Induction of prenylated isoflavonoids and stilbenoids in legumes

Siti Aisyah

Thesis committee

Promotor
Prof. Dr H. Gruppen
Professor of Food Chemistry
Wageningen University

Co-promotor
Dr J.-P. Vincken
Associate professor, Laboratory of Food Chemistry
Wageningen University

Other members
Prof. Dr P.J.G.M. de Wit, Wageningen University
Prof. Dr E.J. Smid, Wageningen University
Prof. Dr S. de Saeger, Ghent University, Belgium
Dr H.W.M. Hilhorst, Wageningen University

This research was conducted under the auspices of the Graduate School VLAG (Advanced studies in Food Technology, Agrobiotechnology, Nutrition and Health Sciences).

Induction of prenylated isoflavonoids and stilbenoids in legumes

Siti Aisyah

Thesis

submitted in fulfilment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus
Prof. Dr A. P. J. Mol,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Tuesday 13 October 2015
at 11 a.m. in the Aula.

Siti Aisyah
Induction of prenylated isoflavonoids and stilbenoids in legumes
156 pages.

PhD thesis, Wageningen University, Wageningen, NL (2015)
With references, with summary in English

ISBN: 978-94-6257-481-6

Abstract

The germination of legume seeds in the presence or absence of stress factors was studied with respect to compositional changes in prenylated isoflavonoids and stilbenoids. Different strategies were applied using (i) different types of legume seed, (ii) different stress factors i.e. biotic, abiotic and their combination, and (iii) different time point of application of the fungus. Mass spectrometric tools to better characterize the position of prenyl groups in the molecules were optimized. Isoflavonoids and stilbenoids appeared more inducible than flavonoids. Fungus was a more effective stress factor than light and wounding. The impact of fungus might be enhanced by combining it with other stress factors; the combination of fungus and light was more promising than that of fungus and wounding. The seeds of various legume species appeared to respond differently towards elicitation by *Rhizopus* during germination. The kind of molecules induced followed the phylogenetic relationship of the various species, but their amounts induced during germination, alone or combined with elicitation, did not. In terms of quantities of compounds induced, some species such as *Glycine max*, *Phaseolus* spp., *Lupinus* spp. and *Arachis hypogaea* were more promising than *Vigna* spp., *Lablab purpureus* and *Psophocarpus tetragonolobus*. Moreover, the fact that *Rhizopus* and *Aspergillus* could metabolize the stilbenoids induced during the process of simultaneous germination and elicitation of peanut seedlings showed that the type of fungus was a crucial parameter for optimizing accumulation of potentially bioactive compounds.

Table of contents

Chapter 1

General Introduction

BACKGROUND

The family of Leguminosae comprises most of the common edible seed species. During germination of legume seeds, often compounds are accumulated, which either support or protect the growing seedlings.[1] The accumulation of these molecules can be further induced by exposing the germinating seeds to stress. The stress factors can be of a different nature, such as microbial elicitors, UV irradiation or chemicals. Activation of plant defense responses results in accumulation of anti-microbial compounds, so-called phytoalexins.[2] It is generally accepted that phytoalexins have health-promoting potential. This is not only because they often have anti-oxidant activity, but also because some of them are regarded as hormone look-alikes that offer opportunities for treating hormone-related diseases.[3] Due to the interest in those health-promoting compounds, a method to increase isoflavonoid content and diversity of legume seeds has been previously developed in our laboratory.[4] The method consisted of two steps, malting and challenging of the seedlings by fungus, performed under controlled conditions using a micro-malting machine, often employed in the brewing industry. The method was applied to soybean using *Rhizopus microsporus* as an elicitor. The composition and total content of isoflavonoids in the elicited soybean seedlings were changed drastically upon the treatment. The elicited soybean seedlings accumulated phytoalexins belonging to the isoflavonoid subclasses of pterocarpans and coumestans, most of which were prenylated.[5] These phytoalexins appeared to have promising health-promoting or pharmaceutical properties, the estrogenic potential of which (i.e. the ability to bind to human estrogen receptors to direct transcriptional activity, as observed with the female sex hormone estradiol) was particularly of interest to us.[6] To obtain a larger set of molecules for unravelling the structure-activity relationships of prenylated isoflavonoids with respect to estrogenic potential, the induction method was optimized and extrapolated to various legume species. The research described in this PhD thesis deals with the structural elucidation and quantification of the compounds induced with the protocol of combined malting and elicitation by fungus.

TAXONOMIC RELATIONSHIP OF ECONOMICALLY IMPORTANT LEGUMES

The Leguminosae (legume) represents one of the most exploited plant families worldwide by human.[7] Legumes have been domesticated for food and non-food purposes. Based on their main use, legume seeds can be categorized into five groups: dry-grain legumes, green-vegetable legumes, whole pod legumes, oil-bearing legumes and nitrogen-fixation legumes (**Table 1**). Dry-grain legumes refer to legumes of which the dry seed is consumed. Most of common legumes, such as *Cicer arietinum* (chickpea), *Lens esculenta* (lentils) and *Vicia faba* (broad bean), are in this category. This category differs from green-vegetable legumes, which are consumed while the seeds are still green and not dried, such as *Pisum sativum* (green peas). The whole pod legumes are also harvested when they are very young, but they are consumed as a whole, including the pods, such as *Psophocarpus tetragonolobus* (winged beans). The oil-bearing legumes are mainly used for oil extraction, such as *Glycine max* (soybean) and *Arachis hypogaea* (peanut). *Trifolium* species (clover) and *Medicago sativa* (alfalfa) are used mainly for nitrogen fixation. Nevertheless, the classification is not unambiguous, as in common use, some legumes might be categorized differently. The 2013 production quantities of the legume commodities in the world are shown in **Table 1**. Amongst legume commodities, soybean is the most cultivated one, up to 70% (w/w) of the total production of legume crops (**Table 1**).

The family of Leguminosae is the third most species-rich among flowering plants, comprising approximately 19,325 species and 727 genera.[8] Three subfamilies are distinguished in the Leguminosae: Caesalpinioideae, Mimosoideae and Papilionoideae. These three subfamilies are further divided into 36 tribes.[8] The Papilionoideae is the largest subfamily with 28 tribes, 478 genera and 13,800 species, including economically important commodity legumes. The legumes used in this PhD research were phylogenetically scattered over several different tribes within the Papilionoideae.[7, 9] For instance, *Lupinus* and *Arachis* genera are in the Genisteae and Dalbergieae tribes, respectively. *Phaseolus*, *Vigna*, *Lablab*, *Psophocarpus* and *Glycine* genera are in the Phaseoleae tribe, whereas *Lens*, *Vicia* and *Pisum* genera are in the Fabeae tribe.

Table 1. Production of legume commodities in 2013. Source: Food and Agriculture Organization of the United Nations (FAO) database (http://faostat3.fao.org).

FAO categories	Main use categories	Production ($x10^3$ tonnes)	Species belonging to the FAO categories	
			Scientific names	Common names
Alfalfa	N_2 fixation	n.a[a]	*Medicago sativa*	Alfalfa
Bambara beans	Dry-grain	244	*Vigna subterranea*	Bambara groundnut
Beans	Dry-grain	23139		
			Phaseolus aconitifolius	Moth bean
			Phaseolus acutifolius	Tepary bean
			Phaseolus aureus	Mungo bean
			Phaseolus calcaratus	Rice bean
			Phaseolus coccineus	Scarlet runner bean
			Phaseolus lunatus	Lima bean
			Phaseolus vulgaris	Kidney bean
			Vigna angularis	Adzuki bean
			Vigna mungo	Black gram
			Vigna radiata	Mung bean
Broad beans	Dry-grain	3398		
			Vicia faba var. equina	Horse bean
			Vicia faba var. major	Broad bean
			Vicia faba var. minor	Field bean
Chick peas	Dry-grain	13102	*Cicer arietinum*	Chick pea
Clover	N_2 fixation	n.a[a]	*Trifolium* spp.	Clover
Cow peas	Dry-grain	5718	*Vigna unguiculata*	Cowpea
Groundnut	Oil bearing	45225	*Arachis hypogaea*	Groundnut
Lentils	Dry-grain	4952	*Lens esculenta*	Lentil
Lupins	Dry-grain	786	*Lupinus spp.*	Lupin
Peas	Green-vegetable	10980		
			Pisum arvense	Field pea
			Pisum sativum	Garden pea
Pigeon peas	Dry-grain	4742	*Cajanus cajan*	Pigeon pea
Pulses	Dry-grain	5212		
			Canavalia spp.	Jack bean
			Cyamopsis tetragonoloba	Guar bean
			Pachyrrhizus erosus	Yam bean
			Stizolobium spp.	Velvet bean
			Lablab spp.	Lablab
Soybeans	Oil bearing	276406	*Glycine max*	Soybeans
Vetches	Dry-grain	735	*Vicia sativa*	Spring vetch

[a] n.a.: Data were not available.

PHENOLIC COMPOUNDS IN LEGUME SEEDS

The major phenolic compounds of legume seeds belong to the classes of phenolic acids and (iso)flavonoids.[1, 10] Other classes of phenolics are found less frequently in legumes seeds.

In this thesis, we focus on flavonoids, isoflavonoids and stilbenoids. These classes are derived from the same precursor, a polyketide unit, which is built by elongation of one cinnamoyl coenzyme A (cinnamoyl-CoA) unit (derived from the shikimate pathway) with three molecules of malonyl-CoA (derived from the acetate pathway) (**Figure 1**).

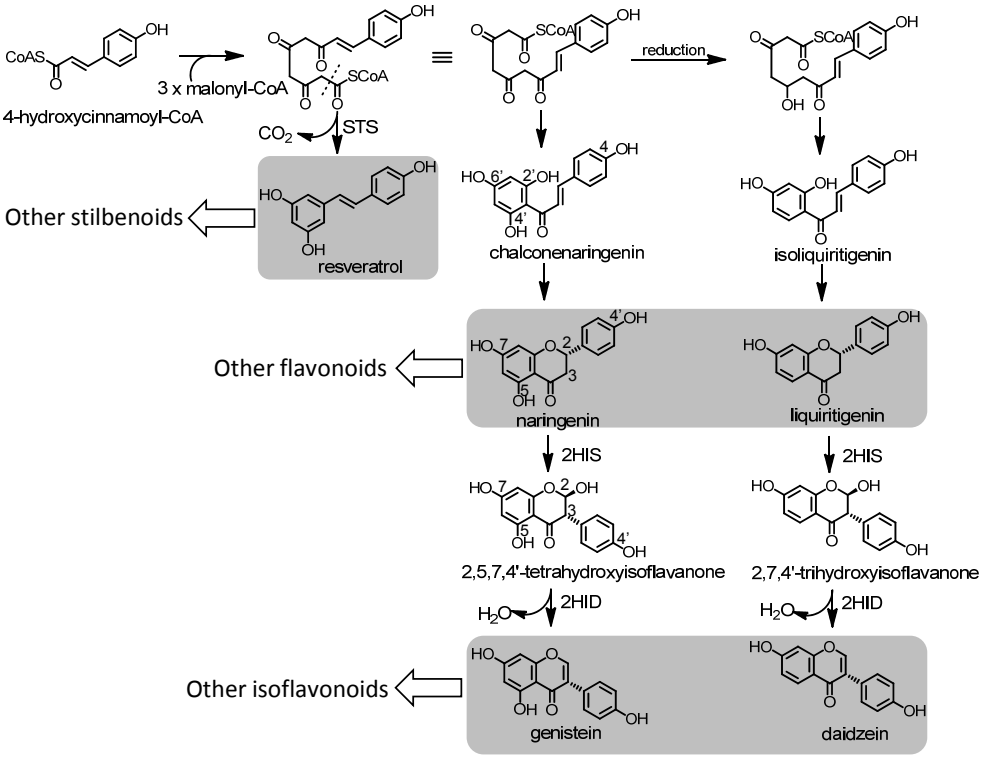


Figure 1. Biosynthetic formation of the related classes: stilbenoid, flavonoid and isoflavonoid, dealt with in this thesis.

For flavonoids, the polyketide precursor undergoes an intramolecular Claisen condensation, resulting in chalconenaringenin (2',4',6',4-tetrahydroxychalcone) or, via an additional reduction step, in isoliquiritigenin (2',4',4-trihydroxychalcone). This reduction step has been associated with reductase acting in concert with chalcone synthase.[11] The two chalcones are transformed into flavanones naringenin (5,7,4-trihydroxyflavanone) and liquiritigenin (7,4'-dihydroxyflavanone), and subsequently into an array of flavonoid derivatives (**Figure 1**).[11, 12] Isoflavonoids contain a rearranged flavonoid skeleton in which the shikimate-derived ring (B-ring) has migrated to the adjacent carbon of the heterocycle

(C-ring).[12] Isoflavones are derived from the flavanones naringenin and liquiritigenin by two consecutive steps. The first step, the so-called aryl rearrangement, converts the flavanones naringenin and liquiritigenin into 2,5,7,4'-tetrahydroxyisoflavanone and 2,7,4'-trihydroxy-isoflavanone, respectively (**Figure 1**).[6, 13, 14] The first step, performed by isoflavanone synthase (2HIS), involves the *C*-3 hydrogen abstraction of the benzopyran moiety, followed by migration of the aromatic B-ring from the *C*-2 to the *C*-3 with a concomitant *C*-2 hydroxylation.[6, 13, 14] The second step consists of a dehydration reaction, performed by isoflavanone dehydratase (2HID), converting the isoflavanones into the isoflavones genistein or daidzein (**Figure 1**).[6, 13] These two isoflavones are considered as the key elements in the structural diversification of isoflavonoids. Stilbenoids result from an aldol condensation in the polyketide precursor (**Figure 1**). Different from (iso)flavonoids, the skeleton of a stilbenoid is shortened by one carbon unit through decarboxylation to form resveratrol (**Figure 1**).[12] The formation of stilbenes is known to use one single enzyme, stilbene synthase (STS).[15]

Flavonoids

A wide variety of flavonoids (2-phenyl benzopyrans) is found in legumes.[1] Five subclasses are made from the basic flavanone skeleton: flavone, flavonol, flavanol, flavanonol and anthocyanidin (**Figure 2**).[11, 12] Modifications, such as hydroxylation, alk(en)ylation (including methylation and prenylation) and glycosylation, increase the range of compounds.[16]

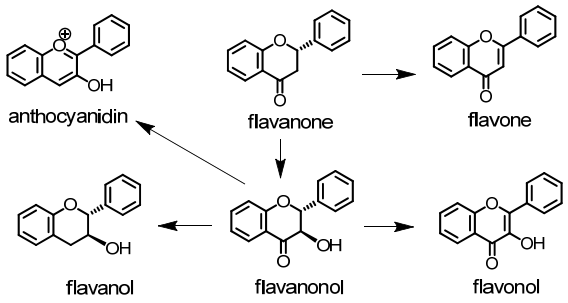


Figure 2. The biosynthetic relationship among several representative flavonoid subclasses.

Flavonoids found in the seeds or seed coats of legumes occur mainly in glycosylated form. *Phaseolus vulgaris* seeds, for instance, contains mainly flavonol glycosides, i.e. kaempferol and quercetin *O*-glycosides,[17] whereas *Vigna radiata* contains mainly flavone glycosides, i.e. apigenin *C*-/*O*-glycosides. The total content of flavonoids in legume seeds varies by species, but often it is in the range of 0 to 0.3 mg/g dry weight[18] (recalculated from fresh

weight using a water content of 65-70% (w/w))[19]. Nevertheless, some legume seeds, such as *Phaseolus vulgaris* and *Vigna radiata*, have been reported to contain larger amounts of flavonoids, i.e. 0.6 to 2.5 and 0.6 mg/g dry weight, respectively.[17, 20]

Isoflavonoids

The class of isoflavonoids (3-phenyl benzopyrans) is characterized by large structural variation due to different degrees of oxidation and the presence of extra heterocyclic rings, i.e. the subclasses of pterocarpans, coumestans, coumaronochromones and rotenoids are characterized by an additional D-ring (**Figure 3**).[8, 21, 22] These latter variations are not encountered in the class of flavonoids. The loss of the 5-hydroxyl group on the isoflavonoid skeleton, hydroxylation, methylation, methylenedioxy-bridge formation, prenylation, and glycosylation enlarge the structural variation.[23]

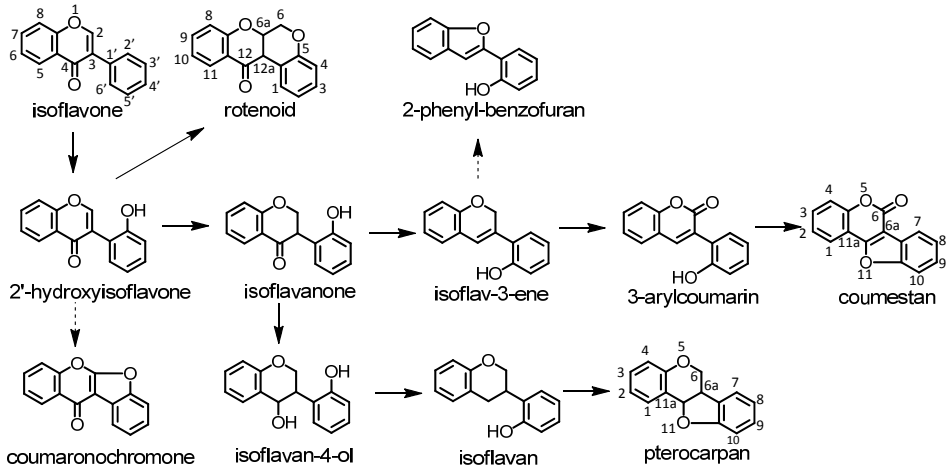


Figure 3. The biosynthetic relationship among several representative isoflavonoid subclasses. Subclasses without numbering system have the same system as in isoflavone.

Different from flavonoids, the majority of isoflavonoids has been found in the family of Leguminosae.[8] Nonetheless, at least fifty-nine non-leguminous families have been reported to contain isoflavonoids.[24] The occurrence of isoflavonoids in legume seeds is mainly associated with the plant's defense mechanism. The isoflavone content of legume seeds can be affected by variety (cultivar) and environmental factors.[25] Soybeans, for instance, are known as one of the richest sources of isoflavones. The major isoflavones found in soybean are conjugated forms (glucosides, acetylglucosides, or malonylglucosides) of daidzein and genistein. The isoflavone content is highly variable and

ranges usually between 0.2 and 2.0 mg/g dry weight, with occasional extremes up to 9.5 mg/g dry weight.[26]

Stilbenoids

Stilbenoids do not have a wide distribution in the plant kingdom. Phytochemical investigation has hitherto focused mainly on a few families, such as Dipterocarpaceae, Vitaceae, Cyperaceae, Gnetaceae and Leguminosae.[27, 28] Peanut is the prominent example of legumes that contains stilbenoids. Stilbenoids can be divided into two categories: monomeric and oligomeric stilbenoids, the distribution of which varies depending on the plant family. Monomeric stilbenoids have never been isolated from any of the Dipterocarpaceae species, whereas stilbenoids found in Leguminoseae are predominantly in monomeric form.[27] **Figure 4** shows the structures of the monomeric stilbenoids, i.e. resveratrol, isorhapotigenin, piceatannol, oxyresveratrol. These four monomeric units can be combined into a variety of homo- and hetero-oligomers.[28] The two monomeric units might be assembled in different ways, with up to four *C-C* and/or *C-O-C* cross-links between them (**Figure 5**).[28] This, combined with the various possible units, produces structures with complex configurations and different degrees of oligomerization, creating a huge number of oligomeric structures.[28]

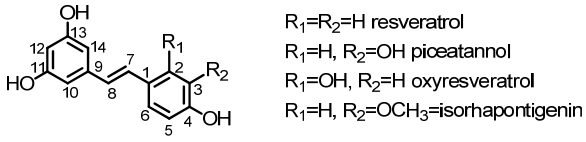


Figure 4. Monomeric stilbenoids involved in formation of oligomeric stilbenoids.

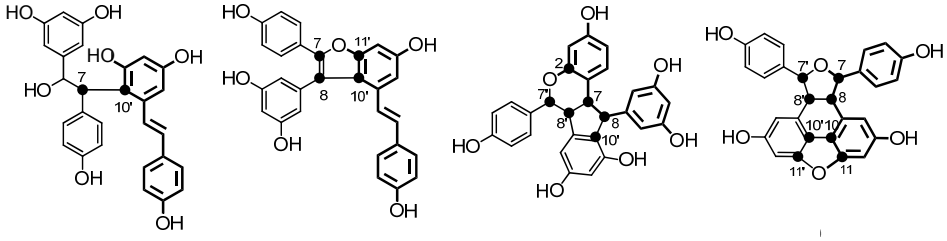


Figure 5. Oligomeric stilbenoids assembled with different types of crosslinking.

Stilbenoids have attracted interest for their potential in therapeutic or preventive applications in human health.[28] Similar to isoflavonoids, the occurrence of stilbenoids in

legume seeds is inducible. The resveratrol content in several peanut cultivars ranges between 0.02-1.79 μg/g dry weight of peanut.[29]

DERIVATIZATION OF PHENOLIC COMPOUNDS IN LEGUME SEEDS

Besides the more common substitutions, such as hydroxyl and *O*-methyl groups, phenolic compounds can also be derivatized with larger substituents, such as prenyl groups and glycosyl residues.

Prenylation of phenolic compounds

Prenylation refers to the substitution of a molecule with a five-carbon isoprenoid (prenyl; 3-methyl-but-2-en-1-yl) group. In a more broad sense, prenylation also refers to the substitution with other moieties originating from the isoprenoid pathway, including C_{10}-isoprenoid (geranyl; 3,7-dimethyl-2,6-octadien-1-yl) or C_{15}-isoprenoid (farnesyl; 3,7,11-trimethyldodeca-2,6,10-trien-1-yl). The prenyl groups are mainly attached to the *C*-atoms of (iso)flavonoids, although *O*-prenylation also occurs in few cases.[30, 31] With isoflavonoids, *C*-prenylation occurs most frequently on ring A at positions *C*-6/*C*-8 and, sometimes, on the B-ring at positions *C*-2'/*C*-3' (**Figure 3**). Although this carbon numbering accounts for most isoflavonoids, it should be noted that, according to IUPAC, the carbon numbering of isoflavones differs from that of, for instance, pterocarpans and coumestans (**Figure 3**). This might erroneously hint at different substitution patterns of isoflavonoids, whereas they are actually the same. In pterocarpans and coumestans, the A-ring *C*-6/*C*-8 and B-ring *C*-2'/*C*-5' positions of isoflavones are numbered *C*-2/*C*-4 and *C*-7/*C*-10, respectively. The most frequent type of prenylation is represented by the 3,3-dimethylallyl chain. Other forms of chains are known as well (**Figure 6**).[31] The chain prenyl substituent can be modified by oxidation or reduction, and subsequently by dehydration or cyclization. The latter modification leads to either five (furan) or six-membered (pyran) rings. Interestingly, some of the modifications were suggested to be performed by fungi.[32]

Prenylation is catalyzed by prenyltransferases. These are membrane proteins that are located in plastids.[33] Only few (iso)flavonoid prenyltransferases from Leguminosae have been characterized. These include (-)glycinol 4-methylallyltransferase (G4DT) from *Glycine max*, naringenin 8-dimethylallyltransferase (SfN8DT) from *Sophora flavescens*, genistein prenyltransferase (*SfG6DT*) from *S. flavescens*, isoliquiritigenin prenyltransferase (*SfiLDT*) from *S. flavescens* and isoflavone prenyltransferase (LaPT1) from *Lupinus albus*.[33-36] Some of these prenyltransferases have been reported to be substrate- and/or regio-specific.[34] LaPT1 seems to have a preference for the isoflavones genistein and 2'-hydroxygenistein as an acceptor substrate, whereas isoflavones daidzein, formononetin, biochanin A and 7-hydroxyisoflavone are less good acceptor substrates.[33] G4DT

exclusively prenylates the *C*-4 position of glycinol, a 6a-hydroxypterocarpan, and not the *C*-2 position, another common position for prenylation of 6a-hydroxypterocarpans from soybean seedlings.[34]

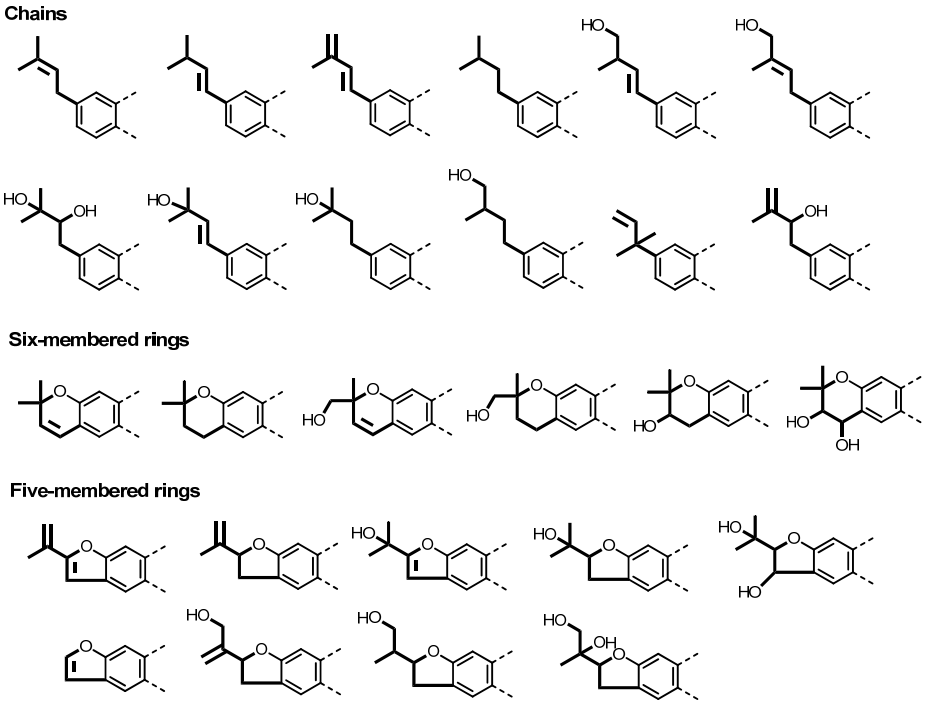


Figure 6. C5-isoprenoid forms of phenolics naturally occurring in plants.

Glycosylation of phenolic compounds

Glycosylation is a key decoration in natural products from plants, resulting in chemical complexity and diversity of compounds. It influences their chemical properties and bioactivities.[37] Glycosylation enhances water solubility. It provides stability through the protection of reactive nucleophilic groups of the natural products and facilitates their storage and accumulation in plant cells.[38, 39] Glycosylation is also known as one of the major factors determining natural product's bioactivity and bioavailability.[40] In plants, glycosylation involves uridine diphosphate glycosyl transferases (UGTs), a member of family 1 glycosyl transferases (GTs).[37] The majority of the characterized UGTs utilize UDP-glucose as the favored donor substrate. Other UDP-sugars, such as UDP-galactose,

UDP-glucuronic acid, UDP-xylose and UDP-rhamnose, are also used by plant UGTs, albeit less commonly.[37] Glycosylation might occur on several types of atoms, including O-, C-, N-, and S-atoms. Nevertheless, it remains to be established whether the same GT1 glycosyl transferases are involved. In this thesis, only *O*- and *C*-glycosylation are relevant. Different from *O*-glycosylation, in *C*-glycosylation the anomeric carbon of the glycosyl moiety is directly attached to the phenolic skeleton. This results in resistance to hydrolysis by glycosidases.[41]

IDENTIFICATION OF (ISO)FLAVONOIDS AND STILBENOIDS BY UHPLC-MS

Reversed-phase ultra-high-performance liquid chromatography (RP-U(H)PLC) coupled to diode array detection (DAD) and/or mass spectrometry (MS) or tandem MS offers good selectivity and sensitivity to analyze constituents of a complex extract without the need for extensive sample preparation.[42, 43] DAD is an essential tool for the identification and quantification of phenolic compounds. This technique records chromatograms at different wavelengths simultaneously, resulting in UV-visible spectral data that can be compared to library/references for either full identification or a compound's subclass determination.[44, 45]

Mass spectrometry is one of the most sensitive methods of molecular analysis. The mass spectrum is produced by plotting the mass-to-charge (*m/z*) ratio of ions to their (relative) abundance.[46] "Soft" ionization techniques, such as electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI), have been frequently used to generate ions from small plant metabolites, such as (iso)flavonoids. Soft ionization involves either the protonation ($[M+H]^+$, positive ion (PI) mode) or deprotonation ($[M-H]^-$, negative ion (NI) mode) of the analyte.[47, 48] However, information about the *m/z* of the molecular ion is insufficient to determine the structure. Therefore, collision-induced dissociation (CID) is often employed to induce fragmentation of the charged analyte. In this way, structural information can be retrieved upon analysis of the fragments by e.g. hybrid quadrupole time of flight (Q-TOF) or ion trap (IT).[44] It is noteworthy that in certain cases, the mass spectrometers mentioned generated different fragmentation patterns. Thus, the data created by different methods are not necessarily comparable.[44]

Identification of (iso)flavonoids

The UV spectral data of (iso)flavonoids are characterized by two absorbance bands that are commonly referred to as Bands I and II. Band I (300-550 nm) is considered to be associated with the absorption of the B-ring cinnamoyl system, whereas Band II (240-280 nm) involves the A-ring benzoyl system (**Figure 7**).[49, 50] The intensity and maximum absorbance of the bands are influenced mainly by the size of the conjugated system, and, to

some extent, by the oxygenation pattern of the moieties (**Table 2**).[49, 51] Hence, the UV spectral data can be useful information for subclass determination (**Table 2**).

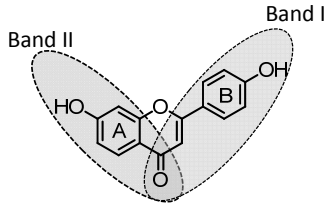
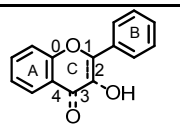


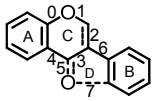
Figure 7. The two absorbance bands, referred to as Band I (associated with absorption of the B-ring cinnamoyl system) and Band II (associated with absorption of the A-ring benzoyl system).

Table 2. Representative spectral data of the main (iso)flavonoid subclasses.

UV



Flavonoid



Isoflavonoid

Band I	Band II	*Retro*-Diels Alder (RDA) fragments[b]	Subclass
Flavonoid			
300-380	240-280	$^{1,3}A/B^+$, $^{0,2}B^+$, $^{0,4}B^+$	Flavone
300-380	240-280	$^{1,3}A^+$, $[^{1,3}B\text{-}2H]^+$, $^{0,2}A/B^+$, $[^{1,4}B+2H]^+$	Flavonol
300-360[a]	270-295	$^{1,3}A^+$, $[^{1,3}B\text{-}2H]^+$, $[^{0,4}B\text{-}H_2O]^+$	Flavanone
	269-279	$[^{1,2}A\text{-}H_2O]^+$, $^{1,2}B^+$, $^{1,3}A^+$, $[^{0,4}B\text{-}H_2O]^+$	Flavan-3-ol
475-545	267-275	$^{0,3}A^+$, $^{0,2}A/B^+$	Anthocyanidin
Isoflavonoid			
300-340[a]	245-270	$^{1,3}A/B^+$, $^{1,4}A/B^+$	Isoflavone
300-360[a]	270-295	$^{1,3}A/B^+$, $^{0,4}B^+$, $^{2,3}B^+$	Isoflavanone
	270-285	$^{1,3}A/B^+$, $^{1,4}A/B^+$, $^{2,3}A/B^+$	Isoflavan
	280-310	$^{2,4}A/B^+$, $^{1,4}A/B^+$, $^{5,6}A/B^+$	Pterocarpan
340-350	260-268[a]	-	Coumestan
330-347	260-268, 280-289	-	Coumaronochromone

[a] Shoulder or inflection.
[b] The numbers of the RDA fragmentation refer to the bond numbers as indicated.

The fragmentation pattern of many (iso)flavonoids has been systematically studied. Generally, fragment ions resulting from the cleavage of the C-ring (often referred to as *retro*-Diels Alder (RDA) reaction) and the neutral/radical losses occurring are used to delineate the compound's structure. Cleavage of the C-ring results in A-ring and B-ring

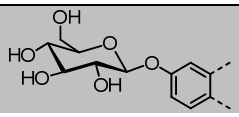
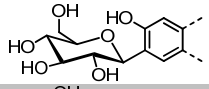
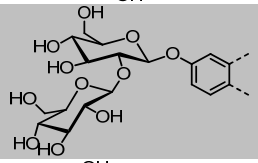
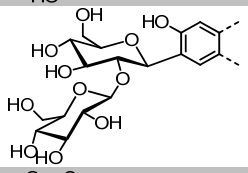
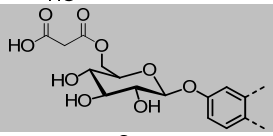
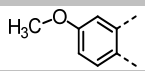
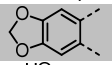
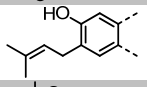
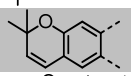
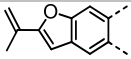
fragment ions that provide information on the number and type of substituents of these rings.[42] The nomenclature used for fragmentation of the C-ring is based on the nomenclature of carbohydrate fragmentation.[52] A-ring and B-ring fragments are indicated by either $^{i,j}A^{+}$ and $^{i,j}B^{+}$ or by $^{i,j}A^{-}$ and $^{i,j}B^{-}$ for PI or NI mode fragmentation, respectively.[52] The superscripts i,j represent the bonds that are cleaved in the C-ring, as indicated in **Table 2**. In the structural analysis of (iso)flavonoids, PI mode spectra are used more often than NI mode spectra. The latter are considered to be more difficult to interpret than the former.[42] The most common C-ring cleavage of (iso)flavonoids in PI mode is at the 1/3 bonds (**Table 2**). Nevertheless, the fragmentation of (iso)flavonoids appears to follow specific degradation pathways, characteristic for each subclass, which is attractive to use for identification purposes. Flavones and flavonols showed the fragmentation of the 0/2 bonds that lack in the fragmentation of flavanones.[53] Isoflavans and isoflavanones showed the characteristic fragmentation of the C-ring through the 2/3 bonds in PI mode.[54] The neutral/radical losses are frequently related to the characteristic cleavage of substituents. Thus, some studies (**Table 3**) have suggested the diagnostic neutral/radical losses to determine groups attached to the (iso)flavonoid skeleton, e.g. glycosyl, malonyl, prenyl, methoxyl and methylenedioxyl groups.

LC-MS has been used for identification and quantification of (iso)flavonoids in crude extracts of various plant parts of legume species.[55, 56] This technique was also applied to screen the change in content and composition of isoflavonoids during the induction process.[5, 57] Nevertheless, little information on fragmentation behavior of (iso)flavonoids, prenylated forms in particular, is available. Recently, the diagnostic neutral losses have been suggested to distinguish different type of prenyl substituents.[4, 58] However, the position of the prenyl substituent cannot be determined.

Identification of stilbenoids

Stilbenoids usually show UV absorption maxima at 280-336 nm.[59] As with many phenolics, the UV spectral data of stilbenoids can be shifted by its substituents attached. Furthermore, different isomers can occur as a result of the different configuration around the double bond connecting the two rings (*cis*- or *trans*-isomers). These isomers can be distinguished by their retention behavior with reversed-phase liquid chromatography and UV absorption maxima. Generally, *trans*-stilbenoids are more polar and show a UV absorption maximum at higher wavelength than *cis*-stilbenoids.[60] The fragmentation patterns of stilbenoids, other than those of resveratrol and piceatannol, have not been described yet in any detail, particularly when they are substituted with prenyl or other substituents, like arachidins.[61, 62]

Table 3. Neutral/radical losses in PI mode of some representative substituents attached to (iso)flavonoids.

Substituent	Position	Neutral losses	Ref
O-Hexosyl-		162	[56]
C-Hexosyl-		90, 120	[56]
O,*O*-Dihexosyl (1→2″)		162	[63, 64]
O,*C*-Dihexosyl (1→2″)		180, 162	[63, 64]
Malonyl-		86	[56, 65]
Methoxyl-		15, 32	[66]
Methylenedioxyl-		58	[67]
Prenyl chain		56	[55, 58]
2,2-Dimethylpyran ring		42, 70, 54, 15	[55, 58]
2-Isopropenyl-dihydrofuran ring		42, 70, 54, 15	[55, 58]

MODIFYING THE COMPOSITION OF PHENOLICS OF VARIOUS LEGUME SEEDLINGS

The opportunities to modulate the content and composition of phenolic compounds are the principle idea behind the research described in this thesis.[68] Germination has been reported to increase the level of phenolics in legume seeds.[19, 69-71] The extent of enrichment in

phenolics varies, depending on the type of legume seed, growth conditions and length of germination period.[70-73] The content and composition of phenolics can be modulated further by combining germination with stimulation of the defense response of the legume.[5] The exposure of seedlings to stress, which comprises both mechanical barriers and toxic chemicals, can result in the mobilization of the plant's defense mechanisms. The latter mechanism includes a complex system of inducible defense molecules aimed at stopping herbivores and pathogens, so-called phytoalexins.[74] Phytoalexins are defined as low molecular mass (usually below 1000 Da) secondary metabolites, formed in plants via a metabolic sequence induced either biotically or in response to chemical or environmental factors.[2] Some plants do not produce phytoalexins when challenged by pathogens, but release chemicals that are normally stored as less toxic glycosides (so-called phytoanticipins).[2, 75, 76] Activation of these compounds involves hydrolysis by glycosidases, the activity of which can be induced by elicitation.[2, 77, 78]

Stimulation of defense response in various legume species

The compounds induced are often structurally unique and have chemotaxonomic potential. Hence, they have been used to describe the relationships among geni or speci.[79-82] Typical phytoalexins of Leguminosae are isoflavonoids with characteristic species-specific modifications in both their skeletons and their decorations (**Table 4**).[34] The phytoalexin isoflavonoid skeletons include isoflavone, isoflavanone, pterocarpan, isoflavan, coumestan and coumaranochromone subclasses. Some species of Leguminosae produced non-isoflavonoid types of phytoalexins, e.g. stilbenoid, as in the case of *Arachis hypogaea*.[2, 81, 83] In terms of decoration, prenyl substituents have been frequently associated with the plant's defense mechanism against microorganisms.[34] The attachment of prenyl substituents increases the lipophilicity of the compounds, thereby enhancing their capacity to penetrate biological membranes, and hence, to increase their toxicity towards microorganisms.[84]

Table 4. Subclasses of phenolic compounds induced in some species of Leguminosae.

Species	Plants tissue	Induced phenolic compounds	Ref
Arachis hypogaea	Sliced seeds	Stilbenoids	[85]
Lupinus albus	Roots	Isoflavones, coumaronochromones	[86, 87]
Glycine max	Leaves	6a-Hydroxypterocarpans, coumestans	[88]
Phaseolus vulgaris	Cotyledons	Isoflavanones, isoflavans	[89]
Psophocarpus tetragonolobus	Stems	Pterocarpans	[90]
Trigonella spp.	Leaves	Pterocarpans, isoflavans	[79]
Apios tuberosa	Leaves	Isoflavones, pterocarpans	[91]
Medicago spp.	Leaves	Pterocarpans, isoflavans	[92]

The compounds induced are known to be plant tissue-dependent.[93] The use of detached leaflets in phytoalexin studies has been more popular than that of other plant tissues, such

as seedlings.[79-81, 84, 92, 94-96] The phytoalexins of various species representing 37 genera of the Phaseoleae tribe, for instance, have been investigated using detached leaflets.[81] Studies with seedlings might yield different results than those with leaves.[97] In addition, the age of the seed/seedlings at the moment of application of the elicitor has been suggested to affect the content and composition of phenolics.[98]

Elicitors to induce phenolics in legume seeds

The content and composition of phytoalexins are not only affected by the legume species and plant tissue (as described above), but also by other factors, including type of elicitor and induction method. The elicitors can be of different nature, i.e. biotic elicitors and abiotic elicitors. Biotic elicitation can be due to bacteria, fungi and yeasts, as well as oligosaccharides, lipid and protein derived from microbial and plant cell walls.[99-102] Biotic elicitation has been shown to induce different responses, including increasing the content of phenolics and diversifying the composition of phenolics.[103] The wounded cotyledons of *Phaseolus vulgaris* responded differently to elicitation with different rhizobacteria and pathogenic fungi. Nine out of fifteen rhizobacteria induced the production of three phytoalexins, i.e., kievitone, phaseollin and phaseollinisoflavan, in *P. vulgaris*, whereas the phytoalexins were not detected when the other rhizobacteria were used.[97] Similarly, different biotic factors elicited production of stilbenoids in peanut, but the content and composition of these phytoalexins varied considerably. *Aspergilli* were more potent elicitors of stilbenoids than *Cladosporium* spp., *Saccharomyces cerevisiae*, *Bacillus subtilis*, and *Rhizobium leguminosarum*.[104] Additionally, microorganisms seem capable of metabolizing or detoxifying phytoalexins, thereby altering the profile of compounds induced.[105]

Abiotic elicitation can be induced by wounding, chemicals, light and UV irradiation, leading to similar phytoalexins as with biotic elicitation.[99-102] Wounding by slicing or exposure to hydrogen peroxide, for instance, induced the production of stilbenoids in peanuts. Under similar incubation conditions, the stilbenoid content in wounded peanuts was significantly lower than that in peanuts elicited with hydrogen peroxide.[104] The presence of light during germination has also been suggested to increase the phenolic content of legume seeds.[106, 107] The effect of light on the content of phenolic compounds in legume species depended on the light parameters employed, including intensity, quality (wavelength), direction and duration of the exposure.[106, 108, 109] In combination, the biotic and abiotic elicitor treatments can either antagonize or harmonize with each other.[103] Taken together, the modulation of the content and composition of phenolics in legume seeds can be influenced by several factors, i.e. legume species, plant tissue, nature of the elicitor, elicitor dose, and time of application of the elicitor. As these factors (and combinations thereof) have not been systematically investigated, it is fair to assume that

there is room for optimization of the induction process. Nevertheless, such studies are laborious.

AIM AND OUTLINE OF THIS THESIS

The demand for food products or supplements with health-promoting activities is increasing. As legume species have the potential to produce an array of secondary metabolites that offer such activities, it seems attractive to employ these species for this purpose. Nevertheless, the production of bioactive compounds in legumes is often inadequate, both from a qualitative and a quantitative point of view. By exposing the legume seeds to stress during germination, the production of bioactive compounds might be enhanced. This induction process has already been successfully implemented for soybean, although there might be room for enhancing the content of isoflavonoids even further by combining biotic and abiotic stress factors. Furthermore, we are interested in increasing our collection of prenylated isoflavonoids to perform future structure-activity relationship (SAR) with respect to estrogenic potential. In this thesis we aim to (i) extrapolate the induction process established for soybean to various other legume species, with respect to enhancing the content and molecular diversity of prenylated compounds, (ii) investigate whether a change in biotic and abiotic stress factors, in particular light, wounding, time point of application of biotic stress, different kinds of fungus, can enhance the efficiency of the induction process. In this respect, efficiency relates to both quantity of, and variety (skeleton and decorations) in, phytoalexins produced.

In **Chapter 2**, the effects of wounding and light on (iso)flavonoid content and composition of *Rhizopus*-elicited soybeans is described. The discussion is extended to the impact of light in mediating the prenylation of pterocarpans. In **Chapter 3**, the changes in (iso)flavonoid content and composition in three edible lupine species during *Rhizopus*-elicitation is described. Also a tool to characterize the position of prenyl group of prenylated isoflavones is described. In addition, the estrogenic activities of extracts from elicited lupine, as well as those of fractions enriched in prenylated isoflavones, were determined towards human estrogen receptors. The application of *Rhizopus* to induce prenylated (iso)flavonoids was extended to other species of Leguminosae. The amount and type of phytoalexins induced in those species are described in **Chapter 4**. In **Chapter 5**, the effect of two food-grade fungi, *Rhizopus oryzae* and *Aspergillus oryzae*, applied at two different time points, on the stilbenoid composition of peanut seedlings was investigated. In **Chapter 6**, the content and composition of the various phytoalexins, induced in all species studied in this thesis, are correlated with the phylogenetic relationships between these species. In addition, key factors that can improve the production of phytoalexins as found in this PhD research are highlighted.

REFERENCES

[1] P. A. Ndakidemi, F. D. Dakora, Legume seed flavonoids and nitrogenous metabolites as signals and protectants in early seedling development, *Functional Plant Biology* **2003**, *30*, 729-745.

[2] R. J. Grayer, T. Kokubun, Plant-fungal interactions: The search for phytoalexins and other antifungal compounds from higher plants, *Phytochemistry* **2001**, *56*, 253-263.

[3] S. M. Boue, T. E. Cleveland, C. Carter-Wientjes, B. Y. Shih, D. Bhatnagar, J. M. McLachlan, M. E. Burow, Phytoalexin-enriched functional foods, *Journal of Agricultural and Food Chemistry* **2009**, *57*, 2614-2622.

[4] R. Simons, J.-P. Vincken, M. C. Bohin, T. F. M. Kuijpers, M. A. Verbruggen, H. Gruppen, Identification of prenylated pterocarpans and other isoflavonoids in *Rhizopus* spp. elicited soya bean seedlings by electrospray ionisation mass spectrometry, *Rapid Communications in Mass Spectrometry* **2011**, *25*, 55-65.

[5] R. Simons, J.-P. Vincken, N. Roidos, T. F. H. Bovee, M. Van Iersel, M. A. Verbruggen, H. Gruppen, Increasing soy isoflavonoid content and diversity by simultaneous malting and challenging by a fungus to modulate estrogenicity, *Journal of Agricultural and Food Chemistry* **2011**, *59*, 6748-6758.

[6] R. Simons, H. Gruppen, T. F. H. Bovee, M. A. Verbruggen, J.-P. Vincken, Prenylated isoflavonoids from plants as selective estrogen receptor modulators (phytoSERMs), *Food and Function* **2012**, *3*, 810-827.

[7] G. The Legume Phylogeny Working, Legume phylogeny and classification in the 21st century: Progress, prospects and lessons for other species-rich clades, *Taxon* **2013**, *62*, 217-248.

[8] N. C. Veitch, Isoflavonoids of the Leguminosae, *Natural Product Reports* **2007**, *24*, 417-464.

[9] J. J. Doyle, Phylogenetic perspectives on nodulation: Evolving views of plants and symbiotic bacteria, *Trends in Plant Science* **1998**, *3*, 473-478.

[10] W. D. Loomis, J. Battaile, Plant phenolic compounds and the isolation of plant enzymes, *Phytochemistry* **1966**, *5*, 423-438.

[11] P. M. Dewick, The biosynthesis of shikimate metabolites, *Natural Product Reports* **1990**, *7*, 165-189.

[12] P. M. Dewick, The biosynthesis of shikimate metabolites, *Natural Product Reports* **1992**, *9*, 153-182.

[13] M. F. Hashim, T. Hakamatsuka, Y. Ebizuka, U. Sankawa, Reaction mechamism of oxidative rearrangement of flavanone in isoflavone biosynthesis, *FEBS Letters* **1990**, *271*, 219-222.

[14] Y. Sawada, K. Kinoshita, T. Akashi, T. Aoki, S.-I. Ayabe, Key amino acid residues required for aryl migration catalysed by the cytochrome P450 2-hydroxyisoflavanone synthase, *The Plant Journal* **2002**, *31*, 555-564.

[15] J. Chong, A. Poutaraud, P. Hugueney, Metabolism and roles of stilbenes in plants, *Plant Science* **2009**, *177*, 143-155.

[16] N. C. Veitch, R. J. Grayer, Flavonoids and their glycosides, including anthocyanins, *Natural Product Reports* **2008**, *25*, 555-611.

[17] G. Dinelli, A. Bonetti, M. Minelli, I. Marotti, P. Catizone, A. Mazzanti, Content of flavonols in Italian bean (*Phaseolus vulgaris* L.) ecotypes, *Food Chemistry* **2006**, *99*, 105-114.

[18] S. Prati, V. Baravelli, D. Fabbri, C. Schwarzinger, V. Brandolini, A. Maletti, P. Tedeschi, S. Benvenuti, M. Macchia, I. Marotti, A. Bonetti, P. Catizone, G. Dinelli, Composition and content of seed flavonoids in forage and grain legume crops, *Journal of Separation Science* **2007**, *30*, 491-501.

[19] H. Shi, P. K. Nam, Y. Ma, Comprehensive profiling of isoflavones, phytosterols, tocopherols, minerals, crude protein, lipid, and sugar during soybean (*Glycine max*) germination, *Journal of Agricultural and Food Chemistry* **2010**, *58*, 4970-4976.

[20] D. Tang, Y. Dong, N. Guo, L. Li, H. Ren, Metabolomic analysis of the polyphenols in germinating mung beans (*Vigna radiata*) seeds and sprouts, *Journal of the Science of Food and Agriculture* **2014**, *94*, 1639-1647.

[21] N. C. Veitch, Isoflavonoids of the Leguminosae, *Natural Product Reports* **2009**, *26*, 776-802.

[22] N. C. Veitch, Isoflavonoids of the Leguminosae, *Natural Product Reports* **2013**, *30*, 988-1027.

[23] R. Hegnauer, R. J. Gpayer-Barkmeijer, Relevance of seed polysaccharides and flavonoids for the classification of the Leguminosae: A chemotaxonomic approach, *Phytochemistry* **1993**, *34*, 3-16.

[24] O. Lapčík, Isoflavonoids in non-leguminous taxa: A rarity or a rule?, *Phytochemistry* **2007**, *68*, 2909-2916.

[25] M. J. Morrison, E. R. Cober, M. F. Saleem, N. B. McLaughlin, J. Frégeau-Reid, B. L. Ma, L. Woodrow, Seasonal changes in temperature and precipitation influence isoflavone concentration in short-season soybean, *Field Crops Research* **2010**, *117*, 113-121.

[26] R. Simons, Prenylated isoflavonoids from soya and licorice : Analysis, induction and *in vitro* estrogenicity, PhD thesis, Wageningen University (Wageningen, The Netherlands), **2011**.

