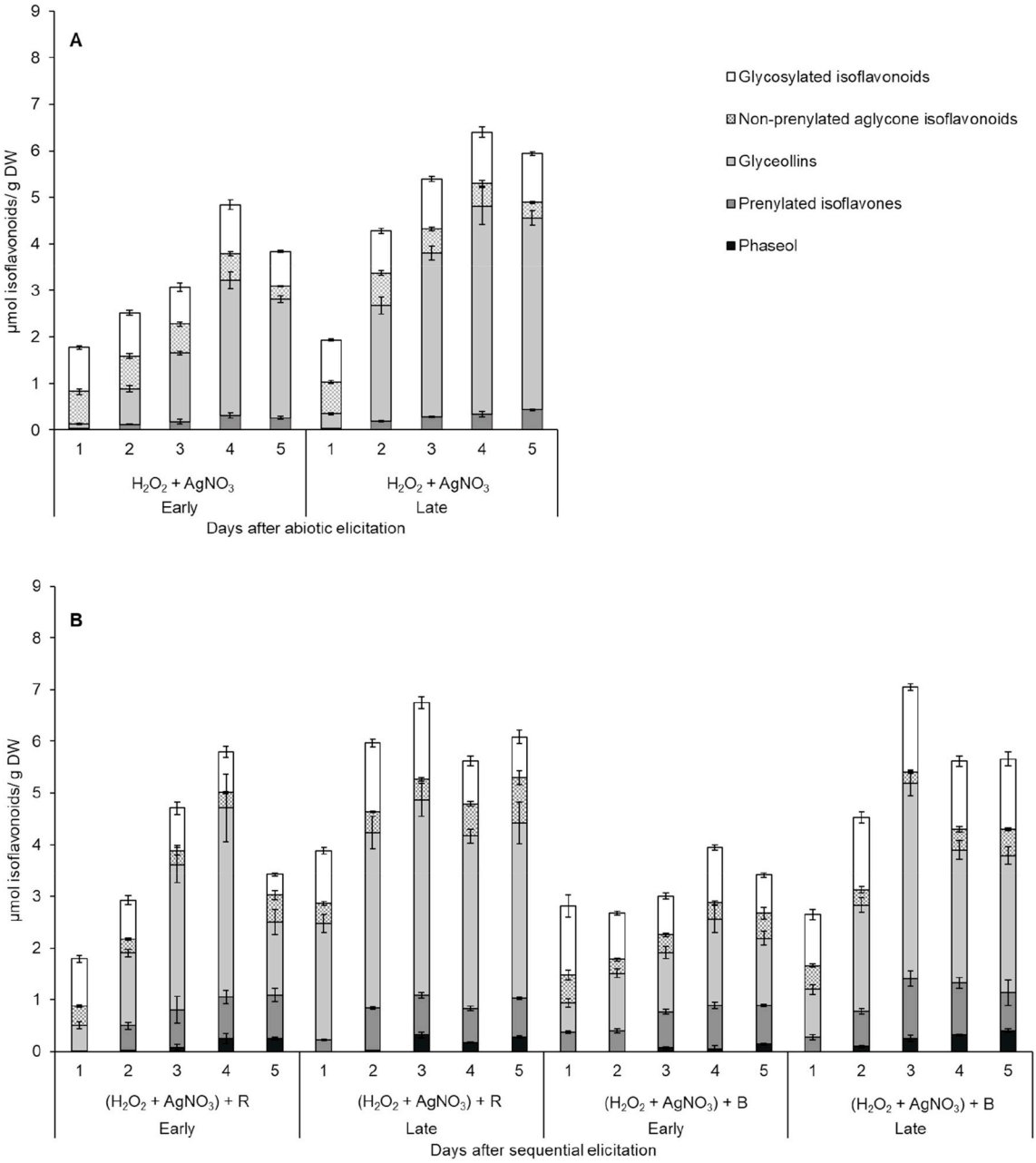

Fig. 4. Isoflavonoid content (μmol/g DW) of "early" and "late" (H_2O_2 + $AgNO_3$)-elicited (**A**) and (H_2O_2 + $AgNO_3$)-elicited prior to *R*- or *B*- elicited (**B**) soybean seedlings over five days. Isoflavonoids are classified into four main families (from top to bottom); ie. glycosylated isoflavonoids (white), non-prenylated aglycones (patterned), glyceollins (light grey) and prenylated isoflavones (dark grey). Phaseol contents (black) are also depicted for sequential elicitation treatments (**B**). Error bars indicate the standard deviation of three biological replicates. Quantification of the individual isoflavonoids over time per treatment can be found in Tables S2–S4. Statistical analysis (Tukey's test, $p < 0.05$) of the over-time differences in each isoflavonoid subclass within the same treatment can be found in Table S5.

ROS + *R* in the induction of glyceollins (4.6 ± 0.3 μmol glyceollins/g DW) when applied in 4d- and 3d-old seedlings, respectively, of the same soybean cultivar (Fig. 4A & Fig. S1). This finding suggests that the purely chemical treatment resembles more to elicitation than to priming, despite the short exposure of the seedlings to the agents. Furthermore, the "Late" (H_2O_2 + $AgNO_3$)-elicitation resulted in similar induction of *C*2- and *C*4- glyceollins (48:52, Table S3) whereas the "Late" ROS + *R* was found to favour more the production of *C*4- glyceollins (36:64) (Kalli et al., 2020) on the optimal day of the treatments. This finding shows that the newly proposed abiotic treatment might also lead to higher diversity of the induced glyceollins compared to the recently published ROS + *R*.

Along with the increased levels of glyceollins, the treatment with H_2O_2+ $AgNO_3$ alone slightly triggered the production of approximately 0.3 ± 0.05 μmol/g DW prenylated isoflavones (on the 4th day after elicitation), regardless the time of application of elicitation ("Early" or "Late") (Fig. 4A).

Last, seedlings treated with H_2O_2+ $AgNO_3$ consistently contained more than 50% less glycosylated isoflavonoids (Fig. 4A) compared to ROS + *R* (Fig. S1) (Kalli et al., 2020). The most affected compound was 6″-O-malonyldaidzin (**2**) (Table S2), which was approximately 75% less in the (H_2O_2+ $AgNO_3$)-treatment. This seems to corroborate the hypothesis that $AgNO_3$ stimulates specific deglycosylation of 6″-O-malonyldaidzin and subsequent generation of precursors for prenylation (Farrell et al., 2017).

Overall, we suggest the potential of a combined, purely abiotic elicitation treatment (H_2O_2+ $AgNO_3$) to circumvent microbial agents (ROS + *R*) in the induction of glyceollins. Since all plants seem to share a similar signal transduction pathway, elicitation with chemicals will enable a more universally applicable and controllable approach than approaches involving biotic agents.

2.3. Microbial elicitation after (H_2O_2+ $AgNO_3$)-elicitation boosts the production of prenylated isoflavones and of the prenylated coumestan, phaseol

Elicitation with (H_2O_2+ $AgNO_3$) without a subsequent microbial elicitation had a stimulatory effect on the induction of glyceollins and a smaller triggering effect on the production of isoflavones. When chemical elicitation was followed by microbial elicitation, the biosynthesis of prenylated isoflavones was enhanced further by 3–4 times. *Bacillus subtilis* was more effective in inducing prenylated isoflavones than *Rhizopus* spp. when applied "late" in (H_2O_2+ $AgNO_3$)-elicited seedlings, at all time-points (Fig. 4B). On the contrary, both microorganisms induced similar glyceollin levels in (H_2O_2 + $AgNO_3$)-elicited seedlings over time (Fig. 4B). Interestingly, *Rhizopus* spp. (phytopathogenic) generally outperformed *Bacillus subtilis* (symbiont) in terms of glyceollin production, when these were applied alone without prior elicitation (Fig. S2). This discrepancy in the effects of the microorganisms, when used as direct elicitors or after elicitation, has not been reported before. It might be related to the differential recognition of *B. subtilis* as a symbiont or as a pathogen by non-elicited or already elicited soybeans, respectively. This differential recognition may have an effect on the balance between maintaining plant's vitality and inducing the production of defensive metabolites (Berg, 2009; González-Lamothe et al., 2009).