[27] S. Sotheeswaran, V. Pasupathy, Distribution of resveratrol oligomers in plants, *Phytochemistry* **1993**, *32*, 1083-1092.
[28] T. Shen, X.-N. Wang, H.-X. Lou, Natural stilbenes: An overview, *Natural Product Reports* **2009**, *26*, 916-935.
[29] T. H. Sanders, R. W. McMichael Jr, K. W. Hendrix, Occurrence of resveratrol in edible peanuts, *Journal of Agricultural and Food Chemistry* **2000**, *48*, 1243-1246.
[30] S. I. Khalivulla, B. A. K. Reddy, D. Gunasekar, A. Blond, B. Bodo, M. M. Murthy, T. P. Rao, A new di-*O*-prenylated isoflavone from *Tephrosia tinctoria*, *Journal of Asian natural products research* **2008**, *10*, 953-955.
[31] D. Barron, R. K. Ibrahim, Isoprenylated flavonoids: A survey, *Phytochemistry* **1996**, *43*, 921-982.
[32] S. Tahara, R. K. Ibrahim, Prenylated isoflavonoids: An update, *Phytochemistry* **1995**, *38*, 1073-1094.
[33] G. Shen, D. Huhman, Z. Lei, J. Snyder, L. W. Sumner, R. A. Dixon, Characterization of an isoflavonoid-specific prenyltransferase from *Lupinus albus*, *Plant Physiology* **2012**, *159*, 70-80.
[34] T. Akashi, K. Sasaki, T. Aoki, S. I. Ayabe, K. Yazaki, Molecular cloning and characterization of a cDNA for pterocarpan 4-dimethylallyltransferase catalyzing the key prenylation step in the biosynthesis of glyceollin, a soybean phytoalexin, *Plant Physiology* **2009**, *149*, 683-693.
[35] K. Sasaki, K. Mito, K. Ohara, H. Yamamoto, K. Yazaki, Cloning and characterization of naringenin 8-prenyltransferase, a flavonoid-specific prenyltransferase of *Sophora flavescens*, *Plant Physiology* **2008**, *146*, 1075-1084.
[36] K. Sasaki, Y. Tsurumaru, H. Yamamoto, K. Yazaki, Molecular characterization of a membrane-bound prenyltransferase specific for isoflavone from *Sophora flavescens*, *Journal of Biological Chemistry* **2011**, *286*, 24125-24134.
[37] X. Wang, Structure, mechanism and engineering of plant natural product glycosyltransferases, *FEBS Letters* **2009**, *583*, 3303-3309.
[38] P. Jones, T. Vogt, Glycosyltransferases in secondary plant metabolism: Tranquilizers and stimulant controllers, *Planta* **2001**, *213*, 164-174.
[39] C. M. Gachon, M. Langlois-Meurinne, P. Saindrenan, Plant secondary metabolism glycosyltransferases: The emerging functional analysis, *Trends in Plant Science* **2005**, *10*, 542-549.
[40] D. Bowles, J. Isayenkova, E.-K. Lim, B. Poppenberger, Glycosyltransferases: Managers of small molecules, *Current Opinion in Plant Biology* **2005**, *8*, 254-263.
[41] O. Talhi, A. M. S. Silva, Advances in *C*-glycosylflavonoid research, *Current Organic Chemistry* **2012**, *16*, 859-896.
[42] F. Cuyckens, M. Claeys, Mass spectrometry in the structural analysis of flavonoids, *Journal of Mass Spectrometry* **2004**, *39*, 1-15.
[43] A. Wojakowska, D. Muth, D. Narozna, C. Madrzak, M. Stobiecki, P. Kachlicki, Changes of phenolic secondary metabolite profiles in the reaction of narrow leaf lupin (*Lupinus angustifolius*) plants to infections with *Colletotrichum lupini* fungus or treatment with its toxin, *Metabolomics* **2013**, *9*, 575-589.
[44] A. Marston, K. Hostettmann, Separation and quantification of flavonoids, in *Flavonoids: Chemistry, biochemistry and applications* (Eds.: O. M. Andersen, K. R. Markham), CRC Press Boca Raton, FL, USA, **2006**, pp. 1-36.
[45] K. Hostettmann, B. Domon, D. Schaufelberger, M. Hostettmann, On-line high-performance liquid chromatography: Ultraviolet-visible spectroscopy of phenolic compounds in plant extracts using post-column derivatization, *Journal of Chromatography A* **1984**, *283*, 137-147.
[46] W. M. A. Niessen, A. P. Tinke, Liquid chromatography-mass spectrometry: General principles and instrumentation, *Journal of Chromatography A* **1995**, *703*, 37-57.
[47] E. de Rijke, P. Out, W. M. A. Niessen, F. Ariese, C. Gooijer, U. A. T. Brinkman, Analytical separation and detection methods for flavonoids, *Journal of Chromatography A* **2006**, *1112*, 31-63.
[48] E. de Rijke, H. Zappey, F. Ariese, C. Gooijer, U. A. T. Brinkman, Liquid chromatography with atmospheric pressure chemical ionization and electrospray ionization mass spectrometry of flavonoids with triple-quadrupole and ion-trap instruments, *Journal of Chromatography A* **2003**, *984*, 45-58.
[49] V. T. J. Mabry, K. R. Markham, M. B. Thomas, *The systematic identification of flavonoids*, Springer-Verlag, New York, NY, USA, **1971**.
[50] T. Fossen, Ø. M. Andersen, Spectroscopic techniques applied to flavonoids, in *Flavonoids: Chemistry, biochemistry and applications* (Eds.: O. M. Andersen, K. R. Markham), CRC Press, Boca Raton, FL, USA, **2006**, pp. 37-142.
[51] C. A. Williams, J. B. Harborne, Isoflavonoids, in *Methods in plant biochemistry, Vol. 1*, Academic Press San Diego, CA, USA, **1989**, pp. 421-449.

[52] B. Domon, C. Costello, A systematic nomenclature for carbohydrate fragmentations in FAB-MS/MS spectra of glycoconjugates, *Glycoconjugate Journal* **1988**, *5*, 397-409.
[53] B. Abad-García, L. A. Berrueta, S. Garmón-Lobato, B. Gallo, F. Vicente, A general analytical strategy for the characterization of phenolic compounds in fruit juices by high-performance liquid chromatography with diode array detection coupled to electrospray ionization and triple quadrupole mass spectrometry, *Journal of Chromatography A* **2009**, *1216*, 5398-5415.
[54] R. Maul, N. Schebb, S. Kulling, Application of LC and GC hyphenated with mass spectrometry as tool for characterization of unknown derivatives of isoflavonoids, *Analytical and Bioanalytical Chemistry* **2008**, *391*, 239-250.
[55] R. Simons, J.-P. Vincken, E. J. Bakx, M. A. Verbruggen, H. Gruppen, A rapid screening method for prenylated flavonoids with ultra-high-performance liquid chromatography/electrospray ionisation mass spectrometry in licorice root extracts, *Rapid Communications in Mass Spectrometry* **2009**, *23*, 3083-3093.
[56] A. Wojakowska, A. Piasecka, P. M. García-López, F. Zamora-Natera, P. Krajewski, L. Marczak, P. Kachlicki, M. Stobiecki, Structural analysis and profiling of phenolic secondary metabolites of Mexican lupine species using LC-MS techniques, *Phytochemistry* **2013**, *92*, 71-86.
[57] D. Durango, N. Pulgarin, J. Gil, G. Escobar, F. Echeverri, W. Quiñones, Differential accumulation of defense-related isoflavonoids in hypocotyls/roots of common bean (*Phaseolus vulgaris* L.) cultivars treated with salicylic acid and structurally related compounds, *Boletín Latinoamericano y del Caribe de Plantas Medicinales y Aromáticas* **2014**, *13*, 381-405.
[58] M.-J. Xu, B. Wu, T. Ding, J.-H. Chu, C.-Y. Li, J. Zhang, T. Wu, J. Wu, S.-J. Liu, S.-L. Liu, W.-Z. Ju, P. Li, Simultaneous characterization of prenylated flavonoids and isoflavonoids in *Psoralea corylifolia* L. by liquid chromatography with diode-array detection and quadrupole time-of-flight mass spectrometry, *Rapid Communications in Mass Spectrometry* **2012**, *26*, 2343-2358.
[59] J. Gorham, Stilbenes and phenanthrenes, in *Methods in plant biochemistry, Vol. 1*, Academic Press, San Diego, CA, USA, **1989**, pp. 159-196.
[60] S. Gocan, Analysis of stilbenes in wine by HPLC: Recent approaches, *Journal of Liquid Chromatography & Related Technologies* **2009**, *32*, 1598-1643.
[61] L. Stella, M. De Rosso, A. Panighel, A. D. Vedova, R. Flamini, P. Traldi, Collisionally induced fragmentation of $[M-H]^-$ species of resveratrol and piceatannol investigated by deuterium labelling and accurate mass measurements, *Rapid Communications in Mass Spectrometry* **2008**, *22*, 3867-3872.
[62] L. Vlase, B. Kiss, S. E. Leucuta, S. Gocan, A rapid method for determination of resveratrol in wines by HPLC-MS, *Journal of Liquid Chromatography & Related Technologies* **2009**, *32*, 2105-2121.
[63] F. Ferreres, A. Gil-Izquierdo, P. B. Andrade, P. Valentão, F. A. Tomás-Barberán, Characterization of *C*-glycosyl flavones *O*-glycosylated by liquid chromatography-tandem mass spectrometry, *Journal of Chromatography A* **2007**, *1161*, 214-223.
[64] F. Ferreres, R. Llorach, A. Gil-Izquierdo, Characterization of the interglycosidic linkage in di-, tri-, tetra- and pentaglycosylated flavonoids and differentiation of positional isomers by liquid chromatography/electrospray ionization tandem mass spectrometry, *Journal of Mass Spectrometry* **2004**, *39*, 312-321.
[65] D. Muth, E. Marfisden-Edwards, P. Kachlicki, M. Stobiecki, Differentiation of isomeric malonylated flavonoid glyconjugates in plant extracts with UPLC-ESI/MS/MS, *Phytochemical Analysis* **2008**, *19*, 444-452.
[66] Y. L. Ma, Q. M. Li, H. Van Den Heuvel, M. Claeys, Characterization of flavone and flavonol aglycones by collision-induced dissociation tandem mass spectrometry, *Rapid Communications in Mass Spectrometry* **1997**, *11*, 1357-1364.
[67] L. Z. Lin, X. G. He, M. Lindenmaier, J. Yang, M. Cleary, S. X. Qiu, G. A. Cordell, LC-ESI-MS study of the flavonoid glycoside malonates of red clover (*Trifolium pratense*), *Journal of Agricultural and Food Chemistry* **2000**, *48*, 354-365.
[68] M. M. J. Champ, Non-nutrient bioactive substances of pulses, *British Journal of Nutrition* **2002**, *88*, S307-S319.
[69] L. R. Silva, M. J. Pereira, J. Azevedo, R. F. Gonçalves, P. Valentão, P. G. de Pinho, P. B. Andrade, *Glycine max* (L.) Merr., *Vigna radiata* L. and *Medicago sativa* L. sprouts: A natural source of bioactive compounds, *Food Research International* **2013**, *50*, 167-175.
[70] Y. Aguilera, M. F. Díaz, T. Jiménez, V. Benítez, T. Herrera, C. Cuadrado, M. Martín-Pedrosa, M. A. Martín-Cabrejas, Changes in nonnutritional factors and antioxidant activity during germination of nonconventional legumes, *Journal of Agricultural and Food Chemistry* **2013**, *61*, 8120-8125.

[71] Z. Wu, L. Song, S. Feng, Y. Liu, G. He, Y. Yioe, S. Q. Liu, D. Huang, Germination dramatically increases isoflavonoid content and diversity in chickpea (*Cicer arietinum* L.) Seeds, *Journal of Agricultural and Food Chemistry* **2012**, *60*, 8606-8615.
[72] M. L. López-Amorós, T. Hernández, I. Estrella, Effect of germination on legume phenolic compounds and their antioxidant activity, *Journal of Food Composition and Analysis* **2006**, *19*, 277-283.
[73] D. Guajardo-Flores, M. García-Patiño, D. Serna-Guerrero, J. A. Gutiérrez-Uribe, S. O. Serna-Saldívar, Characterization and quantification of saponins and flavonoids in sprouts, seed coats and cotyledons of germinated black beans, *Food Chemistry* **2012**, *134*, 1312-1319.
[74] J. Ebel, E. G. Cosio, Elicitors of plant defense responses, *International Review of Cytology* **1994**, *148*, 1-36.
[75] A. E. Osbourn, Preformed antimicrobial compounds and plant defense against fungal attack, *The Plant Cell* **1996**, *8*, 1821.
[76] M. Naoumkina, M. A. Farag, L. W. Sumner, Y. Tang, C. J. Liu, R. A. Dixon, Different mechanisms for phytoalexin induction by pathogen and wound signals in *Medicago truncatula*, *Proceedings of the National Academy of Sciences of the United States of America* **2007**, *104*, 17909-17915.
[77] M. Rybus-Zajqc, M. Kozlłowska, Activity of β-D-glucosidase of lupin leaves infected with *Pleiochaeta setosa* (Kirchn.) Hughes, *Acta Physiologiae Plantarum* **1996**, *18*, 211-216.
[78] M. Píslewska, P. Bednarek, M. Stobiecki, M. Zielínaka, P. Wojtaszek, Cell wall-associated isoflavonoids and β-glucosidase activity in *Lupinus albus* plants responding to environmental stimuli, *Plant, Cell and Environment* **2002**, *25*, 29-40.
[79] J. L. Ingham, Phytoalexin induction and its chemosystematic significance in the genus *Trigonella*, *Biochemical Systematics and Ecology* **1981**, *9*, 275-281.
[80] J. L. Ingham, Phytoalexin production by *Ononis* species, *Biochemical Systematics and Ecology* **1982**, *10*, 233-237.
[81] J. L. Ingham, Systematic aspects of phytoalexin formation within tribe Phaseoleae of the Leguminosae (subfamily Papilionoideae), *Biochemical Systematics and Ecology* **1990**, *18*, 329-343.
[82] J. L. Ingham, J. B. Harborne, Phytoalexin induction as a new dynamic approach to the study of systematic relationships among higher plants, *Nature* **1976**, *260*, 241-243.
[83] J. L. Ingham, 3,5,4′-Trihydroxystilbene as a phytoalexin from groundnuts (*Arachis hypogaea*), *Phytochemistry* **1976**, *15*, 1791-1793.
[84] J. B. Harborne, J. L. Ingham, L. King, M. Payne, The isopentenyl isoflavone luteone as a pre-infectional antifungal agent in the genus *Lupinus*, *Phytochemistry* **1976**, *15*, 1485-1487.
[85] V. S. Sobolev, Localized production of phytoalexins by peanut (*Arachis hypogaea*) kernels in response to invasion by *Aspergillus* species, *Journal of Agricultural and Food Chemistry* **2008**, *56*, 1949-1954.
[86] H. Gagnon, R. K. Ibrahim, Effects of various elicitors on the accumulation and secretion of isoflavonoids in white lupin, *Phytochemistry* **1997**, *44*, 1463-1467.
[87] S. Tahara, J. L. Ingham, J. Mizutani, New coumaronochromones from white Lupin, *Lupinus albus* L. (Leguminosae), *Agricultural and Biological Chemistry* **1985**, *49*, 1775-1783.
[88] J. L. Ingham, N. T. Keen, L. J. Mulheirn, R. L. Lyne, Inducibly-formed isoflavonoids from leaves of soybean, *Phytochemistry* **1981**, *20*, 795-798.
[89] J. A. Bailey, M. Berthier, Phytoalexin accumulation in chloroform-treated cotyledons of *Phaseolus vulgaris*, *Phytochemistry* **1981**, *20*, 187-188.
[90] J. L. Ingham, Phaseollidin, a phytoalexin of *Psophocarpus tetragonolobus*, *Phytochemistry* **1978**, *17*, 165.
[91] J. L. Ingham, L. J. Mulheirn, Isoflavonoid phytoalexins from fungus-inoculated leaves of *Apios tuberosa*, *Phytochemistry* **1982**, *21*, 1409-1413.
[92] J. L. Ingham, Isoflavonoid phytoalexins of the genus *Medicago*, *Biochemical Systematics and Ecology* **1979**, *7*, 29-34.
[93] M.-C. Gutiérrez-Mellado, R. Edwards, M. Tena, F. Cabello, K. Serghini, J. Jorrín, The production of coumarin phytoalexins in different plant organs of sunflower (*Helianthus annuus* L.), *Journal of Plant Physiology* **1996**, *149*, 261-266.
[94] J. L. Ingham, Medicarpin as a phytoalexin of the genus *Melilotus*, *Zeitschrift fur Naturforschung Section C Biosciences* **1977**, *32*, 449-452.
[95] J. L. Ingham, Isoflavonoid phytoalexins from the fungus-inoculated leaflets of *Erythrina* species, *Biochemical Systematics and Ecology* **1991**, *19*, 497-506.
[96] J. L. Ingham, Flavonoid and isoflavonoid compounds from leaves of sainfoin (*Onobrychis viciifolia*), *Z. Naturforsch* **1978**, *33 c*, 146-148.

[97] R. K. Hynes, J. Hill, M. S. Reddy, G. Lazarovits, Phytoalexin production by wounded white bean (*Phaseolus vulgaris*) cotyledons and hypocotyls in response to inoculation with rhizobacteria, *Canadian Journal of Microbiology* **1994**, *40*, 548-554.
[98] P. A. Abbasi, T. L. Graham, Age-related regulation of induced isoflavonoid responses in soybean lines differing in inherent elicitation competency, *Physiological and Molecular Plant Pathology* **2001**, *59*, 143-152.
[99] T. L. Graham, M. Y. Graham, Signaling in soybean phenylpropanoid responses: Dissection of primary, secondary, and conditioning effects of light, wounding, and elicitor treatments, *Plant Physiology* **1996**, *110*, 1123-1133.
[100] J. Ebel, Phytoalexin synthesis: The biochemical analysis of the induction process, *Annual Review of Phytopathology* **1986**, *24*, 235-264.
[101] A. Ramakrishna, G. A. Ravishankar, Influence of abiotic stress signals on secondary metabolites in plants, *Plant Signaling and Behavior* **2011**, *6*, 1720-1731.
[102] J. W. Wang, J. Y. Wu, Effective elicitors and process strategies for enhancement of secondary metabolite production in hairy root cultures, *Advances in Biochemical Engineering/Biotechnology* **2013**, *134*, 55-89.
[103] N. Baenas, C. García-Viguera, D. A. Moreno, Elicitation: A tool for enriching the bioactive composition of foods, *Molecules* **2014**, *19*, 13541-13563.
[104] V. S. Sobolev, Production of phytoalexins in peanut (*Arachis hypogaea*) seed elicited by selected microorganisms, *Journal of Agricultural and Food Chemistry* **2013**, *61*, 1850-1858.
[105] M. S. C. Pedras, P. W. K. Ahiahonu, Metabolism and detoxification of phytoalexins and analogs by phytopathogenic fungi, *Phytochemistry* **2005**, *66*, 391-411.
[106] E. H. Kim, S. H. Kim, J. I. Chung, H. Y. Chi, J. A. Kim, I. M. Chung, Analysis of phenolic compounds and isoflavones in soybean seeds (*Glycine max* (L.) Merill) and sprouts grown under different conditions, *European Food Research and Technology* **2006**, *222*, 201-208.
[107] A. Lal, S. Warber, A. Kirakosyan, P. B. Kaufman, J. A. Duke, Upregulation of isoflavonoids and soluble proteins in edible legumes by light and fungal elicitor treatments, *Journal of Alternative & Complementary Medicine* **2003**, *9*, 371-378.
[108] A. Kirakosyan, P. Kaufman, R. L. Nelson, M. J. Kasperbauer, J. A. Duke, E. Seymour, S. C. Chang, S. Warber, S. Bolling, Isoflavone levels in five soybean (*Glycine max*) genotypes are altered by phytochrome-mediated light treatments, *Journal of Agricultural and Food Chemistry* **2006**, *54*, 54-58.
[109] Y. Jiao, O. S. Lau, X. W. Deng, Light-regulated transcriptional networks in higher plants, *Nature Reviews Genetics* **2007**, *8*, 217-230.

Chapter 2

Modulation of Isoflavonoid Composition of *Rhizopus oryzae*-Elicited Soybean (*Glycine max*) Seedlings by Light and Wounding

The isoflavonoid profile of soybean was altered in different ways by stimulation of defence response upon germination. The combination of simultaneous germination and induction by *Rhizopus oryzae* increased the total isoflavonoid content of soybeans over two-fold. Pterocarpans became the predominant isoflavonoids, up to 50% (w/w) of total isoflavonoids. To modulate both isoflavonoid content and composition further, the treatment was extended with wounding or light stimuli. The total isoflavonoid content could be increased over three-fold compared to untreated beans by growing fungus-elicited soybean seedlings in light, whereas wounding was less effective. Interestingly, light altered the composition of prenylated pterocarpans by mediating the position of prenylation. The 2-prenylated pterocarpan level increased two-fold, whereas that of 4-prenylated pterocarpan remained similar. Taken together, fungus was the most effective elicitor to alter the isoflavonoid content and composition of soybean seedlings, the impact of which can be further enhanced and mediated by additional stimuli, particularly light.

Based on: Siti Aisyah, Harry Gruppen, Betty Madzora and Jean-Paul Vincken, Modulation of isoflavonoid composition of *Rhizopus oryzae* elicited soybean (*Glycine max*) seedlings by light and wounding, *Journal of Agricultural and Food Chemistry*, **2013**, *61*, 8657.

INTRODUCTION

Isoflavonoids are a class of phenolic compounds mainly found in Leguminosae, comprising amongst others the isoflavone, pterocarpan, and coumestan subclasses.[1] The isoflavonoid structure has similarity with that of mammalian estradiol and, therefore, many isoflavonoids bind to the human estrogen receptors, resulting in estrogenic or anti-estrogenic activities.[2] These features might potentially benefit human health.[3, 4] For instance, soybean seeds can be processed in such a way that they offer a range of isoflavonoids, which might be used as food supplements or as therapeutic agents.[5-7]

Soybean (*Glycine max*) is a rich source of isoflavonoid compounds. The major isoflavonoids found in soybean are conjugated forms (glucoside, acetylglucoside, or malonylglucoside) of daidzein and genistein.[8, 9] The amounts of these isoflavonoids in soybean vary greatly with cultivar and with physiological and developmental stage of the plant.[10-12] In addition, the isoflavonoid profile of soybean can be altered by different factors: germination, fermentation, heat treatment, chemical / enzymatic hydrolysis, and stimulation of plant defence response,[6, 13-15] the latter of which has the largest potential. Such defence response can be induced by exposing germinating seeds to stress. The stress factors can be of a different nature, such as fungal or bacterial elicitors, UV irradiation, and chemicals.[16-18] The activation of plant defence response results in the accumulation of phytoalexins.[19] For example, so-called glyceollins, prenylated-6a-hydroxy-pterocarpans, were the main compounds accumulated in germinating soybean exposed to fungal infection, with glyceollin I-III as the main representatives.[14, 20-23] Both the content and composition of glyceollins induced by fungal infection can vary depending on the experimental conditions of the induction process, soybean varieties, plant tissues, and the fungal genotype.[5, 14] Besides fungal infection, the effect of wounding and light on the isoflavonoid composition of germinating soybean has been investigated. Wounding employed on fungus-treated soybean was reported as a stress factor that affected glyceollin production.[24] The effect of light in combination with wounding was also reported to increase the glyceollin content.[25, 26]

Although the impact of wounding and its combination with light is known to enhance phytoalexin content of fungus-treated soybean, such treatments have not been extensively associated with changes in isoflavonoid composition. In this study, we systematically investigated the effect of light and wounding on isoflavonoid content and composition of fungus-elicited soybean, and we propose how these factors can mediate phytoalexin accumulation in soybean.

MATERIALS AND METHODS

Materials

Soybeans, *Glycine max* (L.) Merrill, were provided by Frutarom Ltd. (Londerzeel, Belgium). The authentic standards of daidzein and genistein were purchased from Sigma Aldrich (St. Louis, MO, USA). UHPLC-MS grade acidified acetonitrile (ACN) and water were obtained from Biosolve BV (Valkenswaard, The Netherlands). Other chemicals were purchased from Merck (Darmstadt, Germany). The fungus, *Rhizopus oryzae* (LU 581), was kindly provided by the Laboratory of Food Microbiology, Wageningen University (Wageningen, The Netherlands).

Soybean treatments

Soybean treatments were performed in a modified sprouting machine (Sprouter micro-farm EQMM; EasyGreen, San Diego, CA, USA), which could accommodate 300 g dry beans. The machine was modified to provide more appropriate experimental conditions. The temperature (25-30 °C) was maintained by a heating mat with thermostat (HMT-A; Bio Green, Bischoffen-Oberweidbach, Germany) placed under the machine, and a styrofoam box covered the machine. Instead of using mist sprayed by the machine, humid air was created by a fog generator (mini fogger; Conrad, Hirschau, Germany) placed in the water compartment of the machine. The generator produced fog every 3 h with a duration 15 min. During this period, a fan attached to the sprouting machine distributed the fog homogenously with a frequency of 4 s per 20 s.

Soybeans were subjected to six different treatments: germination of soybeans in dark (g) and in light (gL), germination of wounded soybeans in dark (gW), germination of fungus-elicited soybeans in dark (gF) and in light (gFL), and germination of fungus-elicited wounded soybeans in dark (gFW) (**Table 1**). In all treatments, soybeans were sequentially subjected to soaking (1 d), germination (2 d) and elicitation (7 d) stages. Prior to the soaking step, soybeans were surface-sterilised by immersing them in a 1% (v/v) hypochlorite solution (5 L/kg beans) for 1 h at room temperature and subsequently rinsed 4 times with Milli-Q water (3 L/kg beans). The sterilized soybeans were soaked for 24 h at 25 °C in sterilized Milli-Q water in the absence of light. Subsequently, the soaked soybeans were put in sterilized plastic cartridges, which were then placed in the modified sprouting machine. Prior to this, the machine was sterilized according to the cleaning protocol provided by the manufacturer. The soybeans were germinated for 2 d at 25 °C and 100% RH. Next, a spore suspension (0.2 mL/g beans) was added to the soybeans, and the soybeans were incubated for 7 d at 30 °C and a RH controlled at 55-85%. Spore suspensions for the inoculation stage were prepared from pure plate cultures of *R. oryzae* grown on malt extract agar (CM59; Oxoid, Basingstoke, UK). The sporangia were scraped

off from the agar plate and suspended in 0.85% (w/v) NaCl solutions (10^7 CFU/mL). For wounding experiments (gW and gFW), the soaked soybeans were wounded prior to the germination stage by cutting the cotyledon individually (longitudinal cut ~6 mm long, opposite side of hilum) with a sterilized knife. In the experiments with light (gL and gFL), an incandescent 55 watt bulb (34.76 µmol/m^2/s) was placed on top of the machine, simulating natural sunlight,[27] 220 mm away from the sample cartridge surface. The light was applied during the germination and elicitation steps for 16 h per d (**Table 1**). All the experiments were performed in triplicates. All treated soybeans were collected after 10 d of treatment and directly stored at -20 °C.

Table 1. Summary of different soybean treatments.

Treatments	Stage			
	Soaking (1 d)	Wounding	Germination (2 d)	Elicitation (7 d)
Untreated	-[a]	-	-	-
g	√[b]	-	√ (dark)	√ (dark, no-fungus)
gW	√	√	√ (dark)	√ (dark, no-fungus)
gL	√	-	√ (light)	√ (light, no-fungus)
gF	√	-	√ (dark)	√ (dark, fungus)
gFW	√	√	√ (dark)	√ (dark, fungus)
gFL	√	-	√ (light)	√ (light, fungus)

[a] The treatment mentioned was not performed.
[b] The treatment mentioned was performed.

Soybean extraction

Soybeans were freeze-dried and milled with a high speed rotor mill (Retsch Ultra Centrifugal Mill ZM 200; Haan, Germany) using a 0.5 mm sieve. The sample extraction was performed using a speed extractor (E-916; Buchi, Flawil, Switzerland). A soybean sample (100 mg) was mixed with sand (granulation 0.3-0.9 mm, dried at 750 °C; Buchi) and placed in a 40 mL stainless steel extraction cell. Cellulose filters (Buchi) were placed at the bottom and top of the extraction cell. Hexane and 70% (v/v) aqueous ethanol (EtOH) were used for defatting and extraction of isoflavonoids, respectively. During extraction, the cell was filled with solvents, pressurized (100 atm) and heated (40 °C). For each extractant, the sample was extracted using two consecutive extraction cycles of 10 min, in which all oil (hexane) and isoflavonoids (70% aqueous EtOH) were recovered. After the second extraction step with each solvent, the cell was flushed with 40 mL solvent and with a flow of nitrogen for 300 s. The extract was collected into a 150 mL glass vial. The extract was evaporated under reduced pressure. The dried extracts were re-solubilised in 5 mL of 70% aqueous EtOH, and stored at -20 °C. All samples were centrifuged (18000 *g*, 5 min; room temperature) prior to analysis. The hexane extract was found to be isoflavonoid-free, and will not be considered further.

Isoflavonoid analysis

The extracts obtained were analysed by UHPLC-MS. An Accela UHPLC system (Thermo Scientific, San Jose, CA, USA) was equipped with a pump, autosampler, and photodiode array (PDA) detector. Sample (1 μL) was injected onto an Acquity UPLC BEH shield RP18 column (2.1 mm ID × 150 mm, 1.7 μm particle size; Waters, Milford, MA, USA) with an Acquity UPLC BEH shield RP18 VanGuard pre-column (2.1 mm ID × 5 mm, 1.7 μm particle size; Waters). Water acidified with 0.1% (v/v) acetic acid, eluent A, and ACN acidified with 0.1% (v/v) acetic acid, eluent B, were used as solvents at a flow rate of 300 μL/min. The temperatures of the autosampler and column oven were controlled at 15 and 35 °C, respectively. The PDA detector was set to monitor the 200-400 nm range. The elution profile was as follows: 0-2 min, linear gradient from 10%-25% (v/v) B; 2-9 min, linear gradient from 25%-50% (v/v) B; 9-12 min, isocratic on 50% B; 12-22 min, linear gradient from 50%-100% (v/v) B; 22-25 min, isocratic on 100% B; 25-27 min, linear gradient from 100%-10% (v/v) B; 27-29 min, isocratic on 10% (v/v) B. Mass spectrometric analysis was performed on a LTQ Velos (Thermo Scientific) equipped with an HESI-MS probe coupled to RP-UHPLC. Nitrogen was used as sheath and auxiliary gas. The spectra were acquired in the *m/z* range of 150-1500. Data-dependent MS^n analysis was performed with a normalized collision energy of 35%. The system was tuned with genistein in both positive (PI) and negative ionisation (NI) mode. For the PI mode, the ion transfer tube (ITT) temperature was 400 °C and the source voltage was 4.50 kV. For NI mode, the ITT temperature was 400 °C and the source voltage was 3.50 kV.

The identification of isoflavonoids was based on UV and MS spectra using the approach reported earlier.[5, 28] MS fragmenter software (Advanced Chemistry Development, Toronto, Canada) was used for further confirmation of glyceollidins isomers. The quantification of isoflavonoids was performed based on their absorption at 280 nm by means of Xcalibur (version 2.1.0, Thermo Scientific). For different compounds eluted at the same retention time, the quantification was based on the ratio of intensity of those peaks in full HESI-MS, assuming that no isomers eluted at the same retention time. As for many compounds no commercial standards were available, the amounts of isoflavonoid were expressed as mg daidzein equivalents per g dry weight of soybeans (mg DE/g DW), in which daidzein was used as a generic standard to make a calibration curve with five data points (0.1-0.001 mg/mL, $R^2 = 0.998$).

Statistical analysis

Statistical analysis was performed using the SPSS Statistics (version 21, IBM, Armonk, NY, USA). Differences in the amounts of isoflavonoid subclasses between treatments were evaluated for significance ($P < 0.05$) with Tukey's *post hoc* multiple comparison test.

RESULTS

Identification of isoflavones in treated and control soybeans

UHPLC analysis of the extracts from untreated and treated soybeans showed that the UV-profiles changed during the treatments (**Figure 1**). The untreated soybeans contained mainly the 7-*O*-glucoside and 7-*O*-(6″-*O*-malonylglucoside) of daidzein and genistein, whereas the induced soybeans contained predominantly other isoflavonoids. Thirty isoflavonoids were identified in treated soybean belonging to isoflavone, pterocarpan, and coumestan subclasses**,** and one compound was identified as flavonoid (**Table 2)**. The identities of most peaks were determined previously in our laboratory,[5] whereas peaks **7, 8**, **14**, and **17** were identified in this study. These peaks were tentatively assigned as isoflavones based on their maximum absorbance of around 260 (±3) nm. Compounds **8** and **17** were tentatively identified as 7-*O*-(6″-*O*-malonylglucoside) formononetin (or 6″-*O*-malonylononin) and formononetin, respectively. Formononetin and ononin have been previously found in soybean.[1] Compound **8** lost 248 Da (corresponding to the malonyl-glucoside moiety) in MS^2 to afford the fragment ion *m/z* 269. This ion was fragmented further in MS^3 to produce the fragment ions *m/z* 254 $[M+H-CH_3]^{+\bullet}$, 237 $[M+H-CH_3OH]^+$, and 213 $[M+H-2CO]^+$, the same fragmentation pattern as that of the corresponding aglycone **17** in MS^2. Additional evidence for the presence of a methoxy group at the B-ring in compounds **8** and **17** was obtained from the abundance of fragment ion *m/z* 254 $[M+H-CH_3]^{+\bullet}$; when the methoxy group is attached to the A-ring, loss of the methyl group is a much more rare event.[29] These compounds have been previously found in soy-based products.[30] Peak **14** was tentatively assigned as prunetin, which has the methoxy group at the A-ring. This compound produced the fragment ions *m/z* 267 $[M+H-H_2O]^+$, 257 $[M+H-CO]^+$, and 229 $[M+H-2CO]^+$, without a predominant methyl loss, matching the prunetin fragmentation pattern described in the literature.[29] Compound **7** was tentatively identified as 7-*O*-(6″-*O*-malonylglucoside) demethyltexasin. Demethyltexasin has been previously found in soybean.[1] Compound **7** provided the aglycone fragment ion *m/z* 271, which was fragmented further in MS^3 to afford the fragment ions *m/z* 253 $[M+H-H_2O]^+$, 215 $[M+H-2CO]^+$, and the *retro*-Diels Alder (RDA) fragment ion *m/z* 153 $^{1,3}A^+$, indicating that the hydroxyl group was attached to the A-ring of daidzin, but different to genistin, which eluted earlier.

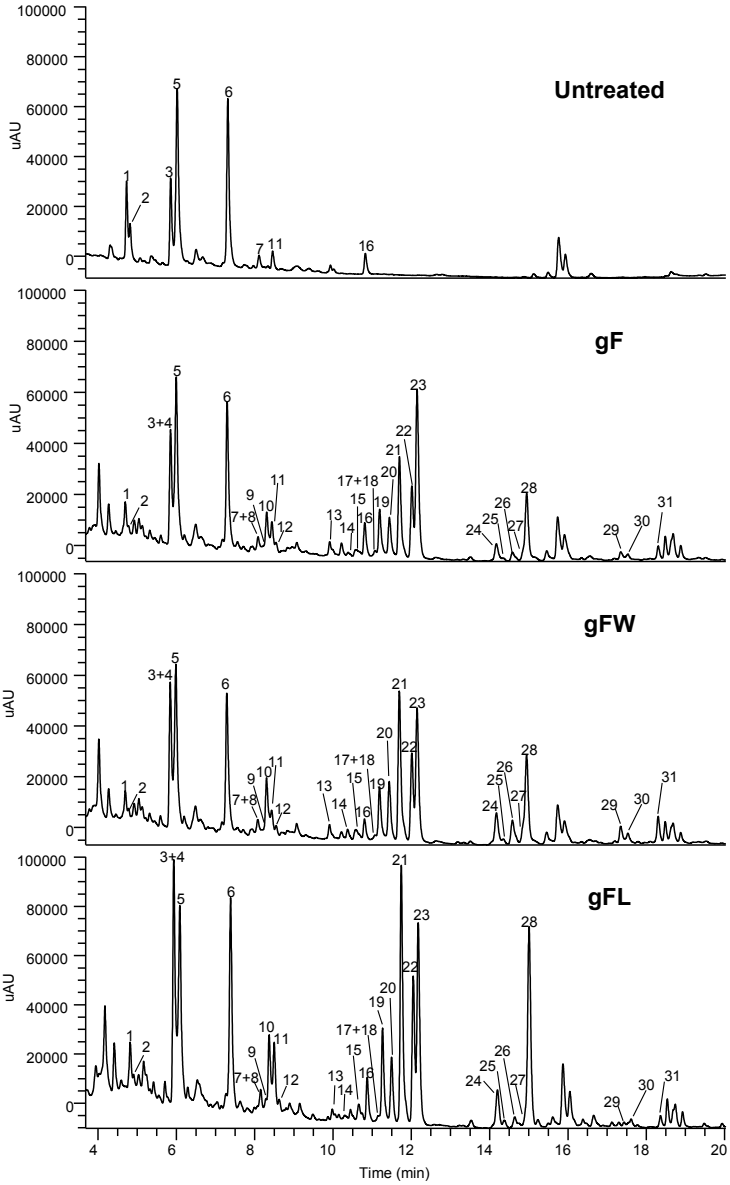


Figure 1. RP-UHPLC-UV profile of 70% aqueous EtOH extracts of untreated and fungus-elicited soybean. Codes (Untreated, gF, gFW, gFL) refer to the treatment in **Table 1**, and peak numbers refer to compounds in **Tables 2** and **4**.

Table 2. Compounds tentatively identified by RP-UHPLC-PDA-MS in elicited soybean extracts.

No[a]	t_R (min)	Compounds[b]	λ_{max} (nm)	$[M-H]^-$	MS^2 NI product ion[c] (rel. abundance)	$[M+H]^+$	MS^2 PI product ion (rel. abundance)
1	4.72	Daidzin	249	415	253 (100)	417	255 (100)
2	4.82	Glycitin	257	445	283 (100)	447	285 (100)
3	5.85	Glycinol	283	271	227 (31), 161 (100)	255[d]	237 (22), 227 (61), 199 (100)
4	5.86	Genistin	260	431	311 (13), 269 (100)	433	271 (100)
5	6.02	6"-*O*-Malonyldaidzin	255	501	253 (100), 225 (73), 197 (31)	503	255 (100)
6	7.31	6"-*O*-Malonylgenistin	259	517	269 (11), 241 (28), 225 (100)	519	433 (8), 271 (100)
7	8.11	7-*O*-(6"-*O*-Malonyl-Glc) demethyltexasin	260	517	241 (43), 225 (100), 209 (56)	519	271 (100)
8	8.11	6"-*O*-Malonylononin	259	515	252 (100)	517	269 (100)
9	8.24	Glycitein	255	283	268 (100)	285	270 (100), 257 (19), 240 (13)
10	8.30	Glyceofuran	257, 291	353	335 (100), 149 (21)	337 [d]	319(82), 309 (100), 188(30)
11	8.44	Daidzein	248	253	225 (89), 209 (100)	255	237 (22), 227 (61), 199 (100)
12	8.54	2'-OH-Genistein	257	285	241 (10), 217 (100), 199 (10)	287	269 (20), 259 (49), 217 (100)
13	9.92	Naringenin	258	271	253 (2), 177 (22), 151 (100)	273	255 (11), 214 (7), 153 (100)
14	10.41	Prunetin	257	283	268 (11), 255 (100), 240 (16)	285	267 (2), 257 (100), 229 (8)
15	10.60	Isotrifoliol	351	297	282 (100)	299	284 (7) 271 (100), 267 (17)
16	10.81	Genistein	260	269	241 (45), 225 (100), 201 (67)	271	253 (29), 243 (73), 215 (69)
17	11.07	Formononetin	260	267	252 (100)	269	254 (100), 237 (30), 213 (32)
18	11.08	Glyceollidin I	284	339	324 (54), 161 (100)	323 [d]	267 (100)
19	11.19	Glyceollidin II	284	339	324 (44), 161 (100)	323 [d]	267 (100)
20	11.44	Coumestrol	304, 343	267	239 (100), 211 (10)	269	241 (100), 225 (28), 197 (22)
21	11.70	Glyceollin III	289	337	319 (100), 149 (17)	321 [d]	306 (73), 279 (100), 251 (64)
22	12.01	Glyceollin II	283	337	319 (100), 149 (40)	321 [d]	306 (55), 279 (100), 251 (53)
23	12.15	Glyceollin I	283	337	319 (100), 149 (86)	321 [d]	306 (80), 303 (100), 293 (37)
24	14.17	Glyceollin VI	278, 317	335	317 (100), 149 (39)	319 [d]	291 (79), 263 (100)
25	14.58	A_{prenyl}-daidzein	253	321	266 (100)	323	267 (100)
26	14.69	A_{prenyl}-2'-OH-daidzein	253	353	285 (100), 284 (100), 267 (33)	355	299 (100)
27	14.84	B_{prenyl}-daidzein	263	321	265 (100), 252 (5)	323	267 (100), 255 (10)
28	14.95	Glyceollin IV	285	353	335 (100), 149 (27)	337 [d]	281 (100), 269 (65)
28	17.35	A_{prenyl}-genistein	262	337	322 (2), 309 (4), 282 (100)	339	283 (100), 257 (6)
30	17.53	B_{prenyl}-genistein	261	337	293 (11), 281 (100), 268 (4)	339	283 (100), 271 (17), 257 (5)
31	18.31	Phaseol	307, 343	335	291 (6), 280 (100)	337	281 (100)

[a] Numbers refer to peaks in **Figures 1** and **4**.

[b] Standard three letters code for Glc (glucoside) are used.

[c] For the pterocarpan subclass only the two most abundant product ions in NI mode are indicated. The complete set of product ions is shown in **Table 3**.

[d] In positive mode ESI-MS, parent ions lost a water molecule to produce $[M+H-H_2O]^+$. The intensity of this ion dominated the $[M+H]^+$ mass spectrum.

Identification of pterocarpans in treated and control soybeans

The pterocarpans detected in fungus-elicited soybean were glycinol (**3**), glyceollidins (**18-19**), and glyceollins (**10**, **21-24**, **28**). Glycinol is the non-prenylated precursor of all prenylated pterocarpans in soybean. The prenyl group can be attached to the 4-position (glyceollidin I, glyceollin I and VI) or the 2-position (glyceollidin II, glyceollin II, III, IV, and glyceofuran) of the A-ring, either as a chain or as a ring (pyran or furan) with an adjacent hydroxyl group. The tentative assignment of these 6a-OH-pterocarpans was based on their maximum absorbance of around 280 (±3) nm in UV spectrum. An additional maximum absorbance was observed at 317 nm in the UV spectrum of **24**. This absorption might be caused by the extra conjugated double bond in the 2"-isoprenyl-furano group of glyceollin VI. Further confirmation of the assignment of the pterocarpans was performed by analysis of their MS/MS fragmentation pattern obtained in both NI and PI mode.

The fragmentation patterns of pterocarpans in NI mode have been elaborated in a previous report,[28] but it was impossible to distinguish the two glyceollidin isomers (I and II) known, which were suggested to elute at the same retention time (t_R). In the present study, two peaks with *m/z* 339 (NI mode) were observed (t_R 11.08 and 11.19), which might correspond to the two glyceollidins isomers. The main fragment ions of *m/z* 339 were *m/z* 324, 295, 270, and 161 (**Table 3**). Interestingly, the previously unreported anion, *m/z* 270, appeared to be more abundant in the mass spectrum at t_R 11.08 than that at t_R 11.19 (**Figure 2**), suggesting that this difference in abundance might be diagnostic for one of the isomers. The fragment ion *m/z* 270 might represent the radical anion originating from homolytic cleavage of the prenyl group from a deprotonated glyceollidin precursor (**Figure 3**). Although the formation of this radical anion is a violation of the 'even-electron rule', exceptions have been reported to occur, especially when the radical anion can be resonance-stabilized by an aromatic ring system.[31-33] We hypothesize that the loss of the radical fragment $C_5H_9^{\bullet}$ from the $[M\text{-}H]^-$ of glyceollidin II is less likely to occur than that of glyceollidin I, as the radical anion of glyceollidin I might be resonance-stabilized (**Figure 3**), which was supported by theoretical fragmentation using MS fragmenter software. Glyceollidin II was at least 5-fold more abundant than glyceollidin I, in line with Zähringer *et al.* stating that glyceollidin I comprised approximately 10% of the glyceollidins mixture (**Table 4**).[34]

Apart from the two glyceollidins, also the isomers glyceollin V and VI could not be distinguished in our previous report.[28] The 3-OH in both glyceollin V and VI is not free, and consequently the fragmentation rules derived for glyceollidin I and II cannot be employed here. By extrapolating the differences observed in the fragmentation patterns of glyceollin I and II (4- and 2-prenylated pterocarpan, respectively), and the series of glyceollin II, III, IV, and glyceofuran (all 2-prenylated pterocarpans, but with different configurations of the A-ring), **24** was tentatively annotated as glyceollin VI, as follows. First, the relative abundance of the RDA fragment ion *m/z* 149 ($^{2,4}B^-$), a distinctive ion

amongst glyceollin isomers, was higher for glyceollin I than for glyceollin II and other 2-prenylated pterocarpans (**Table 3**). Second, prenyl configurations other than the pyran ring seemed to yield fragment ion *m/z* 149 ($^{2,4}B^-$) in lesser abundance for the series of 2-prenylated pterocarpans. Taken together, we speculate that the relatively high abundance of fragment ion *m/z* 149 ($^{2,4}B^-$) suggests that **24** is a 4-prenylated pterocarpan, most likely corresponding to glyceollin VI, assuming that the extra conjugated bond in the prenyl group does not contribute too much to the stability of the fragment ion.

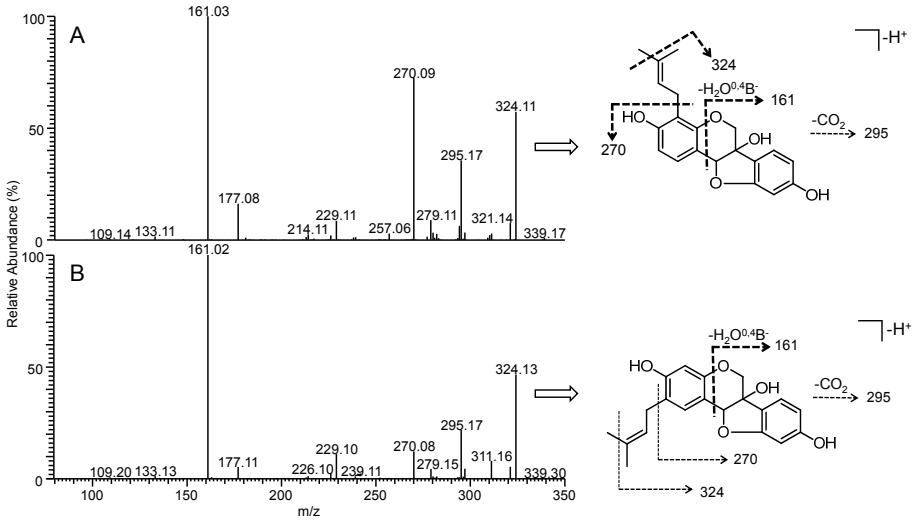


Figure 2. MS^2 spectra of *m/z* 339 in NI mode and proposed cleavage of glyceollidin I eluted at t_R 11.08 (**A**) and of glyceollidin II eluted at t_R 11.19 (**B**). Bold dashed arrows indicate cleavage yielding product ions with relative abundance over 50%.

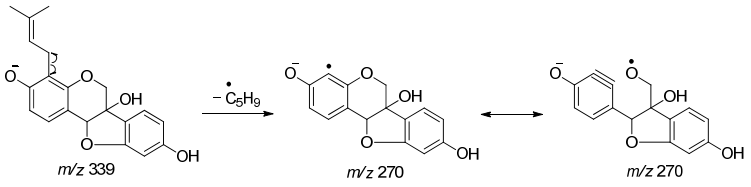


Figure 3. Proposed route for the formation of radical fragment ion *m/z* 270 of glyceollidin I, which is resonance-stabilized.

Table 3. MS^2 product ions obtained from the $[M-H]^-$ precursor ion of pterocarpans.

Product ion	Non-prenylated	4-Prenylated pterocarpans			2-Prenylated pterocarpans				
	Glycinol	Glyceollidin I	Glyceollin I	Glyceollin VI	Glyceollidin II	Glyceollin II	Glyceollin III	Glyceollin IV	Glyceofuran
$[M-H]^-$	271 (1)[a]	339 (1)	337 (0)	335 (0)	339 (0)	337 (0)	337 (0)	353 (0)	353 (0)
$[M-H-CH_3]^{-\bullet}$	256 (28)	324 (54)		320 (1)	324 (44)	322 (2)		338 (1)	338 (5)
$[M-H-H_2O]^-$	253 (3)	321 (5)	319 (100)	317 (100)	321 (4)	319 (100)	319 (100)	335 (100)	335 (100)
$[M-H-CO]^-$	243 (6)	311 (2)	309 (3)	307 (3)	311 (7)	309 (2)	309 (1)		
$[M-H-CO_2]^-$	227 (31)	295 (34)	293 (36)	291 (13)	295 (19)	293 (23)	293 (7)	309 (9)	309 (7)
$[M-H-C_5H_9]^{-\bullet}$		270 (77)	268 (2)	266 (1)	270 (13)	268 (2)	268 (1)		284 (1)
$^{5,6}A^-$			229 (3)						
$^{5,6}B^-$	109 (3)								
$^{2,3,7}A^-$		217 (2)	215 (5)	213 (3)		215 (5)	215 (3)	231 (2)	231 (1)
$^{2,3,7}B^-$	121 (1)		121 (4)	121 (2)		121 (2)	121 (1)	121 (1)	121 (1)
$^{2,4}A^-$	121(0)		187 (9)	213 (3)		187 (17)			
$^{2,4}B^-$			149 (86)	149 (39)		149 (40)	149 (17)	149 (27)	149 (21)
$-H_2O^{6,7}A^-$	161 (100)	229 (8)	227 (1)		229 (9)	227 (4)	227 (1)	243 (1)	
$-H_2O^{0,4}B^-$	161 (100)	161 (100)	161 (18)	161 (8)	161 (100)	161 (6)	161 (3)	161 (5)	161 (2)
$-H_2O^{1,4}A^-$	109 (3)	177 (17)	175 (5)	173 (1)	177 (6)	175 (3)		191 (1)	
$-H_2O^{2,4}B^-+2H$		133 (1)			133 (1)				

[a] *m/z* (relative abundance).