Unlike glyceollins, prenylated isoflavones were already maximally induced upon "early" application of the sequential elicitation treatment. Later application of the treatment did not lead to a consistent increase in prenylated isoflavones. Prenylation of isoflavonoids in soybean is catalysed via two classes of prenyltransferases, the glycinol prenyltransferases yielding glyceollins and the isoflavone prenyltranferases yielding prenylated isoflavones (Fig. 1) (Yoneyama et al., 2016). Early induction of prenyltransferases involved in direct prenylation of isoflavones by $AgNO_3$ has been shown before (Sukumaran et al., 2018).

The increased prenylated isoflavone accumulation in sequentially elicited seedlings, compared to only (H_2O_2+ $AgNO_3$)-elicited ones, can be partially explained by the comparably low levels of glycosylated isoflavonoids observed in both cases (Fig. 4A and B), compared to ROS + *R* (Fig. S1). Nevertheless, the combined treatments seem to additionally upregulate the activity of isoflavone-specific prenyltransferase (s).

Sequential elicitation treatments additionally stimulated the synthesis of phaseol (Fig. 4B). Phaseol (4-prenyl coumestrol) derives from daidzein as a distant precursor (Dewick et al., 1970), similarly to glyceollins and to prenylated isoflavones (Fig. 1). Seedlings accumulated more pronounced levels of phaseol upon late application of sequential elicitation, especially when *B. subtilis* was used as microbial elicitor (Fig. 4B). Even though phaseol has been detected before in soybean seedlings (Caballero et al., 1986; Yuk et al., 2011), there is no information on its antimicrobial potency.

2.4. "Late" elicitation with H_2O_2 + $AgNO_3$ prior to biotic elicitation, the new protocol for induction of a wide array of prenylated isoflavonoid subclasses

Overall, abiotic elicitation prior to microbial elicitation and seedling age seem important in improving the biosynthetic capacity of the soybean seedlings. Since the "Late" treatments resulted in maximum accumulation of all subclasses of prenylated (iso)flavonoids, ie. glyceollins, prenylated isoflavones and the prenylated coumestan, the "Late" treatments are considered as optimal and are further compared (Fig. 5).

Treatments that involved microbial elicitation after (H_2O_2+ $AgNO_3$)-elicitation stimulated the levels of prenylated isoflavones more than 4-fold compared to ROS + *R* (0.2 ± 0.02 μmol/g DW) on their optimal day (3rd after the treatment). Among the studied microorganisms, *B. subtilis* induced the accumulation of 30% more prenylated isoflavones than *Rhizopus* spp. in (H_2O_2 + $AgNO_3$)-elicited seedlings (0.8 ± 0.1 μmol/g DW and 1.2 ± 0.1 μmol/g DW for *R*- and *B*- elicitation, respectively) (Fig. 5). This increase in prenylated isoflavones was mainly attributed to the enhanced accumulation of 6-prenyl daidzein (60% of the total induced prenylated isoflavones, Table S4). Neobavaisoflavone was the second most strongly induced prenylated isoflavone (over 25% of the total induced prenylated isoflavones, Table S4) in sequentially elicited seedlings. Isowighteone and lupiwighteone make up the complete prenylated isoflavone pool (Table S4). Along with prenylated isoflavones, the prenylated coumestan, phaseol was induced at levels of 0.3 ± 0.06 μmol/g DW in sequentially elicited seedlings on the optimal day (3rd after the treatment) (Fig. 5).

Maximal induction of prenylated isoflavones upon the "late" subsequent elicitation treatments occurred without significantly compromising the levels of total induced glyceollins (3.8 ± 0.3 μmol/g DW for both *R*- and *B*- elicitation) compared to ROS + *R* (4.6 ± 0.3 μmol/g DW). This indicates that (H_2O_2+ $AgNO_3$)-elicitation prior to microbial elicitation is able to specifically induce prenylated isoflavones (Fig. 5), whereas ROS-priming prior to *R*-elicitation targeted mainly glyceollin production (Kalli et al., 2020).