Isoflavonoids in fungus-elicited soybean

Simultaneous germination and elicitation by *R. oryzae* (gF) increased the isoflavonoid content of the soybeans from 1.30 to 2.95 mg DE/g DW (**Table 4**). This increase was mainly due to the accumulation of pterocarpans, which were accumulated up to 1.47 mg DE/g DW, constituting 50% (w/w) of the total isoflavonoid content. Besides, lower quantities of isoflavone (1.23 mg DE/g DW) and coumestan (0.25 mg DE/g DW) were found. Amongst the pterocarpans, glyceollin I (**23**) was the most predominant species with a content of 0.44 mg DE/g DW. Glycinol (**3**) was accumulated in lower quantities (0.15 mg DE/g DW). The levels of 4-prenylated pterocarpans (**18**, **23-24**) and 2-prenylated pterocarpans (**10**, **19**, **21-22**, **28**) were 0.53 and 0.79 mg DE/g DW, respectively. Within the isoflavone subclass, the isoflavones glucoside and malonylated glucoside were predominant (82% (w/w) of total isoflavones). Only a small amount of the isoflavones was prenylated (0.12 mg DE/g DW).

Effect of wounding on the isoflavonoid profile of fungus-elicited soybean

A combination of the stress factors fungus and wounding was applied to germinated soybeans (gFW). Compared to the gF treatment, the total isoflavonoid content in the gFW was not notably different, as were the types of compounds present. Interestingly, the content of glycinol was 1.5 times higher than in the gF. It seemed that the procedure of wounding prior to inoculation with fungus triggered the accumulation of phytoalexins by inducing the pterocarpan precursor, but this did not increase the total content of prenylated pterocarpans (**Table 4**). Nevertheless, the composition of prenylated pterocarpans was remarkably different in gFW compared to gF, with glyceollin III becoming equally abundant as glyceollin I. Besides, the content of glyceollin IV, the relatively less abundant isomer, increased 1.6 times in gFW compared to gF. After the treatment, the quantity of 4-prenylated pterocarpans and 2-prenylated pterocarpans was 0.42 and 1.01 mg DE/g DW, respectively. Moreover, the content of neither isoflavones nor coumestan was influenced by wounding. These results showed that fungal elicitation combined with wounding rearranged the pterocarpan composition.

Effect of light on isoflavonoid profile of fungus-elicited soybean

The isoflavonoid level of fungus-inoculated soybeans grown in the light (gFL) increased up to 4.61 mg DE/g DW. Pterocarpans, reaching 58% of the total isoflavonoid content, were mainly responsible for this increase (**Table 4**). Light raised the content of glycinol more than two-fold compared to the gF treatment, as well as the content of 2-prenylated pterocarpans. Surprisingly, light did not boost the level of 4-prenylated pterocarpans in fungus-treated soybean. Hence, the level of 4-prenylated pterocarpans and 2-prenylated pterocarpans became 0.58 and 1.70 mg DE/g DW, respectively. Glyceollin III became the

most abundant pterocarpan, up to 0.58 mg DE/g DW, whereas normally glyceollin I was the most predominant pterocarpan species. Isoflavone content in fungus-treated soybeans were also influenced by light. The total isoflavone level increased up to 1.65 mg DE/ g DW. This increase was characterised by an increase of 6″-*O*-malonylgenistin, genistein and daidzin. Unlike the increase in prenylated pterocarpans, the content of prenylated isoflavones remained the same as in gF. Finally, light did not influence the coumestan level. Thus, light did not only considerably increase the pterocarpan level in fungus-treated soybeans, it also rearranged their pterocarpan composition.

Isoflavonoids profile of germinated soybean in the absence of fungus

In a separate set of experiments, the effect of germination of soybean in the absence of fungus (g, gW, and gL) was investigated with respect to isoflavonoid composition (**Figure 4**).

Germinated soybean (g). The isoflavonoid content in germinated soybeans increased slightly from 1.30 to 1.53 mg DE/g DW. After 9 days of germination, the isoflavonoid profile did not change much and isoflavones were still dominant, equivalent to 93% (w/w) of the total isoflavonoid content (**Table 4**). Pterocarpans and coumestans accumulated to 0.08 and 0.02 mg DE/g DW, respectively. This result showed that germination alone in the dark has much less impact on isoflavonoid content and composition of soybean than in combination with fungus.

Germinated wounded soybean (gW). The isoflavonoid level in gW increased up to 1.75 mg DE/g DW, mainly characterised by the accumulation of isoflavones (**Table 4)**. Interestingly, glyceollins were found up to 0.47 mg DE/g DW, which is the highest amount of glyceollins found in treatments without fungus. It showed that the wounding treatment prior to germination was able to trigger the accumulation of common glyceollins. This result was in line with a previous report.[35] Surprisingly, accumulation of glyceollins was not accompanied by higher accumulation of glycinol, which was found below 0.01 mg DE/g DW. Although wounding alone was much less effective than treatment with fungus, it was sufficient to initiate accumulation of glyceollins in germinated soybean.

Germinated soybean in the light (gL). An increase of isoflavonoid content was observed when the soybeans were germinated in the presence of light. The total isoflavonoid content increased substantially up to 2.60 mg DE/g DW (**Table 4**). Strikingly, the isoflavones were the most affected compounds, accumulating up to 2.28 mg DE/g DW, especially 6″-*O*-malonyldaidzin and 6″-*O*-malonylgenistin.[7, 36] In addition, small quantities of pterocarpans (0.22 mg DE/g DW) and coumestans (0.11 mg DE/g DW) were induced. These results were in line with a previous report, showing that germination alone in light can effectively increase the content of malonylated isoflavones glucoside, but not that of pterocarpans and coumestans.

Table 4. Contents of isoflavonoids in extracts from untreated and variously treated soybeans.

		mg DE/g DW[b]						
No[a]	Compounds	Untreated	g	gW	gL	gF	gFW	gFL
1	Daidzin	0.14±0.02	0.10±0.04	0.10±0.05	0.17±0.04	0.05±0.01	0.06±0.02	0.07±0.01
2	Glycitin	0.06±0.01	0.03±0.01	0.01±0.00	0.05±0.00	0.02±0.00	0.02±0.01	0.02±0.00
4	Genistin	0.16±0.02	0.07±0.01	0.03±0.00	0.07±0.03	0.03±0.01	0.04±0.02	0.10±0.03
5	6''-*O*-Malonyldaidzin	0.43±0.03	0.57±0.15	0.47±0.01	1.08±0.15	0.41±0.05	0.41±0.03	0.46±0.05
6	6''-*O*-Malonylgenistin	0.44±0.06	0.50±0.07	0.40±0.04	0.70±0.08	0.35±0.05	0.35±0.03	0.52±0.01
7	7-*O*-(6''-*O*-Malonyl-Glc) demethyltexasin	0.04±0.01	0.03±0.02	0.01±0.00	0.02±0.01	0.02±0.01	0.02±0.01	0.02±0.01
8	6''-*O*-Malonylononin	-	0.05±0.02	0.05±0.00	0.10±0.02	0.02±0.02	0.02±0.01	0.02±0.01
9	Glycitein	<0.01	<0.01	<0.01	0.01±0.00	<0.01	<0.01	<0.01
11	Daidzein	0.03±0.01	0.04±0.01	0.03±0.02	0.02±0.01	0.09±0.02	0.05±0.00	0.15±0.05
12	2'-OH-Genistein	-	-	<0.01	<0.01	0.01±0.01	0.02±0.00	0.02±0.01
14	Prunetin	-	-	<0.01	<0.01	0.02±0.01	0.02±0.00	0.02±0.00
16	Genistein	0.03±0.01	0.01±0.01	0.02±0.01	0.02±0.01	0.07±0.00	0.04±0.00	0.14±0.05
17	Formononetin	-	0.01±0.01	<0.01	0.01±0.01	0.01±0.01	0.01±0.00	0.01±0.00
25	A_{prenyl}-daidzein	-	0.01±0.00	0.04±0.00	0.01±0.01	0.05±0.02	0.03±0.02	0.03±0.01
26	A_{prenyl}-2'-OH daidzein	-	<0.01	0.02±0.00	<0.01	0.01±0.00	0.04±0.02	0.01±0.00
27	B_{prenyl}-daidzein	-	<0.01	0.02±0.00	<0.01	0.01±0.00	0.03±0.01	0.02±0.01
29	A_{prenyl}-genistein	-	0.01±0.01	0.02±0.00	0.01±0.00	0.03±0.01	0.04±0.01	0.01±0.01
30	B_{prenyl}-genistein	-	<0.01	<0.01	<0.01	0.02±0.00	0.02±0.00	0.02±0.01
Total isoflavones		**1.30±0.11a**	**1.43±0.31a**	**1.18±0.10a**	**2.28±0.35b**	**1.23±0.05a**	**1.22±0.05a**	**1.65±0.12a,b**
3	Glycinol	-	-	<0.01	0.02±0.01	0.15±0.03	0.23±0.03	0.41±0.05
10	Glyceofuran	-	<0.01	0.01±0.00	0.01±0.01	0.10±0.01	0.13±0.02	0.17±0.01
18	Glyceollidin I	-	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
19	Glyceollidin II	-	<0.01	0.02±0.01	0.01±0.01	0.07±0.03	0.12±0.01	0.16±0.05
21	Glyceollin III	-	0.01±0.00	0.12±0.01	0.06±0.02	0.25±0.04	0.35±0.02	0.58±0.03
22	Glyceollin II	-	0.01±0.00	0.09±0.05	0.03±0.01	0.23±0.06	0.19±0.02	0.41±0.05
23	Glyceollin I	-	0.04±0.03	0.19±0.05	0.04±0.02	0.44±0.02	0.33±0.02	0.45±0.07
24	Glyceollin VI	-	<0.01	<0.01	0.01±0.01	0.09±0.03	0.09±0.01	0.12±0.01
28	Glyceollin IV	-	<0.01	0.03±0.02	0.04±0.02	0.14±0.02	0.23±0.04	0.38±0.13
Total pterocarpans		**0a**	**0.08±0.05a**	**0.47±0.08b**	**0.22±0.10a,b**	**1.47±0.11c**	**1.67±0.17c**	**2.69±0.23d**
15	Isotrifoliol	-	<0.01	0.01±0.00	0.02±0.00	0.05±0.02	0.03±0.01	0.04±0.01
20	Coumestrol	-	0.01±0.00	0.04±0.01	0.05±0.01	0.15±0.04	0.14±0.01	0.14±0.03
31	Phaseol	-	<0.01	0.04±0.00	0.04±0.01	0.05±0.01	0.06±0.01	0.08±0.01
Total coumestans		**0a**	**0.02±0.00a**	**0.10±0.00a,b**	**0.11±0.01a,b**	**0.25±0.07c**	**0.22±0.02b,c**	**0.26±0.04c**
Total Isoflavonoid		**1.30±0.11a**	**1.53±0.28a**	**1.75±0.03a**	**2.60±0.24b**	**2.95±0.19b**	**3.11±0.23b**	**4.61±0.32c**

[a] Numbers refer to compounds in **Table 2**.

[b] Data are the means ± SD of experiments performed in triplicate. Values within the same row with different letters show significant differences (Tukey's test, $P < 0.05$).

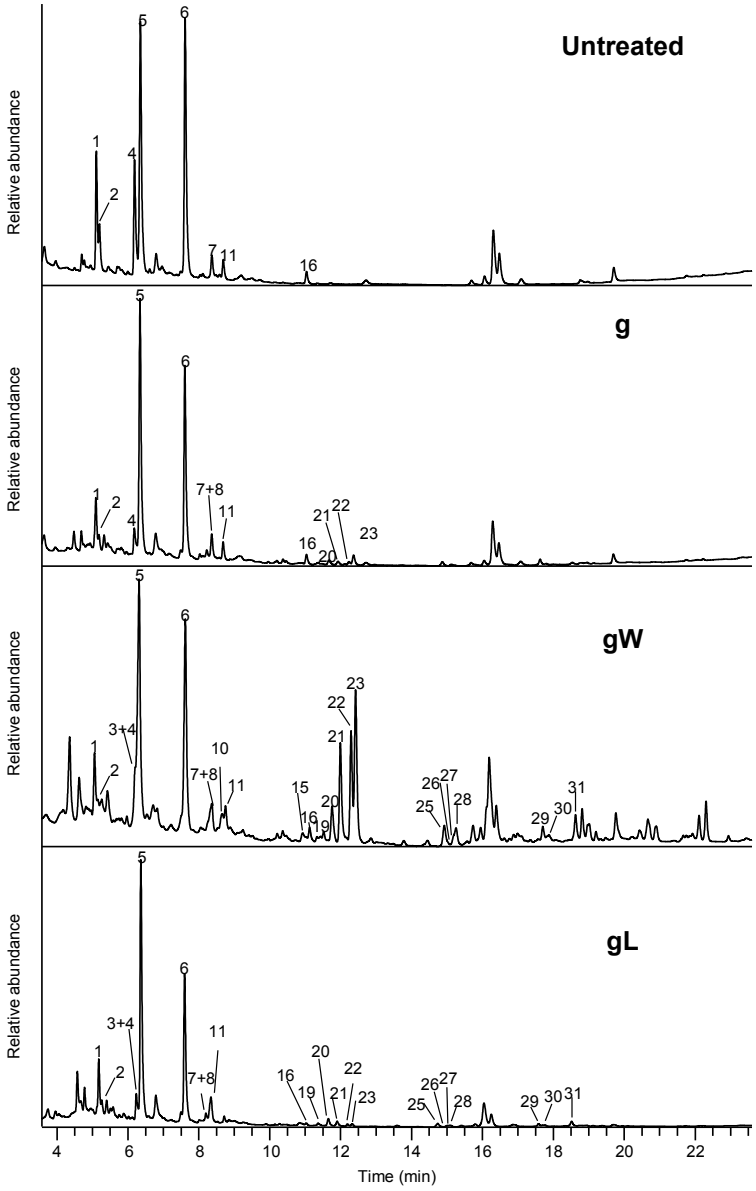


Figure 4. RP-UHPLC-UV profile of 70% aqueous EtOH extracts of untreated and germinated soybean in the absence of fungus. Codes (Untreated, g, gW, gL) refer to the treatment in **Table 1**, and peak numbers refer to compounds in **Table 2** and **4**.

DISCUSSION

Six different treatments with soybean seedlings were performed to investigate whether the accumulation of phytoalexins can be directed towards larger amounts of these molecules and towards specific compositions of mixtures of them. It appeared that wounding and treatment with fungus were essential to induce the accumulation of mainly (prenylated) pterocarpans, with the fungus being the best elicitor of the two. Prenylation always coincided with induction of molecules from the pterocarpan and coumestan subclasses. Besides, light appeared to be a key factor in boosting the total amount of isoflavonoids, the kind of which strongly depended on whether fungus was applied (**Figure 5**).

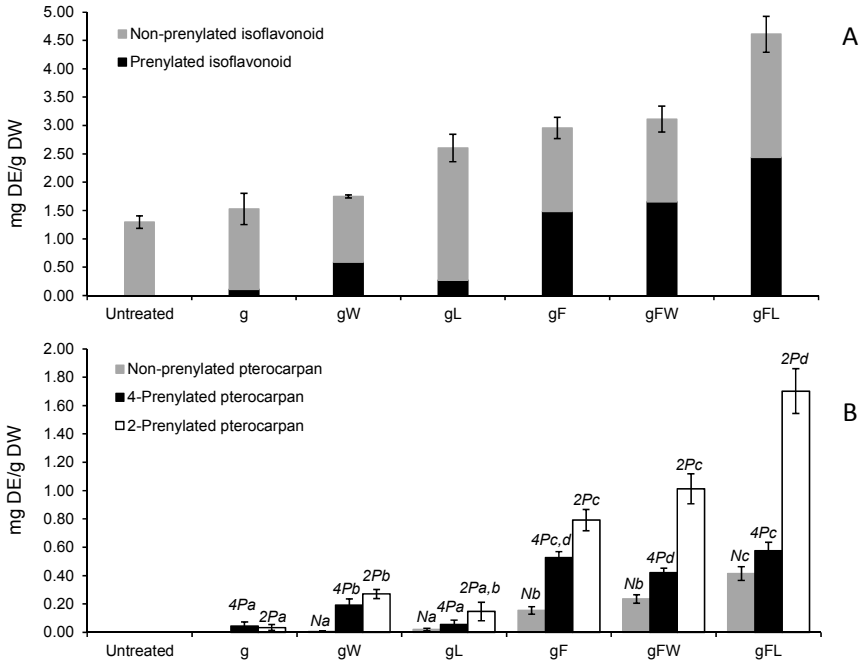


Figure 5. (**A**) Total non-prenylated and prenylated isoflavonoid contents of untreated and treated soybean. (**B**) Total non-prenylated, 4-prenylated, and 2-prenylated pterocarpan content of untreated and treated soybean. Codes (Untreated, g, gW, gL, gF, gFW, and gFL) refer to the treatment in **Table 1.** All contents are expressed in mg daidzein equivalent (DE) per g dry weight (DW) of soybean. Data are the means ± SD of experiments performed in triplicate. Bars with different letters (a-d) show significant differences (Tukey's test, $P < 0.05$, conducted for the three types of pterocarpans differing in prenylation (N: non-prenylated, 4P: 4-prenylated, 2P: 2-prenylated)).

Wounding in addition to fungus did not boost isoflavonoid content

The combination of fungus and wounding did not increase the isoflavonoid content in germinating soybeans compared to unwounded fungus-elicited soybeans, although a small increase in pterocarpan level was detected (**Figure 5**). Instead, wounding influenced the pterocarpan composition of fungus-elicited soybean. This was in contrast to a previous report showing that the amount of common glyceollins (I, II, and III) in wounded (half-sliced) *Aspergillus*-treated soybean was approximately 10-fold higher than that in unwounded *Aspergillus*-treated soybean, with glyceollin I always being the predominant pterocarpan species.[24] This discrepancy in glyceollin composition might be explained by differences in the variety of soybean, time point of application of the fungus after wounding, or the fungal genotype employed.[37]

Enhancing isoflavonoid content of soybean seedlings by light

Exposure of the fungus-elicited soybean seedlings to light boosted the accumulation of all subclasses of isoflavonoids (**Figure 5**), except coumestans. Moreover, our results suggested that light and fungus are synergistic factors in raising the total pterocarpan content (compare pterocarpan level of gL, gF, and gFL). Light is thought to increase the production of malonyl-CoA and coumaroyl-CoA,[7] thus enhancing the pool size of natural precursors for isoflavonoid production, including daidzein, the first devoted precursor of pterocarpans. Hence, daidzein was available in larger abundance for the production of prenylated pterocarpans. The increase of prenylated pterocarpans in fungus-treated soybean grown in light has been shown before for soybean seedlings exposed to *Phytophthora megasperma*.[26] Our results indicate that the combination of fungus and light holds potential for the production of prenylated isoflavonoids, which might find use as estrogenic and anti-estrogenic food supplements or therapeutics.[2]

Mediating the position of prenylation of pterocarpans by light

The most downstream event in the biosynthesis of pterocarpans is prenylation of glycinol, often followed by cyclization into a pyran or a furan ring. Attachment of the prenyl group to the pterocarpan moiety can occur at the 2- and 4-position of pterocarpans.[34] Our results showed that exposure to light can alter the preference of site of prenylation. The ratio of 4- to 2-prenylated pterocarpans of inoculated beans changed from 1:1.5 in gF to 1:2.9 in gFL, in which the content of 4-prenylated pterocarpans remained the same, and the content of 2-prenylated pterocarpans increased. This is schematically summarized in **Figure 6**. This observation provides strong evidence for regiospecific prenylation of glycinol, in accordance with other reports.[34, 38, 39] The biosynthesis of glyceollin I is known to require the *C*-4 specific prenyltransferase known as G4DT (glycinol 4-dimethylallyltransferase). For prenylation of the 2-position of glycinol, PT3 has been suggested as a candidate of

G2DT (glycinol 2-dimethylallyltransferase). Soybean PT3 is 61% identical at the amino acid level to G4DT, which has been abundantly found in stress-induced soybean seedlings.[38] Taken together, our results suggest that exposure of soybean sprouts elicited with fungus in light not only accumulated more pterocarpans, but also orchestrated prenylation towards a higher proportion of 2-prenylated pterocarpans.

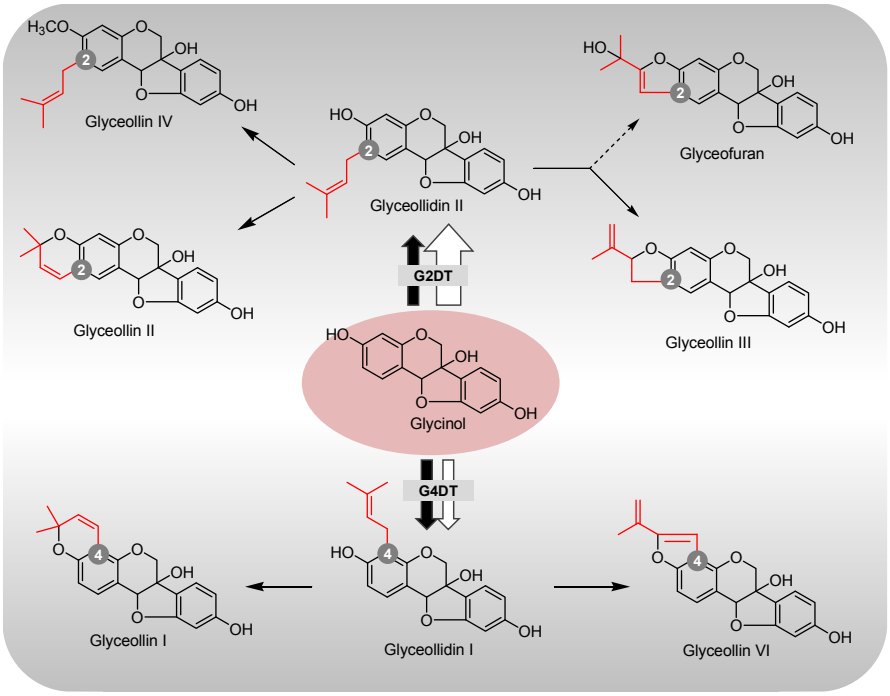


Figure 6. Schematic illustration summarizing the influence of light on the position of prenylation of glycinol in fungus-elicited soybeans. The block arrows with black solid fill (➡) and white solid fill (⇨) represent the flux of prenylated pterocarpan production obtained in dark and light, respectively. The size of the arrow indicates the importance of the flux. Dashed arrows indicate proposed conversions, awaiting further proof.

ACKNOWLEDGEMENTS

This research was financially supported by Directorate General of Higher Education Ministry of National Education of Indonesia. The authors are grateful to Prof. Dr. Michel W.F. Nielen and Ing. Edwin J. Bakx for fruitful discussions on mass spectrometry.

REFERENCES

[1] N. C. Veitch, Isoflavonoids of the Leguminosae, *Natural Product Reports* **2007**, *24*, 417-464.

[2] R. Simons, H. Gruppen, T. F. H. Bovee, M. A. Verbruggen, J.-P. Vincken, Prenylated isoflavonoids from plants as selective estrogen receptor modulators (phytoSERMs), *Food and Function* **2012**, *3*, 810-827.

[3] K. D. R. Setchell, Phytoestrogens: The biochemistry, physiology, and implications for human health of soy isoflavones, *American Journal of Clinical Nutrition* **1998**, *68*, 1333S-1346S.

[4] K. D. R. Setchell, A. Cassidy, Dietary isoflavones: Biological effects and relevance to human health, *Journal of Nutrition* **1999**, *129*, 758S-767S.

[5] R. Simons, J.-P. Vincken, N. Roidos, T. F. H. Bovee, M. Van Iersel, M. A. Verbruggen, H. Gruppen, Increasing soy isoflavonoid content and diversity by simultaneous malting and challenging by a fungus to modulate estrogenicity, *Journal of Agricultural and Food Chemistry* **2011**, *59*, 6748-6758.

[6] S. M. Boue, T. E. Cleveland, C. Carter-Wientjes, B. Y. Shih, D. Bhatnagar, J. M. McLachlan, M. E. Burow, Phytoalexin-enriched functional foods, *Journal of Agricultural and Food Chemistry* **2009**, *57*, 2614-2622.

[7] E. H. Kim, S. H. Kim, J. I. Chung, H. Y. Chi, J. A. Kim, I. M. Chung, Analysis of phenolic compounds and isoflavones in soybean seeds (*Glycine max* (L.) Merill) and sprouts grown under different conditions, *European Food Research and Technology* **2006**, *222*, 201-208.

[8] S. Barnes, M. Kirk, L. Coward, Isoflavones and their conjugates in soy foods: Extraction conditions and analysis by HPLC-mass spectrometry, *Journal of Agricultural and Food Chemistry* **1994**, *42*, 2466-2474.

[9] G. Wang, S. S. Kuan, O. J. Francis, G. M. Ware, A. S. Carman, A simplified HPLC method for the determination of phytoestrogens in soybean and its processed products, *Journal of Agricultural and Food Chemistry* **1990**, *38*, 185-190.

[10] M. Naim, B. Gestetner, S. Zilkah, Y. Birk, A. Bondi, Soybean isoflavones: Characterization, determination, and antifungal activity, *Journal of Agricultural and Food Chemistry* **1974**, *22*, 806-810.

[11] K.-M. Oksman-Caldentey, D. Inzé, Plant cell factories in the post-genomic era: New ways to produce designer secondary metabolites, *Trends in Plant Science* **2004**, *9*, 433-440.

[12] S. J. Lee, J. K. Ahn, S. H. Kim, J. T. Kim, S. J. Han, M. Y. Jung, I. M. Chung, Variation in isoflavone of soybean cultivars with location and storage duration, *Journal of Agricultural and Food Chemistry* **2003**, *51*, 3382-3389.

[13] S. B. Agrawal, S. Singh, M. Agrawal, Ultraviolet-B induced changes in gene expression and antioxidants in plants, in *Advances in Botanical Research, Vol. 52*, **2009**, pp. 47-86.

[14] S. W. Banks, P. M. Dewick, Biosynthesis of glyceollins I, II and III in soybean, *Phytochemistry* **1983**, *22*, 2729-2733.

[15] P. McCue, K. Shetty, Health benefits of soy isoflavonoids and strategies for enhancement: A review, *Critical Reviews in Food Science and Nutrition* **2004**, *44*, 361-367.

[16] M. Montesano, G. Brader, E. T. Palva, Pathogen derived elicitors: Searching for receptors in plants, *Molecular Plant Pathology* **2003**, *4*, 73-79.

[17] A. K. Mishra, K. Sharma, R. S. Misra, Elicitor recognition, signal transduction and induced resistance in plants, *Journal of Plant Interactions* **2012**, *7*, 95-120.

[18] R. A. Dixon, N. L. Paiva, Stress-induced phenylpropanoid metabolism, *Plant Cell* **1995**, *7*, 1085-1097.

[19] R. J. Grayer, T. Kokubun, Plant-fungal interactions: The search for phytoalexins and other antifungal compounds from higher plants, *Phytochemistry* **2001**, *56*, 253-263.

[20] R. L. Lyne, L. J. Mulheirn, Minor pterocarpinoids of soybean, *Tetrahedron Letters* **1978**, *19*, 3127-3128.

[21] M. Yoshikawa, K. Yamauchi, H. Masago, Glyceollin: Its role in restricting fungal growth in resistant soybean hypocotyls infected with *Phytophthora megasperma* var. sojae, *Physiological Plant Pathology* **1978**, *12*, 73-82.

[22] P. Moesta, H. Grisebach, Investigation of the mechanism of glyceollin accumulation in soybean infected by *Phytophthora megasperma* f. sp. *glycinea*, *Archives of Biochemistry and Biophysics* **1981**, *212*, 462-467.

[23] S. M. Boué, C. H. Carter, K. C. Ehrlich, T. E. Cleveland, Induction of the soybean phytoalexins coumestrol and glyceollin by *Aspergillus*, *Journal of Agricultural and Food Chemistry* **2000**, *48*, 2167-2172.

[24] H. J. Kim, H. J. Suh, J. H. Kim, S. C. Kang, S. Park, C. H. Lee, J. S. Kim, Estrogenic activity of glyceollins isolated from soybean elicited with *Aspergillus sojae*, *Journal of Medicinal Food* **2010**, *13*, 382-390.

[25] T. L. Graham, M. Y. Graham, Signaling in soybean phenylpropanoid responses: Dissection of primary, secondary, and conditioning effects of light, wounding, and elicitor treatments, *Plant Physiology* **1996**, *110*, 1123-1133.
[26] E. W. B. Ward, R. I. Buzzell, Influence of light, temperature and wounding on the expression of soybean genes for resistance to *Phytophthora megasperma* f.sp. *glycinea*, *Physiological Plant Pathology* **1983**, *23*, 401-409.
[27] H. Shi, P. K. Nam, Y. Ma, Comprehensive profiling of isoflavones, phytosterols, tocopherols, minerals, crude protein, lipid, and sugar during soybean (*Glycine max*) germination, *Journal of Agricultural and Food Chemistry* **2010**, *58*, 4970-4976.
[28] R. Simons, J.-P. Vincken, M. C. Bohin, T. F. M. Kuijpers, M. A. Verbruggen, H. Gruppen, Identification of prenylated pterocarpans and other isoflavonoids in *Rhizopus* spp. elicited soya bean seedlings by electrospray ionisation mass spectrometry, *Rapid Communications in Mass Spectrometry* **2011**, *25*, 55-65.
[29] P. J. A. Madeira, C. M. Borges, M. H. Florêncio, Electrospray ionization Fourier transform ion cyclotron resonance mass spectrometric and semi-empirical calculations study of five isoflavone aglycones, *Rapid Communications in Mass Spectrometry* **2010**, *24*, 3432-3440.
[30] M. Bustamante-Rangel, M. M. Delgado-Zamarreño, R. Carabias-Martínez, J. Domínguez-Álvarez, Analysis of isoflavones in soy drink by capillary zone electrophoresis coupled with electrospray ionization mass spectrometry, *Analytica Chimica Acta* **2012**, *709*, 113-119.
[31] M. Karni, A. Mandelbaum, The 'even-electron rule', *Organic Mass Spectrometry* **1980**, *15*, 53-64.
[32] Y. Cai, Z. Mo, N. S. Rannulu, B. Guan, S. Kannupal, B. C. Gibb, R. B. Cole, Characterization of an exception to the 'even-electron rule' upon low-energy collision induced decomposition in negative ion electrospray tandem mass spectrometry, *Journal of Mass Spectrometry* **2010**, *45*, 235-240.
[33] S. S. Quadri, R. E. Stratford, S. M. Boué, R. B. Cole, Screening and identification of glyceollins and their metabolites by electrospray ionization tandem mass spectrometry with precursor ion scanning, *Analytical Chemistry* **2013**, *85*, 1727-1733.
[34] U. Zähringer, J. Ebel, L. J. Mulheirn, R. L. Lyne, H. Grisebach, Induction of phytoalexin synthesis in soybean. Dimethylallylpyrophosphate:trihydroxypterocarpan dimethylallyl transferase from elicitor-induced cotyledons, *FEBS Letters* **1979**, *101*, 90-92.
[35] S. M. Boué, F. F. Shih, B. Y. Shih, K. W. Daigle, C. H. Carter-Wientjes, T. E. Cleveland, Effect of biotic elicitors on enrichment of antioxidant properties and induced isoflavones in soybean, *Journal of Food Science* **2008**, *73*, H43-H49.
[36] S. J. Lee, J. K. Ahn, T. D. Khanh, S. C. Chun, S. L. Kim, H. M. Ro, H. K. Song, I. M. Chung, Comparison of isoflavone concentrations in soybean (Glycine max (L.) Merrill) sprouts grown under two different light conditions, *Journal of Agricultural and Food Chemistry* **2007**, *55*, 9415-9421.
[37] P. A. Abbasi, M. Y. Graham, T. L. Graham, Effects of soybean genotype on the glyceollin elicitation competency of cotyledon tissues to *Phytophthora sojae* glucan elicitors, *Physiological and Molecular Plant Pathology* **2001**, *59*, 95-105.
[38] T. Akashi, K. Sasaki, T. Aoki, S. I. Ayabe, K. Yazaki, Molecular cloning and characterization of a cDNA for pterocarpan 4-dimethylallyltransferase catalyzing the key prenylation step in the biosynthesis of glyceollin, a soybean phytoalexin, *Plant Physiology* **2009**, *149*, 683-693.
[39] N. C. Veitch, Isoflavonoids of the Leguminosae, *Natural Product Reports* **2013**, *30*, 988-1027.

Chapter 3

Compositional Changes in (Iso)Flavonoids and Estrogenic Activity of Three Edible *Lupinus* Species by Germination and *Rhizopus*-elicitation

The effects of germination and elicitation on (iso)flavonoid composition of extracts from three edible lupine species (*Lupinus luteus*, *L. albus*, *L. angustifolius*) were determined by RP-UHPLC-MS^n. The total (iso)flavonoid content of lupine increased over 10-fold upon germination, with the total content and composition of isoflavonoids more affected than those of flavonoids. Glycosylated isoflavones were the most predominant compounds found in lupine seedlings. Lesser amounts of isoflavone aglycones, including prenylated ones, were also accumulated. Elicitation with *Rhizopus oryzae*, in addition to germination, raised the content of isoflavonoids further: the total content of 2'-hydroxygenistein derivatives was increased considerably, without increasing that of genistein derivatives. Elicitation by fungus triggered prenylation of isoflavonoids, especially of the 2'-hydroxygenistein derivatives. The preferred positions of prenylation differed among the three lupine species. The change in isoflavone composition increased the agonistic activity of the extracts towards the human estrogen receptors, whereas no antagonistic activity was observed.

Based on: Siti Aisyah, Jean-Paul Vincken, Silvia Andini, Zahara Mardiah and Harry Gruppen, Compositional Changes in (Iso)Flavonoids and Estrogenic Activity of Three Edible *Lupinus* Species by Germination and *Rhizopus*-elicitation, **2015**, *Submitted*.

INTRODUCTION

Isoflavonoids have been associated with several health-promoting effects, including reduced risks of various cancers and alleviating effects of hormone replacement therapy. These effects are (partially) exerted by binding of isoflavonoids to human estrogen receptors (hER).[1] Previously, we have developed an effective method to elevate the isoflavonoid content of soybeans by performing germination and fungal elicitation simultaneously.[2] The treatment increased the total isoflavonoid content of soybeans by up to 2-fold, accompanied by compositional changes. The total amount of prenylated isoflavonoids was boosted up to 13-fold. As a result, up to 50% (w/w) of total isoflavonoids were prenylated pterocarpans, i.e. glyceollins.[2] Glyceollin I, a major prenylated pterocarpan in elicited soybean, has been suggested as a novel therapeutic agent for hormone dependent tumors.[3]

Lupine (*Lupinus*) is a genus of the legume family, consisting of around 200-400 species.[4] Contrary to most genera of legumes, roots and leaves of lupine can produce a variety of prenylated isoflavonoids in addition to glycosylated ones and aglycones.[5, 6] Further investigation on the ability of lupine to generate defense metabolites upon stress showed that lupine failed to induce isoflavonoids other than the constitutive ones.[5] Nevertheless, it has been observed that the isoflavonoid content of *Lupinus angustifolius* can be boosted in response to fungal infection.[7] Moreover, fungal infection is known to increase the ratio of aglycones to glycosylated isoflavonoids of *L. albus,* which was linked to an increase of β-glucosidase activity.[8, 9]

Contrary to studies on the content of isoflavonoids in leaf and root parts, the induction of isoflavonoids in lupine seedlings has not been extensively investigated. Furthermore, most studies focused on aglycones.[10] Additional to soybean, germinated or elicited lupine seeds might be a source of bioactive isoflavonoids. Hence, in the present study, the seeds of three edible lupine species, *L. luteus*, *L. albus* and *L. angustifolius*, were subjected to the process of simultaneous germination and elicitation by fungus, which has been successfully applied to soybean seeds previously.[2, 11, 12] It is hypothesized that isoflavonoid content and molecular diversity of isoflavonoids in lupine seedlings, as well as the estrogenic potential of lupine seedling extracts, will change upon the treatment. Thereby, it will provide novel lead molecules for therapeutic purposes when compared to extracts obtained from soybean seedlings. The compositional changes during treatment were monitored by LC-MS/MS analysis with emphasis on prenylated isoflavonoids. For this, the current diagnostic tools for characterizing prenylation of isoflavonoids in complex extracts were extended using MS/MS fragmentation data.[11, 13, 14]

MATERIALS AND METHODS

Materials

The seeds of three lupine species (*L. albus*, *L. angustifolius*, and *L. luteus*) were purchased from Vreeken's Zaden (Dordrecht, Netherlands). Standards of wighteone and lupiwighteone were purchased from Plantech UK (Berkshire, UK). Daidzein, genistein, L-leucine, L-histidine and 17β-estradiol (E2) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acidified acetonitrile (ACN) (ULC/MS and HPLC-R grade), water (ULC/MS grade), HOAc (ULC/MS grade), acetonitrile (ACN) (ULC/MS and HPLC-R grade), methanol (ULC/MS and HPLC-R grade) and silica gel (60 Å, 70−230 mesh) were purchased from Biosolve BV (Valkenswaard, The Netherlands). Water for other purposes than UHPLC was prepared using a Milli-Q water purification system (Millipore, Molsheim, France). Yeast nitrogen base without amino acids and without ammonium sulphate and agar were obtained from Becton-Dickinson (Franklin Lakes, NJ, USA). The reporter yeast strain was provided by RIKILT (Wageningen, The Netherlands). *Rhizopus oryzae* (LU 581) was kindly provided by the Laboratory of Food Microbiology, Wageningen University, Wageningen, The Netherlands. Other chemicals were purchased from Merck (Darmstadt, Germany) and Sigma Aldrich Chemie (Zwijndrecht, The Netherlands).

Lupine seed treatments

The treatment of lupine seeds was performed in an EQMM sprouting machine (EasyGreen, San Diego, CA, USA), which was modified as described previously.[2] The seeds were sequentially subjected to a soaking (1 d) and a germination (7 d) stage. Prior to soaking, seeds were surface-sterilized by immersing them in a 1% (v/v) hypochlorite solution (5 L/kg beans) for 10 min at room temperature and subsequently rinsed 4 times with Milli-Q water (3 L/kg beans). The sterilized seeds were soaked for 24 h at 25 °C in sterilized Milli-Q water. Subsequently, the soaked seeds were transferred into plastic cartridges (sterilized by soaking them in hypochlorite 1% (v/v) for 2 h, and subsequently rinsing them with Milli-Q water), which were then placed in the sprouting machine. Prior to this, the machine was sterilized according to the cleaning protocol provided by the manufacturer. The lupine seeds were germinated for 7 d at 25 °C and 100% RH. In another set of sprouting experiments, the lupine seeds were also subjected to fungal elicitation. A spore suspension (0.2 mL/g beans) was added to the seeds after 2 d of germination. Spore suspensions for the inoculation stage were prepared from pure plate cultures of *R. oryzae* grown on malt extract agar (CM59; Oxoid, Basingstoke, UK). The sporangia were scraped off from the agar plate and suspended in a 0.85% (w/v) NaCl solution (10^7 CFU/mL). The fungus-elicited seeds were incubated for 5 d at 30 °C and a RH controlled at 55-85%. The seeds were collected after the treatment and directly stored at -20 °C.

Extraction of (iso)flavonoids from lupine seeds

The extracts of untreated and treated lupine seeds were prepared as described previously for soybean,[2] with the modification that 80% (v/v) aqueous methanol (MeOH) was used for extraction of (iso)flavonoids from lupine. For (iso)flavonoid profiling of the lupine, the dried extracts were re-solubilized in 80% (v/v) aqueous methanol to a concentration of 5 mg/mL and subjected to LC-MS/MS analysis. Prior to the estrogenic activity assay, alkaloids were removed from the dried extracts by solid phase extraction (SPE), because lupine is known to contain alkaloids of the quinolizidine group that showed *in-vivo* estrogenic activity in rats.[15, 16] The SPE was performed on a cation-exchange column (Supelclean LC-SCX, 500 mg, Sigma-Aldrich) according to a procedure described elsewhere,[17] with the exception that the extract was solubilized in 0.05 M HCl instead of water. The extracts were solubilized to a concentration of 20 mg/mL. Prior to sample application, the column was pre-conditioned using 5 mL methanol followed by 5 mL water. Samples (2 mL) were percolated through the columns. After washing with water (5 mL), the column was eluted with methanol (5 mL), followed by 2 M ammonium in methanol (10 mL). Both methanol and ammoniated methanol fractions were collected and subsequently evaporated under reduced pressure. The dried fractions were resolubilized to the required concentration in 80% (v/v) aqueous methanol (LC-MS/MS analysis) or DMSO (estrogenicity assay). All samples were centrifuged (18,000 *g*, 5 min; room temperature) prior to analysis. The mass chromatograms of the crude extract from *L. albus* seedlings before SPE, and the alkaloid fraction retained on the column, are shown in **Figure S1** (Supporting Information).

Fractionation of elicited *L. angustifolius* extract

The fungal elicitation process of *L. angustifolius* was scaled up in a two-tank steep germinator (Custom Laboratory Products, Keith, UK). The elicitation process of *L. angustifolius* consisted of the same steps as described for the sprouting machine. The soaking step was performed at 22 ^{o}C, involving three subsequent steps: 6 h immersing in water, 12 h resting in humid air and 6 h immersing in water. The germination step was performed at 22 ºC and a humidity of 95-98%, whereas the elicitation with fungus was performed at 30 ºC and a humidity of 70-90%. The elicited *L. angustifolius* seedlings were freeze-dried and milled (Retsch Ultra Centrifugal Mill ZM 200; Haan, Germany). The dried powder was extracted with 80% (v/v) aqueous methanol with ratio 1:20 (w/v) under influence of sonication at 40 °C for 30 min. The extract was evaporated under reduced pressure and then freeze-dried. A Reveleris Flash system (Grace, Deerfield, IL, USA) was used to obtain fractions enriched in prenylated genistein and prenylated 2'-hydroxygenistein derivatives. To this end, dry extract (250 mg) was mixed with 1.2 g of silica gel. The mixture was transferred into an empty 5 g cartridge and closed with a plunger. The

cartridge was placed upstream of a 12 g Reveleris C18 RP column (particle size 38.6 μm) (Grace Davison Discovery Science, Columbia, MD, USA). Water (Milli-Q) acidified with 1% (v/v) HOAc (HPLC grade) + 1% (v/v) ACN (HPLC grade), eluent A, and methanol (HPLC-grade), eluent B, were used as eluents. The flow rate was 30 mL/min and the experiment was performed at room temperature. The following elution profile was used: 0-1 min, isocratic on 0% B; 1-3 min, linear gradient from 0-60% B; 3-5 min, isocratic on 60% B; 5-6 min, linear gradient from 60-70% B; 6-9 min, isocratic on 70% B; 9-10 min, linear gradient from 70-80% B; 10-13 min, isocratic on 80% B; 13-17 min, linear gradient from 80-100% B. The eluate was monitored at 260 nm. Fractions (10 mL) were analyzed with UHPLC-MS. Fractions containing similar prenylated genistein or prenylated 2'-hydroxygenistein derivatives were pooled. The pools were evaporated under vacuum, frozen and freeze-dried. The pools were solubilized in 80% (v/v) aqueous methanol for analysis with UHPLC-MS and solubilized in DMSO to determine their estrogenicity.

RP-UHPLC-MS analysis

The lupine extracts obtained were analyzed by LC-MS as described previously.[2] Quantification of (iso)flavonoids was performed based on their absorption at 260 nm by means of Xcalibur (version 2.1.0, Thermo Scientific, San Jose, CA, USA). For compounds eluted at the same retention time, the quantification was based on the ratio of intensity of the peaks corresponding to those compounds in full HESI-MS, assuming that no isomers eluted at the same retention time. As for many compounds no commercial standards were available, the amounts of (iso)flavonoid were expressed as mg genistein equivalents per g dry weight (mg GE/g DW). Genistein was used as a generic standard to make a calibration curve with five data points (0.1-0.001 mg/mL, $R^2 = 0.998$).

Determination of estrogenic activity

To determine estrogenic activity of the extracts, a yeast-based bioassay was used[18] with slight modifications as described previously.[19] Dilution series of each sample were prepared in DMSO. The final concentration of DMSO in the assay did not exceed 1% (v/v). A range of 0.1-10 μg/mL was assayed for the estrogenicity of elicited lupine extracts on ERα, whereas a range of 0.01-1 μg/mL was prepared for ERβ. Pools containing prenylated 2'-hydroxygenistein derivatives and prenylated genistein derivatives were tested in the range of 0.0007-0.7 and 0.0005-0.5 μg/mL, respectively, on ERα, and in the range of 0.00007-0.07 and 0.00005-0.05 μg/mL, respectively, on ERβ. EC_{50} calculations were performed in Sigma Plot (8.02, SPSS Inc., Chicago, IL, USA). The yeast-based assay was validated with a dilution series of estradiol. The EC_{50} values of estradiol in the ERα and ERβ bioassay were 0.74 nM and 0.16 nM, respectively, which were in line with those reported previously.[18]

RESULTS

Chromatographic profiling of different lupine extracts

UHPLC-UV analysis of the *L. albus* extract showed that the extract from untreated seeds contained only a single peak (**Figure 1A**), whereas both germination and elicitation by fungus generated an array of compounds (**Figures 1B** and **1C**). The untreated seeds of two other *Lupinus* species contained two peaks, one of which was eluted at the same retention time as that of *L. albus* (data not shown). Both other species showed a similar response to the treatments (germination and elicitation) as *L. albus*, although they accumulated different sets of compounds (**Figures 1D** and **1E**). In total, sixty-one peaks were used for further analysis. Within each chromatogram, the peaks analyzed represented more than 95% of the total UV response at 260 nm of the chromatogram. The identification showed that the (iso)flavonoids identified belong to the flavone, flavanone, isoflavone and coumaronochromone subclasses. The molecules were present in aglyconic, glycosylated and prenylated forms. The identification of aglycones and glycosylated derivatives was based on comparison of spectral data obtained from UHPLC-UV-ESI-MS/MS (including fragmentation patterns) with literature data (**Table 1**).[20-30] Spectral analysis of LC-MS/MS data of the majority of prenylated derivatives, not previously described in the literature, is elaborated upon in the present study.

C- and *O*-glycosides of (Iso)flavonoids

Twenty-seven non-prenylated (iso)flavonoids glycosides were tentatively identified in treated *Lupinus*, comprising the flavone, flavanone and isoflavone subclasses. The different subclasses were discriminated on the basis of UV absorption. Typical λ_{max} values of 270 (±5) and 330-365 nm for flavones, 290 (±5) nm for flavanones, and 260 (±5) nm for isoflavones were observed.[31] The glycosylated derivatives had one to three glycosyl residues, some of which were malonylated. The glycosyl residues found previously in lupine were mainly glucosyl, xylosyl, and rhamnosyl, attached to hydroxyl groups or directly to a *C*-atom.[21] In the present study, the *C*- and *O*-glycosylated (iso)flavonoids were distinguished by their characteristic fragmentation patterns resulting from the cleavage of the sugar moieties in MS^2.[20, 32] Neutral losses of 162/146/132 Da are ascribed to *O*-glucoside/*O*-rhamnoside/*O*-xyloside residues, respectively, whereas neutral losses of 90 and 120 Da are ascribed to *C*-glucosides of (iso)flavonoids in PI or NI mode.[20, 32, 33] Moreover, neutral losses of 150/164/180 Da in NI mode are attributed to *O*,*C*-diglycosides, namely *O*-xylosyl-*C*-glucosyl/ *O*-rhamnosyl-*C*-glucosyl/ *O*-glucosyl-*C*-glucosyl(iso)flavonoids, respectively.[33] A high relative abundance of these neutral losses was ascribed to the attachment of one of these glycosyl residues at the 2"-OH of the *C*-

glucosyl residue.[33] In addition, a malonyl group was identified by neutral losses of 44 or 86 Da, in NI or PI mode, respectively.[21, 23]

Prenyl configuration of isoflavonoids in extracts of lupine seedlings

Twenty-five prenylated isoflavonoids were found in treated lupines, including prenylated isoflavonoid glycosides. The UV spectra indicated that most of the prenylated derivatives were isoflavones. Two compounds were identified as prenylated coumaronochromones (**58** and **59**), which were distinguished from isoflavones by their UV spectrum, exhibiting λ_{max} values of around 257 (±2), 284 (±2) (*sh*), and 338 (±3) nm.[14] The prenyl group can be attached in different configurations to the A- and/or B-rings (**Table 2**). A neutral loss of 56 Da (C_4H_8) was used to distinguish a prenyl chain from a ring-closed prenyl in PI mode.[13, 34] Major neutral losses of 42 Da (C_3H_6) and, to a lesser extent, 70 Da ($C_3H_6 + H_2O$), 54 Da (C_4H_6) and 15 Da (CH_3•) were used to identify both 2,2-dimethylpyran and 2-isopropenyldihydrofuran rings.[13] Neutral losses of 18 Da (H_2O) and 72 Da (C_4H_8O) were indicative of a 2-(1-hydroxy-1-methylethyl)-dihydrofuran ring.[13] Based on these neutral losses, peaks **41**, **45**, **47**, **51**-**55** and **57**-**59** were classified as prenyl chain isoflavonoid derivatives. It was impossible to distinguish between isoflavonoid isomers with a pyran or furan substituent by mass spectrometry, as both of them provided almost the same fragmentation patterns. Nevertheless, these isomers were tentatively assigned on the basis of the literature, accounting for their elution behavior and their abundance in previous studies.[6, 22, 35-38] Peaks **42** and **48**-**50** were assigned as 2,2-dimethylpyran isoflavonoids, whereas **33** and **46** were assigned as 2-isopropenyl dihydrofuran and 2-(1-hydroxy-1-methylethyl)-dihydrofuran isoflavonoid, respectively. Moreover, lupine generated diprenylated isoflavonoids as well. The fragmentation behavior of diprenylated isoflavonoid was similar to that of monoprenylated isoflavonoid, only the diagnostic losses occurred twice: in MS^2 and MS^3.[39] As a result, peaks **56** and **61** were annotated as isoflavonoids containing two prenyl chains, whereas peak **60** was assigned as an isoflavonoid containing a prenyl chain and a pyran-ring. In addition, five out of twenty-five prenylated isoflavonoids were in *O*-glycosylated form (peaks **13**, **26**, **30**, **34**, and **35**), no *C*-glycosylated form was found. This is consistent with the fact that prenylation and *C*-glycosylation can occur at the same positions. The type of prenyl attached to these glycosides of prenylated isoflavonoids can be determined from fragmentation of the aglycone product ions in either in MS^3 or MS^4. As a result, all glycosides of prenylated isoflavonoids were annotated as isoflavonoids containing a prenyl chain.

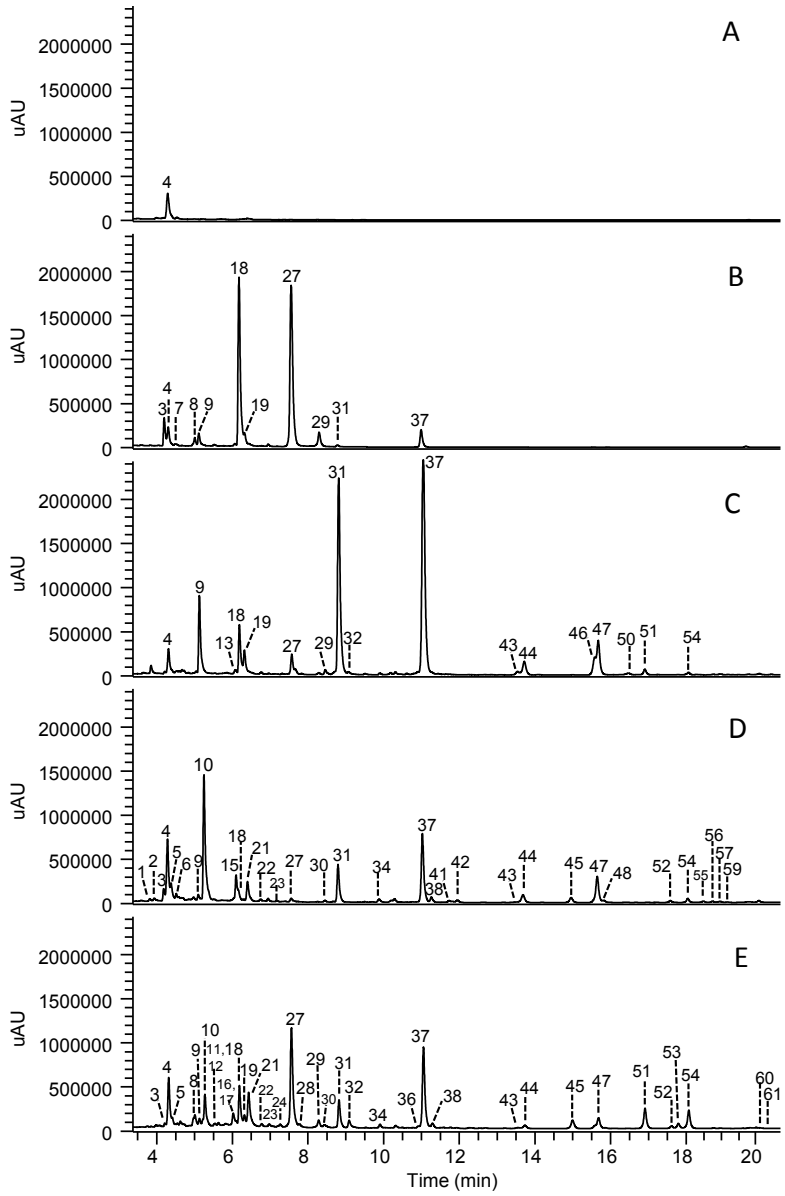


Figure 1. RP-UHPLC-UV profiles at 260 nm of 80% (*v/v*) aqueous methanol of a crude extract of untreated *L. albus* (**A**), germinated *L. albus* (**B**), elicited *L. albus* (**C**), elicited *L.* luteus (**D**) and elicited *L. angustifolius* (**E**). Peak numbers refer to compounds in **Table 1**.

Table 1. Compounds tentatively identified by RP-UHPLC-PDA-MS in *Lupinus* extracts.

No[a]	t_R (min)	λ_{max}[b] (nm)	Tentative compound[c]	$[M+H]^+$	MS^2 PI mode (rel. abundance)	MS^3 PI mode (rel. abundance)	$[M-H]^-$	MS^2 NI mode (rel. abundance)	MS^3 NI mode (rel. abundance)
Isoflavones									
1	3.85	257	2'-Hydroxygenistein 4',7-*O*-diglucoside	611	287 (100), 449 (97)	217 (100), 259 (67), 245 (66), 153 (59), 231 (30), 269 (17), 175 (16), 161 (14), 149 (13)	609	447 (100), 489 (12), 471 (11), 285 (9)	285 (100), 379 (17), 217 (8)
2	3.96	260	Genistein 8-*C*-7-*O*-diglucoside	595	433 (100), 415 (12)	415 (100), 397 (24), 367 (24), 337 (11), 313 (21)	593	473 (100)	445 (100), 310 (86), 282 (43), 311 (36), 283 (13),
3	4.20	259	Genistein 4',7-*O*-diglucoside	595	271 (100), 433 (53)	153 (100), 215 (78), 253 (37), 159 (18), 187 (10)	593	431 (100)	269 (100)
6	4.53	260	2'-Hydroxygenistein 8-*C*-glucoside	449	431 (100), 329 (36), 413 (29), 311 (29), 383 (16)	413 (100), 383 (64), 311 (60), 299 (32), 353 (24), 395 (15)	447	327 (100), 357 (34), 429 (5)	299 (100), 259 (55), 217 (43), 241 (37), 255 (34), 193 (18), 231 (15), 257 (13), 201 (13), 213 (11)
8	5.00	259	Genistein 4'-*O*-glucoside-7-*O*-(6"-*O*-malonyl) glucoside	681	271 (100), 519 (52), 433 (37)	215 (100), 243 (91), 253 (41), 187 (12), 225 (11)	679	431 (100)	268 (100), 269 (30), 341 (4)
9	5.10	257	2'-Hydroxygenistein 7-*O*-glucoside	449	287 (100)	217 (100), 259 (67), 245 (66), 153 (59), 231 (30), 269 (17), 175 (16), 161 (14), 149 (13)	447	285 (100), 327 (28), 309 (17), 387 (4)	217 (100), 241 (12), 199 (11), 151 (9)
10	5.25	261	Genistein 8-*C*-glucoside	433	415 (100), 367 (15), 397 (15), 313 (14)	397 (100), 367 (96), 337 (75), 295 (37), 379 (16)	431	311 (100)	283 (100)
12	5.54	261, 283	2'-Hydroxygenistein 8-*C*-(6"-*O*-malonyl) glucoside	535	517 (100), 329 (78), 311 (11)	311 (100), 499 (31), 353 (29), 473 (26), 455 (11), 395 (11)	nr[d]	-	-
13	6.07	259	Luteone 4',7-*O*-diglucosides	679	517 (100), 461 (8)	461 (100), 355 (51), 299 (20)	677	515 (100), 353 (19), 557 (12)	353 (100), 446 (6)
14	6.07	260	Genistein 4', 7-*O*-diglucoside dimalonylated	767	519 (100), 271 (73), 681 (11)	271 (100), 433 (10)	nr[d]	-	-
18	6.17	260	Genistein 7-*O*-glucoside	433	271 (100)	153 (100), 215 (78), 243 (76), 253 (35), 149 (27), 145 (19), 159 (17)	431	268 (100), 269 (88), 311 (14), 341 (5)	269 (100), 224 (43), 240 (32), 226 (12)
19	6.31	258	2'-Hydroxygenistein 7-*O*-(6"-*O*-malonyl) glucoside	535	287 (100), 449 (10), 517 (1), 491 (1)	153 (100), 269 (78), 217 (61), 259 (37), 245 (36), 231 (20), 175 (11)	**533**, 489[e]	285 (100), 309 (42), 327 (37), 339 (9), 471 (5), 369 (1)	217 (100), 151 (54), 241 (23), 199 (13), 257 (7), 243 (4)

21	6.46	261	Genistein 8-*C*-(6"-*O*-malonyl) glucoside	519	501 (100) 313 (86), 295 (14), 483 (11)	295 (100), 483 (37), 337 (30), 457 (17), 439 (14)	**517**, 473[e]	413 (100), 311 (47)	311 (100), 353 (83), 323 (62), 341 (52), 281 (31), 325 (25), 307 (24), 269 (19), 297 (15)
22	6.77	261	Genistein 6-*C*-(6"-*O*-malonyl) glucoside	519	501 (100), 397 (57), 367 (57), 295 (56), 313 (49), 379 (34), 423 (33), 283 (26), 457 (15), 337 (14)	99 (100)	**517**, 473[e]	413 (100), 311 (11)	323 (100), 335 (12), 295 (11), 269 (10), 395 (4)
23	6.92	257	Genistein 4'-*O*-glucoside	433	271 (100)	243 (100), 215 (90), 153 (71), 253 (61)	431	268 (100), 269 (37), 341 (4), 323 (2)	224 (100), 240 (79), 226 (33), 212 (22), 196 (10), 250 (3)
26	7.40	261	Luteone 4',7-*O*-diglucoside malonylated	765	603 (100), 559 (9)	559 (100), 355 (51), 517 (41), 547 (41), 503 (26), 299 (21), 585 (13)	763	557 (100), 599 (11), 353 (9)	353 (100), 352 (17), 488 (14)
27	7.54	260	Genistein 7-*O*-(6"-*O*-malonyl) glucoside	519	271 (100), 433 (10)	215 (100), 153 (51), 149 (43), 243 (41), 272 (38), 253 (22), 225 (10)	**517**, 269[e]	269 (100), 225 (24)	270 (100), 269 (94), 225 (35), 181 (10)
29	8.26	260	Genistein 4'-*O*-(6"-*O*-malonyl) glucoside	519	271 (100), 475 (5)	153 (100), 243 (51), 149 (37), 253 (24), 145 (15), 225 (11), 229 (8), 203 (5)	517	270 (100), 225 (92), 201 (33), 227 (31), 181 (27), 241 (19), 149 (9), 151 (12)	226 (100), 198 (32), 228 (29), 202 (29), 182 (28), 251 (17), 184 (14), 242 (13)
30	8.39	261	Luteone 7-*O*-glucoside	517	355 (100), 299 (22) , 461 (2)	299 (100)	515	353 (100)	284 (100), 309 (93), 285 (87), 219 (66), 298 (43), 267 (43), 325 (28), 201 (27), 199 (20), 151 (15), 310 (13), 175 (11)
31	8.74	258	2'-Hydroxygenistein	287	217 (100), 259 (63), 153 (61), 245 (60), 231 (30), 175 (17), 269 (16), 161 (14), 203 (10)	189 (100)	285	217 (100), 241 (7), 199 (7), 175 (3), 257 (2)	173 (100), 175 (62), 189 (29), 199 (18), 161 (6), 149 (4), 131 (2), 109 (1)
33	9.82	256	Lupinisoflavone B	371	353 (100), 299 (59), 354 (17), 311 (15), 300 (10)	219 (100), 335 (75), 311 (75), 299 (40), 325 (40), 283 (19), 338 (10)	369	299 (100), 351 (15), 311 (10), 235 (10),	231 (100), 213 (19), 255 (15)
34	9.82	256	Wighteone 7-*O*-glucoside	501	339 (100)	283 (100)	499	337 (100), 336 (72)	282 (100), 293 (43), 281 (9)
35	10.38	261	Licoisoflavone A 7-*O*-glucoside	517	461 (100), 355 (69), 299 (20), 462 (5)	299 (100)	515	353 (100)	219 (100), 309 (92), 325 (34), 285 (22), 284 (20), 201 (17), 298 (17), 267 (15), 310 (15), 297 (11),

37	10.94	260	Genistein	271	153 (100), 243 (69), 215 (67), 253 (31), 149 (26), 145 (20)	127 (100), 111 (59), 124 (51)	269	225 (100), 181 (69), 201 (58), 241 (38), 224 (36), 197 (34), 227 (24), 169 (19)	181 (100), 197 (52), 196 (27), 183 (17), 210 (11), 180 (10), 169 (10), 151 (2)
39	11.63	257, 372	Orobol	287	269 (46), 259 (33), 258 (33), 241 (100), 231 (43),213 (67), 165 (79), 153 (45)	213 (100)	285	267 (59), 257 (81), 256 (24), 243 (50), 241 (62), 229 (62), 213 (63), 185 (41), 169 (49), 151 (100)	107 (100)
40	11.64	265, 357	3'-*O*-Methylorobol	301	286 (100)	258 (100)	299	256 (100), 257 (57), 285 (28), 229 (14), 241 (13), 213 (12), 239 (10), 151 (20)	229 (100), 228 (62), 212 (45), 213 (28), 211 (20), 227 (16), 163 (14)
41	11.72	262	Barpisoflavone B	369	313 (100)	178 (100), 295 (27), 179 (7), 241 (7), 298 (4), 296 (4), 109 (4), 149 (3)	367	323 (100), 298 (13), 233 (1)	308 (100), 255 (17), 280 (14), 267 (4)
42	11.85	264	Barpisoflavone C	367	337 (100), 349 (49), 233 (11), 352 (9)	203 (100), 319 (24)	365	231 (100), 350 (63), 349 (35), 321 (24), 333 (23), 322 (21), 337 (19), 306 (14), 347 (11)	216 (100), 163 (59), 187 (48), 131 (30), 198 (23), 172 (17), 155 (7)
45	14.79	264	2,3-Dehydrokievitone	355	299 (100)	165 (100), 281 (20), 257 (13), 229 (11)	353	284 (100), 285 (99), 298 (31), 267 (34), 309 (26), 151 (7)	267 (100), 216 (13), 256 (9), 230 (8), 242 (4)
46	15.44	262	Lupinisoflavone A	353	219 (100), 335 (77), 299 (62), 311 (47), 325 (36), 283 (23), 297 (10), 338 (9)	177 (100), 201 (30), 191 (28), 165 (12), 151 (7), 163 (5), 204 (4), 133 (2)	351	217 (100), 307 (55), 323 (27), 265 (18), 201 (13), 333 (12), 283 (8), 309 (4)	173 (100), 147 (34), 175 (25), 149 (17), 131 (11), 189 (2), 129 (2)
47	15.50	264	Luteone	355	299 (100)	165 (100), 281 (52), 183 (9)	353	309 (100), 219 (61), 285 (47), 325 (36), 310 (31), 297 (12), 335 (11)	265 (100), 267 (58), 281 (41), 291 (34), 294 (23), 199 (22), 241 (19), 263 (12)
48	15.68	266	Parvisoflavone A	353	219 (100), 335 (82), 283 (34), 325 (25), 311 (16), 338 (14), 297 (11)	177 (100), 201 (33), 191 (23), 123 (13), 109 (13), 165 (5), 204 (4), 163 (4)	nr[d]	-	-
49	16.31	267	Parvisoflavone B	353	219 (100), 335 (87), 299 (63), 311 (52), 325 (34), 283 (21), 297 (14)	177 (100), 191 (30), 201 (29), 123 (13), 109 (11), 165 (11), 204 (4), 163 (4)	351	217 (100), 307 (78), 323 (48), 201 (25), 265 (22), 333 (20)	173 (100), 175 (36), 149 (24), 131 (18)

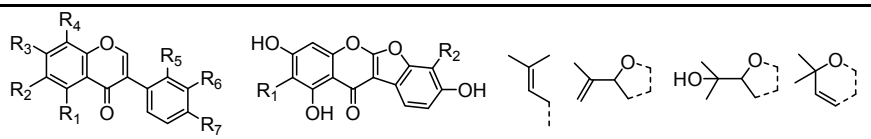
50	16.59	263	Licoisoflavone B	353	311 (100), 325 (33), 299 (23), 335 (20), 283 (6)	283 (100), 255 (49), 153 (20)	351	283 (100), 265 (13), 307 (12), 336 (3)	239 (100), 175 (32), 255 (16), 265 (15), 241 (11), 268 (9)
51	16.77	262	Licoisoflavone A	355	299 (100)	271 (100), 147 (58), 243 (45), 245 (40), 281 (32), 217 (29), 253 (26), 153 (25), 191 (24), 173 (20)	353	285 (100), 284 (59), 267 (17)	216 (100), 241 (13), 256 (11)
52	17.45	264	Lupiwighteone	339	283 (100)	241 (100), 213 (10), 255 (9), 265 (2)	337	282 (100), 309 (2), 175 (2)	253 (100), 238 (39), 189 (20), 264 (16), 267 (12), 161 (4)
53	17.65	261	Isowighteone	339	283 (100)	255 (100)	337	281 (100), 282 (22), 294 (11), 293 (11)	237 (100), 267 (91), 253 (95), 209 (84), 213 (43), 239 (43), 225 (41), 238 (40), 254 (23), 252 (20), 281 (16)
54	17.92	264	Wighteone	339	283 (100)	255 (100), 165 (98), 121 (24), 227 (17), 265 (13), 199 (13)	337	282 (100), 322 (3),	254 (100), 238 (40), 267 (9), 226 (6)
55	18.32	267	Lupisoflavone	369	313 (100)	297 (100), 269 (15)	367	352 (100)	309 (100), 297 (21), 324 (17), 284 (5)
56	19.34	268	8-Prenylluteone	423	367 (100)	311 (100), 233 (2)	421	287 (100), 309 (57), 377 (45), 219 (36), 393 (30), 366 (27), 267 (27), 201 (26), 323 (19), 335 (14), 353 (12)	243 (100), 219 (21), 259 (9), 201 (9)
57	19.58	257	5-Methylwighteone	353	297 (100)	255 (100), 273 (29), 227 (56), 241 (22)	351	323 (100), 307 (69), 295 (24), 283 (13), 309 (11), 336 (6)	279 (100), 305 (76), 149 (75), 268 (44), 280 (39), 295 (35), 255 (33), 281 (28), 175 (15), 267 (6)
60	20.64	266	Angustone B	421	365 (100)	165 (100), 347 (65), 219 (49), 311 (39), 201 (39), 337 (30), 323 (26)	419	350 (100), 375 (53), 351 (30), 401 (23), 269 (21), 267 (17), 391 (17), 404 (12)	305 (100), 216 (94), 306 (54), 322 (46), 241 (36), 307 (30), 244 (29)
61	20.71	267	Angustone A	423	367 (100)	311 (100)	421	352 (100), 377 (20), 353 (20), 219 (17), 267 (13), 269 (12)	

Coumaronochromones									
44	13.53	256, 281*sh*, 334	Lupinalbin A	285	257 (100), 213 (12), 229 (6), 241 (4)	229 (100)	283	255 (100), 239 (27), 265 (19), 227 (2), 195 (1)	227 (100), 211 (9), 237 (3)
58	19.72	257, 284*sh*, 338	Lupinalbin B/D	353	297 (100), 285 (86), 298 (16), 269 (13)	269 (100), 270 (13), 241 (16)	351	323 (100), 295 (48), 307 (21), 333 (18), 324 (13), 296 (13), 334 (11)	295 (100)
59	19.81	257, 339	Lupinalbin B/D	353	297 (100)	269 (100), 241 (40), 213 (22), 297 (15)	351	323 (100), 351 (55), 295 (46), 307 (20), 283 (10)	295 (100), 279 (8), 267 (8)
Flavones									
4	4.30	271, 335	Apigenin *C*-(2"-*O*-xylosyl) glucoside- *C*-glucoside	727	577 (100), 595 (53), 709 (41), 457 (31), 607 (24), 475 (24), 559 (20)	457 (100), 559 (56), 499 (29), 511 (21), 541 (20), 481 (20)	725	605 (100), 455 (26), 575 (32), 635 (15)	455 (100), 335 (41), 473 (29), 383 (26), 485 (21)
5	4.39	271, 334	Apigenin 7-*O*-glucosyl-glucoside	595	271 (100), 433 (53)	153 (100), 243 (76), 215 (72), 253 (32), 227 (3)	593	268 (100), 403 (5)	240 (100), 224 (97), 212 (18)
7	4.53	270, 338	Apigenin 6-*C*-glucoside 8*C*- (2"-*O*-rhamnosyl) glucoside	741	577 (100), 723 (55), 595 (33), 457 (31), 621 (26), 475 (24), 603 (23), 705 (11)	457 (100), 559 (53), 499 (30), 511 (23), 481 (21), 541 (20), 529 (17), 523 (15), 427 (11), 409 (11)	739	619 (100), 575 (90), 455 (69), 649 (17), 335 (15), 485 (11), 721 (3)	455 (100), 335 (30), 499 (16)
15	6.09	267, 337	Apigenin 7-*O*-rhamnosylglucoside	579	271 (100), 433 (13)	153 (100), 225 (58), 229 (44), 203 (44), 145 (35), 271 (20)	577	269 (100)	225 (100), 197 (25), 149 (24), 269 (22), 201 (20), 181 (16), 182 (15), 151 (14)
17	6.15	259, 340	Apigenin 7-*O*-xylosylglucoside	565	433 (100), 271 (70)	271 (100)	563	269 (100), 431 (17), 269 (5), 443 (4)	225 (100), 197 (25), 227 (22), 149 (19), 183 (18), 201 (17), 151 (12), 181 (11)
20	6.45	256, 331	Luteolin *O*-xylosylglucoside malonylated	667	635 (100), 452 (12), 510 (11), 617 (10)	452 (100), 617 (53), 478 (35), 401 (22)	665	633 (100), 508 (65), 647 (58), 605 (49), 615 (17), 476 (10)	-
25	7.27	254, 339	Acacetin *O*-glucoside	447	285 (100)	257 (100), 213 (10)	445	282 (100)	254 (100), 238 (67)
28	7.74	255, 344	Chrysoeriol 3-*O*-(6"-*O*-malonyl) glucoside	549	301 (100), 463 (26), 505 (6)	286 (100)	547	503 (100), 299 (31)	299 (100)
16	6.15	259, 340	Chrysoeriol *O*-xylosylglucoside	595	463 (44), 301 (100)	286 (100)	593	299 (100)	284 (100)
24	7.22	256, 344	Chrysoeriol 3-*O*-xylosyl -(6"-*O*-malonyl) glucoside	681	301 (100), 549 (65), 595 (11), 286 (9)	286 (100)	679	635 (100)	299 (100), 593 (16), 284 (14)

32	9.00	257	Luteolin	287	153 (100), 269 (62), 241 (48), 259 (45), 231 (37), 258 (30), 217 (29), 245 (14), 149 (15), 161 (15), 224 (11)	67 (100), 129 (28), 114 (29), 127 (16), 123 (13),	285	257 (100), 256 (59), 241 (14), 217 (57)	-
36	10.84	255, 346	Chrysoeriol	301	286 (100)	258 (100)	299	284 (100)	256 (100), 255 (49), 240 (22), 228 (16), 227 (29), 216 (13), 212 (37)
38	11.17	262, 342	Apigenin	271	153 (100), 243 (24), 215 (18), 145 (14), 253 (12), 149 (6)	129 (100), 111 (67)	269	225 (100), 149 (35), 201 (24), 151 (22), 227 (16), 183 (12), 181 (11)	181 (100), 183 (43), 197 (41), 169 (12)
11	5.54	261, 285	Eriodictyol *O*-(6"-*O*-malonyl) glucoside	537	519 (100), 289 (62), 401 (53), 501 (21), 331 (15), 271 (9)	271 (100), 501 (17)	535	287 (100), 463 (62), 259 (17)	259 (100), 243 (18), 269 (6), 201 (5)
Chromone									
43	13.36	256	Lupichromone	315	283 (100), 287 (15)	255 (100)	313	298 (100)	242 (100), 270 (92), 281 (91), 269 (32), 226 (27), 198 (24)

[a] Numbers refer to peaks in **Figure 1**.
[b] *sh*: Shoulder.
[c] Compounds with name underlined are prenylated.
[d] nr: Not relevant, no good mass spectrometric data were obtained in NI mode.
[e] The parent ion showed in-source fragmentation in NI mode MS. The bold *m/z* represents the [M-H]- ion.

Table 2. Structures of tentatively identified prenylated isoflavonoids found in the *Lupinus* extracts.



Isoflavone (IF) Coumaronochromone (CC) Prenyl-C Prenyl-F1 Prenyl-F2 Prenyl-P

No[a]	Moiety	R_1	R_2	R_3[b]	R_4	R_5	R_6	R_7[b]
13	IF	OH	prenyl-C	*O*-Glc	H	OH	H	*O*-Glc
26	IF	OH	prenyl-C	*O*-Glc-mal	H	OH	H	*O*-Glc
30	IF	OH	prenyl-C	*O*-Glc	H	OH	H	OH
33	IF	OH	prenyl-F2		H	OH	H	OH
34	IF	OH	prenyl-C	*O*-Glc	H	H	H	OH
35	IF	OH	H	*O*-Glc	H	OH	prenyl-C	OH
41	IF	OCH_3	H	OH	prenyl-C	OH	H	OH
42	IF	OCH_3	H	prenyl-P		OH	H	OH
45	IF	OH	H	OH	prenyl-C	OH	H	OH
46	IF	OH	prenyl-F1		H	OH	H	OH
47	IF	OH	prenyl-C	OH	H	OH	H	OH
48	IF	OH	H	prenyl-P		OH	H	OH
49	IF	OH	prenyl-P		H	OH	H	OH
50	IF	OH	H	OH	H	OH	prenyl-P	
51	IF	OH	H	OH	H	OH	prenyl-C	OH
52	IF	OH	H	OH	prenyl-C	H	H	OH
53	IF	OH	H	OH	H	H	prenyl-C	OH
54	IF	OH	prenyl-C	OH	H	H	H	OH
55	IF	OH	prenyl-C	OH	H	H	OCH_3	OH
56	IF	OH	prenyl-C	OH	prenyl-C	OH	H	OH
57	IF	OCH_3	H	OH	prenyl-C	H	H	OH
58/59	CC	H	prenyl-C	-	-	-	-	-
58/59	CC	prenyl-C	H	-	-	-	-	-
60	IF	OH	prenyl-C	OH	H	OH	prenyl-P	
61	IF	OH	prenyl-C	OH	H	OH	prenyl-C	OH

[a] Number refers to compounds in **Table 1**.
[b] Standard three-letter codes for monosaccharide and malonyl are used.

A- or B-ring prenylation in isoflavonoids

Analysis of *retro*-Diels-Alder (RDA) fragment ions in PI mode from isoflavone isomers was used to determine the position of the prenyl substituent (A- or B-ring).[26] RDA fragments diagnostic for A- or B-ring prenylation with a prenyl chain were obtained upon fragmentation of the ion $[M+H-C_4H_8]^+$ in MS^3 (**Figures 2A** and **2B**). The diagnostic fragments $^{1,3}A^+–C_4H_8$ and $^{1,3}B^+–C_4H_8$ still contained one carbon reminiscent of the prenyl chain.[13, 36] On the other hand, RDA fragmentation of isoflavones with a ring-closed prenyl moiety mostly occurred upon MS^2 fragmentation of the parent ion with the prenyl substituent still intact, yielding the diagnostic $^{1,3}A^+$ and $^{1,3}B^+$ RDA fragments (**Figure 2C**). As a result, it is concluded that all monoprenylated isoflavones were A-ring prenylated, except **35**, **50**, **51** and **53** that were B-ring prenylated. Similarly, RDA fragmentation of diprenylated isoflavones with two chains was obtained upon fragmentation of the ion $[M+H-2C_4H_8]^+$ in MS^4, whereas RDA fragmentation of diprenylated isoflavones with a chain and a ring-closed was performed on the $[M+H-C_4H_8]^+$ ion in MS^3. As a consequence, peak **56** was annotated as diprenylated isoflavone with two A-ring prenyl chains, whereas peaks **60** and **61** were annotated as diprenylated isoflavones with an A-ring prenyl chain plus a B-ring ring-closed prenyl, and with an A-ring plus B-ring prenyl chain, respectively.

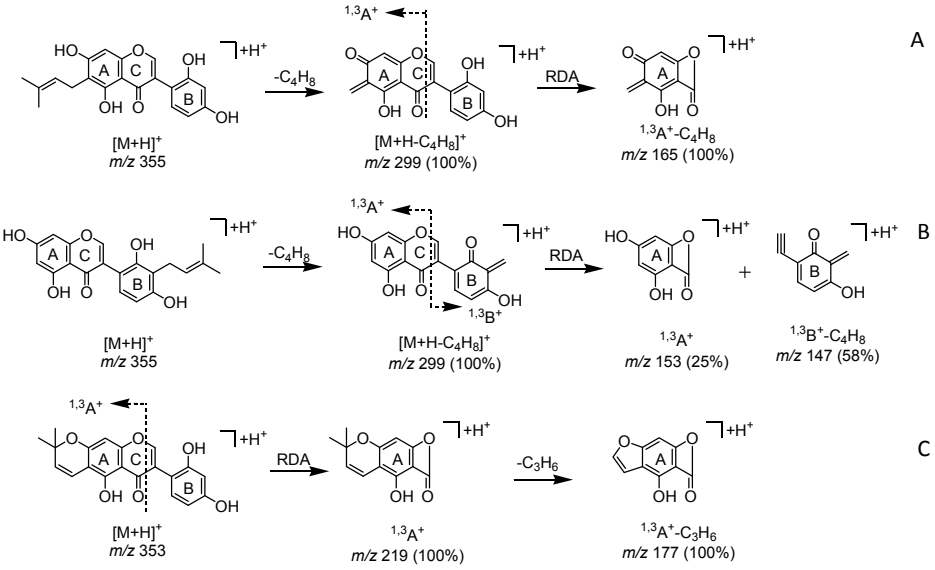


Figure 2. Proposed RDA-fragmentation pattern of luteone (**A**), licoisoflavone A (**B**) and parvisoflavone B (**C**) in PI mode.

Different to prenylated isoflavones, two prenylated coumaronochromones (**58** and **59**) showed almost no RDA fragmentation. This observation was different to a previous report showing that RDA fragmentation of some monoprenylated coumaronochromones occurred at positions 0/4, albeit with low relative intensity (<1-10%). Nevertheless, our observation corresponded with the fragmentation behavior of other prenylated coumaronochromones, such as lupinalbin D and E, that did not show RDA fragmentation in that study.[40] Hence, the prenyl positions of coumaronochromones were not determined.

Position of prenylation within the A-ring of isoflavones

With respect to A-ring prenylation, two positional isomers are possible, i.e. attachment of the prenyl substituent to the *C*-6 or to the *C*-8 position. We investigated the fragmentation behavior of two authentic standards, wighteone and lupiwighteone, which are chain-prenylated at *C*-6 and *C*-8, respectively. In PI mode MS^2, both compounds produced $[M+H-C_4H_8]^+$ with *m/z* 283 at similar relative intensities. In MS^3, the $[M+H-C_4H_8]^+$ ion of the two compounds fragmented differently. The *C*-8 prenylated isoflavone almost exclusively yielded a fragment ion with a neutral loss of 42 Da (C_2H_2O), whereas the *C*-6 prenylated isoflavone gave that fragment ion with less than 1% relative intensity (**Figure 3**). Therefore, we considered the fragment ion $[M+H-C_4H_8-C_2H_2O]^+$ in MS^3 diagnostic for distinguishing *C*-6 and *C*-8 chain-prenylation. This principle was applied to the extracts of elicited lupine seedlings. As a result, peaks **47**, **54** and **55** were classified as *C*-6 chain-prenylated isoflavones, whereas **41**, **45**, **52** and **57** were classified as *C*-8 chain-prenylated isoflavones. For glycosides of prenylated isoflavones, the fragmentations of the aglycone product ions in MS^3 or MS^4 of PI mode were compared to their respective prenylated isoflavone and used to determine the chain-prenyl position. Accordingly, peaks **13**, **26**, **30** and **34** were classified as *C*-6 chain-prenylated isoflavones.

As for the position of ring-closed prenylation (pyran, furan), it was observed that the neutral loss of 54 Da in either PI mode MS^2 or MS^3 was found in relatively high abundance (intensity of $[M+H-54]^+$ >20%) with some molecules and in lower abundance (intensity of $[M+H-54]^+$ <5%) with others, putatively diagnostic for *C*-6 or *C*-8 ring-closed prenylation, respectively. Similar observations were done before for closed-ring prenylated flavones/flavonols.[39] Consequently, peaks **33**, **46** and **49** were identified as *C*-6 ring-closed prenylated isoflavones, whereas peaks **42** and **48** were annotated as *C*-8 ring-closed prenylated isoflavones.

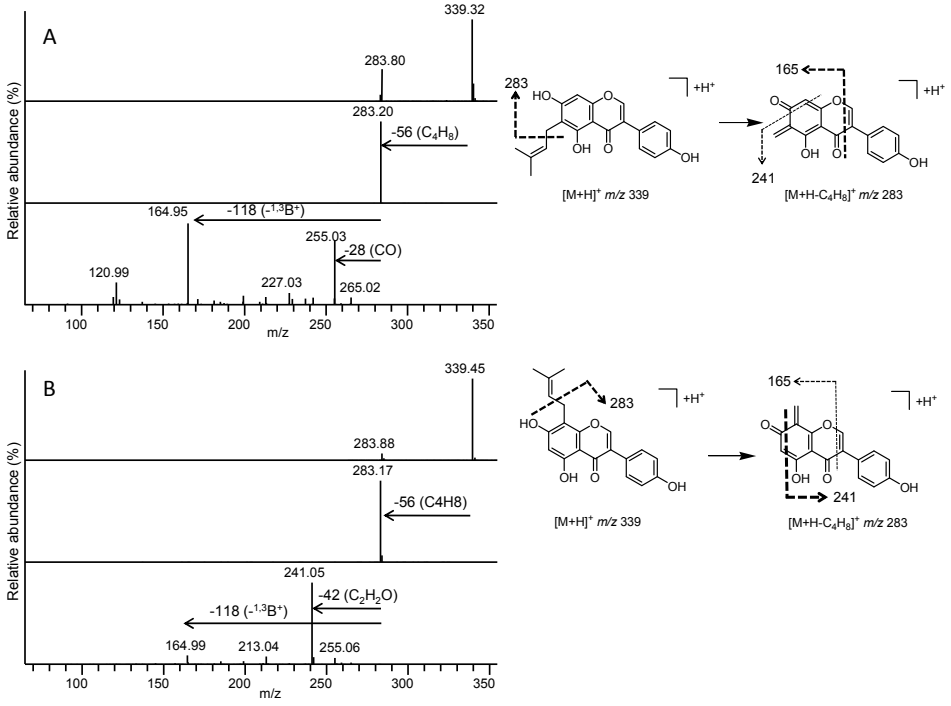


Figure 3. Full ESI-MS, MS^2 and MS^3 spectra of *m/z* 339 in PI mode and proposed cleavage of wighteone (**A**) and lupiwighteone (**B**). Bold dashed arrows indicate cleavage, yielding product ions with relative abundance over 50%.

Content and composition of (iso)flavonoids of lupine seedlings

The (iso)flavonoid profiles of the three *Lupinus* species changed extensively upon germination. The total flavonoid and isoflavonoid content increased from 0.28-0.46 mg GE/g DW to 4.11-5.27 mg GE/g DW, dominated by glycosylated derivatives, which accounted for up to 90% (w/w) of total (iso)flavonoids (**Table S1** in Supporting Information). A more than 300-fold increase was observed for total isoflavonoid content, whereas only a 3-fold increase was noted for total flavonoid content. The total isoflavonoid content reached up to 3.45-4.85 mg GE/g DW. Isoflavone was the predominant isoflavonoid subclass found in lupine seedlings, mainly consisting of genistein derivatives and smaller amounts of 2'-hydroxygenistein derivatives. The genistein derivatives were dominated by their glycosylated forms, up to 90% (w/w). Typically, *O*-glycosylated isoflavones were accumulated in all lupine seedlings, whereas *C*-glycosylated isoflavones were absent in *L. albus* seedlings.

The total flavonoid content increased after germination, from 0.28-0.46 to 0.42-1.08 mg GE/g DW, mainly caused by accumulation of glycosylated compounds (more than 95% (w/w) of total flavonoids) (**Table S1** in Supporting Information). Flavone and, to a lesser extent, flavanone were the main flavonoid subclasses found in lupine seedlings, with *C*-glycosylated apigenin derivatives as the predominant compounds. Small amounts of flavonoid aglycones were found in lupine seedlings, prenylated flavonoids were not detected.

Content and composition of (iso)flavonoids of elicited lupine seedlings

Compared to the lupine seedlings, the total flavonoid and isoflavonoid content of elicited lupine increased from 4.11-5.27 up to 5.99-8.78 mg GE/g DW (**Table S2** in Supporting Information). This was caused by the accumulation of isoflavonoids, particularly isoflavones, as the flavonoid content was not considerably altered. The fungal elicitation modified the total content and relative abundance of the group of genistein derivatives and the group of 2'-hydroxygenistein derivatives, to different extents. The total content of 2'-hydroxygenistein derivatives of elicited lupine seedlings increased 6 to 20 times compared to that in non-elicited lupine seedlings, whereas the total amount of genistein derivatives remained the same. Nevertheless, the relative abundance of genistein derivatives changed during fungal elicitation just as that of the 2'-hydroxygenistein derivatives. The content of *O*-glycosylated genistein derivatives decreased, coinciding with the increase of genistein aglycone (**Table S1** in Supporting Information). It is worth noting that no decrease in the amount of *C*-glycosylated genistein derivatives was detected.

The change in the total content and relative abundance of isoflavones was different amongst the three lupine species. The increase in total amount of 2'-hydroxygenistein derivatives in *L. albus* was much more pronounced than that in the two other species. Surprisingly, the level of prenylated 2'-hydroxygenistein derivatives was almost the same in all species. The decrease in the content of *O*-glycosylated genistein derivatives was higher in *L. albus* than in the other species. As a result, the elicited *L. albus* contained the highest amount of the aglycone genistein. On the contrary, the highest content of prenylated genistein derivatives was observed in *L. angustifolius* (**Table S1** in Supporting Information).

Estrogenic activity of lupine extracts and fractions

The extracts from elicited lupine seedlings that were free from alkaloids were tested for the estrogenic activity towards ERα or ERβ. Results were expressed with the DMSO control set to 0% and 0.2 nM 17β-estradiol set to 100% (**Figure 4**). The extracts tested showed a clear dose-dependent agonistic activity towards both ERα and ERβ. The estrogenic activity towards ERβ was around 10-fold higher than towards ERα. Of the extracts analyzed, the elicited *L. albus* extract showed the highest estrogenic potency towards both ERs (**Figures**

4). The extracts showed no anti-estrogenic activity (data not shown). In addition, no estrogenic activity of extracts from untreated lupine seeds and alkaloid fractions was observed (data not shown).

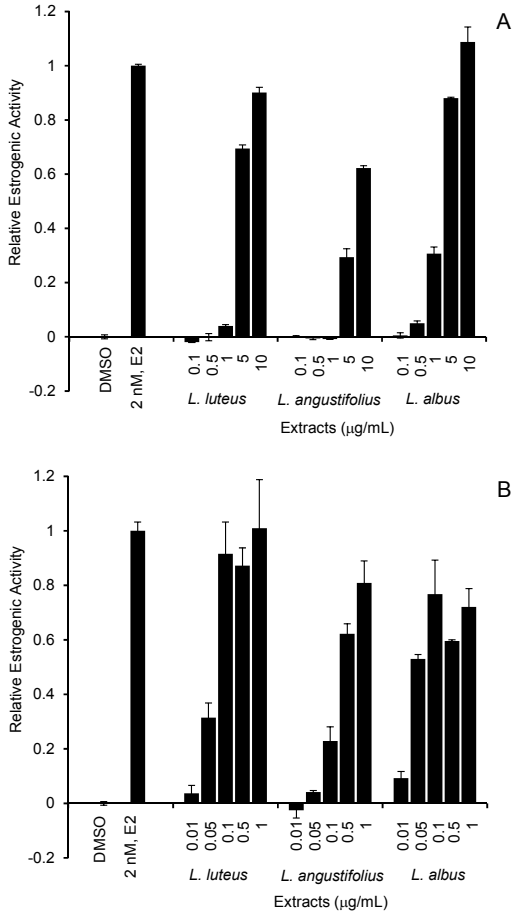


Figure 4. Estrogenicity of extracts of fungus-elicited lupine seedlings towards ERα (**A**) and ERβ (**B**). The maximum ER response with estradiol was expressed as 1.0, and the relative estrogenic activity was calculated as a ratio of this.

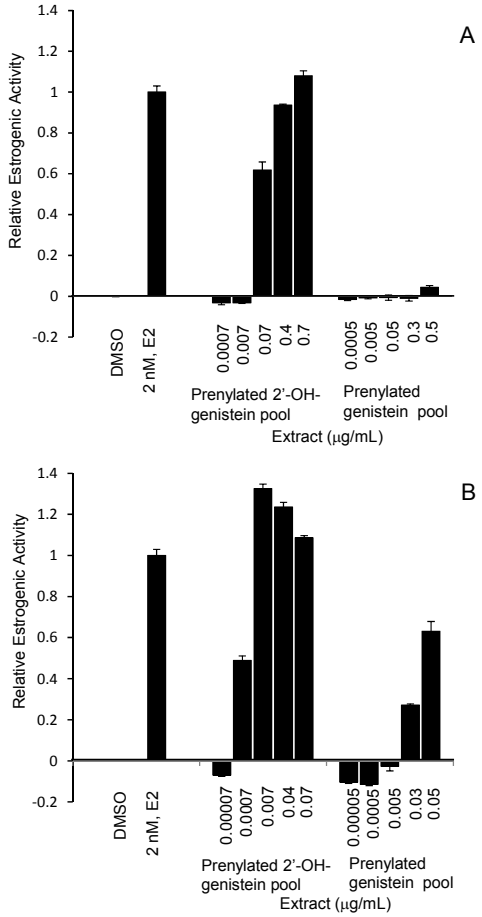


Figure 5. Estrogenicity towards ERα (**A**) and ERβ (**B**) of fractions enriched in prenylated derivatives of 2'-hydroxygenistein and in prenylated derivatives of genistein from an extract of elicited *L. angustifolius* seedlings. The maximum ER response with estradiol was expressed as 1.0, and the relative estrogenic activity was calculated as a ratio of this.

To investigate the estrogenicity of prenylated isoflavones, the extract of elicited *L. angustifolius* was fractionated using Flash chromatography. All fractions were analyzed by UHPLC-MS. Two pools enriched in either prenylated genistein or prenylated 2'-hydroxygenistein derivatives were made based on the compositional analysis. The pool of prenylated genistein derivatives mainly consisted of wighteone (29%), whereas the pool of prenylated 2'-hydroxygenistein derivatives mainly consisted of luteone (32%) and 2,3-dehydrokievitone (25%) (**Table S2** in Supporting Information). The two pools were tested

for (anti)estrogenic activity towards ERα or ERβ. The pool of prenylated 2'-hydroxygenistein derivatives showed a ~70-fold higher estrogenic activity towards ERβ than that of prenylated genistein derivatives (**Figure 5B**). The estrogenic activity of prenylated 2'-hydroxygenistein derivatives towards ERα was ~100-fold lower than that towards ERβ, whereas no estrogenic activity of prenylated genistein derivatives towards ERα was observed at the concentrations tested (**Figure 5A**). No anti-estrogenic activity was observed for either fraction (data not shown).

DISCUSSION

Germination alone or in combination with elicitation by fungus altered the total content and composition of (iso)flavonoids in seed(ling)s of the three lupine species studied. The total content of (iso)flavonoids increased after 7 days of germination, and increased further when the germination was performed in the presence of fungus. During the treatments, isoflavonoids appeared much more inducible than flavonoids, and the compositional changes in isoflavonoids were species-dependent. Moreover, these compositional changes affected the estrogenic activity of the extracts from the elicited lupine seedlings.

Elicitation of seedlings by fungus induces accumulation of particularly 2'-hydroxygenistein derivatives

Elicitation by fungus increased the total content of 2'-hydroxygenistein derivatives of lupine seedlings, whereas no such increase was observed for that of genistein derivatives (**Figure 6**). The accumulation of the 2'-hydroxygenistein derivatives was most prominent with *L. albus*, whereas the other two lupine species showed a two-fold lower increase. The pool of 2'-hydroxygenistein derivatives did not seem to grow at the expense of that of genistein derivatives during elicitation, suggesting *de novo* synthesis of these isoflavonoids. Nevertheless, compositional changes in the pool of genistein derivatives occurred, i.e. due to deglycosylation of the *O*-glycosides and, to lesser extent, to prenylation. It has previously been shown that elicitation by fungus increased β-glucosidase activity, evidenced by a higher amount of aglycones, which is in line with our results.[8, 9] The higher β-glucosidase activity in *L. albus* than in the other two species is in accordance with a previous report stating that the increase of β-glucosidase activity in *L. albus* leaves after infection with *Pleiochaeta setosa* was higher than that in *L. angustifolius*.[9] No β-glucosidase activity in *L. luteus* has been reported. As expected, the amount of isoflavone *C*-glycosides remained constant, as β-glucosidase is not known to cleave carbon-carbon linkages.

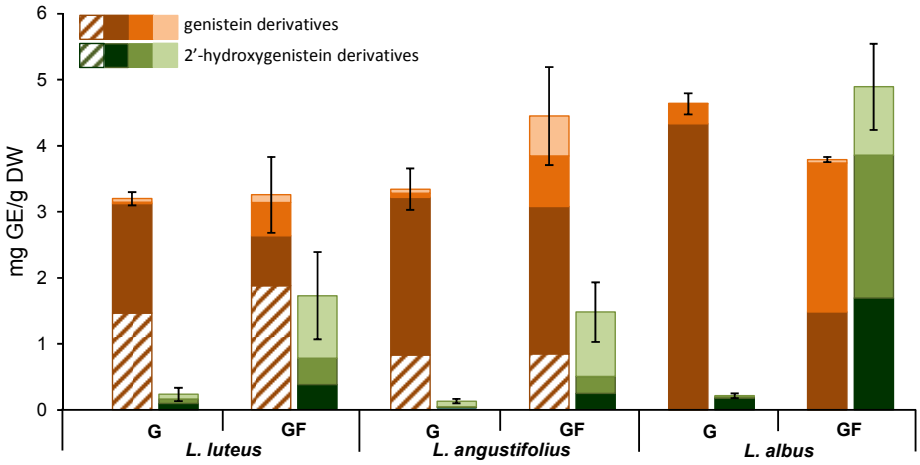


Figure 6. Total contents of genistein derivatives and 2'-hydroxygenistein derivatives of germinated seeds (G) and *Rhizopus*-elicited seedlings (GF) of three lupine species. The color shades indicate from darkest to lightest, *O*-glycosylated, aglycone, and prenylated isoflavonoids (including glycosides of prenylated isoflavonoids). The hatched color pattern indicates *C*-glycosylated compounds. Data are expressed in mg genistein equivalent (GE) per g dry weight (DW) of *Lupinus*. Data are the mean value of measurements performed in duplicate. Error bars shows standard deviation of total content genistein derivatives and 2'-hydroxygenistein derivatives.

Prenylation features in three lupine species compared

Accumulation of prenylated isoflavones was more pronounced during elicitation when compared to germination. Except in *L. angustifolius*, 2'-hydroxygenistein seemed to be the preferred substrate for prenyltransferases (**Figure 7**), as prenylated 2'-hydroxygenistein was more abundant in *L. albus* and *L. luteus* than prenylated genistein, despite the lower abundance of its respective unprenylated precursor. This is consistent with the fact that the isoflavonoid prenyltransferase *LaPT1* isolated from *L. albus* is 20% more active towards 2'-hydroxygenistein than towards genistein,[41] although no such data were available for isoflavone prenyltransferase from either *L. angustifolius* or *L. luteus*. It might be speculated that the prenyltransferase(s) from *L. angustifolius* are more promiscuous with respect to their acceptor substrates (genistein and 2'-hydroxygenistein) than that (those) of *L. luteus* and *L. albus*.

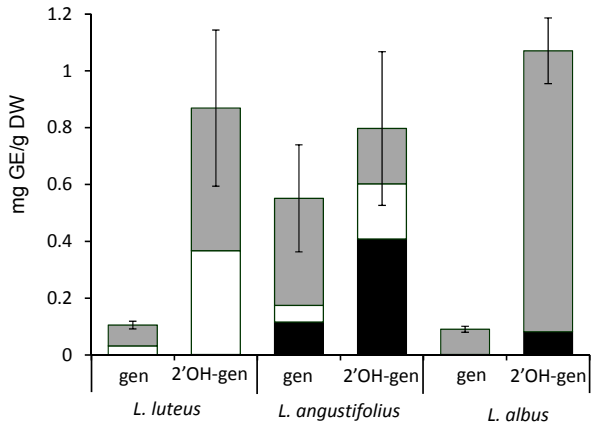


Figure 7. The composition of prenylated genistein (gen) and prenylated 2'-hydroxygenistein (2'OH-gen) of elicited lupine seedlings. The three prenyl positions, *C*-6, *C*-8 and *C*-3', were indicated by different shading patterns, grey, white and black, respectively. All different kinds of prenylation (chain, pyran, and furan) were accounted for. Data are the mean value of measurements performed in duplicate. Error bars shows standard deviation of total content of prenylated genistein and 2'-hydroxygenistein derivatives.

Another interesting finding was that the pattern of isoflavonoid prenylation differed among the three lupine species (**Figure 7**). *L. angustifolius* isoflavones had the most versatile prenylation pattern, with *C*-6:*C*-8:*C*-3' equalling 7:1:2 and 1:1:2, for prenylated genistein and 2'-hydroxygenistein, respectively. With *L. albus* and *L. luteus*, prenylation seemed to be more specific with one (mainly *C*-6) or two (*C*-6, and to a lesser extent *C*-8) preferred positions for prenylation, respectively. From various reports on *L. albus* in the literature, it might be deduced that each of the three positions for attachment of a prenyl chain requires a distinct prenyltransferase.[41, 42] Our results also hint at specific prenyltransferases for each position, the expression of which seems to be lupine species-dependent. Nevertheless, in-depth biochemical characterization of these prenyltransferases is required to establish such region-specificity further.