Overall, we propose elicitation of 4d-old (instead of 2d-old) soybean seedlings with H_2O_2 and $AgNO_3$ before microbial elicitation for the simultaneous enhanced production of three subclasses of prenylated isoflavonoids (ie. pterocarpans, isoflavones and coumestans) in soybean seedlings. The symbiont, *B. subtilis* was shown to be more effective than the phytopathogenic, *Rhizopus* spp. in the induction of prenylated isoflavones in sequentially elicited seedlings.

As might be expected, the use of $AgNO_3$ can rise safety and environmental concerns as metallic silver is anticipated to be deposited on the seedlings in the presence of H_2O_2 (Nishimoto et al., 2018) and subsequently in the rinsing water (Section 4.1.6). Cost-effective, simple technologies that recover silver from waste waters, minimizing its environmental impact are available (for an example, see Zou et al., 2007 and for a review, see Syed, 2016). Furthermore, co-extraction of any

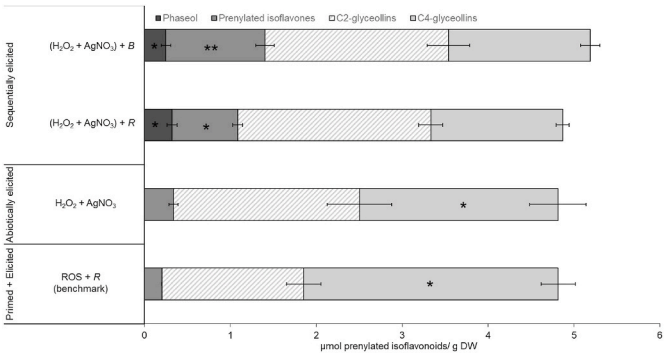

Fig. 5. Content (μmol/g DW) of prenylated isoflavonoids in (H_2O_2 + $AgNO_3$)-elicited (and subsequently *B. subtilis* (*B*)- or *Rhizopus* spp. (*R*)- elicited) soybean seedlings in comparison to the recently proposed priming and elicitation treatment, ROS + *R* published by Kalli et al., 2020. Prenylated isoflavonoids were classified into (from right to left): *C*4-glyceollins (light grey), *C*2-glyceollins (light grey striped), prenylated isoflavones (grey), and phaseol (dark grey). Treatments are shown at their optimum day (4d for (H_2O_2 + $AgNO_3$)-elicited and 3d for the sequentially elicited or ROS + *R* treatments) after "late" application with respect to maximum prenylated isoflavonoid accumulation. Error bars indicate the standard deviation of three biological replicates. Asterisks signify a statistically higher accumulation of a specific prenylated isoflavonoid subclass compared to the rest of the treatments ($p < 0.05$).

residual silver present in the seedlings with the compounds of interest should be limited when pure organic solvents (eg. ethyl acetate), known to maximize the extractability of prenylated isoflavonoids, are used (Simons et al., 2009).

2.5. QSAR-based prediction of the antibacterial potency of 6-prenyl daidzein and phaseol

In this study, treated seedlings were extracted with 96% (v/v) EtOH to obtain and study the entire range of isoflavonoid families (glycosylated, aglycone and prenylated). As the antimicrobial activity of crude extracts (containing glycosylated and aglycone isoflavonoids) is low (Araya-Cloutier et al., 2017), enrichment of the extracts in prenylated isoflavonoids or purification of prenylated isoflavonoids are suitable strategies to obtain potent, natural antimicrobials. Several reports on the antibacterial activity of purified prenylated isoflavonoids is available in literature (Araya-Cloutier et al., 2018; Djeussi et al., 2015; Mbaveng et al., 2015). In particular, certain prenylated isoflavones have been shown to be powerful antibacterial agents against the food-borne Gram-positive pathogen *L. monocytogenes* and the clinically-relevant Gram-negative pathogen *E.coli* (Araya-Cloutier et al., 2018). Among the most abundant prenylated isoflavones induced by our proposed sequential elicitation protocol, neobavaisoflavone has showed high potency towards *E.coli* (20 μg/mL, 59 μM), in the presence of an efflux pump inhibitor, and more moderate activity against *L. monocytogenes* (50 μg/mL, 155 μM) (Araya-Cloutier et al., 2018).

The most strongly induced prenylated isoflavone by sequential elicitation, 6-prenyl daidzein, and the prenylated coumestan, phaseol, have not been tested for their antimicrobial potency yet. To predict their antibacterial potency, the structures of molecules were inputed to MOE (Molecular Operating Environment) and molecular descriptors corresponding to these structures were calculated (Table S6). With these descriptors, the antibacterial potency of these two compounds against *L. monocytogenes* and *E. coli* was predicted by using QSAR models already developed against these two bacteria (Eq. (2) and Eq. (3), Section 4.1.6). The predicted antibacterial activity of 6-prenyl daidzein and phaseol can be found in Table 1. 6-Prenyl daidzein was predicted as very active, having a MIC value of 6.9 μg/ml (21 μM) and 9.1 μg/ml (28 μM) against *L. monocytogenes* and *E. coli*, respectively. Phaseol was predicted to be potent having a MIC value of 36 μg/ml (108 μM) and 23 μg/ml (69 μM) against the two bacteria, respectively. Based on these predictions, it is worthwhile to evaluate *in-vitro* the antibacterial activity of the purified compounds.

Table 1
Antibacterial activity of 6-prenyl daidzein and phaseol as predicted by using already developed QSAR models for *L. monocytogenes* and *E. coli* (Araya-Cloutier et al., 2018).

Bacterium	Predicted antibacterial activity (MIC, μM)	
	6-Prenyl daidzein	Phaseol
L. monocytogenes	21	108
E. coli[a]	28	69

[a] Activity predicted in the presence of an efflux pump inhibitor (PAβN).

3. Conclusions

A combination of H_2O_2 and $AgNO_3$ was used to induce different subclasses of prenylated isoflavonoids in microbially-elicited soybean seedlings. (H_2O_2+ $AgNO_3$)-treatment without subsequent microbial elicitation was as effective in inducing glyceollins (4.5 ± 0.3 μmol/g DW) as the recently proposed, priming and elicitation treatment (ROS + *R*). This finding suggests that abiotic elicitation may circumvent the use of microbial agents for glyceollin production. Additionally, treatment with H_2O_2 and $AgNO_3$ triggered the synthesis of prenylated isoflavones, which were substantially boosted (3–4 fold) by subsequent microbial elicitation. Elicitation with the symbiotic bacterium, *Bacillus subtilis,* induced 30% more prenylated isoflavones in (H_2O_2+ $AgNO_3$)-treatment seedlings than with the phytopathogenic fungus, *Rhizopus* spp. This increase was mainly attributed to the production of 6-prenyl daidzein (60% of the total induced prenylated isoflavones). Besides this, the prenylated coumestan, phaseol was boosted by the sequential elicitation treatments. The antibacterial potency of the newly induced 6-prenyl daidzein and phaseol was predicted by previously developed QSAR models. Both compounds were predicated to be promising antibacterials against *L. monocytogenes* and *E.coli*, although 6-prenyl daidzein was predicted to be 2–5 times more potent than phaseol.

We propose elicitation of 4d-old soybean seedlings with H_2O_2 and $AgNO_3$ before elicitation with *B. subtilis* for the simultaneous induction of the three isoflavonoid subclasses: prenylated pterocarpans (glyceollins), isoflavones and coumestans. These results show that sequential elicitation can be used to produce extracts containing prenylated isoflavonoids from different subclasses, which, after enrichment or purification, may be used as natural antibacterial agents.

4. Experimental

4.1. General experimental procedures

4.1.1. Application of (H_2O_2+ $AgNO_3$)-treatments to soybean seedlings

Soybeans were treated in a modified sprouting machine (MikroFarm™ EQMM; Easy-Green, San Diego, CA, USA) as described previously (Kalli et al., 2020). In all treatments, 50 g of seeds (approximately 250 seeds) were subjected to three phases: soaking (1 day), germination (2 or 4 days), elicitation (1–5 days) as described by (Kalli et al., 2020).

Elicitation treatments were sequentially applied on 2-day old ("Early" application) or 4-day old ("Late" application) germinated seedlings. The (iso)flavonoid content of the sequentially elicited seedlings was subsequently monitored daily for five days. A time-line of the experimental set-up can be found in Fig. 2.