Besides the position of prenylation, also the extent of the different kinds of prenylation varied among the three lupine species. The ratio of chain : pyran : furan prenylation was approximately 13:3:1 for *L. luteus*, 1:0:0 for *L. angustifoliu*s, and 4:0:1 for *L. albus*. This suggests that the occurrence of cyclization reactions subsequent to the actual prenylation step can differ among the three species.

Accumulation of phytoestrogens during elicitation of seedlings by fungus

The estrogenic activity of extracts from lupine seed(ling)s increased after elicitation by fungus. The *L. albus* extract displayed the highest estrogenic activity on both estrogen receptors, followed by the *L. luteus* and the *L. angustifolius* extracts. The *L. albus* extract contained the largest amount of aglycones (**Figure 6**), i.e. genistein and 2'-hydroxygenistein, which might explain its higher estrogenic activity.[1, 43] Nevertheless, the relationship of estrogenic potential of the extracts and their isoflavonoid composition might be more complex than proportion of aglycones. The prenylation pattern of isoflavonoids differed between extracts, and it is not unlikely that this influences the estrogenic activity.[1] Besides, *C*-glycosylated isoflavonoids (e.g. puerarin) are known to have estrogenic activity,[44] but it is unknown whether this also holds for the isoflavonoid *C*-glycosides from lupine. More in-depth studies with purified compounds are required to establish this further.

This study showed that the process of simultaneous germination and elicitation with fungus, successful in inducing estrogenic isoflavonoids in soybean seedlings, can be extrapolated to lupine seedlings for similar purpose. The estrogenic activity of extracts from lupine and soybean seedlings was comparable. Nevertheless, the set of molecules induced in elicited lupine seedlings differed from that in elicited soybean seedlings. In lupine, the biosynthesis of isoflavonoids comes to a hold at a relatively early stage, i.e. 2'-hydroxygenistein, whereas that of elicited soybeans continues to 6a-hydroxypterocarpans and coumestans.[2, 12] Besides different skeletons (and therewith planarity), also the prenylation patterns between lupine and soybean are different, in that lupine showed more chain prenylation than soybean (pyran and single prenyl). Therefore, prenylated isoflavonoids from lupine might be an interesting complementary set of molecules to those from soybean in studying modulation of estrogenic responses. In this respect, it is required to determine EC_{50} and IC_{50} values of purified molecules in different bioassays (e.g. yeast assay used in this study, and MCF-7 cells), in order to determine whether the molecules show agonistic, antagonistic or SERM behavior.[1]

ACKNOWLEDGEMENTS

This research was financially supported by Directorate General of Higher Education Ministry of National Education of Indonesia. We would like to thank Dr. Toine Bovee of RIKILT-Institute of Food Safety for providing the yeast estrogen bioassays, Monique Bettonvil for the preliminary experiments with *Lupinus* and Milou van de Schans, Carla Araya Cloutier and Wouter de Bruijn for fruitful discussions.

REFERENCES

[1] R. Simons, H. Gruppen, T. F. H. Bovee, M. A. Verbruggen, J.-P. Vincken, Prenylated isoflavonoids from plants as selective estrogen receptor modulators (phytoSERMs), *Food and Function* **2012**, *3*, 810-827.

[2] S. Aisyah, H. Gruppen, B. Madzora, J.-P. Vincken, Modulation of isoflavonoid composition of *Rhizopus oryzae* elicited soybean (*Glycine max*) seedlings by light and wounding, *Journal of Agricultural and Food Chemistry* **2013**, *61*, 8657-8667.

[3] M. C. Zimmermann, S. L. Tilghman, S. M. Boué, V. A. Salvo, S. Elliott, K. Y. Williams, E. V. Skripnikova, H. Ashe, F. Payton-Stewart, L. Vanhoy-Rhodes, J. P. Fonseca, C. Corbitt, B. M. Collins-Burow, M. H. Howell, M. Lacey, B. Y. Shih, C. Carter-Wientjes, T. E. Cleveland, J. A. McLachlan, T. E. Wiese, B. S. Beckman, M. E. Burow, Glyceollin I, a novel antiestrogenic phytoalexin isolated from activated soy, *Journal of Pharmacology and Experimental Therapeutics* **2010**, *332*, 35-45.

[4] C. A. Williams, A. Demissie, J. B. Harborne, Flavonoids as taxonomic markers in old world *Lupinus* species, *Biochemical Systematics and Ecology* **1983**, *11*, 221-231.

[5] J. B. Harborne, J. L. Ingham, L. King, M. Payne, The isopentenyl isoflavone luteone as a pre-infectional antifungal agent in the genus *Lupinus*, *Phytochemistry* **1976**, *15*, 1485-1487.

[6] S. Tahara, J. L. Ingham, S. Nakahara, J. Mizutani, J. B. Harborne, Fungitoxic dihydrofuranoisoflavones and related compounds in white lupin, *Lupinus albus*, *Phytochemistry* **1984**, *23*, 1889-1900.

[7] A. Wojakowska, D. Muth, D. Narozna, C. Madrzak, M. Stobiecki, P. Kachlicki, Changes of phenolic secondary metabolite profiles in the reaction of narrow leaf lupin (*Lupinus angustifolius*) plants to infections with *Colletotrichum lupini* fungus or treatment with its toxin, *Metabolomics* **2013**, *9*, 575-589.

[8] M. Pîslewska, P. Bednarek, M. Stobiecki, M. Zielínaka, P. Wojtaszek, Cell wall-associated isoflavonoids and β-glucosidase activity in *Lupinus albus* plants responding to environmental stimuli, *Plant, Cell and Environment* **2002**, *25*, 29-40.

[9] M. Rybus-Zajqc, M. Kozlłowska, Activity of β-D-glucosidase of lupin leaves infected with *Pleiochaeta setosa* (Kirchn.) Hughes, *Acta Physiologiae Plantarum* **1996**, *18*, 211-216.

[10] P. Wojtaszek, M. Stobiecki, Differential secretion and accumulation of isoflavonoids in *Lupinus albus* in response to fungal elicitor and $CuCl_2$, *Plant Physiology and Biochemistry* **1997**, *35*, 129-135.

[11] R. Simons, J.-P. Vincken, M. C. Bohin, T. F. M. Kuijpers, M. A. Verbruggen, H. Gruppen, Identification of prenylated pterocarpans and other isoflavonoids in *Rhizopus* spp. elicited soya bean seedlings by electrospray ionisation mass spectrometry, *Rapid Communications in Mass Spectrometry* **2011**, *25*, 55-65.

[12] R. Simons, J.-P. Vincken, N. Roidos, T. F. H. Bovee, M. Van Iersel, M. A. Verbruggen, H. Gruppen, Increasing soy isoflavonoid content and diversity by simultaneous malting and challenging by a fungus to modulate estrogenicity, *Journal of Agricultural and Food Chemistry* **2011**, *59*, 6748-6758.

[13] M.-J. Xu, B. Wu, T. Ding, J.-H. Chu, C.-Y. Li, J. Zhang, T. Wu, J. Wu, S.-J. Liu, S.-L. Liu, W.-Z. Ju, P. Li, Simultaneous characterization of prenylated flavonoids and isoflavonoids in *Psoralea corylifolia* L. by liquid chromatography with diode-array detection and quadrupole time-of-flight mass spectrometry, *Rapid Communications in Mass Spectrometry* **2012**, *26*, 2343-2358.

[14] S. Tahara, J. L. Ingham, J. Mizutani, New coumaronochromones from white Lupin, *Lupinus albus* L. (Leguminosae), *Agricultural and Biological Chemistry* **1985**, *49*, 1775-1783.

[15] M. De Cortes Sánchez, P. Altares, M. M. Pedrosa, C. Burbano, C. Cuadrado, C. Goyoaga, M. Muzquiz, C. Jiménez-Martínez, G. Dávila-Ortiz, Alkaloid variation during germination in different lupin species, *Food Chemistry* **2005**, *90*, 347-355.

[16] M. Elghamry, I. Shihata, Estimation of estrogenic and toxic properties of *Lupinus termis* in mice, *Planta Medica* **1964**, *12*, 155-159.

[17] T. Aniszewski, D. Ciesiołka, K. Gulewicz, Equilibrium between basic nitrogen compounds in lupin seeds with differentiated alkaloid content, *Phytochemistry* **2001**, *57*, 43-50.

[18] T. F. H. Bovee, R. J. R. Helsdingen, I. M. C. M. Rietjens, J. Keijer, R. L. A. P. Hoogenboom, Rapid yeast estrogen bioassays stably expressing human estrogen receptors α and β, and green fluorescent protein: a comparison of different compounds with both receptor types, *The Journal of Steroid Biochemistry and Molecular Biology* **2004**, *91*, 99-109.

[19] M. G. M. van de Schans, J.-P. Vincken, T. F. H. Bovee, A. David Cervantes, M. J. Logtenberg, H. Gruppen, Structural changes of 6a-hydroxy-pterocarpans upon heating modulate their estrogenicity, *Journal of Agricultural and Food Chemistry* **2014**, *62*, 10475-10484.

[20] P. Kachlicki, Ł. Marczak, L. Kerhoas, J. Einhorn, M. Stobiecki, Profiling isoflavone conjugates in root extracts of lupine species with LC/ESI/MSn systems, *Journal of Mass Spectrometry* **2005**, *40*, 1088-1103.

[21] A. Wojakowska, A. Piasecka, P. M. García-López, F. Zamora-Natera, P. Krajewski, L. Marczak, P. Kachlicki, M. Stobiecki, Structural analysis and profiling of phenolic secondary metabolites of Mexican lupine species using LC-MS techniques, *Phytochemistry* **2013**, *92*, 71-86.

[22] M. Stobiecki, C. Malosse, L. Kerhoas, P. Wojlaszek, J. Einhorn, Detection of isoflavonoids and their glycosides by liquid chromatography/electrospray ionization mass spectrometry in root extracts of lupin (*Lupinus albus*), *Phytochemical Analysis* **1999**, *10*, 198-207.

[23] D. Muth, E. Marfisden-Edwards, P. Kachlicki, M. Stobiecki, Differentiation of isomeric malonylated flavonoid glyconjugates in plant extracts with LC-ESI/MS/MS, *Phytochemical Analysis* **2008**, *19*, 444-452.

[24] R. Frański, P. Bednarek, D. Siatkowska, P. Wojtaszek, M. Stobiecki, Application of mass spectrometry to structural identification of flavonoid monoglycosides isolated from shoot of lupin (*Lupinus luteus* L.), *Acta Biochimica Polonica* **1999**, *46*, 459-473.

[25] R. Frański, P. Bednarek, P. Wojtaszek, M. Stobiecki, Identification of flavonoid diglycosides in yellow lupin (*L. luteus* L.) with mass spectrometric techniques, *Journal of Mass Spectrometry* **1999**, *34*, 486-495.

[26] F. Cuyckens, M. Claeys, Mass spectrometry in the structural analysis of flavonoids, *Journal of Mass Spectrometry* **2004**, *39*, 1-15.

[27] F. Kuhn, M. Oehme, F. Romero, E. Abou-Mansour, R. Tabacchi, Differentiation of isomeric flavone/isoflavone aglycones by MS^2 ion trap mass spectrometry and a double neutral loss of CO, *Rapid Communications in Mass Spectrometry* **2003**, *17*, 1941-1949.

[28] P. Bednarek, L. Kerhoas, J. Einhorn, R. Frański, P. Wojtaszek, M. Rybus-Zając, M. Stobiecki, Profiling of flavonoid conjugates in *Lupinus albus* and *Lupinus angustifolius* responding to biotic and abiotic stimuli, *Journal of Chemical Ecology* **2003**, *29*, 1127-1142.

[29] N. C. Veitch, Isoflavonoids of the Leguminosae, *Natural Product Reports* **2009**, *26*, 776-802.

[30] N. C. Veitch, Isoflavonoids of the Leguminosae, *Natural Product Reports* **2013**, *30*, 988-1027.

[31] A. Marston, K. Hostettmann, Separation and quantification of flavonoids, in *Flavonoids: Chemistry, biochemistry and applications* (Eds.: O. M. Andersen, K. R. Markham), CRC Press Boca Raton, FL, USA, **2006**, pp. 1-36.

[32] P. Kachlicki, J. Einhorn, D. Muth, L. Kerhoas, M. Stobiecki, Evaluation of glycosylation and malonylation patterns in flavonoid glycosides during LC/MS/MS metabolite profiling, *Journal of Mass Spectrometry* **2008**, *43*, 572-586.

[33] F. Ferreres, A. Gil-Izquierdo, P. B. Andrade, P. Valentão, F. A. Tomás-Barberán, Characterization of *C*-glycosyl flavones *O*-glycosylated by liquid chromatography-tandem mass spectrometry, *Journal of Chromatography A* **2007**, *1161*, 214-223.

[34] R. Simons, J.-P. Vincken, E. J. Bakx, M. A. Verbruggen, H. Gruppen, A rapid screening method for prenylated flavonoids with ultra-high-performance liquid chromatography/electrospray ionisation mass spectrometry in licorice root extracts, *Rapid Communications in Mass Spectrometry* **2009**, *23*, 3083-3093.

[35] S. Tahara, Y. Hashidoko, J. L. Ingham, J. Mizutani, New 5-0-methylisoflavones in the roots of yellow lupin (*Lupinus luteus* L. cv. Barpine), *Agricultural and Biological Chemistry* **1986**, *50*, 1809-1819.

[36] Y. Hashidoko, S. Tahara, J. Mizutani, New complex isoflavones in the roots of yellow lupin (*Lupinus luteus* L., cv. Barpine) *Agricultural and Biological Chemistry* **1986**, *50*, 1797-1807.

[37] S. Tahara, Y. Katagiri, J. L. Ingham, J. Mizutani, Prenylated flavonoids in the roots of yellow lupin, *Phytochemistry* **1994**, *36*, 1261-1271.

[38] S. Tahara, S. Orihara, J. L. Ingham, J. Mizutani, Seventeen isoflavonoids from *Lupinus albus* roots, *Phytochemistry* **1989**, *28*, 901-911.

[39] M. Takayama, T. Fukai, Y. Hano, T. Nomura, Mass spectrometry of prenylated flavonoids, *Heterocycles* **1992**, *33*, 405-434.

[40] F. Hanawa, S. Tahara, J. Mizutani, Retro-Diels-Alder fragmentation of coumaronochromones and complete assignments ^{13}C NMR data for lupinalbin A and ayamenin B, *Heterocycles* **1991**, *32*, 1563-1570.

[41] G. Shen, D. Huhman, Z. Lei, J. Snyder, L. W. Sumner, R. A. Dixon, Characterization of an isoflavonoid-specific prenyltransferase from *Lupinus albus*, *Plant Physiology* **2012**, *159*, 70-80.

[42] P. Laflamme, H. Khouri, P. Gulick, R. Ibrahim, Enzymatic prenylation of isoflavones in white lupin, *Phytochemistry* **1993**, *34*, 147-151.

[43] S. B. Ateba, D. Njamen, S. Medjakovic, M. Zehl, H. Kaehlig, A. Jungbauer, L. Krenn, Lupinalbin A as the most potent estrogen receptor alpha-and aryl hydrocarbon receptor agonist in *Eriosema laurentii* de Wild. (Leguminosae), *BMC complementary and alternative medicine* **2014**, *14*, 294-303.

[44] Y. P. Hwang, H. G. Jeong, Mechanism of phytoestrogen puerarin-mediated cytoprotection following oxidative injury: Estrogen receptor-dependent up-regulation of PI3K/Akt and HO-1, *Toxicology and Applied Pharmacology* **2008**, *233*, 371-381.

Supporting Information

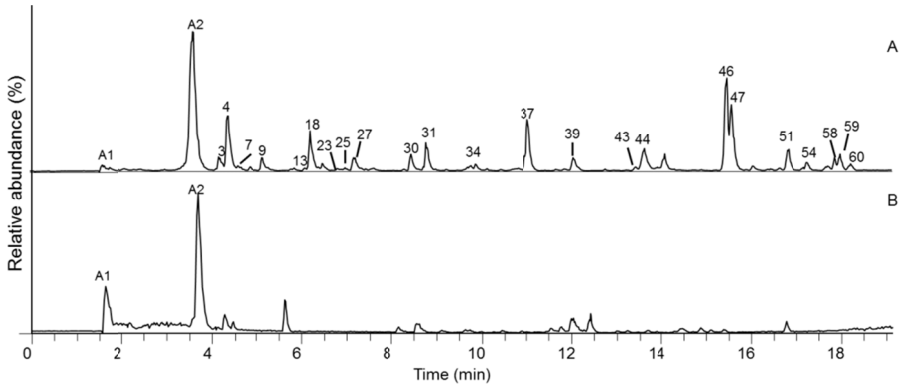


Figure S1. Mass chromatogram of the crude extract of *L. albus* seedlings before SPE (**A**) and the alkaloid fraction retained on the column (**B**). Peak numbers refer to compounds in **Table 1**. Alkaloids are labelled A1 and A2.

Table S1. Contents[a] of (iso)flavonoids in *Lupinus* extracts.

Compounds	Prenyl position	*L. luteus*			*L. angustifolius*			*L. albus*		
		Un[b]	G	GF	Un	G	GF	Un	G	GF
Glycosylated 2'-hydroxygenistein derivatives										
2'-Hydroxygenistein 8-*C*-glucoside	-[c]	-[d]	-	0.05±0.01	-	0.01±0.00	0.02±0.00	-	-	-
2'-Hydroxygenistein 8-*C*-(6"-*O*-malonyl-) glucoside	-	-	-	0.01±0.01	-	0.03±0.01	0.01±0.01	-	-	-
2'-Hydroxygenistein 4',7-*O*-diglucoside	-	-	0.03±0.01	0.04±0.02	-	-	-	-	0.01±0.00	0.04±0.04
2'-Hydroxygenistein 7-*O*-glucoside	-	-	0.02±0.00	0.13±0.07	-	-	0.07±0.02	-	0.14±0.02	0.72±0.01
2'-Hydroxygenistein 7-*O*-(6"-*O*-malonyl) glucoside	-	-	0.02±0.00	0.09±0.08	-	-	0.12±0.04	-	-	0.64±0.37
Luteone 4',7-*O*-diglucosides	C6	-	-	-	-	-	-	-	0.03±0.00	0.05±0.01
Luteone 4',7-*O*-diglucoside malonylated	C6	-	-	0.01±0.01	-	-	-	-	-	-
Luteone 7-*O*-glucoside	C6	-	0.03±0.00	0.03±0.01	-	-	0.02±0.01	-	-	0.05±0.00
Licoisoflavone A 7-*O*-glucoside	C3′	-	-	<0.01	-	-	-	-	-	0.06±0.03
Aglycone 2'-hydroxygenistein derivatives										
2'-Hydroxygenistein	-	-	0.06±0.04	0.38±0.01	-	0.02±0.00	0.25±0.03	-	0.03±0.01	2.06±0.20
Lupinisoflavone B	C6	-	<0.01	0.03±0.00	-	-	-	-	-	-
Barpisoflavone B	C8	-	-	0.03±0.02	-	-	-	-	-	-
Barpisoflavone C	C8	-	0.01±0.00	0.10±0.07	-	-	-	-	-	-
2,3-Dehydrokievitone	C8	-	<0.01	0.15±0.07	-	0.02±0.01	0.15±0.03	-	-	-
Lupinisoflavone A	C6	-	<0.01	0.02±0.02	-	-	0.02±0.01	-	<0.01	0.24±0.04
Luteone	C6	-	0.05±0.04	0.43±0.06	-	0.02±0.00	0.17±0.03	-	0.01±0.01	0.56±0.08
Parvisoflavone A	C8	-	-	0.06±0.03	-	-	0.02±0.01	-	-	-
Parvisoflavone B	C6	-	-	<0.01	-	-	-	-	<0.01	0.01±0.00
Licoisoflavone B	C3'	-	-	-	-	-	0.03±0.03	-	<0.01	0.01±0.01
Licoisoflavone A	C3'	-	-	-	-	0.03±0.01	0.38±0.12	-	<0.01	0.07±0.00
8-Prenylluteone	C6,C8	-	<0.01	0.01±0.00	-	0.02±0.00	-	-	-	-
Angustone B	C6,C3'	-	-	-	-	-	0.02±0.02	-	-	-
Angustone A	C6,C3'	-	-	-	-	<0.01	0.03±0.03	-	-	-

Glycosylated genistein derivatives										
Genistein 8-*C*-7-*O*-diglucoside	-	-	0.06±0.00	0.06±0.03	-	-	-	-	-	-
Genistein 8-*C*-glucoside	-	-	0.69±0.02	1.10±0.12	-	0.23±0.00	0.29±0.00	-	-	-
Genistein 6- or 8-*C*-(6"-*O*-malonyl) glucoside	-	-	0.64±0.03	0.49±0.29	-	0.55±0.06	0.45±0.07	-	-	-
Genistein 6- or 8-*C*-(6''-*O*-malonyl) glucoside	-	-	0.07±0.01	0.07±0.05	-	0.04±0.00	0.03±0.01	-	0.01±0.00	0.01±0.01
Genistein 4',7-*O*-diglucoside	-	<0.01	0.54±0.00	0.17±0.08	0.01±0.00	0.01±0.00	0.02±0.00	0.01±0.00	0.20±0.01	0.03±0.01
Genistein 4'-*O*-glucoside-7-*O*-(6"-*O*-malonyl) glucoside	-	-	0.18±0.01	0.10±0.06	-	0.12±0.00	0.13±0.00	-	0.07±0.00	-
Genistein 4', 7-*O*-diglucoside dimalonylated	-	-	-	-	-	0.17±0.03	0.10±0.01	-	-	-
Genistein 7-*O*-glucoside	-	-	0.31±0.00	0.20±0.11	-	0.33±0.01	0.40±0.04	-	1.85±0.14	0.59±0.16
Genistein 4'-*O*-glucoside	-	-	0.05±0.00	0.03±0.01	-	0.02±0.00	0.01±0.01	-	0.02±0.00	0.01±0.00
Genistein 7-*O*-(6"-*O*-malonyl) glucoside	-	-	0.53±0.03	0.17±0.13	-	1.62±0.18	1.41±0.29	-	2.02±0.06	0.63±0.24
Genistein 4'-*O*-(6"-*O*-malonyl) glucoside	-	-	0.05±0.00	0.02±0.01	-	0.12±0.01	0.11±0.03	-	0.16±0.02	0.05±0.03
Wighteone 7-*O*-glucoside	C6	-	-	<0.01	-	-	0.03±0.01	-	-	0.05±0.05
Aglycone genistein derivatives										
Genistein	-	<0.01	0.02±0.00	0.49±0.28	<0.01	0.06±0.02	0.73±0.20	<0.01	0.30±0.11	2.18±0.84
Lupiwighteone	C8	-	<0.01	0.03±0.01	-	0.01±0.01	0.05±0.02	-	-	-
Isowighteone	C3'	-	-	-	-	0.01±0.00	0.12±0.05	-	-	0.01±0.00
Wighteone	C6	-	0.01±0.00	0.05±0.00	-	0.04±0.01	0.34±0.12	-	<0.01	0.04±0.01
Lupisoflavone	C6	-	0.02±0.00	0.02±0.00	-	-	-	-	-	-
5-Methylwighteone	C8	-	0.02±0.00	<0.01	-	<0.01	0.01±0.00	-	-	-
Lupinalbin (coumaronochromone) derivatives										
Lupinalbin A	-	-	0.01±0.00	0.12±0.00	-	-	0.05±0.00	-	<0.01	0.28±0.07
Lupinalbin B/D	C3'/C6	-	-	-	-	<0.01	<0.01	-	-	-
Lupinalbin B/D	C3'/C6	-	<0.01	0.03±0.01	-			-	-	0.04±0.01
Miscellaneous isoflavonoids										
Orobol	-	-	-	-	-	-	-	-	<0.01	-
3'-*O*-Methylorobol	-	-	<0.01	0.02±0.00	-	<0.01	0.01±0.00	-	-	-
Apigenin derivatives										
Apigenin *C*-diglucoside *O*-xyloside	-	0.36±0.03	0.56±0.08	0.66±0.10	0.33±0.03	0.33±0.01	0.54±0.11	0.27±0.04	0.21±0.01	0.26±0.01
Apigenin 4',7-*O*-diglucoside	-	0.09±0.01	0.10±0.02	0.23±0.05	0.06±0.01	0.04±0.00	0.07±0.01	-	-	-
Apigenin 6-*C*-glucoside 8*C*- (2"-*O*-rhamnosyl) glucoside	-	-	-	-	-	-	-	-	0.02±0.00	0.03±0.00
Apigenin 7-*O*-(2"-*O*-rhamnosyl) glucoside	-	-	0.40±0.06	0.30±0.11	-	-	-	-	-	-
Apigenin 7-*O*-xylosylglucoside	-	-	-	-	-	-	0.01±0.01	-	-	-
Apigenin	-	-	-	0.04±0.02	-	0.01±0.01	0.6±0.00	-	-	-

Chrysoeriol derivatives												
Chrysoeriol *O*-xylosylglucoside	-	-	-	-	-	-	-	0.04±0.00	-	-	-	-
Chrysoeriol 3-*O*-xylosyl -(6"-*O*-malonyl) glucoside	-	-	-	-	-	-	0.07±0.03	0.04±0.01	-	-	-	-
Chrysoeriol 3-*O*-(6"-*O*-malonyl) glucoside	-	-	-	-	-	-	0.16±0.02	0.03±0.03	-	-	-	-
Chrysoeriol	-	-	-	-	-	-	-	0.01±0.00	<0.01	<0.01	0.02±0.02	
Miscellaneous flavonoids												
Eridictyol *O*-glucoside malonylated	-	-	0.02±0.00	-	-	-	-	-	-	0.01±0.00	-	
Luteolin *O*-xylosylglucoside malonylated	-	-	-	-	-	-	-	-	-	0.13±0.03	-	
Acacetin *O*-glucoside	-	-	-	-	-	-	-	-	-	-	0.01±0.00	
Luteolin	-	-	-	-	-	-	-	0.06±0.03	-	-	-	

[a] Expressed in mg genistein equivalent (GE) per g dry weight (DW) of *Lupinus* seed(ling). Data are the means of two replicates with standard deviation.
[b] Extracts tested were seeds (Un), seedling (G) and fungus-elicited seedling (GF) of lupine.
[c] The compound did not contain prenyl group.
[d] The compound was not found in the extract.

Table S2. Relative abundance of prenylated isoflavonoids in the prenylated 2'-hydroxygenistein and prenylated genistein pools.

Identification	Area (%) based on MS chromatogram of pools 1 and 2
Prenylated 2'-hydroxygenistein (pool 1)	
Lupinalbin A	3
2,3-Dehydrokievitone	25
Luteone	32
Licoisoflavone A	3
Lupinalbin B and D	7
Others (including saponin)	30
Prenylated genistein (pool 2)	
Lupiwighteone	8
Isowighteone	8
Wighteone	29
Other (including saponin)	55

Chapter 4

Variation in Accumulation of Isoflavonoids between Phaseoleae Seedlings Elicited by *Rhizopus*

Seeds from seven species of the tribe of Phaseoleae, including *Phaseolus*, *Vigna*, *Lablab* and *Psophocarpus*, were investigated for inducibility of isoflavonoids by germination with or without subsequent elicitation with *Rhizopus oryzae*. Germination alone poorly induced isoflavonoid production in Phaseoleae (in the range of 0.08-0.72 mg DE/g DW), whereas application of *Rhizopus* onto the seedlings increased the isoflavonoid content considerably (in the range of 0.43-2.27 mg DE/g DW). The inducibility of different subclasses of isoflavonoids in seedlings with *Rhizopus* varied per species. Isoflavones and isoflavanones were mainly found in elicited seedlings of *Phaseolus*, *Vigna* and *Lablab* species, whereas pterocarpans were mainly observed in those of *Psophocarpus*. Isoflavones were mainly found in either glycosylated or aglyconic form, whereas isoflavanones and pterocarpans were primarily accumulated in prenylated form. Moreover, for all species, prenylation of the main isoflavonoids mainly occurred on the A-ring, except for *Psophocarpus* for which B-ring prenylation was predominant. Thus, despite their phylogenetic relatedness, the seeds of various species within the Phaseoleae tribe appeared to respond differently towards elicitation by *Rhizopus* during germination. The kind of molecules induced followed the phylogenetic relationship of the various species, but their amounts induced during germination, alone or combined with elicitation, did not.

Based on: Siti Aisyah, Harry Gruppen, Silvia Andini, Monique Bettonvil, Edouard Severing and Jean-Paul Vincken, Variation in Accumulation of Isoflavonoids between Phaseoleae Seedlings Elicited by *Rhizopus*, **2015**, *Submitted*.

INTRODUCTION

Flavonoids and isoflavonoids are major plant secondary metabolites that are synthesized via the phenylpropanoid pathway.[1] These compounds play important roles in many essential physiological processes of plants, such as protecting against UV light, herbivores, microbes or competing plants, and attracting pollinators.[2] Besides, a range of human health-promoting activities has been shown for (iso)flavonoids through *in vitro* and *in vivo* studies, i.e. many isoflavonoids are regarded as hormone look-alikes that bind to the human estrogen receptors, resulting in estrogenic and anti-estrogenic activity. This feature of these so-called phytoestrogens might offer opportunities in therapies for hormone-dependent diseases.[3, 4] Various subclasses of isoflavonoids have gained attention, and particularly isoflavonoid subclasses with prenyl substituents showed interesting estrogenic properties.[3, 5] Due to our interest in these estrogenic properties, we aim to identify the potential of various legume seeds to induce a collection of isoflavonoids, which differ in skeleton and (kind, number and position of) substituents, particularly the prenyl group.

Flavonoids are widely distributed in plants, whereas the majority of isoflavonoids were found in Leguminosae family.[6] The content and composition of isoflavonoids of legume seeds have been reported to differ between legume species and to change by subjecting the seeds to different treatments.[7, 8] Our previous research on soybean and lupine has shown that the content of isoflavonoids can be enhanced by germinating the seeds in the presence of fungus.[9-11] Besides, the diversity in isoflavonoid skeletons increases, and many of the compounds induced are prenylated.[9, 11] The presence of the fungus seemed particularly important for boosting the content and altering the isoflavonoid composition.[11-13]

Phaseoleae is a diverse legume tribe containing over eighty genera, including some popular edible legumes seeds, such as soybean (*Glycine max*), kidney bean (*Phaseolus vulgaris*), and mung bean (*Vigna radiata*).[14, 15] Species other than soybean within this tribe have been reported amenable to induction of isoflavonoids, using wounding, fungal elicitors, bacterial elicitors and chemicals.[12-14, 16] However, the inducibility of isoflavonoids in these seeds during germination with concomitant elicitation by fungus has never been systematically compared. In this study, we investigated the compositional changes in isoflavonoids and flavonoids (in terms of total content and molecular diversity) of seven common edible Phaseoleae seeds that were germinated in presence or absence of the food grade *Rhizopus oryzae*. It was hypothesized that these closely related species responded similarly to the treatment of germination under stress.

MATERIALS AND METHODS

Materials

Seeds from seven edible Phaseoleae species (from four different genera) were purchased from Vreeken's Zaden (Dordrecht, The Netherlands): *Phaseolus vulgaris, P. coccineus, Lablab purpureus, Vigna angularis, V. unguiculata, V. radiata,* and *Psophocarpus tetragonolobus*. The authentic standards of daidzein and genistein were purchased from Sigma Aldrich (St. Louis, MO, USA). ULC-MS grade acidified acetonitrile (ACN), water, methanol and acetic acid (HOAc) were obtained from Biosolve BV (Valkenswaard, The Netherlands). The fungus, *Rhizopus oryzae* (LU 581), was kindly provided by the Laboratory of Food Microbiology, Wageningen University (The Netherlands). Other chemicals were purchased from Merck (Darmstadt, Germany) and Sigma Aldrich Chemie (Zwijndrecht, The Netherlands).

Treatments with Phaseoleae seeds

The treatment of seeds was performed in an EQMM sprouting machine (EasyGreen, San Diego, CA, USA), which was modified as described previously.[11] The seeds were consecutively subjected to soaking (1 d) and germination (7 d) stages. Prior to the soaking step, seeds were surface-sterilized by immersing them in a 70% (v/v) aqueous ethanol (5 L/kg beans) for 10 min at room temperature, and subsequently rinsing them 4 times with Milli-Q water (3 L/kg beans). The sterilized seeds were soaked for 24 h at 25 °C in sterilized Milli-Q water. Subsequently, the soaked seeds were put into sterilized plastic cartridges (sterilized by soaking them in hypochlorite 1% (v/v) for 2 h, then rinsing them with Milli-Q water) that were covered with autoclaved filter paper. Next, they were placed in the modified sprouting machine. Prior to this, the machine was sterilized according to the cleaning protocol provided by the manufacturer. The seeds were germinated for 7 d at 25 °C and 100% RH. In another set of experiments, the seeds were also subjected to fungal elicitation. A spore suspension (0.2 mL/g beans) was added to 2 d-old selected seedlings. The non-germinated seeds were discarded. The fungus-inoculated seeds were incubated for 5 d at 30 °C, and a RH controlled at 55-85%. Spore suspensions for the inoculation stage were prepared from pure plate cultures of *R. oryzae* grown on malt extract agar (CM59; Oxoid, Basingstoke, UK). The sporangia were scraped off from the agar plate and suspended in 0.85% (w/v) NaCl solutions (approximately 10^7 CFU/mL). The seeds were collected after the treatment and directly stored at -20 °C.

Extraction of isoflavonoids from Phaseoleae seeds and seedlings

The extracts of untreated, germinated and elicited Phaseoleae seeds were prepared as described previously for soybean, with the modification that 80% (v/v) aqueous methanol

(MeOH) was used for extraction of (iso)flavonoids.[11] The dried extracts were re-solubilized in 80% (v/v) aqueous methanol to a concentration of 5 mg/mL. All samples were centrifuged (18,000 × *g*, 5 min; room temperature) prior to analysis.

RP-UHPLC-MS analysis

The extracts obtained were analyzed by UHPLC-MS. An Accela UHPLC system (Thermo Scientific, San Jose, CA, USA) was equipped with a pump, autosampler, and photodiode array (PDA) detector. Samples (1 µL) were injected onto an Acquity UPLC BEH shield RP18 column (2.1 mm ID × 150 mm, 1.7 µm particle size; Waters, Milford, MA, USA) with an Acquity UPLC BEH shield RP18 VanGuard pre-column (2.1 mm ID × 5 mm, 1.7 µm particle size; Waters). Water acidified with 0.1% (v/v) acetic acid, eluent A, and ACN acidified with 0.1% (v/v) acetic acid, eluent B, were used as eluents at a flow rate of 300 µL/min. The temperatures of the autosampler and column oven were controlled at 15 and 35 °C, respectively. The PDA detector was set to monitor the 200-400 nm range. The elution profile was as follows: 0-2 min, linear gradient from 10%-25% (v/v) B; 2-9 min, linear gradient from 25%-50% (v/v) B; 9-12 min, isocratic on 50% B; 12-22 min, linear gradient from 50%-100% (v/v) B; 22-24 min, isocratic on 100% B; 24-25 min, linear gradient from 100%-10% (v/v) B; 25-30 min, isocratic on 10% (v/v) B. Mass spectrometric analysis was performed on a LTQ Velos (Thermo Scientific) equipped with an HESI-MS probe coupled to the RP-UHPLC. Nitrogen was used as sheath and auxiliary gas. The spectra were acquired in the *m/z* range of 150-1,500. Data-dependent MS^n analysis was performed with normalized collision energy of 35%. The system was tuned with genistein in both positive (PI) and negative ionisation (NI) mode. For the PI mode, the ion transfer tube (ITT) temperature was 400 °C and the source voltage was 4.50 kV. For NI mode, the ITT temperature was 400 °C and the source voltage was 3.50 kV.

Quantification of isoflavonoids was performed based on their absorption at 280 nm by means of Xcalibur (version 2.1.0, Thermo Scientific). For different compounds eluted at the same retention time, the quantification was based on the ratio of intensity of the peaks in full HESI-MS, assuming that no isomers eluted at the same retention time. As for many compounds no commercial standards were available, the amounts of (iso)flavonoid were expressed as mg daidzein equivalents per g dry weight (mg DE/g DW). Daidzein was used as a generic standard to make a calibration curve with five data points (0.1-0.001 mg/mL, $R^2 = 0.998$).

Phylogenetic analysis of Leguminoceous species

The *Matk* encoding regions were extracted from the following NCBI nucleotide sequences: *Lablab purpureus*: gb|EU717408.1 (725-2239); *Phaseolus coccineus*: gb|DQ445964.1 (654-2198); *Phaseolus vulgaris*: gi|139387430 (4964-6505); *Psophocarpus tetragonolobus*:

gi|378757903 (full); *Vigna angularis* gi|501594910 (5024-6538); *Vigna radiata*: gi|289066804 (4996-6510); *Vigna unguiculata*: gb|AY589510.1 (692-2206); *Glycine max*: gb|AF142700.1 (723-2240). These nucleotide sequences were translated into protein sequences using the on-line version of TransSeq (http://www.ebi.ac.uk/Tools/st/emboss_transeq/). Protein sequences were aligned using muscle version 3.8.31.[17] The resulting alignments were converted to codon alignments by replacing each amino acid by its corresponding codon and extending each gap to 3. The jModelTest version was used for finding the appropriate evolutionary model for maximum likelihood tree construction.[18] The maximum likelihood trees were constructed using PhyML version 3.1 using the parameters indicated by jModelTest with the addition that the number of bootstrap samples was set to 100.[19]

RESULTS

Flavonoid and isoflavonoid subclasses of Phaseoleae

UHPLC analysis of extracts from seeds and seedlings showed that UV-profiles changed during the induction process. **Figure 1** shows (iso)flavonoid profiles of *P. coccineus* during germination and elicitation with *Rhizopus*. The other Phaseoleae species were analyzed in a similar way, which revealed that different types of compounds were induced, and that their onset of induction varied. Flavonoids and isoflavonoids were distinguished by their UV/vis absorption spectra,[20] the shape of which is largely determined by the presence/absence of a double bond in the C-ring, and the position of the B-ring at the C-ring. Generally, both classes have two absorbance bands that are commonly referred to as band I and II. Flavones and flavonols with a double bond in the C-ring exhibited intense bands in the 240-280 nm range (characteristic for the A-ring benzoyl system, band II) and in the 300-380 nm range (characteristic for the B-ring cinnamoyl system, band I). The absence of the double bond in C-ring of flavanones reduces the intensity of band I (in some cases to a shoulder (*sh*) on band II), and only an intense band in the range of 270-295 nm is visible, representing band II. Isoflavonoids without conjugation between the A- and B-ring normally show an intense band in the range 245-290 nm (band II) with little absorption above 300 nm (band I) (**Table 1**).[21-23] Nevertheless, coumestans show a distinct intense band at 345 nm, with a very low intensity band or no band below 300 nm.[23] The exact UV absorption maxima of individual (iso)flavonoids mostly depend on the level and position of oxygenation (**Table S1** in Supporting Information).

Mass spectral data, representing cleavage in the C-ring, can be used to classify the compound's subclass.[20, 24] Often cleavage in the C-ring resulted in diagnostic fragment ions, most of which could be rationalized by *retro*-Diels−Alder (*r*DA) reactions. Generally, most (iso)flavonoids gave fragment ions resulting from C-ring cleavage in either PI or NI

mode. The position of these cleavages in PI mode depended on the subclasses.[20] Nevertheless, all of the subclasses can be fragmented at the 1/3 position (**Table 1**). Flavones and flavonols showed the characteristic cleavage at the C-ring through 0/2 bonds in PI mode,[20] while isoflavanes and isoflavanones showed the fragmentation through 2/3 bond,[25] which is in line with previous data. On the other hands, coumestans hardly yielded any fragment ions resulting from C-ring cleavage.[26] In total, nineteen peaks were classified as flavonoids, dominated by the flavonol subclass, whereas fifty-four peaks were classified as isoflavonoids, dominated by isoflavanone and isoflavone subclasses (**Table S1** in Supporting Information).

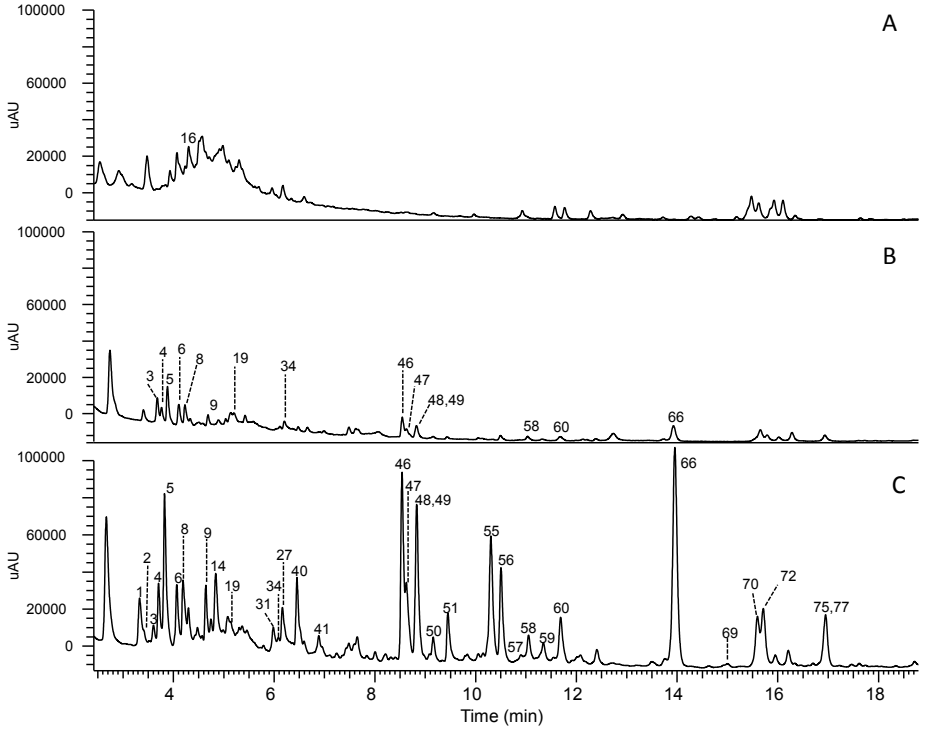


Figure 1. RP-UHPLC-UV profile of a 80% (v/v) aqueous methanol extract of seeds (**A**), germinated seeds (**B**), and fungus-elicited seedlings (**C**) of *P. coccineus*. Peak numbers refer to compounds in **Table S1**.

Table 1. Summary of the UV spectral and mass spectrometric characteristics of (iso)flavonoid subclasses.

UV		RDA fragmentation	Subclass
Band I	Band II		
300-380	240-280	$^{1,3}A/B^+$, $^{0,2}B^+$, $^{0,4}B^+$	Flavone
300-380	240-280	$^{1,3}A/B^+$, $^{0,2}A/B^+$, $[^{1,4}B+2H]^+$	Flavonol
300-360[a]	270-295	$^{1,3}A/B^+$, $^{0,4}B^+$	Flavanone
300-340[a]	245-270	$^{1,3}A/B^+$, $^{1,4}A/B^+$	Isoflavone
300-360[a]	270-295	$^{1,3}A/B^+$, $^{0,4}B^+$, $^{2,3}B^+$	Isoflavanone
	270-285	$^{1,3}A/B^+$, $^{1,4}A/B^+$, $^{2,3}A/B^+$	Isoflavan
	280-310	$^{2,4}A/B^+$, $^{1,4}A/B^+$, $^{5,6}A/B^+$	Pterocarpan
340-350	260-268[a]	-	Coumestan
330-347	260-268, 280-289	-	Coumaronochromone

[a] Shoulder or inflection.

Type of substituents attached to the flavonoid and isoflavonoid skeleton

Glycosyl, prenyl, hydroxyl, and methoxyl groups are common substituents of the (iso)flavonoid skeleton. The presence of these groups can be derived from the losses of neutral and/or radical fragments from parent ions in mass spectra. Glycosylated (iso)flavonoids showed fragments originating from the loss of a glycosyl residue in MS^2 as the most abundant species in both NI and PI modes. The neutral losses of 162/146/132 Da indicated the presence of *O*-hexosyl/-rhamnosyl/-pentosyl isoflavonoids, respectively. The neutral losses of 90 and 120 Da followed by several consecutive losses of 18 Da were observed for *C*-hexosyl (iso)flavonoids. Often the glycosyl residues contained a malonyl group, which was characterized by a neutral loss of 44 or 86 Da, in NI or PI mode, respectively.[27] In this study, most of flavonoids were found to be glycosylated, whereas glycosylation occurred less frequently with isoflavonoids (**Table S1** in Supporting Information).

Different to glycosylation, prenylation was restricted to isoflavonoids. The prenyl group could be attached to the isoflavonoid skeleton in different configurations. A neutral loss of 56 Da (C_4H_8) in PI mode was used to distinguish a prenyl chain from a ring-closed prenyl (or 2,2-dimethylpyran ring), as reported before.[28, 29] Major neutral losses of 42 Da (C_3H_6), and to a lesser extent 70 Da ($C_3H_6 + H_2O$), 54 Da (C_4H_6), and 15 Da (CH_3•) were used to identify the 2,2-dimethylpyran and 2-isopropenyldihydrofuran rings.[29] Neutral losses of 18 Da (H_2O) and 72 Da (C_4H_8O) were indicative of the 2,2-dimethyl-3-hydroxy dihydrofuran ring.[29] Remarkably, the isoflavonoid skeleton affected the intensity of fragment ions resulting from the loss of prenyl group. The intensity of the fragment ion $[M+H\text{-prenyl}]^+$ was predominant in MS^2 spectra of prenylated isoflavones, whereas the fragment ions

produced by C-ring cleavage dominated in MS^2 spectra of prenylated isoflavans (**Table S1** in Supporting Information). For prenylated isoflavanones, often the neutral loss of 18 Da was more favored than that of a prenyl substituent (56 Da or 42 Da). The relative intensities of $[M+H-C_4H_8]^+$ and $[M+H-H_2O]^+$ in MS^2 spectra of kievitone, for instance, were 40% and 100%, respectively. Twenty-five out of fifty-four isoflavonoids were prenylated, of which six were glycosylated.

Position (A- or B-ring) of prenylation of isoflavonoids

Fragments obtained after C-ring cleavage were used to determine the position of the prenyl group in the isoflavonoid skeleton.[30, 31] C-ring cleavage yielded fragment ions containing the A- or B-ring and part of the C-ring. The C-ring cleavage at the 1/3 position of prenylated isoflavones was mainly observed in MS^3 spectra with molecular ions containing one carbon reminiscent of the prenyl chain, resulting in the fragment ions $^{1,3}A^+$, $^{1,3}A\text{-}C_4H_8^+$, and/or $^{1,3}B\text{-}C_4H_8^+$. Based on these fragment ions, three out of four prenylated isoflavones (**52**, **69**, and **78**) were categorized as A-ring prenylated. Besides the 1/3 C-ring cleavage, prenylated isoflavanones also showed 2/3 C-ring cleavage. Based on both C-ring cleavage patterns, all prenylated isoflavanones (**28**, **30**, **32**, **37**, **42**, **50**, **53**, **61** and **66)**, were categorized as A-ring prenylated. Prenylated isoflavans showed mainly C-ring cleavage at the 2/3 position in MS^2. Based on the fragment ion $^{2,3}B^+$, being the base peak in MS^2 spectra, phaseollinisoflavan (**72**) and 2'-*O*-methyl phaseollidinisoflavan (**76**) were categorized as B-ring prenylated. The extra D-ring in the skeleton of prenylated pterocarpans changed the fragmentation pattern. The fragmentation of prenylated pterocarpan found in the extracts was extrapolated from that of prenylated 6a-OH-pterocarpans reported elsewhere.[26] In the PI mode, the $^{5,6}B\text{-}C_4H_8^+$ ion was detected in MS^2 spectra of phaseollidin and its isomer (**70** and **73**, respectively), and the $^{0,4}B\text{-}C_3H_6^+$ ion was detected as the base peak of MS^2 spectra of phaseollin and its isomer (**77** and **71**, respectively), confirming their B-ring prenylation.

Anthraquinones

Five peaks were tentatively identified as anthraquinones, showing λ_{max} in the range of 220-350 nm, and close to 400 nm, depending on the nature and position of the substituents.[32] Of the five peaks, four anthraquinones (**40**, **49**, **51** and **59**) were characterized by the neutral loss of 28 Da yielding the base peak $[M-H-CO]^-$ in MS^2, which was followed by a subsequent neutral loss of 44 Da $[M-H-CO-CO_2]^-$ in MS^3.[33] In case of the fifth peak (**55**), the neutral loss of 44 Da $[M-H-CO_2]^-$ was observed in MS^2, followed by a loss of a methyl radical to yield fragment ion *m/z* 194 in MS^3, indicating the presence of a methoxyl group.[33, 34] Peaks **51** and **59** were tentatively identified as emodin and an isomer thereof,[34]

whereas peak **55** was tentatively annotated as methoxylated anthraquinone. The identities of the two anthraquinone peaks **40** and **49** were not further established.

Changes in (iso)flavonoid composition during germination

UHPLC analysis showed that, generally, flavonoids were more commonly found in the seeds than isoflavonoids. Glycosides of quercetin, kaempferol and apigenin were the main flavonoids of the seeds. Nevertheless, among the seven species studied, only *V. radiata* and *P. tetragonolobus* accumulated above 0.1 mg DE/g DW. The total (iso)flavonoid content of seeds increased after 7 days of germination, up to 0.1- 1.0 mg DE/g DW. As an exception, the total (iso)flavonoid content of *V. radiata* and *P. tetragonolobus* decreased, due to the reduction in the flavonoids content during germination. The content of *C*-glycosylated apigenin derivatives (± 1.7 mg DE/g DW) in the *V. radiata* seeds, for instance, decreased up to 90% (w/w) after germination. The same observation has been reported before for *V. radiata* sprouts.[35] The increase of isoflavonoid content during germination was more pronounced than that of flavonoids. Isoflavonoids were accumulated in the range of 0.1 to 0.7 mg DE/g DW, whereas for flavonoids this was in the range of 0.01 to 0.44 mg DE/g DW. Isoflavone and isoflavanone subclasses were the predominant isoflavonoid subclasses found in sprouts, mainly in the form of glycosides. Kievitone (a prenylated isoflavanone) was found up to 0.2 mg DE/g DW in the sprouts, except in *P. tetragonolobus* (**Table S2** in Supporting Information).