Treatment with H_2O_2+ $AgNO_3$ was performed by first immersing the seedlings in 1 mM $AgNO_3$ solution (10 mL/g dry seed) for 30 min under continuous swirling. Then, the seedlings were drained from the metal

ion solution and subsequently immersed in a 1 M H_2O_2 solution (10 mL/g dry seed) for another 30 min and continuously swirled. Subsequently, the H_2O_2 solution was drained and the seedlings thoroughly rinsed with water. (H_2O_2 + $AgNO_3$)-treated seedlings were subjected to microbial elicitation with either with the fungus, *Rhizopus oligosporus/oryzae* (in brief, *Rhizopus* spp.) or with the rhizobacterium, *Bacillus subtilis*. For the fungal inoculation, a suspension of fungal sporangia was prepared by scrapping off the *Rhizopus* spp. culture, grown in malt extract agar plates, for 7 days at 30 °C in the dark (approximately 10^7 CFU/mL). Bacterial cell suspensions were prepared by streaking *Bacillus subtilis* from a −80 °C glycerol stock onto a brain heart infusion (BHI) agar plate and incubated for 24 h at 30 °C in the dark. One colony was transferred to BHI broth (10 mL) and further incubated for 18 h at 30 °C in the dark. The overnight bacterial culture was diluted with BHI (approximately 10^7 CFU/mL). The microbial spore/cell suspensions (0.4 mL/g dry seed) were poured over the germinated seedlings and the inoculated seedlings were incubated for a maximum of 5 days at 30 °C and 100% RH. All treatments were performed in the dark and in three independent replicates.

An overview of the elicitation treatments discussed in this work can be found in Table 2.

4.1.2. Ethanolic extraction of defatted soybean seedlings

Seedlings were extracted according to an established protocol (Kalli et al., 2020). In short, seedlings were freeze-dried and milled. The powder was defatted with hexane and extracted with 96% (*v/v*) aqueous ethanol. The ethanol extract was dried under reduced pressure, resolubilised in methanol at a concentration of 5 mg/mL and stored at −20 °C until analysis. All samples were centrifuged (15,000*g*, 5 min; room temperature) prior to analysis.

4.1.3. Compositional analysis of ethanolic seedling extracts by RP-UHPLC-PDA-ESI-MS

Samples were analysed by RP-UHPLC-MS on an Accela UHPLC system (Thermo Scientific, San Jose, CA, USA) equipped with a pump, autosampler, PDA detector, and ESI-ion trap-MS. Identical column, eluents and gradient elution program were used as reported by Kalli et al., 2020.

Mass spectrometric analysis was performed on a Velos Pro (Thermo Scientific) equipped with an heated ESI-MS probe coupled *in-line* to the RP-UHPLC system as described elsewhere (Kalli et al., 2020).

4.1.4. Tentative annotation and quantification of isoflavonoids

Annotation and quantification of phytochemicals was performed as described previously by Kalli et al. (2020). In short, isoflavonoids were tentatively annotated based on MS spectral data and identified based on the ultraviolet (UV) absorbance at 280 nm.

A standardized six-point (1–100 μg/mL) calibration curve based on an external standard of daidzein ($R^2 = 0.995$) was used for the quantification of (iso)flavonoids. Compounds were first converted to mg daidzein equivalents per g of dry weight of the seedling (mg DE/g DW).

Table 2
Elicitation treatments performed on 2d-old ("Early" application) and 4d-old ("Late" application) soybean seedlings.

Process	Treatment[a]
Abiotic elicitation	H_2O_2+ $AgNO_3$
Microbial elicitation	*R*
	B
Sequential elicitation	(H_2O_2 + $AgNO_3$) + *R*
	(H_2O_2 + $AgNO_3$) + *B*

[a] *R*: *Rhizopus* spp., *B*: *Bacillus subtilis*.

Then, the quantities of each compound were corrected for the differences in molar extinction coefficients between the standards and the compounds of interest, using Lambert-Beer's law (Eq. (1)) and the molar extinction coefficients reported at Kalli et al. (2020).

$$\varepsilon_A C_A = \varepsilon_B C_B \quad \text{(Eq.1)}$$

Ultimately, the quantities of the compounds were expressed in μmol isoflavonoid per gram of seedling's dry weight (μmol/g DW).

4.1.5. Statistical analysis

Statistical analysis was performed using the SPSS Statistics software package (version 23, IBM, Armonk, NY, USA). Differences in the amounts of isoflavonoid subclasses between pairs of treatments were evaluated for significance ($p < 0.05$) with independent samples *t*-test. Over-time differences in the amounts of isoflavonoid subclasses within the same treatment were assessed with Tukey's *post hoc* multiple comparison test ($p < 0.05$).

4.1.6. Prediction of antibacterial potency of 6-prenyl daidzein and phaseol

The antibacterial potency of 6-prenyl daidzein and phaseol was predicted based on two already developed QSAR models for the bacteria, *L. monocytogenes* (Gram-positive) and *E. coli* (Gram-negative) (Araya-Cloutier et al., 2018). First, chemical structures were drawn in the modelling software (Molecular Operating Environment, MOE, v.2019.08, Chemical Computing Group). A conformational search (LowModeMD, RSM gradient 0.1 kcal/mol/Å, other settings default) was performed and the conformation with the lowest energy was further refined using MOPAC force field (RSM gradient 0.01 kcal/mol/Å). Optimized chemical structures were used to calculate different molecular descriptors available in MOE.

Already proposed QSAR models for the prediction of prenylated (iso) flavonoids against *L. monocytogenes* (Eq. (2)) and *E. coli* (Eq. (3)) were used to calculate the minimum inhibitory concentrations (MICs) of 6-prenyl daidzein and phaseol.

$$y = 2.71 + 0.4*KierA3 + 1.13*rsynth - 0.07*vsurf\_DD12 - 0.16*vsurf\_IW4 + 0.29*vsurf\_ID8 \quad \text{(Eq.2)}$$

$$y = 1.60 + b_{count}*0.06 + 1.54*std_{dim3} + 0.74*vsurf\_IW2 + 0.80*rgyr - 0.28*vsurf\_IW4 \quad \text{(Eq.3)}$$

Both compounds were found to fit within the applicability domains of the models, as determined by the standardization method (Roy et al., 2015).

4.2. Materials

Soybeans (*Glycine max* (L.) Merrill, Leguminosae) from the cultivar Envy were purchased from Vreeken's Zaden (Dordrecht, the Netherlands). Tempeh starter culture (mixture of *Rhizopus* spp. *oligosporus* and *Rhizopus* spp. *oryzae*) was purchased from TopCultures (Zoersel, Belgium). H_2O_2 (30% (*w/w*) and standards of daidzein (≥98%) and genistein (≥98%) were purchased from Sigma Aldrich Chemie B.V. (Zwijndrecht, The Netherlands). ULC-MS grade acetonitrile (ACN) with 0.1% (*v/v*) formic acid (FA), water with 0.1% (*v/v*) FA, and methanol (MeOH) were purchased from Biosolve BV (Valkenswaard, The Netherlands). $AgNO_3$ (≥99.0%) was purchased from VWR International B.V. (Amsterdam, The Netherlands) and NaCl was purchased from Sigma Aldrich Chemie B.V.. *n*-Hexane and 96% (*v/v*) aqueous ethanol were obtained from VWR International B.V.. *Bacillus subtilis* (ATCC 6633) was kindly provided by the Laboratory of Food Microbiology of Wageningen University and Research (Wageningen, The Netherlands). Brain heart infusion broth was purchased from BD (Franklin Lakes, NJ, USA), malt extract agar (CM59) and bacteriological agar from Oxoid Ltd (Basingstoke, UK). Peptone physiological salt solution (PPS) was

purchased from Tritium Microbiologie (Eindhoven, The Netherlands). Standards of prenylated isoflavones (lupiwighteone, isowighteone and neobavaisoflavone) were purchased from Plantech UK (Berkshire, UK).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors are thankful to Yiran Lin for her help in performing screening elicitation experiments.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.phytochem.2020.112496.

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