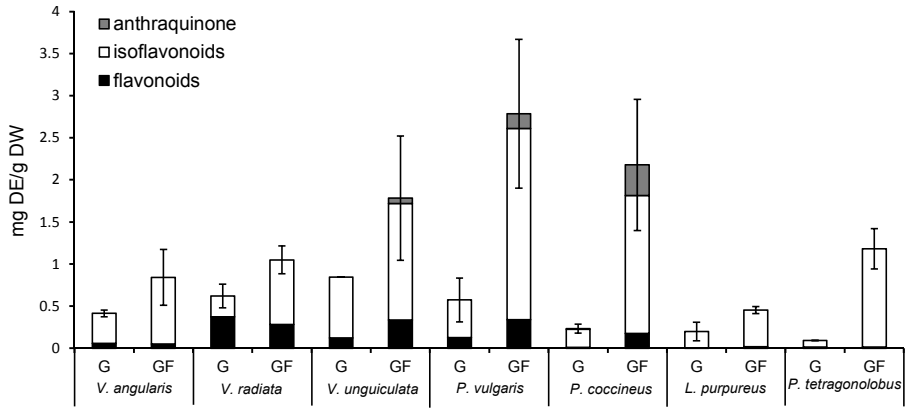


Figure 2. Total isoflavonoid and flavonoid contents of germinated seeds (G) and elicited seedlings (GF) of species of the tribe of Phaseoleae. All contents are expressed in mg daidzein equivalents (DE) per g dry weight (DW) of seedling. Data are the mean ± SD of duplicates.

Changes in (iso)flavonoid and anthraquinone composition during elicitation

The total (iso)flavonoid content increased further when the germination was performed in the presence of *R. oryzae* (**Figure 2**). *Rhizopus* boosted the total isoflavonoid content of the sprouts 2- to 15-fold, whereas no substantial increase in the total flavonoid content was observed (**Table S3** in Supporting Information). After elicitation, the total isoflavonoid content of *Vigna* species was around 0.7-1.4 mg DE/g DW, whereas that of *Phaseolus* species was higher, up to 1.8-2.4 mg DE/g DW. The total isoflavonoid contents of *Psophocarpus* and *Lablab* were around 1.2 and 0.4 mg DE/g DW, respectively (**Figure 3A**). The isoflavonoid composition was also affected by fungal elicitation. Generally, the amount of glycosylated isoflavonoids decreased, whereas those of prenylated and aglyconic ones increased almost in all elicited seedlings (**Figure 3B**). In addition, anthraquinones were found only in elicited *Phaseolus* species and *Vigna unguiculata*, around 0.1-0.5 mg DE/g DW. The occurrence of anthraquinones in *Phaseolus vulgaris* has been reported before, in the range of 0.0-0.34 mg/g DW.[36]

Five isoflavonoid subclasses, i.e. isoflavone, isoflavanone, isoflavan, pterocarpan, and coumestan, were observed in the elicited seedlings (**Figure 3A**). Elicited *Phaseolus* seedlings contained mainly isoflavones and isoflavanones. Isoflavanones were mainly found in prenylated form, exceeding 40% (w/w) of the total isoflavanone content. In contrast, isoflavones were mostly found in glycosylated form, above 50% (w/w) of the total isoflavone content. Other subclasses were accumulated less in elicited *Phaseolus*. Isoflavans, found primarily in prenylated form, for instance, were accumulated in elicited *Phaseolus* species, around 0.1 mg DE/g DW, and coumestans and pterocarpans were accumulated less than 0.1 mg DE/g DW. Similar to *Phaseolus*, isoflavone and isoflavanone subclasses were predominant in *Vigna* and *Lablab* species. The isoflavanone found in these two genera mainly occurred in prenylated form, whereas the isoflavones were found mainly in unprenylated form. Other subclasses were found less than 0.1 mg DE/g DW in elicited *Vigna* and *Lablab*. Isoflavans were observed only in elicited *V. unguiculata*, around 0.02 mg DE/g DW. It is worth to note that the glycosides of prenylated isoflavanones, i.e. prenylated kievitone, were found in *P. vulgaris* and *V. unguiculata*. In contrast to the previous genera, fungus-elicited *Psophocarpus* seedlings contained mainly pterocarpans that accumulated mostly in prenylated form, up to 90% (w/w) of the total isoflavonoid content, whereas prenylated pterocarpans were accumulated in the range of 2-17% (w/w) of the total isoflavonoid content in other species.

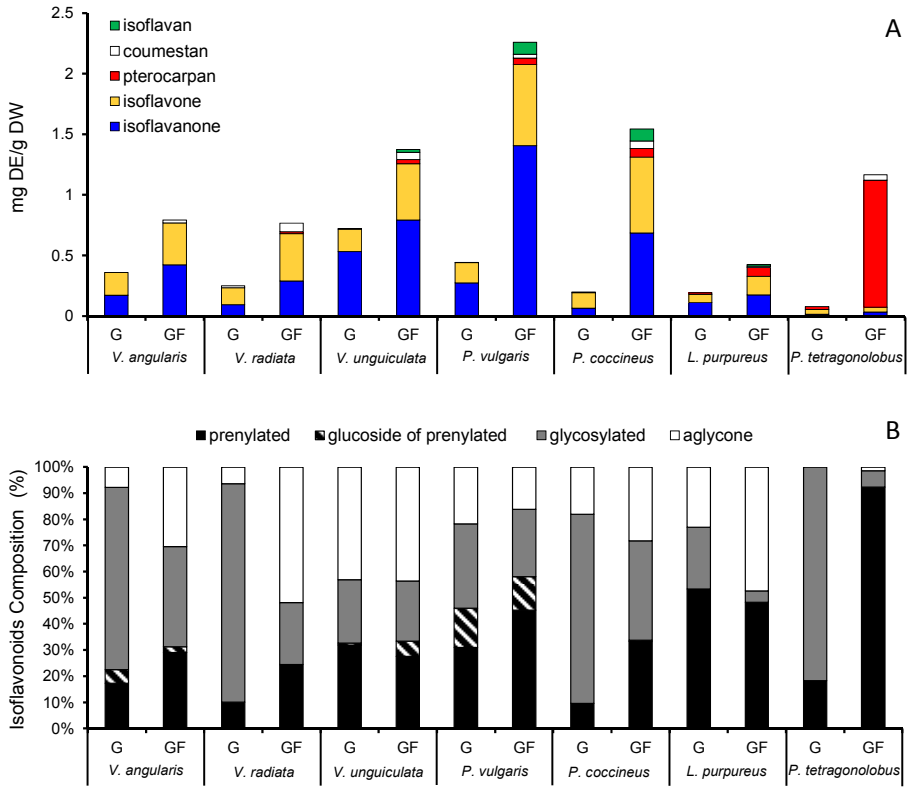


Figure 3. (**A**) Total content of isoflavonoid subclasses of germinated seeds (G) and elicited seedlings (GF) of Phaseoleae species. All contents are expressed in mg daidzein equivalents (DE) per g dry weight (DW) of seedlings. (**B**) The composition (in percentage) of prenylated, glycoside of prenylated, glycosylated and aglyconic isoflavonoids of germinated seeds (G) and elicited seedlings (GF) of Phaseoleae species.

DISCUSSION

The induction of isoflavonoids in the seedlings of seven common edible legume species during germination and subsequent elicitation was investigated. The relationship among the various species of the tribe Phaseoleae was plotted in a phylogenetic tree (**Figure 4**), with addition of *Glycine max* (soybean). *Glycine* is also a member of the tribe of Phaseoleae and the induction of isoflavonoids in this species has been investigated previously.[9, 11] The phylogenetic tree was constructed using the *Matk* encoding regions as described in Materials and Methods section. According to the phylogenetic tree, genera *Phaseolus*, *Vigna* and *Lablab* are more closely related to each other (clustered into the subtribe

Phaseolinae) than *Glycine* (subtribe Glycininae) and *Psophocarpus* (not assigned to a subtribe) (**Figure 4**).[15] The genetic relatedness between the species is discussed in terms of inducibility of isoflavonoids, more in particular their content and molecular diversity.

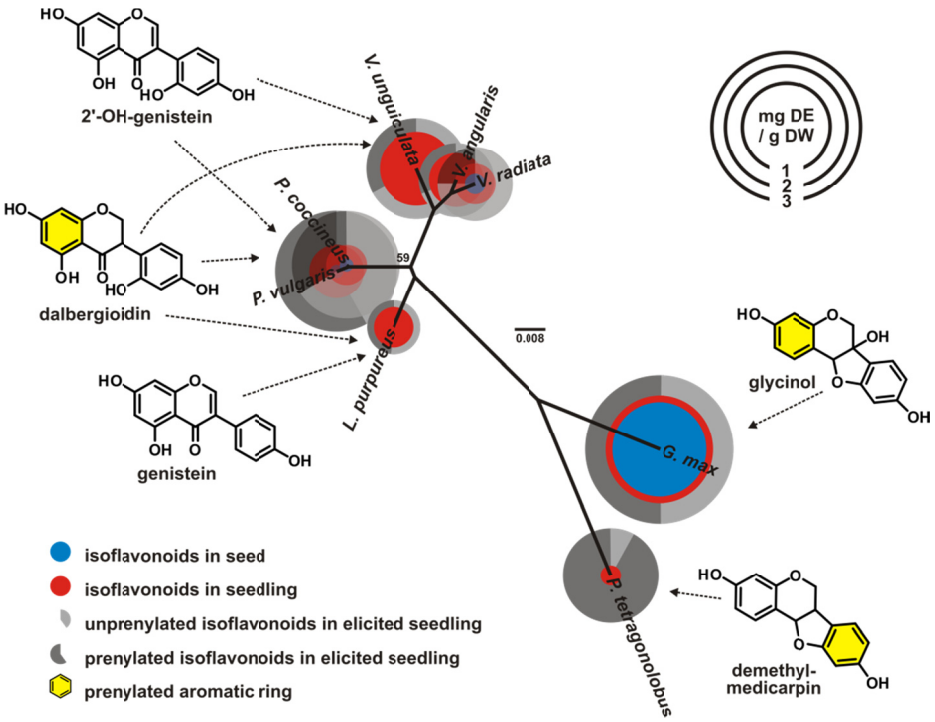


Figure 4. Overview of the inducibility of isoflavonoids in Phaseoleae species during various treatments. The phylogenetic relationship amongst species is described by the un-rooted tree that was constructed using maximum likelihood method. Number at branch point in the tree represents deviating bootstrap support value (%), the other bootstrap support values were 100%. The scale bar (0.008) indicates branch length. The range of total isoflavonoid content of the respective seeds, seedlings and *Rhizopus*-elicited seedlings are indicated by colored circles. The structures indicate the major isoflavonoid induced, when the sum of all compounds with that skeleton represented more than 30% (w/w) of the total isoflavonoid content in *Rhizopus*-elicited seedlings of the tribe Phaseoleae. The colored aromatic rings represent the most favored ring for prenylation.

Inducibility of isoflavonoid content in Phaseoleae seedlings does not follow lineage

The change in isoflavonoid content in the seeds during the treatments is represented in **Figure 4**. Soybean was the only species that already contained a large amount of isoflavonoids in the seeds. The isoflavonoid content was inducible during germination, but the species studied responded differently. Generally, an increase in isoflavonoid content was observed during germination of speci in the subtribe of Phaseolinae, contrary to *G. max* and *P. tetragonolobus* (**Figure 4**). In the presence of *Rhizopus*, the isoflavonoid content developed differently among the speci of the subtribe of Phaseolinae. *Phaseolus* spp. seemed more inducible than *Vigna* spp., whereas *L. purpureus* poorly responded to *Rhizopus*. On the other hand, the isoflavonoid contents of *G. max* and *P. tetragonolobus* that were hardly induced by germination was increased during elicitation by fungus. Thus, our results show that the isoflavonoid content is best boosted by germination in presence of fungus, and that the extent of inducibility is not necessarily linked to genetic relatedness.

Molecular diversity of isoflavonoids induced in Phaseoleae seedlings follows lineage

The main isoflavonoid skeleton induced in elicited seedlings was subtribe-dependent (**Figure 4**). Species belonging to the subtribe of Phaseolinae accumulated mainly isoflavones (genistein and 2'-hydroxygenistein derivatives) and isoflavanones (dalbergioidin derivatives), whereas *G. max* and *P. tetragonolobus* were dominated by 6a-hydroxypterocarpans (glycinol derivatives) and pterocarpans (demethylmedicarpin derivatives), respectively. This indicates that *G. max* and *P. tetragonolobus* are more capable of synthesizing the more downstream compounds of the isoflavonoid pathway during fungal elicitation than *Phaseolus* spp., *Vigna* spp., and *L. purpureus*.

The extent of, the preferred isoflavonoid subclass for, and position of prenylation varied amongst species. Generally, the species of the Phaseolinae subtribe accumulated the prenylated isoflavonoids in the range of 25-50% (w/w) of total isoflavonoids, whereas this was around 50 and 90% (w/w) for *G. max* and *P. tetragonolobus*, respectively. With respect to preferred isoflavonoid subclass for prenylation, pterocarpans and isoflavanones were prenylated to an extent of 60-95% and 35-80% (w/w), respectively, whereas this was only 5-25% (w/w) for isoflavones (**Figure 4**). Furthermore, the position of prenylation of the main compounds induced differed. A-ring prenylation was more favored for dalbergioidin and glycinol, whereas B-ring prenylation was favored for the demethylmedicarpin (**Figure 4**). Consequently, the species of the Phaseolinae subtribe and *G. max* accumulated more A-ring than B-ring prenylated isoflavonoids, whereas *P. tetragonolobus* was dominated by B-ring prenylated isoflavonoids. Our results suggest that the type of skeleton induced upon

elicitation might be deduced from the phylogenetic relationship of the species, whereas this is less clear for the extent and position of prenylation.

ACKNOWLEDGEMENTS

This research was financially supported by Directorate General of Higher Education Ministry of National Education of Indonesia.

REFERENCES

[1] R. A. Dixon, L. Achnine, P. Kota, C.-J. Liu, M. S. S. Reddy, L. Wang, The phenylpropanoid pathway and plant defence: A genomics perspective, *Molecular Plant Pathology* **2002**, *3*, 371-390.

[2] L. Ralston, S. Subramanian, M. Matsuno, O. Yu, Partial reconstruction of flavonoid and isoflavonoid biosynthesis in yeast using soybean type I and type II chalcone isomerases, *Plant Physiology* **2005**, *137*, 1375-1388.

[3] R. Simons, H. Gruppen, T. F. H. Bovee, M. A. Verbruggen, J.-P. Vincken, Prenylated isoflavonoids from plants as selective estrogen receptor modulators (phytoSERMs), *Food and Function* **2012**, *3*, 810-827.

[4] D. C. Vitale, C. Piazza, B. Melilli, F. Drago, S. Salomone, Isoflavones: Estrogenic activity, biological effect and bioavailability, *European Journal of Drug Metabolism & Pharmacokinetics* **2012**, 1-11.

[5] M. C. Zimmermann, S. L. Tilghman, S. M. Boué, V. A. Salvo, S. Elliott, K. Y. Williams, E. V. Skripnikova, H. Ashe, F. Payton-Stewart, L. Vanhoy-Rhodes, J. P. Fonseca, C. Corbitt, B. M. Collins-Burow, M. H. Howell, M. Lacey, B. Y. Shih, C. Carter-Wientjes, T. E. Cleveland, J. A. McLachlan, T. E. Wiese, B. S. Beckman, M. E. Burow, Glyceollin I, a novel antiestrogenic phytoalexin isolated from activated soy, *Journal of Pharmacology and Experimental Therapeutics* **2010**, *332*, 35-45.

[6] N. C. Veitch, Isoflavonoids of the Leguminosae, *Natural Product Reports* **2007**, *24*, 417-464.

[7] P. B. Kaufman, J. A. Duke, H. Brielmann, J. Boik, J. E. Hoyt, A comparative survey of leguminous plants as sources of the isoflavones, genistein and daidzein: Implications for human nutrition and health, *The Journal of Alternative and Complementary Medicine* **1997**, *3*, 7-12.

[8] W. M. Mazur, J. A. Duke, K. Wähälä, S. Rasku, H. Adlercreutz, Isoflavonoids and lignans in legumes: Nutritional and health aspects in humans, *The Journal of Nutritional Biochemistry* **1998**, *9*, 193-200.

[9] R. Simons, J.-P. Vincken, N. Roidos, T. F. H. Bovee, M. Van Iersel, M. A. Verbruggen, H. Gruppen, Increasing soy isoflavonoid content and diversity by simultaneous malting and challenging by a fungus to modulate estrogenicity, *Journal of Agricultural and Food Chemistry* **2011**, *59*, 6748-6758.

[10] S. Aisyah, H. Gruppen, S. Andini, Z. Mardiah, J.-P. Vincken, Compositional changes in (iso)flavonoids and estrogenic activity of three edible *Lupinus* species by germination and *Rhizopus*-elicitation, *Submitted* **2015**.

[11] S. Aisyah, H. Gruppen, B. Madzora, J.-P. Vincken, Modulation of isoflavonoid composition of *Rhizopus oryzae* elicited soybean (*Glycine max*) seedlings by light and wounding, *Journal of Agricultural and Food Chemistry* **2013**, *61*, 8657-8667.

[12] M. J. O'Neill, S. A. Adesanya, M. F. Roberts, I. R. Pantry, Inducible isoflavonoids from the lima bean, *Phaseolus lunatus*, *Phytochemistry* **1986**, *25*, 1315-1322.

[13] I. M. Whitehead, P. M. Dey, R. A. Dixon, Differential patterns of phytoalexin accumulation and enzyme induction in wounded and elicitor-treated tissues of *Phaseolus vulgaris*, *Planta* **1982**, *154*, 156-164.

[14] J. L. Ingham, Systematic aspects of phytoalexin formation within tribe Phaseoleae of the Leguminosae (subfamily Papilionoideae), *Biochemical Systematics and Ecology* **1990**, *18*, 329-343.

[15] S. Stefanović, B. E. Pfeil, J. D. Palmer, J. J. Doyle, Relationships among Phaseoloid legumes based on sequences from eight chloroplast regions, *Systematic Botany* **2009**, *34*, 115-128.

[16] J. Doherty, R. Buescher, Occurrence of the phytoalexin phaseollin in pods of *Phaseolus vulgaris*, *Journal of the Science of Food and Agriculture* **1978**, *29*, 853-856.

[17] R. C. Edgar, MUSCLE: Multiple sequence alignment with high accuracy and high throughput, *Nucleic Acids Research* **2004**, *32*, 1792-1797.

[18] D. Posada, K. A. Crandall, MODELTEST: Testing the model of DNA substitution, *Bioinformatics (Oxford, England)* **1998**, *14*, 817-818.

[19] S. Guindon, O. Gascuel, A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood, *Systematic Biology* **2003**, *52*, 696-704.
[20] E. de Rijke, P. Out, W. M. A. Niessen, F. Ariese, C. Gooijer, U. A. T. Brinkman, Analytical separation and detection methods for flavonoids, *Journal of Chromatography A* **2006**, *1112*, 31-63.
[21] A. Marston, K. Hostettmann, Separation and quantification of flavonoids, in *Flavonoids: Chemistry, biochemistry and applications* (Eds.: O. M. Andersen, K. R. Markham), CRC Press Boca Raton, FL, USA, **2006**, pp. 1-36.
[22] E. Corradini, P. Foglia, P. Giansanti, R. Gubbiotti, R. Samperi, A. Lagana, Flavonoids: Chemical properties and analytical methodologies of identification and quantitation in foods and plants, *Natural Product Research* **2011**, *25*, 469-495.
[23] C. A. Williams, J. B. Harborne, Isoflavonoids, in *Methods in plant biochemistry, Vol. 1*, Academic Press San Diego, CA, USA, **1989**, pp. 421-449.
[24] B. Abad-García, L. A. Berrueta, S. Garmón-Lobato, B. Gallo, F. Vicente, A general analytical strategy for the characterization of phenolic compounds in fruit juices by high-performance liquid chromatography with diode array detection coupled to electrospray ionization and triple quadrupole mass spectrometry, *Journal of Chromatography A* **2009**, *1216*, 5398-5415.
[25] R. Maul, N. Schebb, S. Kulling, Application of LC and GC hyphenated with mass spectrometry as tool for characterization of unknown derivatives of isoflavonoids, *Analytical and Bioanalytical Chemistry* **2008**, *391*, 239-250.
[26] R. Simons, J.-P. Vincken, M. C. Bohin, T. F. M. Kuijpers, M. A. Verbruggen, H. Gruppen, Identification of prenylated pterocarpans and other isoflavonoids in *Rhizopus* spp. elicited soya bean seedlings by electrospray ionisation mass spectrometry, *Rapid Communications in Mass Spectrometry* **2011**, *25*, 55-65.
[27] F. Ferreres, A. Gil-Izquierdo, P. B. Andrade, P. Valentão, F. A. Tomás-Barberán, Characterization of *C*-glycosyl flavones *O*-glycosylated by liquid chromatography-tandem mass spectrometry, *Journal of Chromatography A* **2007**, *1161*, 214-223.
[28] R. Simons, J.-P. Vincken, E. J. Bakx, M. A. Verbruggen, H. Gruppen, A rapid screening method for prenylated flavonoids with ultra-high-performance liquid chromatography/electrospray ionisation mass spectrometry in licorice root extracts, *Rapid Communications in Mass Spectrometry* **2009**, *23*, 3083-3093.
[29] M.-J. Xu, B. Wu, T. Ding, J.-H. Chu, C.-Y. Li, J. Zhang, T. Wu, J. Wu, S.-J. Liu, S.-L. Liu, W.-Z. Ju, P. Li, Simultaneous characterization of prenylated flavonoids and isoflavonoids in *Psoralea corylifolia* L. by liquid chromatography with diode-array detection and quadrupole time-of-flight mass spectrometry, *Rapid Communications in Mass Spectrometry* **2012**, *26*, 2343-2358.
[30] F. Cuyckens, M. Claeys, Mass spectrometry in the structural analysis of flavonoids, *Journal of Mass Spectrometry* **2004**, *39*, 1-15.
[31] N. Fabre, I. Rustan, E. De Hoffmann, J. Quetin-Leclercq, Determination of flavone, flavonol, and flavanone aglycones by negative ion liquid chromatography electrospray ion trap mass spectrometry, *Journal of the American Society for Mass Spectrometry* **2001**, *12*, 707-715.
[32] E. H. Anouar, C. P. Osman, J.-F. F. Weber, N. H. Ismail, UV/Visible spectra of a series of natural and synthesised anthraquinones: Experimental and quantum chemical approaches, *SpringerPlus* **2014**, *3*, 233.
[33] L. Rafaëlly, S. Héron, W. Nowik, A. Tchapla, Optimisation of ESI-MS detection for the HPLC of anthraquinone dyes, *Dyes and Pigments* **2008**, *77*, 191-203.
[34] R. Song, H. Lin, Z. Zhang, Z. Li, L. Xu, H. Dong, Y. Tian, Profiling the metabolic differences of anthraquinone derivatives using liquid chromatography/tandem mass spectrometry with data-dependent acquisition, *Rapid Communications in Mass Spectrometry* **2009**, *23*, 537-547.
[35] D. Tang, Y. Dong, N. Guo, L. Li, H. Ren, Metabolomic analysis of the polyphenols in germinating mung beans (*Vigna radiata*) seeds and sprouts, *Journal of the Science of Food and Agriculture* **2014**, *94*, 1639-1647.
[36] S. O. Mueller, M. Schmitt, W. Dekant, H. Stopper, J. Schlatter, P. Schreier, W. K. Lutz, Occurrence of emodin, chrysophanol and physcion in vegetables, herbs and liquors. Genotoxicity and anti-genotoxicity of the anthraquinones and of the whole plants, *Food and Chemical Toxicology* **1999**, *37*, 481-491.

Supporting Information

Table S1. Compounds tentatively identified by RP-UHPLC-PDA-MS in extracts from legume seeds and seedlings.

No[a]	t_R (min)	λ_{max} (nm)	$[M-H]^-$	MS^{2b}	MS^{3b}	$[M+H]^+$	MS^{2b}	MS^{3b}	Tentative annotation	Prenyl position
Flavanones										
9	4.55	288	449	287, 269, 259	259, 243, 269 201	nr[d]			Unknown flavanone *O*-glucoside	
12	4.77	288	449	329, 287, 359, 269, 431, 259	167, 161	451	433, 415, 331	415, 355, 397, 385	Unknown flavanone *C*-glucoside	
22	5.41	nd[c]	563	255	135, 119, 153	nr[d]			Liquiritigenin *O*-rhamnosyl glucoside	
54	10.17	288	271	151, 177, 227, 119	107	273	153, 147	111, 129, 127, 125, 74, 71	Naringenin	
Flavones										
2	3.40	nr[d]	595	433, 403, 271	271, 253, 405	nr[d]			Apigenin *O*-glucosyl glucoside	
20	5.26	268, 336	431	311, 341	283, 191	433	415, 367, 397, 313, 337	397, 367, 337, 295, 379	Apigenin *C*-glucoside	
23	5.48	269, 337	577	311, 341, 395	283	579	433, 415, 313	415, 367, 313, 337, 397	Apigenin *C*-glucoside *O*-rhamnoside	
26	5.63	270, 336	431	311, 341, 413	283	433	415, 367, 397, 313, 337, 379	367, 397, 337, 295, 379, 283	Apigenin *C*-glucoside	
38	6.33	266, 334	517	473	311, 413, 283	519	501, 313, 475, 457, 295, 483, 337	457, 295, 483, 337, 439, 415, 379, 361	Apigenin *C*-glucoside malonylated	
Flavonols										
10	4.69	255, 295*sh*[e], 353	757	301, 300, 625, 607, 343, 475, 271, 739, 595, 255	179, 151, 273, 272, 257, 256	759	465, 597, 303, 627, 435, 579, 741, 489, 507, 447, 742, 477	447, 345, 369, 399, 303, 429, 411	Quercetin *O*-xylosyl glucoside *O*-glucoside	
11	4.73	266, 321	739	593	285, 284, 327, 255	741	433, 287, 595	287	Kaempferol *O*-rhamnoside-*O*-rhamnosylglucoside	
13	4.85	264, 329	695	651, 533, 489	489, 285, 447, 531	697	287, 449, 535	153, 269, 241, 231, 259, 213, 165, 217, 245, 149	Kaempferol *O*-diglucoside malonylated	
14	5.02	255, 302*sh*[e], 355	625	301, 300, 343, 302	178, 257, 273	627	303, 465, 447	257, 229, 165, 285, 247, 274, 275, 163	Quercetin *O*-diglucoside	
21	5.33	267, 342	609	285, 327, 447, 255, 229	257, 241, 255, 267, 151, 229, 213, 197, 199, 163	611	287, 449, 431	241, 165, 213, 153, 231, 121, 259, 133, 197, 145	Kaempferol *O*-diglucoside	

25	5.56	255, 353	609	301, 300, 302	179, 151, 257, 273	611	303, 465, 449	257, 285, 229, 165, 247, 275, 153, 274, 137, 149	Quercetin *O*-rhamnosyl glucoside	
27	5.64	250*sh*[e], 368	463	301	151, 179, 273	465	303	257, 285, 229, 165, 247, 137, 275, 153	Quercetin *O*-glucoside	
29	5.82	265, 348	593	285, 286	257, 267, 241, 229, 213, 163, 199	595	287, 449	241, 165, 213, 269, 153, 231, 121, 259	Kaempferol *O*-rhamnosyl glucoside	
31	5.96	255, 355	711	667, 463	463, 301, 505	713	303, 465, 345, 695, 447, 677, 551, 533	137, 151, 179, 275, 285	Quercetin *O*-diglucoside malonylated	
44	7.65	264, 350	533	285, 489	257, 267, 229, 241, 213, 197, 163, 239	535	287	241, 165, 213, 153, 231, 259, 269, 217, 245, 121, 133, 197	Kaempferol *O*-glucoside malonylated	
Isoflavans										
41	6.89	272	271	243	109, 149, 133, 135, 199, 121, 123	273	255, 227, 137	227, 199	Isovestitol	
76	16.95	282	339	217, 121, 135, 324	202, 159, 162 147	341	205, 219, 231, 285, 123, 149	163, 149, 175, 177, 135, 137, 173	2′-*O*-Methyl phaseollidinisoflavan	B-ring
72	15.70	277	323	135, 147, 201, 213, 175, 109, 279, 121, 305, 308, 239, 281, 148, 215, 187, 253	91, 93	325	189, 123, 203, 215, 149	147, 171	Phaseollinisoflavan	B-ring
Isoflavanones										
6	4.10	283	611	449	287, 161, 329, 311, 381, 359	451, 613	433, 289, 415	415, 271, 253	Dalbergioidin *O*-diglucoside	
28	5.72	285, 342*sh*[e]	841	517, 355, 679, 541, 559	193, 355	843	519, 441, 357, 681	357, 463, 501, 301	Kievitone *O*-triglucoside	A-ring
30	5.85	285, 341*sh*[e]	841	517, 355, 679, 559	193, 355	843	519, 441, 357, 681	357, 463, 501, 301	Kievitone *O*-triglucoside	A-ring
32	6.01	282, 357*sh*[e]	679	517, 635, 541	193, 355, 161	681	357	339, 301, 283, 247	Kievitone *O*-diglucoside	A-ring
37	6.25	282, 339*sh*[e]	679	517, 559, 541	193, 355, 161	681	357	339, 301, 283, 247	Kievitone *O*-diglucoside	A-ring
42	6.97	282, 351*sh*[e]	765	517, 559, 355	193, 355, 475	767	549, 357, 301, 587, 283, 531, 339	301, 283, 531, 513, 495, 179, 255, 325	Kievitone *O*-diglucoside malonylated	A-ring
45	8.02	283	549	505	301	551	533, 345, 369, 303, 515, 411, 327	515, 369, 411, 327, 497, 393	Isoferreirin *O*-glucoside malonylated	
46	8.54	287	287	161, 125	133	289	271, 179, 151, 261	243, 215, 229, 203, 187, 253, 227, 161	Dalbergioidin	

50	9.16	291, 320*sh*[e]	371	209, 161	179, 123, 141, 191, 165, 125, 167	373	355	337, 233, 245	Kievitol	A-ring
53	10.09	284	517	355, 397, 379	193, 161, 124	nr[d]			Kievitone *O*-glucoside	A-ring
56	10.50	286	301	165	137, 121, 109	303	137, 151, 179, 275, 285, 149	107, 109, 125, 123	Isoferreirin	
61	12.42	286	339	161, 177	133	341	285, 231, 323, 313, 257	123, 267, 175	5-Deoxykievitone	A-ring
64	13.48	284	355	299, 125	174	357	289, 301, 339, 151	271, 179, 151, 261	Dihydrolicoisoflavone	B-ring
66	13.91	291, 339*sh*[e]	355	193, 161	124, 149, 151, 137, 109	357	339, 301, 283, 247, 163	283	Kievitone	A-ring
74	16.48	284	339	151, 176, 187	107	341	285, 179, 267, 215, 153	267, 165, 153	Prenylated dihydrogenistein	B-ring
Isoflavones										
1	3.33	259	593	431	269	595	433, 271	271	Genistein *O*-diglucoside	
3	3.68	256	443	361, 237, 425, 161, 383, 281, 219, 399, 143, 179, 189, 159	279, 217, 203, 343, 319, 317, 269, 315	nr[d]			Unknown isoflavone	
4	3.71	248, 295	637, 577[f]	415	253, 295	579	417, 255	255	Daidzein *O*-diglucoside acetylated	
5	3.86	257, 322*sh*[e]	609	447, 489, 471, 285	285, 379, 217	611	287, 449	217, 259, 245, 153, 231, 269, 175, 161, 149	2'-Hydroxygenistein *O*-diglucoside	
7	4.19	258, 334	691	301, 345, 259	241	693	325, 283, 271, 307, 227	243, 307, 227, 281, 265, 283, 301, 185	Unknown isoflavone	
8	4.21	259, 332*sh*[e]	653, 593[f]	431	268, 269, 311	595	271, 433	153, 243, 215, 253, 227	Genistein *O*-diglucoside acetylated	
16	5.03	257	679	431	268, 269	681	271, 519, 433	215, 153, 243, 253, 149, 145, 159, 225	Genistein *O*-glucoside malonylated	
17	5.08	248, 297*sh*[e]	475	253, 425	253, 225, 224, 209, 135, 211	477	255	199, 137, 227, 237, 145	Daidzein *O*-glucoside	
18	5.14	257, 323*sh*[e]	447	285, 327, 309, 387	217, 241, 199, 151	nr[d]			2'-Hydroxygenistein *O*-glucoside	
19	5.25	259, 328*sh*[e]	705	367	331, 303, 305, 285, 349, 287	nr[d]			Unknown isoflavone	
33	6.11	264, 344	577	269, 270	225, 269, 149, 183, 201, 227, 181, 197, 117, 151, 169, 226, 224	579	271, 433	153, 225, 229, 203, 145, 121, 119	Genistein *O*-rhamnosyl-glucoside	

34	6.17	257, 315*sh*[e]	447	379, 285, 217, 357	217, 241	449	287	133, 159, 151	2',4',7,8-Tetrahydroxy isoflavone *O*-glucoside	
35	6.19	260	605	311, 252, 267	267, 296, 252, 201	nr[d]			6,7,4'-Trimethoxy isoflavone *O*-xylosil glucoside	
36	6.21	259	431	268, 269, 311, 341, 371	225, 241, 201, 181, 227, 197	433	271	153, 215, 243, 253, 149, 145, 159	Genistein *O*-glucoside	
39	6.33	257	619	311, 575, 267	267, 296, 252, 201	nr[d]			6,7,4'-Trimethoxy isoflavone *O*-rhamnosyl glucoside	
43	7.63	260	517	269	225, 241, 181, 147, 201, 226	519	271, 433	215, 153, 149, 243, 253, 145, 225	Genistein *O*-glucoside malonylated	
47	8.68	248, 300	253	225, 209, 224, 253, 226, 197, 134, 223, 254, 208	197, 198, 181, 170	255	199, 137, 213, 227, 237, 145, 200, 228, 197	181, 171, 182, 153, 157	Daidzein	
48	8.83	258, 284*sh*[e]	285	217, 241, 199, 175	173, 175, 189, 199, 161, 149, 131	287	217, 259, 153, 245, 231, 175, 269, 161, 203	189	2'-Hydroxygenistein	
52	9.45	265, 284, 305*sh*[e]	369	339, 284, 351, 301, 298, 325	271, 284, 295, 253, 298, 201	371	353	219, 335, 325, 283, 311	Dihydrofuranoisoflavone	A-ring
57	10.91	259	299	284	256, 212, 227, 240, 228, 211, 216, 200	nr[d]			Gliricidin	
58	11.01	260	269	225, 181, 201, 241, 197, 269, 224, 227, 213,	181, 197, 169, 182, 210, 183, 177, 179	271	153, 243, 215, 253, 149, 145	109, 67, 95, 145, 125, 135	Genistein	
69	14.99	264	353	284, 285, 298, 267, 309, 151	267, 216, 256, 230, 242	355	299	165, 281, 257, 229	2,3-Dehydrokievitone	A-ring
75	16.86	261	353	285, 267, 309	216, 241, 267	355	299	271, 147, 243, 245, 281, 217, 153, 253, 191, 173	Phaseoluteone (licoisoflavone A)	B-ring
78	17.62	264	337	282	253, 238, 189, 264, 267, 161	339	283	241, 213, 255, 265	Lupiwighteone	A-ring
Pterocarpans										
24	5.50	292	561	253	253, 211, 225, 209, 135	nr[d]			Anhydroglycinol *O*-rhamnosylglucoside	
63	13.20	288	255	213, 211, 151, 187	185, 169, 145, 171	257	153, 131, 173, 215	67, 111, 109	Demethylmedicarpin	
68	14.63	280, 284	353	309, 338, 294	294, 251, 279	337[g]	281	253, 251, 197, 225	Cristacarpin	B-ring
70	15.6	286	323	308, 254, 267, 268, 280, 279, 255, 281, 305, 309, 295, 177,	253, 265	325	269, 191, 123	251, 241, 215, 159, 223, 227, 147, 213, 161	Phaseollidin	B-ring

71	15.68	293, 306*sh*[e]	321	306, 233, 277, 303, 293, 278, 175, 279, 307, 266, 145, 199	157, 175	323	189, 295, 213, 147, 163, 123, 305	147, 171, 161	Phaseollin isomer	B-ring
73	15.81	283	323	268, 254, 308, 280, 213, 279, 255, 267, 305, 281, 295, 201	253, 224, 240, 225, 223, 239, 226, 159, 209	325	269, 191, 123	251, 241, 215, 159, 223, 227, 147, 213, 161	Phaseollidin isomer	B-ring
77	16.95	279, 314*sh*[e]	321	306, 277, 303, 175, 279, 265	291, 289, 277, 261	323	189, 213, 147, 123, 295, 163, 305, 308	147, 171, 161	Phaseollin	B-ring
Coumaronochromones										
65	13.77	255, 280, 335	283	255, 239, 265, 227	227, 211, 237	285	257, 213, 229, 241	229	Lupinalbin A	
Coumestans										
60	11.65	345	267	239, 267, 240, 223	211, 212	269	241, 225, 197	213	Coumestrol	
67	13.96	355	337	309	253, 254, 240, 266, 265	339	271, 255, 283, 311, 243	243, 215	Unknown coumestan	ND[h]
79	18.35	260*sh*[e], 345	335	279	251, 279, 252	337	269, 281, 253	241, 225, 197, 213	Phaseol/psoralidin/isosojagol	ND[h]
80	18.72	nd[c]	335	280	236, 252	337	281, 309, 269, 237	209, 253, 213, 210, 181	Phaseol/psoralidin/isosojagol	ND[h]
Athraquinones										
40	6.46	267, 296, 443	269	241	197, 213, 199	271	253, 243	224, 225, 197, 253	Unknown anthraquinone	
49	8.83	250*sh*[e], 343, 470	269	241	197, 199, 213, 169	271	243, 215	215, 149	Unknown anthraquinone	
51	9.45	268, 283, 290, 301*sh*[e]	269	241, 225, 197	197, 213, 199, 169	271	243, 253, 215	215, 149	Emodin/its isomer	
55	10.30	287, 375	253	209	167, 194, 181, 182	255	237	209, 227	Methoxy anthraquinone	
59	11.35	284*sh*[e], 321, 475	269	241, 225, 197	197, 213, 199, 169	271	243, 215, 253	215, 149	Emodin/its isomer	
Unknown										
62	12.73	nd[c]	341	167, 323, 231, 341	137, 123, 121, 79, 149, 139	343	325, 175, 215	175, 215, 269, 307, 297, 257, 163, 137	Unknown	ND[h]

[a] Numbers refer to peaks in **Figure 1**.
[b] Daughter ion in MS^2 and MS^3 are listed in order of intensity, first value is the base peak.
[c] nd, Not detected, as no clear UV/vis spectrum was obtained.
[d] nr, Not relevant. Either the *m/z* of the parent ion showed a mismatch between PI and NI mode MS, or the λ_{max} was ambiguous due to overlap signals of multiple compounds.
[e] *sh*, Shoulder.
[f] The parent ion showed in-source fragmentation in NI and PI mode MS. The bold *m/z* represents the $[M-H]^-$ ion.
[g] In positive mode ESI-MS, parent ions lost a water molecule to produce $[M + H-H_2O]^+$. The intensity of this ion dominated the $[M + H]^+$ mass spectrum.
[h] ND, not determined. The prenyl position was not determined.

Table S2. Contents (mg DE/g DW)[a] of (iso)flavonoids in extracts from Phaseoleae seedlings. Data are the means ± standard deviation (SD) of experiments performed in duplicate.

No[b]	Tentatively compounds	*Vigna angularis*		*V. radiata*		*V. unguiculata*		*Phaseolus vulgaris*		*P. coccineus*		*Lablab purpureus*		*Psophocarpus tetragonolobus*	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Flavanones															
9	Unknown flavanone *O*-glucoside	0.04	0.00	0.07	0.02	0.08	0.02	0.01	0.01	0.01	0.00	0.01	0.01	0.02	0.00
12	Unknown flavanone *C*-glucoside	-[c]	-	-	-	0.04	0.01	-	-	-	-	-	-	-	-
22	Liquiritigenin *O*-rhamnosyl glucoside	-	-	-	-	-	-	-	-	-	-	-	-	0.01	0.00
54	Naringenin	-	-	-	-	0.04	0.01	-	-	-	-	-	-	-	-
Flavones															
2	Apigenin *O*-glucosyl glucoside	-	-	-	-	-	-	-	-	0.01	0.00	-	-	-	-
20	Apigenin *C*-glucoside	-	-	0.04	0.01	-	-	-	-	-	-	-	-	<0.01	-
23	Apigenin *C*-glucoside O-rhamnoside	-	-	0.06	0.00	-	-	-	-	-	-	-	-	-	-
26	Apigenin *C*-glucoside	-	-	-	-	-	-	-	-	-	-	-	-	-	-
38	Apigenin *C*-glucoside malonylated	-	-	0.04	0.02	-	-	-	-	-	-	-	-	-	-
Flavonols															
10	Quercetin *O*-xylosyl glucoside *O*-glucoside	-	-	-	-	-	-	-	-	-	-	-	-	-	-
11	Kaempferol *O*-rhamnoside-*O*-rhamnosylglucoside	0.05	0.00	-	-	-	-	-	-	-	-	-	-	-	-
13	Kaempferol *O*-diglucoside malonylated	-	-	-	-	-	-	0.02	0.01	-	-	-	-	-	-
14	Quercetin *O*-diglucoside	-	-	-	-	0.07	0.02	-	-	-	-	-	-	-	-
21	Kaempferol *O*-diglucoside	-	-	-	-	0.02	0.00	-	-	-	-	-	-	-	-
25	Quercetin *O*-rhamnosyl glucoside	-	-	0.17	0.01	-	-	0.06	0.01	-	-	-	-	-	-
27	Quercetin *O*-glucoside	-	-	-	-	-	-	0.01	0.00	-	-	-	-	-	-
29	Kaempferol *O*-rhamnosyl glucoside	-	-	0.08	0.00	-	-	0.04	0.02	-	-	-	-	-	-
31	Quercetin *O*-diglucoside malonylated	-	-	-	-	-	-	-	-	-	-	-	-	-	-
44	Kaempferol *O*-glucoside malonylated	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Isoflavans															
41	Isovestitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-
76	2′-O-Methyl phaseollidinisoflavan	-	-	-	-	-	-	-	-	-	-	-	-	-	-
72	Phaseollinisoflavan	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Isoflavanones															
6	Dalbergioidin *O*-diglucoside	0.04	0.00	-	-	0.02	0.00	0.03	0.02	0.02	0.00	-	-	-	-

28	Kievitone *O*-triglucoside	-	-	-	-	-	-	-	-	-	-	-	-	-	-
30	Kievitone *O*-triglucoside	-	-	-	-	-	-	-	-	-	-	-	-	-	-
32	Kievitone *O*-diglucoside	-	-	-	-	-	-	0.01	0.01	-	-	-	-	-	-
37	Kievitone *O*-diglucoside	-	-	-	-	0.01	0.01	0.05	0.03	-	-	-	-	-	-
42	Kievitone *O*-diglucoside malonylated	-	-	-	-	-	-	0.01	0.01	-	-	-	-	-	-
45	Isoferreirin *O*-glucoside malonylated	-	-	-	-	-	-	-	-	-	-	-	-	-	-
46	Dalbergioidin	0.01	0.00	-	-	0.12	0.03	0.04	0.02	0.01	0.01	0.01	0.01	-	-
50	Kievitol	-	-	-	-	<0.01	-	0.01	0.00	<0.01	-	-	-	-	-
53	Kievitone *O*-glucoside	0.02	0.01	-	-	-	-	-	-	-	-	-	-	-	-
56	Isoferreirin	<0.01	-	-	-	0.03	0.01	0.01	0.01	-	-	-	-	-	-
61	5-Deoxykievitone	-	-	-	-	<0.01	-	<0.01	-	-	-	0.02	0.01	-	-
64	Dihydrolicoisoflavone	-	-	-	-	-	-	-	-	-	-	-	-	-	-
66	Kievitone	0.06	0.01	0.03	0.02	0.22	0.06	0.11	0.05	0.02	0.01	0.07	0.05	-	-
74	Prenylated dihydrogenistein	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Isoflavones															
1	Genistein *O*-diglucoside	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3	Unknown isoflavone	-	-	-	-	-	-	-	-	0.02	0.00	-	-	-	-
4	Daidzein *O*-diglucoside acetylated	0.01	0.00	0.07	0.05	0.01	0.00	<0.01	-	0.01	0.00	-	-	-	-
5	2'-Hydroxygenistein *O*-diglucoside	0.10	0.01	0.03	0.01	0.01	0.01	0.08	0.06	0.03	0.01	-	-	-	-
7	Unknown isoflavone	-	-	0.02	0.02	0.01	0.01	-	-	-	-	-	-	-	-
8	Genistein *O*-diglucoside acetylated	0.03	0.00	-	-	0.01	0.01	0.02	0.01	0.03	0.00	-	-	-	-
16	Genistein *O*-glucoside malonylated	0.02	0.00	-	-	-	-	-	-	-	-	-	-	-	-
17	Daidzein *O*-glucoside	<0.01	-	-	-	-	-	-	-	-	-	-	-	-	-
18	2'-Hydroxygenistein *O*-glucoside	-	-	-	-	-	-	-	-	-	-	0.01	0.01	-	-
19	Unknown isoflavone	-	-	-	-	-	-	-	-	0.02	0.00	-	-	-	-
33	Genistein *O*-rhamnosyl-glucoside	-	-	-	-	-	-	-	-	-	-	-	-	0.01	0.00
34	2',4',7,8-Tetrahydroxy isoflavone *O*-glucoside	-	-	-	-	-	-	-	-	0.01	0.00	-	-	-	-
35	6,7,4'-Trimethoxy isoflavone *O*-xylosil glucoside	-	-	-	-	-	-	-	-	-	-	-	-	0.02	0.01
36	Genistein *O*-glucoside	-	-	-	-	-	-	-	-	-	-	-	-	-	-
39	6,7,4'-Trimethoxy isoflavone *O*-rhamnosyl glucoside	-	-	-	-	-	-	-	-	-	-	-	-	0.01	0.00
43	Genistein *O*-glucoside malonylated	-	-	-	-	-	-	-	-	-	-	0.03	0.00	-	-
47	Daidzein	-	-	-	-	0.03	0.00	-	-	0.01	0.01	-	-	-	-
48	2'-Hydroxygenistein	0.01	0.00	-	-	0.03	0.02	0.03	0.01	0.01	0.00	0.01	0.01	-	-
52	Dihydrofuranoisoflavone	-	-	-	-	-	-	-	-	-	-	-	-	-	-

57	Gliricidin	-	-	-	-	-	-	-	-	-	-	-	-	-	-
58	Genistein	0.01	0.00	-	-	0.09	0.00	0.01	0.00	<0.01	-	0.01	0.00	-	-
69	2,3-dehydrokievitone	-	-	-	-	-	-	-	-	-	-	-	-	-	-
75	Phaseoluteone (licoisoflavone A)	-	-	-	-	-	-	0.02	0.01	-	-	-	-	-	-
78	Lupiwighteone	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Pterocarpans															
24	Anhydroglycinol *O*-rhamnosylglucoside	-	-	-	-	-	-	-	-	-	-	-	-	0.01	0.00
63	Demethylmedicarpin	-	-	-	-	-	-	-	-	-	-	-	-	-	-
68	Cristacarpin	-	-	-	-	-	-	-	-	-	-	-	-	-	-
70	Phaseollidin	-	-	-	-	-	-	-	-	-	-	0.01	0.01	0.01	0.01
71	Phaseollin isomer	-	-	-	-	-	-	-	-	-	-	-	-	<0.01	-
73	Phaseollidin isomer	-	-	-	-	-	-	-	-	-	-	-	-	-	-
77	Phaseollin	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Coumaronochromone															
65	Lupinalbin A	-	-	-	-	-	-	<0.01	-	<0.01	-	<0.01	-	-	-
Coumestans															
60	Coumestrol	-	-	0.02	0.01	0.01	0.00	<0.01	-	0.01	0.00	-	-	-	-
67	Unknown coumestan	-	-	-	-	-	-	-	-	-	-	-	-	-	-
79	Phaseol/psoralidin/isosojagol	-	-	-	-	-	-	-	-	-	-	-	-	-	-
80	Phaseol/psoralidin/isosojagol	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Anthraquinones															
40	Unknown anthraquinone	-	-	-	-	-	-	-	-	-	-	-	-	-	-
49	Unknown anthraquinone	-	-	-	-	-	-	-	-	-	-	-	-	-	-
51	Emodin/its isomer	-	-	-	-	-	-	-	-	-	-	-	-	-	-
55	Methoxy anthraquinone	-	-	-	-	-	-	-	-	-	-	-	-	-	-
59	Emodin/its isomer	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Unknown															
62	Unknown	-	-	-	-	-	-	-	-	-	-	-	-	-	-

[a] Data are expressed in mg daidzein equivalent/g dry weight.
[b] Numbers refer to compounds in **Table S1**.
[c] The compound was not found in the extract.

Table S3. Contents (mg DE/g DW)[a] of (iso)flavonoids in extracts from elicited Phaseoleae seedlings. Data are the means ± standard deviation (SD) of experiments performed in duplicate.

No[b]	Tentatively compounds	*Vigna angularis*		*V. radiata*		*V. unguiculata*		*Phaseolus vulgaris*		*P. coccineus*		*Lablab purpureus*		*Psophocarpus tetragonolobus*	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Flavanones															
9	Unknown flavanone *O*-glucoside	0.05	0.01	0.07	0.01	0.08	0.04	0.09	0.02	0.06	0.00	0.01	0.00	0.03	0.01
12	Unknown flavanone *C*-glucoside	-[c]	-	-	-	0.04	0.02	-	-	-	-	-	-	-	-
22	Liquiritigenin *O*-rhamnosyl glucoside	-	-	-	-	-	-	-	-	-	-	-	-	<0.01	-
54	Naringenin	-	-	0.01	0.01	0.02	0.01	-	-	-	-	0.02	0.02	-	-
Flavones															
2	Apigenin *O*-glucosyl glucoside	-	-	-	-	-	-	-	-	0.02	0.01	-	-	-	-
20	Apigenin *C*-glucoside	-	-	0.09	0.03	-	-	-	-	-	-	-	-	0.01	0.01
23	Apigenin *C*-glucoside O-rhamnoside	-	-	0.06	0.02	-	-	-	-	-	-	-	-	-	-
26	Apigenin *C*-glucoside	-	-	-	-	-	-	-	-	-	-	-	-	-	-
38	Apigenin *C*-glucoside malonylated	-	-	0.05	0.01	-	-	-	-	-	-	-	-	-	-
Flavonols															
10	Quercetin *O*-xylosyl glucoside *O*-glucoside	-	-	-	-	0.05	0.02	-	-	-	-	-	-	-	-
11	Kaempferol *O*-rhamnoside-*O*-rhamnosylglucoside	0.05	0.02	-	-	-	-	-	-	-	-	-	-	-	-
13	Kaempferol *O*-diglucoside malonylated	-	-	-	-	-	-	0.14	0.02	-	-	-	-	-	-
14	Quercetin *O*-diglucoside	-	-	-	-	0.21	0.03	-	-	0.08	0.04	-	-	-	-
21	Kaempferol *O*-diglucoside	-	-	-	-	0.05	0.02	-	-	-	-	-	-	-	-
25	Quercetin *O*-rhamnosyl glucoside	-	-	0.05	0.02	-	-	0.05	0.04	-	-	-	-	-	-
27	Quercetin *O*-glucoside	-	-	-	-	-	-	0.02	0.02	0.06	0.02	-	-	-	-
29	Kaempferol *O*-rhamnosyl glucoside	-	-	0.03	0.01	-	-	0.06	0.01	-	-	-	-	-	-
31	Quercetin *O*-diglucoside malonylated	-	-	-	-	-	-	0.02	0.02	0.02	0.01	-	-	-	-
44	Kaempferol *O*-glucoside malonylated	-	-	-	-	-	-	0.04	0.00	-	-	-	-	-	-
Isoflavans															
41	Isovestitol	-	-	-	-	-	-	-	-	0.01	0.01	-	-	-	-
76	2'-O-Methyl phaseollidinisoflavan	-	-	-	-	0.02	0.01	-	-	-	-	-	-	-	-
72	Phaseollinisoflavan	-	-	-	-	-	-	0.10	0.01	0.09	0.02	-	-	-	-
Isoflavanones															
6	Dalbergioidin *O*-diglucoside	0.03	-	-	-	0.06	0.03	0.14	0.01	0.08	0.01	-	-	-	-

28	Kievitone *O*-triglucoside	-	-	-	-	-	-	0.02	0.01	-	-	-	-	-	-
30	Kievitone *O*-triglucoside	-	-	-	-	-	-	0.04	0.03	-	-	-	-	-	-
32	Kievitone *O*-diglucoside	-	-	-	-	0.01	0.01	0.04	0.01	-	-	-	-	-	-
37	Kievitone *O*-diglucoside	-	-	-	-	0.07	0.02	0.13	0.03	-	-	-	-	-	-
42	Kievitone *O*-diglucoside malonylated	-	-	-	-	-	-	0.06	0.02	-	-	-	-	-	-
45	Isoferreirin *O*-glucoside malonylated	-	-	-	-	-	-	0.01	0.00	-	-	-	-	-	-
46	Dalbergioidin	0.08	0.04	0.05	0.02	0.16	0.07	0.14	0.07	0.13	0.06	0.03	0.00	-	-
50	Kievitol	-	-	-	-	-	-	0.07	0.02	0.03	0.01	-	-	-	-
53	Kievitone *O*-glucoside	0.02	0.01	-	-	-	-	0.01	0.01	0.01	0.01	-	-	-	-
56	Isoferreirin	0.02	0.01	0.04	0.02	0.07	0.03	0.07	0.03	0.08	0.03	0.02	0.00	-	-
61	5-Deoxykievitone	-	-	0.02	0.01	0.01	0.01	0.06	0.03	-	-	0.03	0.02	-	-
64	Dihydrolicoisoflavone	-	-	-	-	-	-	0.01	0.00	-	-	-	-	-	-
66	Kievitone	0.23	0.12	0.12	0.06	0.29	0.14	0.53	0.21	0.30	0.12	0.09	0.04	-	-
74	Prenylated dihydrogenistein	-	-	-	-	-	-	-	-	-	-	0.01	0.01	-	-
Isoflavones															
1	Genistein *O*-diglucoside	-	-	-	-	-	-	-	-	0.06	0.00	-	-	-	-
3	Unknown isoflavone	-	-	-	-	-	-	-	-	0.02	0.01	-	-	-	-
4	Daidzein *O*-diglucoside acetylated	0.02	0.01	0.01	0.01	0.03	0.02	-	-	0.04	0.02	-	-	-	-
5	2'-Hydroxygenistein *O*-diglucoside	0.12	0.00	0.03	0.02	0.05	0.03	0.27	0.07	0.18	0.01	-	-	-	-
7	Unknown isoflavone	-	-	0.03	0.03	0.04	0.04	-	-	-	-	-	-	-	-
8	Genistein *O*-diglucoside acetylated	0.04	0.01	-	-	0.01	0.01	0.06	0.03	0.08	-	-	-	-	-
16	Genistein *O*-glucoside malonylated	0.01	0.01	-	-	-	-	-	-	-	-	-	-	-	-
17	Daidzein *O*-glucoside	0.03	0.01	-	-	-	-	-	-	-	-	-	-	-	-
18	2'-Hydroxygenistein *O*-glucoside	-	-	-	-	-	-	-	-	-	-	0.01	0.01	-	-
19	Unknown isoflavone	-	-	-	-	-	-	-	-	0.04	0.01	-	-	-	-
33	Genistein *O*-rhamnosyl-glucoside	-	-	-	-	-	-	-	-	-	-	-	-	0.01	0.00
34	2′,4′,7,8-Tetrahydroxy isoflavone *O*-glucoside	-	-	-	-	0.01	0.00	-	-	0.01	0.00	-	-	-	-
35	6,7,4′-Trimethoxy isoflavone *O*-xylosil glucoside	-	-	-	-	-	-	-	-	-	-	-	-	0.01	0.01
36	Genistein *O*-glucoside	-	-	0.05	0.01	-	-	-	-	-	-	-	-	-	-
39	6,7,4′-Trimethoxy isoflavone *O*-rhamnosyl glucoside	-	-	-	-	-	-	-	-	-	-	-	-	0.01	0.00
43	Genistein *O*-glucoside malonylated	-	-	-	-	-	-	-	-	-	-	0.01	0.01	-	-
47	Daidzein	0.02	0.00	0.10	0.06	0.05	0.01	-	-	0.07	0.05	0.01	0.01	<0.01	-
48	2'-Hydroxygenistein	0.06	0.03	0.08	0.03	0.12	0.04	0.11	0.05	0.06	0.04	0.04	0.01	0.01	0.01
52	Dihydrofuranoisoflavone	-	-	-	-	-	-	0.04	0.02	-	-	-	-	-	-

57	Gliricidin	-	-	-	-	-	-	-	-	0.01	0.00	-	-	-	-
58	Genistein	0.04	0.02	0.09	0.01	0.14	0.02	0.02	0.00	0.03	0.02	0.07	0.02	-	-
69	2,3-dehydrokievitone	-	-	0.01	0.01	-	-	0.02	0.01	0.01	0.00	-	-	-	-
75	Phaseoluteone (licoisoflavone A)	-	-	-	-	-	-	0.14	0.05	0.05	0.02	-	-	-	-
78	Lupiwighteone	-	-	-	-	-	-	-	-	-	-	0.03	0.03	-	-
Pterocarpans															
24	Anhydroglycinol *O*-rhamnosylglucoside	-	-	-	-	-	-	-	-	-	-	-	-	0.01	0.00
63	Demethylmedicarpin	-	-	-	-	-	-	-	-	-	-	0.03	0.02	-	-
68	Cristacarpin	-	-	-	-	-	-	-	-	-	-	-	-	0.09	0.05
70	Phaseollidin	-	-	0.01	0.01	-	-	-	-	0.05	0.03	0.05	0.02	0.22	0.04
71	Phaseollin isomer	-	-	-	-	-	-	-	-	-	-	-	-	0.69	0.11
73	Phaseollidin isomer	-	-	-	-	0.03	0.02	-	-	-	-	-	-	-	-
77	Phaseollin	-	-	-	-	-	-	0.05	0.01	0.02	0.00	-	-	0.01	0.01
Coumaronochromone															
65	Lupinalbin A	-	-	-	-	0.01	0.01	0.01	0.01	0.01	0.00	0.01	0.00	-	-
Coumestans															
60	Coumestrol	0.02	0.00	0.04	0.01	0.04	0.02	0.03	0.01	0.06	0.02	0.01	0.01	0.01	0.00
67	Unknown coumestan	-	-	-	-	-	-	-	-	-	-	-	-	0.03	0.01
79	Phaseol/psoralidin/isosojagol	<0.01	-	0.02	0.01	0.02	0.01	-	-	0.01	0.01	-	-	0.04	0.00
80	Phaseol/psoralidin/isosojagol	-	-	0.01	0.01	-	-	0.01	0.00	-	-	-	-	-	-
Anthraquinones															
40	Unknown anthraquinone	-	-	-	-	-	-	0.08	0.01	0.09	0.01	-	-	-	-
49	Unknown anthraquinone	-	-	-	-	-	-	-	-	0.08	0.03	-	-	-	-
51	Emodin/its isomer	-	-	-	-	-	-	-	-	0.06	0.00	-	-	-	-
55	Methoxy anthraquinone	-	-	-	-	0.07	0.07	0.07	0.07	0.10	0.10	-	-	-	-
59	Emodin/its isomer	-	-	-	-	-	-	0.02	0.02	0.03	0.01	-	-	-	-
Unknown															
62	Unknown	-	-	-	-	-	-	-	-	-	-	0.01	0.01	-	-

[a] Data are expressed in mg daidzein equivalent/g dry weight.
[b] Numbers refer to compounds in **Table S1**.
[c] The compound was not found in the extract.

Chapter 5

Modification of Prenylated Stilbenoids in Peanut Seedlings by the Same Fungi that Elicited Them: The Fungus Strikes Back

Aspergillus oryzae and *Rhizopus oryzae* were compared for inducing the production of prenylated stilbenoids in peanut (*Arachis hypogaea*) seedlings. The fungus was applied at two different time points: directly after soaking (day 1) or after 2 days of germination (day 3). After the treatments, aqueous methanolic extracts of the elicited peanut seedlings were analyzed by LC-PDA-MS. *Aspergillus*- and *Rhizopus*-elicited peanut seedlings accumulated an array of prenylated stilbenoids, with overlap in compounds induced, but also with compounds specific to the fungal treatment. The differences were confirmed to be due to modification of prenylated stilbenoids by the fungus itself. Each fungus appeared to deploy different strategies for modification, i.e. glycosylation by *Rhizopus* and oxidative cleavage by *Aspergillus*. The content of prenylated stilbenoids modified by fungi accounted for around 4% to 39% (w/w) of total stilbenoids. The contents of modified prenylated stilbenoids by *Aspergillus* and *Rhizopus* were 1.9-fold and 2.6-fold higher, respectively, when the fungus was applied on day 1 instead of day 3. Furthermore, the time point of application of the fungus affected the content of unmodified prenylated stilbenoids of peanut seedlings elicited by *Rhizopus* and *Aspergillus* differently. Early application (day 1) of *Rhizopus* decreased the amount of unmodified prenylated stilbenoids of the seedlings up to 3.8-fold compared to application at day 3, but that of *Aspergillus* increased the amount of unmodified prenylated stilbenoids up to 2.4-fold. Taken together, type of fungus and time point of inoculation appeared to be crucial parameters for optimizing accumulation of prenylated stilbenoids in peanut seedlings.

Based on: Siti Aisyah, Harry Gruppen, Mathijs Slager, Bianca Helmink and Jean-Paul Vincken, Modification of Prenylated Stilbenoids in Peanut Seedlings by the Same Fungi that Elicited Them: The Fungus Strikes Back, **2015**, *Submitted*.

INTRODUCTION

By combining malting and fungal stress on legume seeds, phenolic compounds with health-promoting properties, particularly isoflavonoids, can be induced.[1] Upon such biotic stress, the seedlings generate a pool of defense molecules, so-called phytoalexins, to fight the fungus. This method has been applied to soybean and lupine. It appeared that not only the content of isoflavonoids could be increased, but that also the isoflavonoid composition was altered, albeit in different ways. After treatment, soybean seedlings contained prenylated pterocarpans,[2] whereas those of lupine accumulated prenylated isoflavones.[3]

Unlike other members of the legume family, peanuts produce stilbenoid-type phytoalexins instead of isoflavonoids upon elicitation by microorganisms.[4] The major phytoalexins found in peanuts are *trans*-resveratrol, arachidin-1, arachidin-2, arachidin-3, and *trans*-3'-isopentadienyl-3,5,4'-trihydroxystilbene (IPD).[5] The capacity of peanuts to produce stilbenoid phytoalexins has been widely investigated, mainly to improve resistance to pathogenic fungi, such as *Aspergillus flavus* that produces aflatoxin.[4] Control of aflatoxin contamination in peanut is an important issue in food safety.[5] Pathogenic *Aspergillus* species are reported to elicit a strong stilbenoid response in peanut compared to other biotic elicitors such as *Cladosporium* species.[4] Stilbene-1, for instance, has only been found in peanut seedlings elicited by *Aspergillus*.[4] Nevertheless, it is questionable whether stilbene-1 in *Aspergillus*-elicited peanut seedlings is derived from the plant itself, or whether it is a phytoalexin which has been altered by the fungus, e.g. to make it less harmful.[6] In other words, is stilbene-1 a true or a modified phytoalexin?

The potential benefits of peanut phytoalexins to human health have increased the interest in producing such compounds by elicitation with food-grade fungi. However, the induction of peanut phytoalexins by food-grade fungi has not been extensively investigated. To the best of our knowledge, only *Rhizopus oligosporus* has been used to elicit stilbenoid production in peanuts.[7-9] In the present study, we extrapolated the method, which has been applied to soybean and lupine, to peanut. Two different types of food-grade fungi, *Aspergillus oryzae* and *Rhizopus oryzae*, were selected to elicit peanuts. *Rhizopus oryzae* was selected to facilitate comparison with legume species other than peanut, which have been successfully subjected to a similar induction protocol in our laboratory, whereas *Aspergillus oryzae* was selected because of its presumed higher potency to induce phytoalexins. Both fungi have been widely used in food fermentation industry for a long time.[10, 11] It is worth to highlight that *A. oryzae* has low pathogenic potential and does not produce aflatoxins or any other carcinogenic metabolites.[11] The content and compositional changes of stilbenoids in peanuts, peanut seedlings and fungus-elicited peanut seedlings were investigated systematically. It was hypothesized that the stilbenoid profile can be directed by the kind of fungus applied, as well as by the time point of inoculation. Also, we describe, for the first

time, the transformation of prenylated stilbenoids by *A. oryzae* and *R. oryzae*, and discuss the implications of this with respect to generating health-promoting compounds.

MATERIALS AND METHODS

Seeds and chemicals

Dehulled peanuts (*Arachis hypogaea*) were purchased from Vreeken's Zaaden (Dordrecht, The Netherlands). *Trans*-resveratrol and genistein were purchased from Sigma Aldrich (Steinheim, Germany). UHPLC-MS grade acidified water, methanol, acetonitrile and formic acid were obtained from Biosolve BV (Valkenswaard, The Netherlands). Other solvents and D-glucose were purchased from Merck (Darmstadt, Germany) and Sigma Aldrich Chemie (Zwijndrecht, The Netherlands). Neutralized bacteriological peptone and yeast extract were purchased from Oxoid (Basingstoke, UK). Milli-Q water was prepared using an integral water purification system (Millipore, Billerica, MA, USA).

Fungal strains and culture conditions

Rhizopus oryzae (LU581) and *Aspergillus oryzae var. effususs* (LU009) were kindly provided by the Laboratory of Food Microbiology, Wageningen University (Wageningen, The Netherlands). The fungal strains were stored at -80 ^{o}C in 20% (v/v) glycerol. Pure plate cultures of fungi were grown on CM59 malt extract agar (Oxoid, Basingstoke, UK) for 7 days at 30 °C.

Peanut treatments

The treatment of peanuts was performed in an EQMM sprouting machine (EasyGreen, San Diego, CA, USA), which was modified as described previously.[2] The peanuts were sequentially subjected to soaking and germination stages. Prior to soaking, peanuts were surface-sterilized by immersing them in 70% (v/v) aqueous ethanol (2 mL/g peanuts) for 15 min at room temperature and subsequently rinsed 4 times with Milli-Q water (5 mL/g peanut). The sterilized peanuts were soaked for 24 h at 25 °C in sterilized Milli-Q water. Subsequently, the soaked peanuts were peeled and put in plastic cartridges (sterilized by soaking them in hypochlorite 1% (v/v) for 2 h, then rinsing them with Milli-Q water) that were covered with autoclaved filter paper. Next, they were placed in the sprouting machine. Prior to this, the machine was sterilized according to the cleaning protocol provided by the manufacturer. The peanuts were germinated in the dark for 7 days (7G) at 25 °C and 100% RH (**Table 1**). In another set of experiments, the peanuts were also subjected to fungal elicitation. A spore suspension (0.2 mL/g peanuts) was added to the peanuts, either directly after soaking (on day 1, 5R and 5A), or after 2 days of germination (on day 3, 2G-5R and 2G-5A) (**Table 1**). The fungus-inoculated peanuts were incubated for 5 days at 30 °C and a

RH controlled at 55-85%. Spore suspensions for the inoculation stage were prepared from pure plate cultures of fungus grown on malt extract agar. The sporangia were scraped off from the agar plate and suspended in 0.85% (w/v) NaCl solutions to spore concentrations of 10^7 and 10^5 CFU/mL for *R. oryzae* and *A. oryzae*, respectively. After treatment, samples were directly stored at -20 °C.

Table 1. Summary of different peanut treatments.

Treatment codes	Fungus	Stage		
		Soaking (1 day)	Germination (2 days)	Incubation (5 days)
Un	-	-[a]	-	-
7G	-	√[b]	√	√
5R	*Rhizopus oryzae*	√	-	√
2G-5R	*Rhizopus oryzae*	√	√	√
5A	*Aspergillus oryzae*	√	-	√
2G-5A	*Aspergillus oryzae*	√	√	√

[a] The treatment mentioned was not performed.
[b] The treatment mentioned was performed.

Extraction of phenolic compounds

Untreated and treated peanuts were freeze-dried and subsequently ground using a MM2000 bead mill (Retsch, Haan, Germany). The extraction was performed with a E-916 speed extractor (Buchi, Flawil, Switzerland) as described previously,[2] using *n*-hexane and 80% (v/v) aqueous methanol for defatting and extraction of stilbenoids, respectively. The aqueous methanol extract was evaporated under reduced pressure and freeze-dried. The extract was re-solubilized in 80% (v/v) aqueous methanol to a concentration of 10 mg/mL and subjected to LC-MS/MS analysis.

Preparation of extract enriched in prenylated stilbenoids

Freeze-dried and ground *Rhizopus* elicited peanuts were defatted using *n*-hexane (1:10 (w/v)) and subsequently extracted using ethyl acetate (1:20 (w/v)). During defatting and extraction, the mixture was stirred for 1 min and then sonicated for 20 min at 40 °C. The suspension was filtered through a Grade 3 filter paper (Whatman, Buckinghamshire, UK). The defatting and extraction steps were repeated three times. The *n*-hexane extracts were discarded, whereas the ethyl acetate extracts were combined and evaporated under reduced pressure. The dried extract was dissolved in 10% (v/v) aqueous methanol and subjected to solid-phase extraction (SPE) on a Sep-Pak Vac 20 cc (5 g) C_{18} cartridge (Waters, London, UK). Prior to sample application, the cartridge was pre-conditioned using 30 mL of methanol, followed by 30 mL of water. The sample was loaded onto the cartridge, and then washed with 50% (v/v) MeOH (37.5 mL). The following elution profile was used: 60% (v/v) MeOH (15 mL), 70% (v/v) MeOH (22.5 mL), 80% (v/v) MeOH (15 mL), and 100%

(v/v) MeOH (15 mL). The SPE-fractions (7.5 mL) were subjected to LC-MS/MS analysis. Subsequently, fractions containing only prenylated stilbenoids were combined and dried under reduced pressure. The combined fraction was resolubilized in DMSO and used further for a modification experiment by fungus.

Incubation of the fraction enriched in prenylated stilbenoids with fungus

R. oryzae and *A. oryzae* were grown at 30 °C in a cotton-plugged Erlenmeyer flask (100 mL), containing 25 mL of sterilized liquid medium, and shaken at 130 rpm. The liquid medium consisted of glucose (50 g/L), neutral peptone (10 g/L) and yeast extract (1 g/L). The medium was inoculated with either 1 mL of spore suspension of *R. oryzae* or *A. oryzae*. After 4 days, 1 mL of the fraction enriched in prenylated stilbenoids (1 mg/mL) was added to the culture, and the incubation was continued for another 3 days. All experiments were performed in duplicate. Three control samples were used: (i) sterile liquid medium with 1 mL of the fraction enriched in prenylated stilbenoids; (ii) *Rhizopus* culture in liquid medium with 1 mL of DMSO; (iii) *Aspergillus* culture in liquid medium with 1 mL of DMSO. At day 7, the culture was filtered through a Grade 3 filter paper (Whatman). The filtrate was acidified with 0.5 M hydrochloric acid to pH 3.0 and extracted three times with 12.5 mL ethyl acetate. The ethyl acetate fraction was subsequently dried with a saturated NaCl solution and centrifuged (10,000 × *g*, 5 min, 15 °C). The fungal biomass, present on the paper, was also extracted with 12.5 mL ethyl acetate two times, using sonication for 30 min at 40 °C. All ethyl acetate fractions were combined, evaporated under reduced pressure and freeze-dried. The material was re-solubilized in 80% (v/v) methanol to a concentration of 10 mg/mL and subjected to LC-MS/MS analysis.

LC-MS/MS

Stilbenoid analysis was performed using a UHPLC-MS system (Thermo Scientific, San Jose, CA, USA), with a photo diode array (PDA) detector and a mass spectrometer. Samples (1 µL) were injected onto a Hypersyl Gold C18 column (2.1 mm i.d. x 150 mm, 1.9 µm particle size, Thermo Scientific). Water acidified with 0.1% (v/v) formic acid, eluent A, and methanol acidified with 0.1 % (v/v) formic acid, eluent B, were used as solvents at a flow rate of 300 µL/min. The temperatures of the autosampler and column oven were controlled at 15 and 40 °C, respectively. The PDA detector was set to monitor the wavelength range of 200-600 nm. The elution profile was as follows: 0-1 min, isocratic on 0 % B; 1-2 min linear gradient from 0% to 30% B; 2-18 min, linear gradient from 30% to 80 % B; 18-23 min, linear gradient from 80% to 95 % B; 23-24 min, linear gradient from 95% to 100% B; 24-26 min, linear gradient from 100% to 0% B; 26-31 min, isocratic on 0% B. Mass spectrometric analysis was performed on a LTQ Velos (Thermo Scientific) equipped with an HESI-MS probe. Nitrogen was used as sheath and auxiliary gas. The

spectra were acquired in the *m/z* range of 150−1,500. Data-dependent MS^n analysis was performed with a normalized collision energy of 35%. The system was tuned with genistein in both positive (PI) and negative ionization (NI) mode. For the PI mode, the ion transfer tube (ITT) temperature was 400 °C, and the source voltage was 4.50 kV. For NI mode, the ITT temperature was 400 °C and the source voltage was 3.50 kV. Quantification of stilbenoids was based on their absorption at 310 nm by using Xcalibur software (version 2.1.0, Thermo Scientific). As for many compounds no commercial standards were available, the amounts of stilbenoids were expressed as mg *trans*-resveratrol equivalents (RE) per g dry weight of peanut (mg RE/g DW). *Trans*-resveratrol was used as a generic standard to make a calibration curve with six data points (0.0005-0.1 mg/L, R^2=0.997).

RESULTS

Aspergillus and *Rhizopus* elicit different morphological responses in peanut

Four different treatments (5R, 2G-5R, 5A, and 2G-5A) using *Rhizopus* or *Aspergillus* as an elicitor were performed on peanuts. The peanut seedlings responded differently to the kind of fungus and to the time point of inoculation (**Figure 1**). *Rhizopus* mycelium expanded faster and denser at the peanut seedling's surface than that of *Aspergillus*. Moreover, the occurrence of brown lesions on the peanut's surface was more pronounced with *Aspergillus*. Peanut seedlings elicited by fungus on day 1 (5A and 5R) were more mycelium-covered than those on day 3 (2G-5R and 2G-5A).

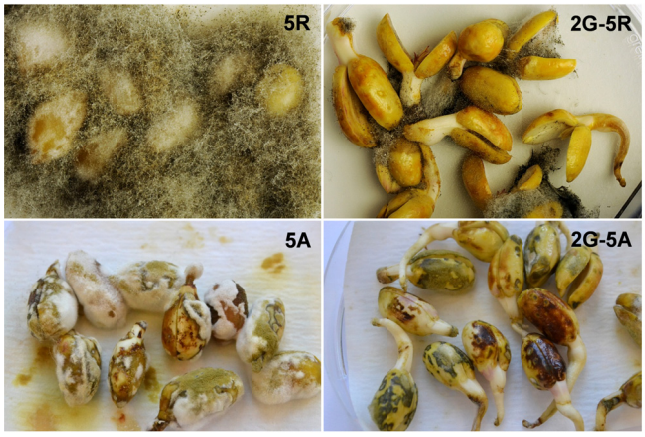


Figure 1. Elicited peanut seedlings, with *Rhizopus* or *Aspergillus* applied at different time points. Codes (5R, 2G-5R, 5A, and 2G-5A) refer to treatments in **Table 1**.

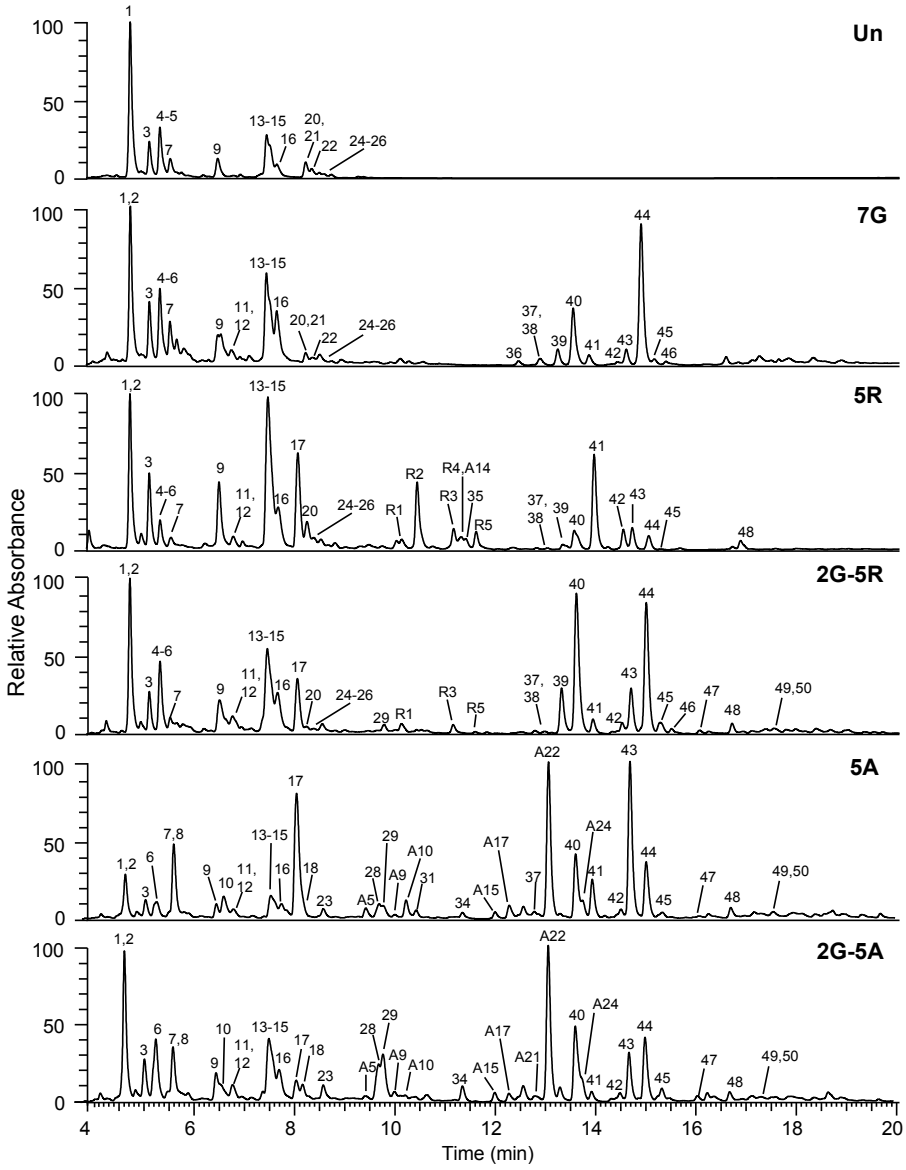


Figure 2. RP-UHPLC-UV chromatogram at 310 nm of 80% (v/v) aqueous methanol extracts of untreated and treated peanuts. Codes (Un, 5R, 2G-5R, 5A, and 2G-5A) refer to the treatment in **Table 1**, and peak numbers refer to compounds in **Table 2**.

Structural elucidation of compounds in extracts from peanut seedlings

Differences between treatments were also observed in the chromatograms of extracts obtained from elicited peanut seedlings (**Figure 2**). The chromatogram of untreated peanuts contained peaks that eluted at retention time (t_R) 0-9 min, whereas in the solely germinated peanuts (7G) also peaks eluted at t_R 13-18 min. For the peanuts elicited by fungi, a group of compounds was eluted between t_R around 9-13 min. In total, 58 peaks were detected in the various extracts from peanuts, based on the UV response at 310 nm (**Figure 2**, **Table 2**). The annotation of many peaks was performed based on a comparison of spectral data from LC-MS/MS (including retention behavior, UV spectra, and fragmentation patterns) to those from the literature (**Table 2**).[6, 12-18] The peaks analyzed represented over 97% of the total UV response in each of the chromatograms.

Phenolic acids. Phenolic acids in the extracts were characterized by their λ_{max} at 315 (±5) nm.[12] Of the 16 peaks, 14 were coutaric acid derivatives, including two in the non-conjugated form. In NI mode, the *trans*- and *cis*-coutaric acids (**1**, **4**), an ester of coumaric acid and tartaric acid, were characterized by the neutral loss of 132 Da in MS^2, characteristic of a tartaric acid moiety.[12, 13] These isomers might be distinguished by their retention behavior in which *trans*-coutaric acid is more polar than *cis*-coutaric acid.[13, 19] The coutaric acid derivatives were formed from coutaric acid conjugated to, amongst others, hydroxycinnamic acids and alkaloids. The conjugates were characterized by specific neutral losses, besides those of coutaric acid. For example, coumaroyl, caffeoyl, sinapoyl, feruloyl and nicotinoyl groups provided mass losses of 146, 162, 206, 176 and 105 Da in MS^2, respectively.[12, 20] Fragmentation of deprotonated coutaric acid derivatives resulted in a characteristic product ion of *m/z* 277 that was assigned as [M-H-conjugate-H_2O] in MS^2.[12] For instance, *p*-coumaroylnicotinoyltartaric acid with *m/z* 400 $[M-H]^-$ produced the fragment ion of *m/z* 277 that was assigned as [M-H-105(nicotinoyl)-H_2O]$^-$ in MS^2. The characteristic product ion of *m/z* 277 was further fragmented to *m/z* 203 by loss of $C_2H_2O_3$, to *m/z* 233 by loss of a CO_2, and to *m/z* 259 by loss of a H_2O in MS^3. [12] Compound **7** was tentatively annotated as *p*-coumaroylcaffeoyltartaric acid-*O*-rhamnoside. The sugar unit was identified from the neutral loss of 146 Da in MS^2. The occurrence of an ester of a glycosylated tartaric acid derivative has been reported before.[21] Compounds **9** and **20** were identified as *p*-coumaric acid and hydroxycinnamic acid-alkaloid derivative, respectively.

Table 2. Compounds tentatively identified by RP-UHPLC-PDA-MS in extracts from elicited peanut seedlings.

No[a]	t_R (min)	Compounds	λ_{max}[b] (nm)	[M-H]$^-$[c]	MS^2 (rel. abundance)	MS^3 (rel. abundance)	[M+H]$^+$	MS^2 (rel. abundance)	MS^3 (rel. abundance)
Phenolic acids									
1	4.71	*trans*-(*p*-Coumaroyl)-tartaric acid	314	295	163 (100)	119 (100)	nr[d]	-[e]	-
3	5.11	*p*-Coumaroylnicotinoyltartaric acid	314	400	277 (100), 254 (3), 163 (2)	203 (100), 233 (31), 259 (24)	402	147 (100), 279 (10)	119 (100)
4	5.35	*cis*-(*p*-Coumaroyl)-tartaric acid	nd	295	163 (100)	119 (100)	nr	-	-
5	5.35	*p*-Coumaroyltartaric acid derivative	313	607	277 (100), 461 (35), 203 (14)	203 (100), 233 (35), 259 (27)	nr	-	-
7	5.57	*p*-Coumaroylcaffeoyltartaric acid-*O*-rhamnoside	309	603	457 (100), 277 (18)	163 (100), 325 (92), 293 (13)	nr	-	-
9	6.50	*p*-Coumaric acid	313	163	119 (100)	91 (100), 93 (95), 135 (69)	nr	-	-
11	6.64	*p*-Coumaroylcaffeoyltartaric acid	313	457	295 (100), 293 (43), 277 (27), 411 (18)	163 (100)	nr	-	-
12	6.68	*p*-Coumaroyltartaric acid derivative	317	579	245 (100), 289 (34), 203 (13)	203 (100)	nr	-	-
13	7.60	di-*p*-Coumaroyltartaric acid	314	441	277 (100), 295 (12), 163 (<1)	203(100), 233 (34), 259 (25)	nr	-	-
14	7.60	*p*-Coumaroylsinapoyltartaric acid	314	501	337 (100), 277 (70)	263 (100), 319 (20), 293 (19)	nr	-	-
15	7.60	*p*-Coumaroyltartaric acid derivative	314	509	463 (100), 441 (22), 395 (18)	299 (100), 277 (96), 349 (63), 331 (42), 273 (14)	nr	-	-
16	7.80	*p*-Coumaroylferuloyltartaric acid	317	471	307 (100), 277 (86), 325 (6)	233 (100), 263 (20), 289 (18)	nr	-	-
19	8.40	di-*p*-Coumaroyltartaric acid	315	441	277 (100), 295 (53), 305 (17), 373 (15)	203 (100), 233 (34), 259 (28), 171 (4)	nr	-	-
20	8.40	Hydroxycinnamic acid-alkaloid derivative	315	680	536 (100), 413 (28), 557 (15)	413 (100)	682	544 (100)	526 (100), 482 (99), 400 (98)
21	8.53	*p*-Coumaroylsinapoyltartaric acid	316	501	337 (100), 277 (91), 355 (30), 295 (12)	263 (100), 319 (22), 293 (20), 265 (4)	nr	-	-
23	8.68	*p*-Coumaroylferuloyltartaric acid	316	471	277 (100), 307 (92), 325 (22), 295 (5)	203 (100), 233 (36), 259 (25)	nr	-	-
(Iso)flavonoids									
2	4.71	Naringenin *C*-glucoside	290	433	313 (100) ,343 (28)	285 (100), 269 (11)	435	417 (100), 389 (41)	218 (100), 179 (93), 161 (55)
6	5.35	Eriodictyol-3-*O*-hexoside	292	449	287 (100), 269 (24), 259 (24)	259 (100)	nr	-	-

18	8.24	4',7-Dihydroxyflavanonol (garbanzol)	278, 314	271	161 (100), 109 (5), 253 (3)	133 (100)	273	163 (100), 255 (55), 245 (55), 135 (48), 139 (17), 123 (15)	135 (100)
24	8.88	Isorhamnetin *O*-rhamnosyl hexoside	267, 344	623	315 (100), 314 (21), 300 (20), 357 (10), 316 (9)	300 (100), 287 (6)	nr	-	-
25	9.05	Isorhamnetin *O*-rhamnosyl hexoside	267, 353	623	315 (100), 314 (21), 300 (20), 357 (10), 316 (9)	300 (100), 287 (6)	nr	-	-
26	9.77	Trihydroxymethoxy-isoflavanone	289	301	269 (100), 273 (10), 191 (5), 283 (3)	241 (100), 225 (6), 197 (2)	303	285 (100), 275 (96), 135 (91), 163 (82), 169 (30)	270 (100), 257 (52), 253 (39), 225 (15), 271 (12), 229 (10)
27	9.85	Aracarpene 1	311, 330, 347	299	284 (100)	256 (100), 228 (75), 227 (15)	301	283 (100), 286 (36), 269 (17), 284 (10)	268 (100), 255 (52), 269 (12), 223 (10)
28	10.51	4'-Methoxy-7-hydroxyflavanonol	277, 311	285	149 (100), 269 (44)	121 (100)	287	259 (100), 135 (70), 162 (45), 269 (21), 149 (17), 137 (16)	135 (100), 227 (38), 149 (20), 241 (11)
29	11.44	7-Methoxy-4'-hydroxyflavanonol	277, 312	285	109 (100)	65 (100)	287	163 (100), 269 (79), 259 (74), 135 (64), 177 (43), 153 (22), 137 (15)	135 (100)
Unmodified stilbenoids									
10	6.63	Piceatannol	320	243	225 (100), 201 (53), 175 (35), 199 (33), 200 (19), 215 (15), 228 (11), 159 (10)	157 (100), 197 (70), 181 (62), 183 (45), 196 (12)	245	135 (100), 227 (87), 199 (21), 107 (11), 201 (11), 161 (11), 209 (11)	107 (100)
17	8.13	*trans*-Resveratrol	305, 318	227	185 (100), 183 (51), 159 (40), 157 (31), 143 (11)	143 (100)	229	135 (100), 211 (29), 119 (18)	107 (100)
22	8.67	Unknown stilbenoid	317	359	315 (100)	285 (100), 271 (20), 241 (14), 297 (12)	361	271 (100), 297 (81), 301 (50), 343 (48), 325 (45), 315 (27), 279 (10)	243 (100)
30	11.50	Unknown stilbenoid	309	601	295 (100), 457 (81), 499 (40), 539 (13)	239 (100), 240 (58), 226 (29), 251 (14)	nr	-	-
31	12.60	4-Isopentadienyl-3,5,3',4'-tetrahydroxystilbene (IPP)	328	309	265 (100), 291(42), 294 (39), 281 (32), 266 (22), 201 (20), 267 (15)	159 (100), 249 (78), 250 (44), 237 (32), 238 (28), 197 (17), 251 (16), 247 (14), 222 (14)	311	283 (100), 201 (57), 269 (37), 135 (26), 293 (26), 175 (18)	173 (100), 255 (97), 135 (63), 241 (60), 265 (49), 123 (28)

32	12.86	Arachidin-1 isomer	329	311	242(100), 241 (73), 255 (45), 267 (34), 293 (24), 243 (19), 224 (13), 269 (12)	172 (100), 224 (31), 213 (22)	313	257 (100)	239 (100), 211 (97), 229 (65), 197 (35), 215 (27), 183 (25), 173 (17), 187 (16)
33	13.06	IPP	351	309	265 (100), 294 (43), 291(40), 281 (31), 266 (24), 201 (17), 267 (15), 159 (12)	238 (100), 159 (98), 249 (78), 250 (44), 237 (43), 222 (21), 247 (15), 197 (12)	311	283 (100), 201 (86), 269 (37), 293 (36), 135 (33), 255 (28)	255 (100), 173 (96), 241 (62), 135 (54), 265 (53), 123 (27)
34	13.38	Arachidin-1 isomer	311	311	242 (100), 241 (71), 255 (44), 267 (44), 243 (42), 293 (29), 224 (14), 269 (13)	172 (100), 224 (25), 213 (21)	313	257 (100)	239 (100), 211 (77), 229 (45)
35	13.69	*trans*-Arachidin-1	339	311	242 (100), 241 (64), 255 (43)	172 (100), 224 (29), 213 (24)	313	257 (100)	239 (100), 211 (72), 229 (45), 197 (35), 215 (21), 173 (19), 183 (17), 147 (17), 187 (16), 201 (15)
36	14.02	*trans*-Arachidin-2	323	295	239 (100), 226 (53), 240 (51), 251 (17), 227 (16), 252 (11), 185 (11)	195 (100), 211 (42), 196 (13)	297	241 (100)	195 (100), 223 (94), 199 (71), 213 (48), 167 (17), 197 (16)
37	14.60	Arachidin-2 isomer	302	295	239 (100), 240 (50), 226 (47), 251 (31), 227 (19), 252 (12), 185 (11)	195 (100), 211 (40), 180 (8), 196 (8)	297	241 (100)	223 (100), 195 (95), 213 (59), 199 (44), 147 (14), 169 (13), 167 (13), 197 (11), 157 (11), 185 (10)
38	14.78	Arachidin-3	335	295	239 (100), 240 (50), 226 (47), 251 (31), 185 (11), 252 (11)	195 (100), 211 (37), 196 (13)	297	241 (100)	223 (100), 213 (21), 195 (12), 147 (2)
39	15.10	*trans*-3′-Isopentadienyl-3,5,4′-trihydroxystilbene (IPD)	298	293	249 (100), 251 (38), 209 (10)	194 (100),180 (45), 234 (24), 193 (22), 181 (17), 206 (17)	295	277 (100), 185 (53), 253 (43), 239 (35), 267 (30), 211 (25), 173 (25), 135 (23)	262 (100), 249 (69), 259 (56), 235 (43), 221 (29), 248 (18), 207 (10), 231 (10)
40	15.33	IPP	343	309	265 (100), 291 (47), 294 (44), 281 (31), 266 (25), 201 (18), 267 (15), 159 (13), 293 (11)	159 (100), 249 (100), 250 (51), 237 (30), 265 (28), 238 (18), 222 (16), 197 (15), 247 (14), 209 (11)	311	201 (100), 283 (55), 135 (34), 177 (32), 293 (29), 269 (25), 187 (25), 202 (22), 175 (16), 123 (15)	173 (100), 183 (40), 159 (39)

41	15.43	IPP	323	309	265 (100), 291 (47), 294 (44), 281 (31), 266 (25), 201 (18), 267 (15), 159 (13), 293 (11)	159 (100), 249 (75), 250 (48), 237 (35), 238 (17), 197 (13), 247 (12), 209 (11)	311	201 (100), 283 (68), 135 (36), 177 (32), 293 (29), 269 (26), 187 (25), 202 (22), 175 (16), 123 (15), 265 (14)	173 (100), 159 (44), 183 (38), 131 (16)
42	15.96	Arahypin-6 isomer	nd	605	511 (100), 309 (30), 495 (28), 587 (21), 483 (19), 536 (16)	493 (100), 442 (82), 401 (63), 467 (52), 399 (36), 455 (33)	607	551 (100), 501 (84), 497 (71), 429 (61), 513 (59), 589 (41), 299 (35), 391 (34), 533 (23), 485 (19)	533 (100), 495 (81), 373 (67), 445 (35), 477 (35), 457 (29), 441 (24)
43	16.79	Arahypin-5	337	293	278 (100), 236 (54), 249 (29)	263 (100), 262 (81), 235 (39)	295	201 (100), 267 (89), 277 (51), 253 (52), 175 (32), 239 (26), 107 (21), 183 (17), 225 (16)	173 (100), 159 (43), 183 (37)
44	17.63	Arahypin-7	334	621	511 (100)	442 (100), 493 (94), 401 (70), 467 (62), 399 (50), 455 (46), 483 (24), 335 (23), 456 (21), 389 (19), 469 (16), 468 (15), 443 (15), 333 (15), 373 (14), 387 (13), 413 (13), 385 (10)	623	513 (100), 501 (81), 391 (49), 567 (41), 445 (38), 605 (35), 499 (30), 299 (29), 457 (15), 335 (12), 549 (12), 500 (11)	457 (100), 335 (13), 403 (11)
45	17.63	Arahypin-6	334	605	511 (100), 495 (24), 309 (21), 483 (16), 512 (16), 587 (15), 536 (14), 414 (12), 561 (12), 453 (12), 413 (11), 479 (10)	493 (100), 442 (87), 467 (65), 401 (56), 399 (41), 455 (36), 483 (25), 389 (23), 456 (19), 335 (19), 443 (19), 468 (18), 469 (18), 333 (17), 373 (14), 387 (12), 494 (12), 385 (12), 424 (11), 363 (9)	607	501 (100), 589 (98), 551 (84), 513 (80), 429 (70), 497 (67), 299 (57), 533 (33), 391 (31), 283 (30), 445 (27), 485 (23), 590 (22), 215 (17)	445 (100), 323 (45), 391 (36), 446 (12)
***Rhizopus*-modified prenylated stilbenoids**									
R1	10.21	Arachidin-1 *O*-hexoside	nd	473	311 (100)	242 (100), 241 (74), 255 (69), 267 (42), 293 (30), 224 (16), 172 (15), 269 (12), 256 (11), 201 (10)	475	313 (100)	257 (100), 258 (4)
R2	10.21	Unknown stilbenoid	nd	319	301 (100)	187 (100), 283 (93), 255 (61), 163 (51), 157 (42), 239 (41), 257 (37)	nr	-	-

R3	10.51	Arachidin-2 *O*-hexoside	322	457	295 (100)	239 (100), 240 (52), 226 (33), 251 (13), 227 (11), 185 (9)	459	297 (100)	241 (100)
R4	11.23	Arachidin-3 *O*-hexoside	335	457	295 (100)	239 (100), 240 (50), 226 (37)	459	297 (100)	241 (100)
R5	11.48	Arachidin-2 or -3 *O*-hexoside	341	457	295 (00)	239 (100), 240 (50), 226 (37)	nr	-	-
R6	11.67	Arachidin-2 or -3 *O*-hexoside	311	457	295(100)	239 (100), 226 (40), 240 (35)	nr	-	-
R7	12.17	Unknown stilbenoid (modified IPD)	310	409	347 (100), 348 (22), 391 (15)	319 (100), 303 (43), 305 (29)	411	393 (100), 375 (23), 347 (14)	347 (100), 375 (92)
R8	12.57	Unknown stilbenoid (modified IPD)	310	409	347 (100), 391 (19), 321 (6)	319 (100), 303 (39), 305 (27), 277 (10)	411	393 (100), 375 (6)	375 (100), 349 (74), 347 (39), 229 (13), 365 (13), 357 (11)
***Aspergillus*-modified non-prenylated stilbenoids**									
8	5.57	Piceatannol lactone	320	**551**, 275	275 (100), 231 (57), 189 (6)	231 (100)	277	289 (100), 190 (8), 199 (6), 155 (6)	161 (100), 171 (5)
***Aspergillus*-modified prenylated stilbenoids**									
A1	8.32	Hydroxyl-IPP	320	327	255 (100), 309 (80), 257 (10)	185 (100), 227 (37)	nr	-	-
A2	8.32	Unknown stilbenoid	320	377	359 (100), 315 (53), 275 (51), 231 (10)	315 (100)	nr	-	-
A3	9.12	Arachidin-1 sulfate	260*sh*, 328	409	391(100)	311(100)	nr	-	-
A4	9.24	Unknown stilbenoid sulfate	308	365	285 (100), 283 (13)	270 (100), 267 (4)	nr	-	-
A5	9.50	Arahypin-2	316	329	241 (100), 311 (44), 253 (4), 293 (3)	199 (100), 197 (34), 173 (28), 171 (16), 157 (13)	331	313 (100)	253 (100), 277 (56), 237 (51), 135 (37), 267 (25), 223 (16), 239 (15), 107 (11)
A6	9.83	Arachidin-1 sulfate	330	409	391(100)	311(100)	nr	-	-
A7	10.08	Arachidin-1 sulfate	nd	409	391(100)	311(100)	nr	-	-
A8	10.08	Unknown	286, 308*sh*	253	254 (100), 209 (50), 225 (32), 224 (26), 253 (23), 197 (13)	164 (100), 181 (80), 180 (58), 112 (32), 143 (27), 166 (24),	255	199 (100), 227 (81), 137 (74), 237 (28), 213 (26), 145 (14)	181 (100), 171 (36), 153 (10)
A9	10.30	Arahypin-3	322	329	253 (100), 241 (38), 311 (11)	209 (100), 185 (92), 225 (31), 211 (27), 157 (24), 143 (12)	331	313 (100), 241 (8), 295 (1)	241 (100), 295 (15)
A10	10.75	Arachidin-2 or -3 sulfate	301	375	295 (100)	239 (100), 226 (57), 240 (50)	nr	-	-

A11	10.91	Arahypin- 3 isomer	290	329	253 (100), 241 (40), 311 (17)	209 (100), 185 (77), 225 (34), 211 (29), 157 (25)	331	313 (100), 241 (7)	241 (100), 295 (15)
A12	10.91	Arachidin-2 or-3 sulfate derivative	290	443	375 (100), 397 (76), 353 (42), 399 (35), 363 (34), 425 (32)	295 (100), 311 (13)	nr	-	-
A13	11.34	Arachidin-2 or -3 sulfate	306	375	295 (100)	240 (100), 239 (94), 251 (55), 226 (47), 253 (20)	nr	-	-
A14	12.09	Stilbene-1 isomer	260, 339	299, 343, **687**	343 (100), 299 (91)	299 (100)	345	327 (100), 271 (92), 299 (39), 285 (38), 281 (32), 243 (11)	309 (100), 281 (32), 271 (27), 253 (13)
A15	12.37	Stilbene-1 isomer	336	299, **343**, 687	299 (100), 255 (5)	255 (100), 257 (49), 281 (14), 230 (12)	345	271 (100), 289 (49), 299 (44), 327 (28), 285 (25)	243 (100), 225 (4)
A16	12.56	Arachidin -2 or -3 sulfate	321	375	295 (100)	239 (100), 226 (57), 240 (50)	nr	-	-
A17	12.56	Unknown stilbenoid	321	315	300 (100), 285 (23), 287 (18), 164 (57), 272 (11)	285 (100), 282 (36), 177 (10)	317	289 (100), 151 (59), 179 (28), 299 (15), 163 (15), 133 (10)	151 (100), 139 (35), 165 (29), 271 (18), 163 (10)
A18	12.87	Hydroxy-IPD	308, 320	311	293 (100), 242 (47), 241 (37), 267 (22), 253 (21), 255 (19)	278 (100), 249 (61), 251 (30), 265 (25), 275 (13)	313	295 (100), 241 (57)	267 (100), 201 (70), 253 (56), 277 (34)
A19	13.15	Stilbene-1	261, 366	299, 343, **687**	343 (100), 299 (91)	299 (100)	345	271 (100), 327 (62), 299 (42), 285 (38), 243 (24), 309 (20), 281 (19)	243 (100)
A20	13.35	1''-Dehydro-stilbene-1	372	297, **341**, 683	297 (100)	253 (100), 255 (42), 279 (16), 269 (11), 238 (10)	343	287 (100), 325 (13), 268 (12), 297 (8)	269 (100), 241 (35), 259 (25)
A21	13.82	1''-Dehydro-stilbene-1	272, 359	297, 341, **683**	341 (100), 297 (85)	297 (100)	343	325 (100), 297 (86), 283 (19), 279 (10)	297 (100), 307 (76), 279 (16)
A22	14.23	Stilbene-1 isomer	350	**299**, 343, 687	255 (100), 283 (90), 299 (85), 237 (20)	200 (100), 199 (83), 186 (71)	345	327 (100), 271 (87), 285 (59), 289 (46), 299 (39)	299 (100), 271 (92), 309 (74), 281 (29), 283 (15)

[a] Numbers refer to peaks in **Figures 2** and **3**.

[b] *sh*, Shoulder in the spectrum; nd, Not detected.

[c] Stilbenoids with lactone ring, such as stilbene-1 and piceatannol lactone, generated in source ions in full ESI-MS of negative mode: $[M-H]^-$, $[M-H-CO_2]^-$, and $[2M-H]^-$. The bold number represents the ion with highest relative abundance, which was fragmented further in MS^2.

[d] nr, Not relevant.

[e] No MS^2 and/or MS^3 data available..

Flavonoids and isoflavonoids. Compounds 2 and 6 were tentatively annotated as flavanones, based on their λ_{max} at 290 nm, whereas **24** and **25** were tentatively annotated as flavones, based on their λ_{max} at 270 (±5) and 330-365 nm.[22] All of them were glycosylated. The sugar unit was attached to either the hydroxyl group or directly to a *C*-atom, distinguished by the neutral losses of 146/162 Da (*O*-rhamnoside or *O*-hexoside, respectively) or 120 Da (*C*-hexoside) in NI mode.[14] Compounds **18**, **28** and **29** were tentatively identified as flavanonols. The typical λ_{max} at 277 (±1) and 311 (±2) nm was characteristic for 5-deoxy flavanonols, such as garbanzol (**18**).[23] The fragment ions *m/z* 163 ($^{1,4}B^+$) and 123 ($^{0,2}B^+$) resulting from the cleavage at the C-ring confirmed the structure of flavanonols.[24] Two other flavanonols were assigned as methoxylated garbanzol derivatives (**28** and **29**), namely 4'-methoxy-7-hydroxyflavanonol and 7-methoxy-4'-hydroxyflavanonol, respectively. The neutral losses of 16 Da $[M\text{-}H\text{-}CH_3\text{-}H]^-$ in NI mode suggested a methoxyl group attached to the skeleton of **28** and **29**.[25] Compound **26** was suggested to have an isoflavanone skeleton based on its λ_{max} at 289 nm and the fragment ion *m/z* 135 ($^{2,3}B^+$) resulting from C-ring cleavage.[22, 26] Compound **27** was tentatively identified as aracarpene 1, based on its λ_{max} at 330 and 347 nm.[17, 22] This compound produced the fragment ion *m/z* 284 in MS^2 that was further fragmented (MS^3) to yield the fragment ions *m/z* 256, 228, and 227. Aracarpene-1 has been reported before in peanut elicited by *Aspergillus*.[17, 22]

Stilbenoids. Stilbenoids found in peanut extracts contained a resveratrol (3,5,4'-trihydroxystilbene) or a piceatannol (3,5,3',4'-tetrahydroxystilbene) moiety, mostly substituted with a prenyl group. These stilbenoids were characterized by a single λ_{max} in the 297-351 nm range and a molecular mass of 294 or 296 Da (prenylated resveratrol derivatives) and 312 Da (prenylated piceatannol derivatives). Fifteen prenylated stilbenoids (**31-45**; **Figure 2**, **Table 2**) were induced in peanuts upon germination without fungus (referred to as unmodified prenylated stilbenoids). The prenyl group can be attached in either isopentenyl (3-methyl-but-1-enyl or 3-methyl-but-2-enyl) or isopentadienyl (3-methyl-but-1,3-dienyl) form.[27, 28] The two isomers of isopentenyl stilbenoids can be distinguished from the UV-spectra.[27] 4-(3-Methyl-but-1-enyl)-resveratrol (arachidin-3) had a ~10 nm higher λ_{max} than 4-(3-methyl-but-2-enyl)-resveratrol (arachidin-2). This might be explained by the position of the double bond in the prenyl chain of arachidin-3, which is conjugated with those of the resorcinol moiety, creating a longer conjugated system in arachidin-3 than in arachidin-2.[27] Isopentenyl- and isopentadienyl-stilbenoids can be differentiated by a mass difference of 2 Da and the neutral losses of 56 Da (C_4H_8) and 42 Da (C_3H_6) in PI mode, respectively. The extra conjugated double bond in isopentadienyl stilbenoids (as in *trans*-3′-isopentadienyl-3,5,4′-trihydroxystilbene (IPD)) was also expected to give a bathochromic shift compared to isopentenyl stilbenoids (such as arachidin-2 or -3). Nevertheless, IPD showed a λ_{max} at 298 nm, which is lower than that of arachidin-3.[28] The structural elucidation of many prenylated stilbenoids (**32**, **34-39** and

42-45) has been described before.[15, 16, 18] Our observations were in line with those results (**Table 2**). Four peaks (**31**, **33**, **40**, and **41**) with a molecular mass of 310 Da have not been identified before. They were tentatively identified as isomeric forms of 4-isopentadienyl-3,5,3',4'-tetrahydroxystilbene (4-isopentadienyl piceatannol, IPP). The 2 Da mass difference to arachidin-1 and a neutral loss of 42 Da in PI mode of IPP confirmed the isopentadienyl form. The isopentadienyl substituent of IPP might be attached at the 4- or 5'-position of the piceatannol skeleton. However, it was hard to determine the position of isopentadienyl of IPP based on mass spectral data, as both rings (catechol and resorcinol moieties) have equal number of hydroxyl groups. We speculated that the isopentadienyl was attached at the 4-position, in analogy to the isopentenyl of arachidin-1. If so, then attachment of an isopentadienyl substituent to a resorcinol moiety (as at the 4-position of IPP) yields the expected bathochromic shift, whereas this is not observed with attachment of an isopentadienyl substituent to a phenol moiety (as at the 3'-position of IPD). Additionally, three dimer prenylated stilbenoids (**42**, **44** and **45**) with the molecular masses of 606 or 622 Da were observed. The structural elucidation of the dimers has been described previously.[16] Our observations are in line with these results (**Table 2**).

Fourteen additional prenylated stilbenoids were observed upon challenging the peanut seedlings with fungi (referred to as modified prenylated stilbenoids). The modified prenylated stilbenoids were characterized by a λ_{max} in the 260-382 nm range and eluted earlier than the unmodified ones. Interestingly, the nature of the modified prenylated stilbenoids was fungus-dependent. The compounds found with *Rhizopus* were annotated as *O*-hexosides of unmodified prenylated stilbenoids, based on their characteristic neutral loss fragment of 162 Da in NI mode MS^2, and the similarities in fragmentation of the aglycone product ions in MS^3 compared to their respective unmodified prenylated stilbenoids.[15, 29, 30] Peaks **R1** and **R3**-**R5** were tentatively identified as *O*-hexosides of arachidin-1, -2 and -3. The compounds found with *Aspergillus* often showed mass differences of +16 or +18 Da (or their multiples) compared to unmodified prenylated stilbenoids. This presumably reflects an extra *O*-atom or hydration of the prenyl double bond of the compounds. Three peaks with the molecular mass of 344 Da were tentatively identified as stilbene-1 (**A19**) and isomers thereof (**A14**, **A15**). The carboxyl group attached to the lactone ring of stilbene-1 gave characteristic neutral losses of 44 Da (CO_2) in NI mode and 46 Da (HCOOH) in PI mode. The presence of a prenyl group in stilbene-1 was concluded from the abundance of fragment ion *m/z* of 271 $[M+H-H_2O-C_4H_8]^+$. Stilbene-1 has been previously found in peanut seeds elicited by *Aspergilli*.[6] Peak **A21** with the molecular mass of 342 Da was tentatively identified as 1"-dehydrostilbene-1. The compound showed similar fragmentation behavior to stilbene-1. The presence of isopentadienyl in 1"-dehydrostilbene-1 was supported by the same reasoning described previously for IPP (**Table 2**). Further confirmation of 1"-dehydrostilbene-1 is required. Two stilbenoids with a dihydroxy prenyl chain (**A5**, **A9**) amounting to a molecular mass of 330 Da were identified.

The presence of two hydroxyl group attached to the prenyl chain was identified from the neutral losses of 18 Da (H_2O), 60 Da (C_3H_8O), 72 Da (C_4H_8O) or 90 Da ($C_4H_{10}O_2$). Peak **A9** was identified as arahypin-3, whereas peak **A5** was suggested to have the same structure as arahypin-2. The arahypin-2 and -3 have been reported previously in peanut seeds elicited by *Aspergillus caelatus*.[15] Peak **A18** was tentatively determined as hydroxyl IPD. The presence of a hydroxyl group on the prenyl chain was identified from the neutral losses of 18 Da (H_2O) and 72 Da (C_4H_8O). The fragmentation pattern of the fragment ion *m/z* 295 $[M+H-H_2O]^+$ of peak **A18** was similar to that of IPD, which supported the structure proposed. One peak (**A13)** was tentatively identified as sulfated arachidin-2 or -3, based on the neutral loss of 80 Da (SO_3) in MS^2 and the fragmentation of the aglycone product ion in MS^3 that was similar to that of arachidin-2 and -3.[31, 32] Compound **A13** was also found in elicited peanut by *Rhizopus*. Typically, the elicited peanut seedlings also contained three non-prenylated stilbenoids, piceatannol (**10**), resveratrol (**17**) and piceatannol lactone (**8**). The latter is referred as modified non-prenylated stilbenoid. The spectral data of those compounds were in line with those from literature.[29, 33]

Fungi are responsible for modification of prenylated stilbenoids

The modified prenylated stilbenoids were believed to be products of fungal metabolism of the actual prenylated stilbenoids produced by peanuts. To verify this hypothesis, a sample enriched in unmodified prenylated stilbenoids was added to cultures with growing fungus. **Figure 3** shows the RP-UHPLC-UV chromatograms of the sample enriched in unmodified prenylated stilbenoids before (**Figure 3A**) and after exposure to fungi (**Figures 3B** and **3C**). It can be seen that *Rhizopus* and *Aspergillus* metabolized the prenylated stilbenoids in different ways. Eight prenylated stilbenoids modified by *Rhizopus* and twenty-two prenylated stilbenoids modified by *Aspergillus* were identified (**Figure 3** and **Table 2**). Glycosides of prenylated stilbenoids were observed predominantly in the *Rhizopus* culture. Five out of the eight *Rhizopus*-modified prenylated stilbenoids were glycosylated, including *O*-hexosides of arachidin-1, -2, and -3, whereas the kind of modification of the compounds represented by the other three peaks remained unknown (**Table 2**). Remarkably, only small amounts of *trans*-arachidin-1 and IPP were converted by *Rhizopus*, leaving almost 90% (w/w) of them unconverted. In contrast, more than 50% (w/w) of arachidin-2, arachidin-3 and IPD were converted by *Rhizopus* (**Figure 4**). With *Aspergillus*, an oxidative product, namely stilbene-1 (**A19**) was predominant. Hydroxylated and *O*-sulfated derivatives of prenylated stilbenoids were also observed, but in much smaller quantities than stilbene-1 (**Figure 3** and **Table 2**). Over 70% (w/w) of all unmodified prenylated stilbenoids were metabolized, including *trans*-arachidin-1 and IPP (**Figure 4**).

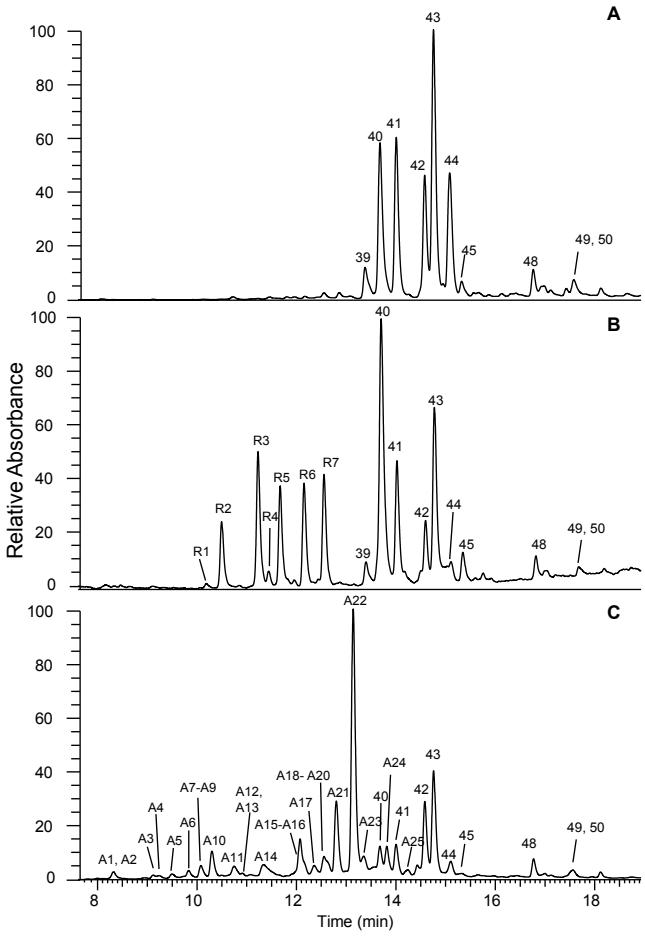


Figure 3. RP-UHPLC-UV chromatogram at 310 nm of a fraction enriched in prenylated stilbenoids, before and after treatment with fungus. Samples comprise treatment without fungus (**A**), with *Rhizopus* (**B**), and with *Aspergillus* (**C**). All peak numbers refer to compounds in **Table 2**.

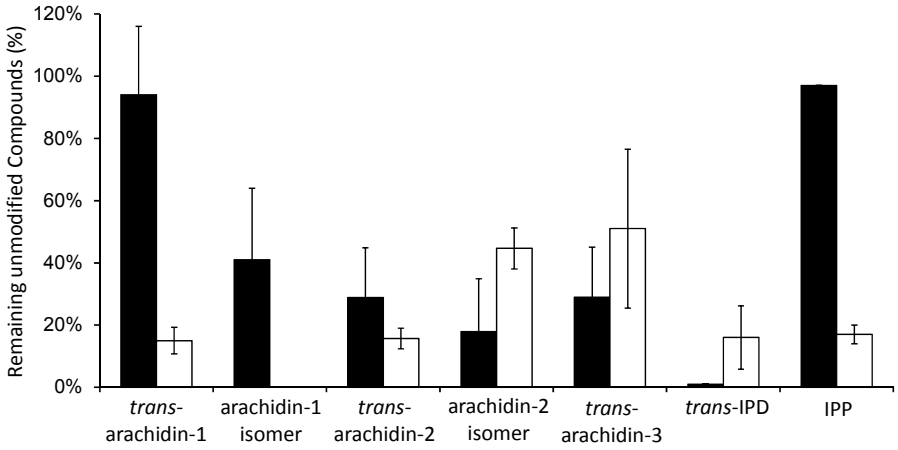


Figure 4. Proportion (%) of unmodified prenylated stilbenoids remaining after incubation of the fraction enriched in prenylated stilbenoids in media inoculated with *Rhizopus* (black) or *Aspergillus* (white). Data are the means ± SD of experiments performed in duplicate.

Level of stilbenoid derivatives

The contents of stilbenoids were quantified using *trans*-resveratrol as an external standard (**Table S1** in Supporting Information). Although not present in the untreated seeds, the total stilbenoid level of germinated peanuts (7G) was around 0.28 mg RE/g DW, comprising only unmodified prenylated stilbenoids. *Rhizopus* inoculation at day 3 (2G-5R) increased the total stilbenoid level of peanut seedlings up to 1.59 mg RE/g DW, of which up to 86% (w/w) were unmodified prenylated stilbenoids (**Figure 5A**). An increase up to 1.80 mg RE/g DW was observed after *Aspergillus* application on day 3 (2G-5A), with unmodified prenylated stilbenoids comprising about 51% (w/w) of the total stilbenoid level (**Figure 5A**). The total level and composition of stilbenoids in elicited peanuts changed when the fungus was applied earlier. The stilbenoid level of peanut elicited by *Rhizopus* on day 1 (5R) was 1.03 mg RE/g DW, of which up to 44% (w/w) was resveratrol (**Figure 5A**). The highest amount of stilbenoids was observed in peanut seedlings elicited by *Aspergillus* on day 1 (5A), up to 5.19 mg RE/g DW. Around 43% (w/w) of the total amount of stilbenoids were unmodified prenylated stilbenoids (**Figure 5A**). It is worth to note that piceatannol lactone was observed only in peanut elicited by *Aspergillus*, accumulated up to 0.14 (2G-5A) and 0.63 (5A) mg RE/g DW (**Table S1** in Supporting Information).

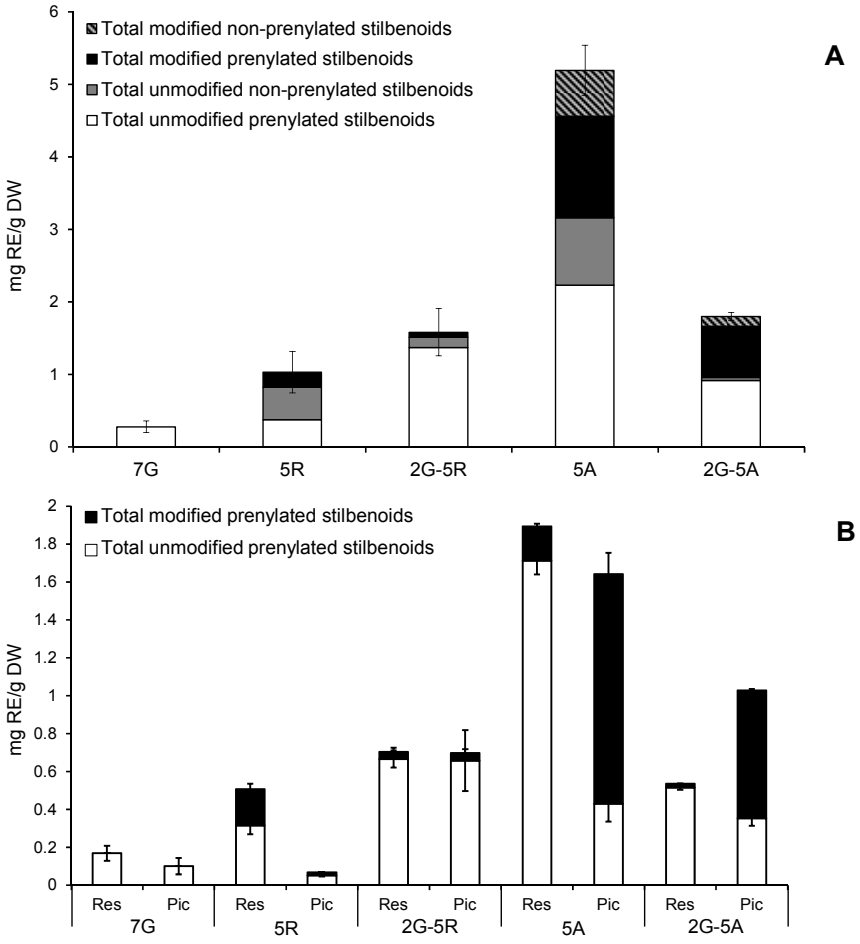


Figure 5. (**A**) Total content (mg resveratrol equivalent (RE)/ g dry weight (DW)) of unmodified non-prenylated stilbenoids, unmodified prenylated stilbenoids, modified non-prenylated stilbenoids and modified prenylated stilbenoids in germinated and elicited peanut seedlings. (**B**) Total content of (unmodified and modified) prenylated stilbenoids with resveratrol (Res) and piceatannol (Pic) skeleton in germinated and elicited peanut seedlings. Codes (7G, 5R, 2G-5R, 5A, and 2G-5A) refer to the treatments in **Table 1**. Data are the means ± SD of experiments performed in duplicate.

DISCUSSION

Due to the fact that the two fungi studied are capable of modifying prenylated stilbenoids, when they are separated from the alive tissue of peanut seedlings, we conclude that our results provide strong evidence for *in planta* modification of phytoalexins by fungi, as suggested before.[34]

Effect of type of fungus and time point of inoculation on the composition of prenylated stilbenoids

Both *Aspergillus* and *Rhizopus* effectively induced prenylated stilbenoids in elicited seedlings. Nevertheless, the ability of *Aspergillus* to modify prenylated stilbenoids was stronger than that of *Rhizopus* (**Figure 5A**). Moreover, the total content of modified prenylated stilbenoids was about twice higher when the fungi were applied early onto the peanuts (5R and 5A) than when it was applied later (2G-5R and 2G-5A) (**Figure 5A**). Because of this, the content of unmodified prenylated stilbenoids was expected to be lower in the former peanut seedlings than that of peanut seedlings elicited later. This was true for those elicited with *Rhizopus* (3.8-fold lower; **Figure 5A**). Surprisingly, the opposite was found with *Aspergillus*, where the content of unmodified prenylated stilbenoids was 2.4-fold higher than that of peanut seedlings elicited later (**Figure 5A**). Furthermore, it appeared that *Aspergillus* more effectively modified piceatannol-based structures than resveratrol-based structures (**Figure 5B**), which is consistent with the fact that dioxygenases require a catechol moiety.[35] It is concluded that the type of fungus and the time point of inoculation of the seed(ling)s with fungus strongly affect the content and composition of stilbenoids in elicited peanut seedlings, in a fungus-specific way.

Aspergillus and *Rhizopus* employ different reactions to modify prenylated stilbenoids

The present study demonstrated that modification of prenylated stilbenoids by *Aspergillus* and *Rhizopus* involved several reactions, i.e. glycosylation, oxidation, hydroxylation and sulfation. The overview of reactions is shown in **Figure 6**. Glycosylation is the main strategy for *Rhizopus* to modify prenylated stilbenoids, a reaction not observed for *Aspergillus*. Although the exact position of glycosylation could not be determined in this study, it was evident that prenylated stilbenoids with a resveratrol skeleton (i.e. arachidin-2 and -3) were preferred substrates for the glycosyl transferases compared to those with a piceatannol skeleton (i.e. arachidin-1 and IPP). Therefore, it is speculated that glycosylation occurred at the hydroxyl group at the 4'-position, and that its vicinal OH (at the 3'-position) hinders this process due to the intramolecular hydrogen bond formation.[36] To the best of our knowledge, this is the first report about glycosylation of prenylated stilbenoids by a fungus, a reaction which has been reported before for flavonoids.[37]

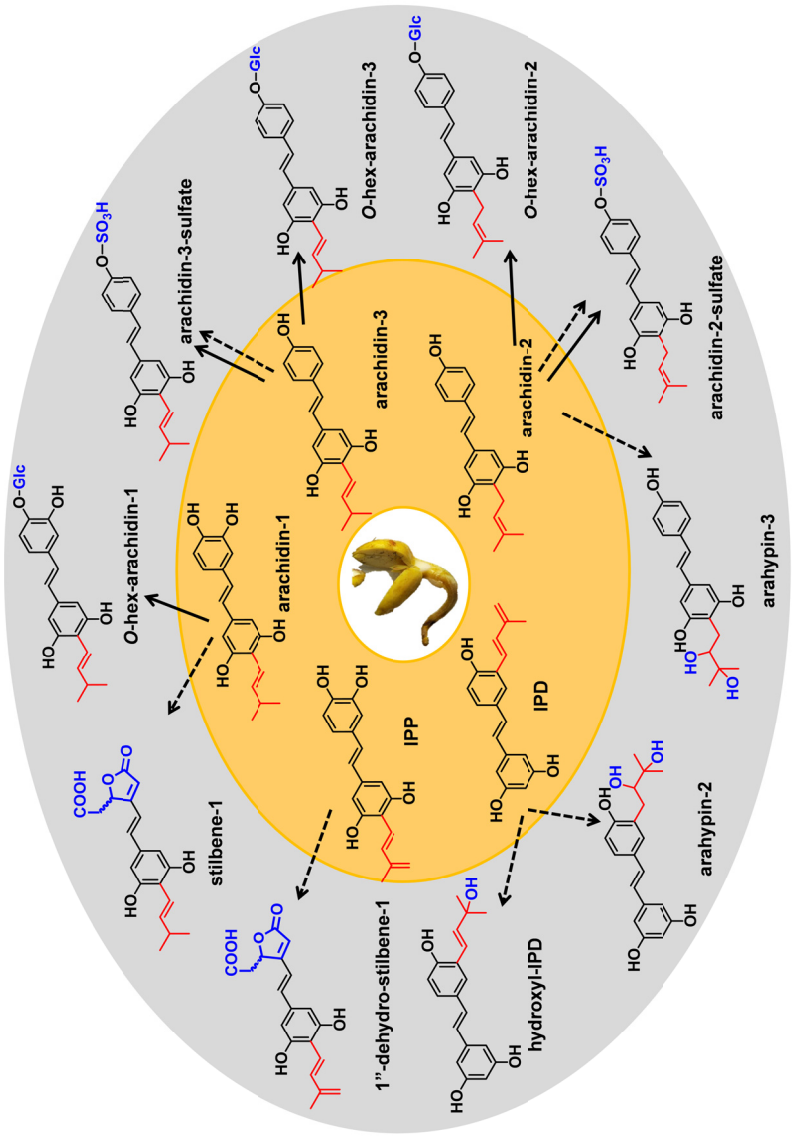


Figure 6. Prenylated stilbenoid structures found in peanut seedlings elicited by fungi. The stilbenoid phytoalexins (in yellow area), referred to as unmodified prenylated stilbenoids in the text, were metabolized by *Rhizopus* (solid arrows) or *Aspergillus* (dashed arrows) resulting in modified prenylated stilbenoids (in grey area). The exact positions of glycosylation, sulfation, and hydroxylation of these modified prenylated stilbenoids were not determined.

Aspergillus employed oxidative cleavage of the catechol ring as main strategy to modify prenylated stilbenoids. As a result, arachidin-1 and IPP were converted to their respective lactone derivatives, stilbene-1 and 1″-dehydro-stilbene-1 (**Figure 6**). Oxidative cleavage was not restricted to prenylated stilbenoids, as also oxidative cleavage products of non-prenylated stilbenoids were found, i.e. piceatannol lactone. Similar observations were reported before, where piceatannol and astringin (piceatannol *O*-glucoside) from Norway spruce (*Picea abies*) were converted into piceatannol lactone and astringin lactone by *Ceratocystis polonica*.[29] *Aspergillus* is known to degrade aromatic substances via the so-called β-ketoadipate pathway, in which a catechol moiety is a prerequisite for entrance.[35, 38] The catechol moiety is converted to a β-carboxymuconolactone ring, such as in stilbene-1.[35] Apparently, there is no such pathway in *Rhizopus oryzae*, as stilbene-1 was not detected in peanut seedlings elicited with this fungus. Nevertheless, stilbene-1 has been reported in black skin peanuts elicited with *Rhizopus oligosporus*.[8] Besides oxidative cleavage, *Aspergillus* also employed sulfation and hydroxylation for modification of prenylated stilbenoids, albeit at lower frequency, considering the lower content of these reaction products. A small amount of prenylated sulfated stilbenoids was also observed in peanut seedlings elicited with *Rhizopus*, indicating that the sulfation was not a fungus-specific modification process.

Unmodified prenylated stilbenoids produced by elicited peanuts have been reported to possess biological activity against *Aspergillus*.[4] Hence, some of the modifications of prenylated stilbenoids might be associated with detoxification of phytoalexins by fungi, i.e. the ability of fungi to metabolize phytoalexins into less inhibitory products.[39] Attachment of glycosyl residues and hydroxyl substituents has been reported to decrease the lipophilicity of phytoalexins and to lower their antimicrobial effect.[40, 41] Oxidative cleavage also has been suggested to decrease the toxicity of the compound.[29] Nevertheless, comparison of the antimicrobial activity of prenylated stilbenoids, before and after modification, remains necessary to prove the detoxification hypothesis.

Optimizing production of bioactive stilbenoids

Our research shows that conversion of potentially bioactive prenylated stilbenoids in elicited peanut seedlings to modified prenylated stilbenoids has to be considered when fungal elicitation is used in the production of pharmaceuticals or ingredients for functional foods. *Aspergillus* metabolized arachidin-1 into stilbene-1, whereas *Rhizopus* used glycosylation as modification strategy. This might lead to diminished bioactivity. The activity of stilbene-1 in a broad spectrum of biological assays, including anti-adipogenic activity, has been reported lower than that of arachidin-1.[8, 42] The catechol moiety might be important to bind to receptors and irreversible destruction of this ring might reduce bioactivity. There are no reports about the bioactivity of prenylated stilbenoid glucosides.

As deglycosylation occurs naturally in the human gastrointestinal tract (as observed with glycosylated (iso)flavonoids), the bioactivity of glycosylated prenylated stilbenoids might be retained.[43] In that respect, elicitation by *Rhizopus* might be a better option than elicitation by *Aspergillus*, although the latter seems to enable higher production levels of potentially bioactive compounds. Based on our results, we conclude that the choice of a fungal elicitor and time point of application of the fungus are crucial parameters to obtain a high yield of bioactive stilbenoids in peanut seedlings.

ACKNOWLEDGEMENT

This research was financially supported by Directorate General of Higher Education Ministry of National Education of Indonesia. We are grateful to Judith Wolkers-Rooijackers (Laboratory of Food Microbiology, Wageningen University, The Netherlands) for providing the fungi and assistance in culturing the fungi in the presence of prenylated stilbenoids.

REFERENCES

[1] R. Simons, J.-P. Vincken, N. Roidos, T. F. H. Bovee, M. Van Iersel, M. A. Verbruggen, H. Gruppen, Increasing soy isoflavonoid content and diversity by simultaneous malting and challenging by a fungus to modulate estrogenicity, *Journal of Agricultural and Food Chemistry* **2011**, *59*, 6748-6758.

[2] S. Aisyah, H. Gruppen, B. Madzora, J.-P. Vincken, Modulation of isoflavonoid composition of *Rhizopus oryzae* elicited soybean (*Glycine max*) seedlings by light and wounding, *Journal of Agricultural and Food Chemistry* **2013**, *61*, 8657-8667.

[3] S. Aisyah, H. Gruppen, S. Andini, Z. Mardiah, J.-P. Vincken, Compositional changes in (iso)flavonoids and estrogenic activity of three edible *Lupinus* species by germination and *Rhizopus*-elicitation, *Submitted* **2015**.

[4] V. S. Sobolev, Production of phytoalexins in peanut (*Arachis hypogaea*) seed elicited by selected microorganisms, *Journal of Agricultural and Food Chemistry* **2013**, *61*, 1850-1858.

[5] R. M. Lopes, T. Da Silveira Agostini-Costa, M. A. Gimenes, D. Silveira, Chemical composition and biological activities of *Arachis* species, *Journal of Agricultural and Food Chemistry* **2011**, *59*, 4321-4330.

[6] V. S. Sobolev, S. T. Deyrup, J. B. Gloer, New peanut (*Arachis hypogaea*) phytoalexin with prenylated benzenoid and but-2-enolide moieties, *Journal of Agricultural and Food Chemistry* **2006**, *54*, 2111-2115.

[7] Z. Liu, J. Wu, D. Huang, New arahypins isolated from fungal-challenged peanut seeds and their glucose uptake-stimulatory activity in 3T3-L1 adipocytes, *Phytochemistry Letters* **2013**, *6*, 123-127.

[8] Z. Liu, J. Wu, D. Huang, New stilbenoids isolated from fungus-challenged black skin peanut seeds and their adipogenesis inhibitory activity in 3T3-L1 cells, *Journal of Agricultural and Food Chemistry* **2013**, *61*, 4155-4161.

[9] Z. Wu, L. Song, D. Huang, Food grade fungal stress on germinating peanut seeds induced phytoalexins and enhanced polyphenolic antioxidants, *Journal of Agricultural and Food Chemistry* **2011**, *59*, 5993-6003.

[10] S. R. Couto, M. A. Sanromán, Application of solid-state fermentation to food industry: A review, *Journal of Food Engineering* **2006**, *76*, 291-302.

[11] P. Barbesgaard, H. P. Heldt-Hansen, B. Diderichsen, On the safety of *Aspergillus oryzae*: A review, *Applied Microbiology and Biotechnology* **1992**, *36*, 569-572.

[12] Y. Ma, A. Kosińska-Cagnazzo, W. L. Kerr, R. Amarowicz, R. B. Swanson, R. B. Pegg, Separation and characterization of phenolic compounds from dry-blanched peanut skins by liquid chromatography-electrospray ionization mass spectrometry, *Journal of Chromatography A* **2014**, *1356*, 64-81.

[13] M. L. Sullivan, Perennial peanut (*Arachis glabrata* Benth.) leaves contain hydroxycinnamoyl-CoA: Tartaric acid hydroxycinnamoyl transferase activity and accumulate hydroxycinnamoyl-tartaric acid esters, *Planta* **2014**, *239*, 1091-1100.

[14] F. Ferreres, A. Gil-Izquierdo, P. B. Andrade, P. Valentão, F. A. Tomás-Barberán, Characterization of *C*-glycosyl flavones *O*-glycosylated by liquid chromatography-tandem mass spectrometry, *Journal of Chromatography A* **2007**, *1161*, 214-223.
[15] V. S. Sobolev, S. A. Neff, J. B. Gloer, New stilbenoids from peanut (*Arachis hypogaea*) seeds challenged by an *Aspergillus caelatus* strain, *Journal of Agricultural and Food Chemistry* **2009**, *57*, 62-68.
[16] V. S. Sobolev, S. A. Neff, J. B. Gloer, New dimeric stilbenoids from fungal-challenged peanut (*Arachis hypogaea*) seeds, *Journal of Agricultural and Food Chemistry* **2010**, *58*, 875-881.
[17] V. S. Sobolev, S. A. Neff, J. B. Gloer, S. I. Khan, N. Tabanca, A. J. De Lucca, D. E. Wedge, Pterocarpenes elicited by *Aspergillus caelatus* in peanut (*Arachis hypogaea*) seeds, *Phytochemistry* **2010**, *71*, 2099-2107.
[18] V. S. Sobolev, T. L. Potter, B. W. Horn, Prenylated stilbenes from peanut root mucilage, *Phytochemical Analysis* **2006**, *17*, 312-322.
[19] S. Gocan, Analysis of stilbenes in wine by HPLC: Recent approaches, *Journal of Liquid Chromatography & Related Technologies* **2009**, *32*, 1598-1643.
[20] V. S. Sobolev, B. W. Horn, T. L. Potter, S. T. Deyrup, J. B. Gloer, Production of stilbenoids and phenolic acids by the peanut plant at early stages of growth, *Journal of Agricultural and Food Chemistry* **2006**, *54*, 3505-3511.
[21] B. Y. Ong, C. W. Nagel, High-pressure liquid chromatographyic analysis of hydroxycinnamic acid-tartaric acid esters and their glucose esters in *Vitis vinifera*, *Journal of Chromatography A* **1978**, *157*, 345-355.
[22] P. M. Dey, J. Harborne, *Methods in plant biochemistry, Vol. 1*, Academic Press, San Diego, CA , USA, **1989**.
[23] V. T. J. Mabry, K. R. Markham, M. B. Thomas, *The systematic identification of flavonoids*, Springer-Verlag, New York, NY, USA, **1971**.
[24] D. Tsimogiannis, M. Samiotaki, G. Panayotou, V. Oreopoulou, Characterization of flavonoid subgroups and hydroxy substitution by HPLC-MS/MS, *Molecules* **2007**, *12*, 593-606.
[25] J. Kang, L. A. Hick, W. E. Price, A fragmentation study of isoflavones in negative electrospray ionization by MS^n ion trap mass spectrometry and triple quadrupole mass spectrometry, *Rapid Communications in Mass Spectrometry* **2007**, *21*, 857-868.
[26] R. Maul, N. Schebb, S. Kulling, Application of LC and GC hyphenated with mass spectrometry as tool for characterization of unknown derivatives of isoflavonoids, *Analytical and Bioanalytical Chemistry* **2008**, *391*, 239-250.
[27] G. E. Aguamah, P. Langcake, D. P. Leworthy, J. A. Page, R. J. Pryce, R. N. Strange, Two novel stilbene phytoalexins from *Arachis hypogaea*, *Phytochemistry* **1981**, *20*, 1381-1383.
[28] C. J. Cooksey, P. J. Garratt, S. E. Richards, R. N. Strange, A dienyl stilbene phytoalexin from *Arachis hypogaea*, *Phytochemistry* **1988**, *27*, 1015-1016.
[29] A. Hammerbacher, A. Schmidt, N. Wadke, L. P. Wright, B. Schneider, J. Bohlmann, W. A. Brand, T. M. Fenning, J. Gershenzon, C. Paetz, A common fungal associate of the spruce bark beetle metabolizes the stilbene defenses of Norway spruce, *Plant Physiology* **2013**, *162*, 1324-1336.
[30] T. Tronina, A. Bartmańska, M. Milczarek, J. Wietrzyk, J. Popłoński, E. Rój, E. Huszcza, Antioxidant and antiproliferative activity of glycosides obtained by biotransformation of xanthohumol, *Bioorganic & Medicinal Chemistry Letters* **2013**, *23*, 1957-1960.
[31] S. H. El-Sharkawy, M. I. Selim, M. S. Afifi, F. T. Halaweish, Microbial transformation of zearalenone to a zearalenone sulfate, *Applied and Environmental Microbiology* **1991**, *57*, 549-552.
[32] S. S. Quadri, R. E. Stratford, S. M. Boué, R. B. Cole, Screening and identification of glyceollins and their metabolites by electrospray ionization tandem mass spectrometry with precursor ion scanning, *Analytical Chemistry* **2013**, *85*, 1727-1733.
[33] L. Stella, M. De Rosso, A. Panighel, A. D. Vedova, R. Flamini, P. Traldi, Collisionally induced fragmentation of $[M-H]^-$ species of resveratrol and piceatannol investigated by deuterium labelling and accurate mass measurements, *Rapid Communications in Mass Spectrometry* **2008**, *22*, 3867-3872.
[34] V. P. W. Miao, H. D. Vanetten, Genetic analysis of the role of phytoalexin detoxification in virulence of the fungus *Nectria haematococca* on chickpea (*Cicer arietinum*), *Applied and Environmental Microbiology* **1992**, *58*, 809-814.
[35] C. S. Harwood, R. E. Parales, The β-ketoadipate pathway and the biology of self-identity, *Annual Reviews in Microbiology* **1996**, *50*, 553-590.
[36] M. Rossi, F. Caruso, C. Opazo, J. Salciccioli, Crystal and Molecular Structure of Piceatannol; Scavenging Features of Resveratrol and Piceatannol on Hydroxyl and Peroxyl Radicals and Docking with Transthyretin, *Journal of Agricultural and Food Chemistry* **2008**, *56*, 10557-10566.

[37] M. Slana, D. Žigon, T. Makovec, H. Lenasi, The response of filamentous fungus *Rhizopus nigricans* to flavonoids, *Journal of Basic Microbiology* **2011**, *51*, 433-441.
[38] Kuswandi, C. F. Roberts, Genetic control of the protocatechuic acid pathway in *Aspergillus nidulans*, *Journal of General Microbiology* **1992**, *138*, 817-823.
[39] M. C. Lucy, P. S. Matthews, H. D. VanEtten, Metabolic detoxification of the phytoalexins maackiain and medicarpin by *Nectria haematococca* field isolates: Relationship to virulence on chickpea, *Physiological and Molecular Plant Pathology* **1988**, *33*, 187-199.
[40] M. S. C. Pedras, P. W. Ahiahonu, M. Hossain, Detoxification of the cruciferous phytoalexin brassinin in *Sclerotinia sclerotiorum* requires an inducible glucosyltransferase, *Phytochemistry* **2004**, *65*, 2685-2694.
[41] P. J. Kuhn, D. A. Smith, D. F. Ewing, 5,7,2′,4′-Tetrahydroxy-8-(3″-hydroxy-3″-methyl-butyl) isoflavanone, a metabolite of kievitone produced by *Fusarium solani* f.sp. phaseoli, *Phytochemistry* **1977**, *16*, 296-297.
[42] V. S. Sobolev, S. I. Khan, N. Tabanca, D. E. Wedge, S. P. Manly, S. J. Cutler, M. R. Coy, J. J. Becnel, S. A. Neff, J. B. Gloer, Biological activity of peanut (*Arachis hypogaea*) phytoalexins and selected natural and synthetic stilbenoids, *Journal of Agricultural and Food Chemistry* **2011**, *59*, 1673-1682.
[43] A. J. Day, M. S. Dupont, S. Ridley, M. Rhodes, M. J. C. Rhodes, M. R. A. Morgan, G. Williamson, Deglycosylation of flavonoid and isoflavonoid glycosides by human small intestine and liver β-glucosidase activity, *FEBS Letters* **1998**, *436*, 71-75.

Supporting Information

Table S1. Contents (mg RE/g DW)[a] of stilbenoids in extracts from germinated and elicited peanuts.

Compounds	7G[b]	5R	2G-5R	5A	2G-5A
Unmodified stilbenoids					
Piceatannol	-[c]	<0.01	<0.01	0.05±0.01	<0.01
trans-Resveratrol	-	0.45±0.27	0.14±0.06	0.88±0.11	0.04±0.02
IPP	0.01±0.00	-	0.01±0.01	-	-
Arachidin-1 isomer	-	0.01±0.00	0.01±0.01	-	-
IPP	0.01±0.00	-	0.01±0.00	-	-
Arachidin-1 isomer	0.02±0.01	0.01±0.00	0.11±0.06	0.02±0.00	0.05±0.01
trans-Arachidin-1	0.05±0.02	0.03±0.00	0.46±0.07	0.42±0.10	0.28±0.02
trans-Arachidin-2	0.01±0.00	0.17±0.00	0.07±0.01	0.22±0.02	0.03±0.01
Arachidin-2-isomer	<0.01	0.05±0.02	0.03±0.01	0.06±0.01	0.04±0.00
trans-Arachidin-3	0.01±0.01	0.06±0.02	0.17±0.01	1.12±0.15	0.20±0.01
trans-IPD	0.14±0.04	0.03±0.01	0.40±0.06	0.30±0.07	0.25±0.01
IPP	0.01±0.00	0.00±0.00	0.04±0.02	0.00±0.00	0.01±0.00
IPP	<0.01	-	0.01±0.01	0.00±0.00	0.01±0.00
Modified stilbenoids					
Piceatannol lactone	-	-	-	0.63±0.18	0.14±0.00
Arachidin-1 *O*-glucoside	-	0.02±0.00	0.03±0.02	-	-
Arachidin-2 *O*-glucoside	-	0.10±0.02	0.01±0.01	-	-
Arachidin-3 *O*-glucoside	-	0.04±0.00	0.02±0.01	-	-
Arachidin 2 or 3 *O*-glucoside	-	0.02±0.01	-	-	-
Arachidin 2 or 3 *O*-glucoside	-	0.03±0.00	0.00±0.00	-	-
Arahypin-2	-	-	-	0.01±0.00	0.01±0.00
Arahypin-3	-	-	-	0.11±0.00	0.02±0.00
Stilbene-1 isomer	-	-	-	0.05±0.01	0.03±0.00
Stilbene-1 isomer	-	-	-	0.11±0.02	0.04±0.00
Hydroxy-IPD	-	-	-	0.07±0.01	
Stilbene-1	-	-	-	1.05±0.08	0.61±0.01
1''-Dehydro-stilbene 1	-	-	-	0.14±0.03	0.09±0.01

[a] Data are the means ± SD of experiments performed in duplicate.
[b] Codes refer to compounds in **Table 1**.
[c] The compound was not found in the extract.

Chapter 6

General Discussion

In previous research at our laboratory, soybean seeds have been used for investigating changes in isoflavonoid content and composition during an induction process, in which the seeds are germinated under biotic stress. As this process both improved the isoflavonoid content and the proportion of isoflavonoid subclasses with promising bioactive potential, in the current research the procedure was extrapolated to twelve other legume seeds. These were selected based on three criteria: 1) edible, 2) available on the market, and 3) belonging to different subtribes of Leguminoseae (phylogenetically scattered). Seeds of the selected legume species were subjected to the same induction process as that employed to soybeans, but using a different machine, i.e. a sprouting machine, modified to provide appropriate experimental conditions. With this new device, the induction process was extended to different types of stress factors. More specifically, two different fungi were used (*Rhizopus oryzae* and *Aspergillus oryzae*) as biotic stress factors, whereas also abiotic stress factors, wounding and exposure to light, were explored. The effects of these stress factors were investigated systematically based on monitoring compositional changes in phenolic compounds in the extracts from the various seedlings, particularly in (iso)flavonoids and stilbenoids. The content and composition of phenolic compounds of unchallenged seedlings were investigated in a separate set of experiments, affording information about the effect of germination alone.

This chapter discusses the main findings presented in this thesis, and addresses prospects and limitations of the methodology used. The former elaborates on the inducibility of particular isoflavonoid and stilbenoid skeletons, and that of prenylation. In addition, it elaborates on the correlation of the changes of isoflavonoids and stilbenoids with the phylogenetic relationships of Leguminosae. Furthermore, modification of phytoalexins by fungus during elicitation is associated to the type of phytoalexin produced and the kind of fungus used. The latter covers the induction process, including the efficiency of a modified sprouting machine, the use of different stress factors and the time point of application of the fungus to seeds or seedlings, and the quantification of phenolic compounds.

IN PLANTA MODIFICATION OF LEGUME PHYTOALEXINS BY FUNGI

An important conclusion of this PhD research was that when performing the induction process with legume seeds, consisting of germination and elicitation by fungus, one should be aware of the possibility that the fungus can modify the phytoalexins accumulated (**Chapter 5**). Potentially, this can lead to loss of bioactive compounds. Consequently, this would be an undesirable side reaction. We evidenced such modification of phytoalexins upon elicitation of *Arachis hypogaea* (peanut) seedlings *in planta*, as well as *in vitro*. The stilbenoids produced by the plant could be modified by the fungus in various ways, of which glycosylation and oxidation were the predominant modification routes. The proportion of modified stilbenoids was affected strongly by the fungus used and the time point of application of the fungus. Moreover, it appeared that certain stilbenoid structures were more prone to modification than others. It was suggested that this modification represents a detoxification strategy of fungi to overcome the effect of phytoalexins.

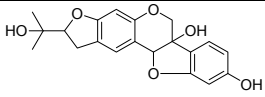
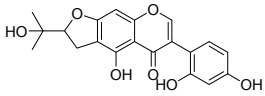
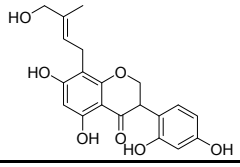
Table 1. Modification of isoflavonoid phytoalexins, found in legume species, by fungi. The experiments were performed *in vitro*, in absence of plant tissue.

Phytoalexins[ref]	Fungal species	Products of metabolism	Reaction involved
Kievitone[1]	*Fusarium solani*	Kievitone hydrate	Hydration
2.3-Dehydrokievitone[2]	*Aspergillus flavus*	Hydroxyl-dihydrofurano 2,3-dehydrokievitone	Epoxidation, cyclisation
		Hydroxyl-dihydropyrano 2,3-dehydrokievitone	Epoxidation, cyclisation
		2,3-Dehydrokievitone glycol	Epoxidation, hydration
Luteone[3]	*Aspergillus flavus*	Lupinisoflavone B	Epoxidation, cyclisation
Maackiain[4]	*Nectria haematococca*	(-)-6a-Hydroxymaackiain	Hydroxylation
		1a-Hydroxymaackiain	Dienone formation, hydroxylation
		Sophorol	Opening D-ring
Medicarpin[5]	*Fusarium proliferatum*	Demethylmedicarpin	Demethylation
Phaseollidin[6]	*Fusarium solani*	Phaseollidin hydrate	Hydration
Phaseollin[5]	*Fusarium solani*	1a-Hydroxyphaseollone	Dienone formation, hydroxylation
Biochanin A[7]	*Rhizopus nigricans*	Biochanin A 7-*O*-Glc	Glycosylation

The *in planta* modification of stilbenoid phytoalexins in the elicited peanut seedlings raised the question whether the modification also occurred in other legumes that produced isoflavonoid phytoalexins. Previous studies have shown that phytoalexins belonging to the class of isoflavonoids can be modified by pathogenic fungi *in vitro* (**Table 1**). The mechanism by which these phytoalexins are modified by fungus might be degradative or non-degradative in nature. The non-degradative mechanisms include the conversion of phytoalexins to more polar products, such as conversion of biochanin A into its

glycosylated form, whereas degradative mechanisms refer e.g. to ring opening, such as the conversion of the pterocarpan maackiain into the isoflavanone sophorol (**Table 1**).[4, 8] The prenyl group is often target of non-degradative modification as well.[9] Hydroxylation of the terminal methyl group and hydration of the double bond of a prenyl chain, for instance, have been reported as reactions involved in the detoxification process by fungi.[9] Therefore, some compounds accumulated in the elicited legume species by *Rhizopus* (**Chapters 2-4**), i.e. glyceofuran, lupinisoflavone B and kievitol, might be considered as isoflavonoid phytoalexins modified by fungus (**Table 2**).

Table 2. Prenylated isoflavonoids considered as fungal modification products, found in the legume species in the research described in this PhD thesis.

Modified prenylated isoflavonoid		Species	Quantity
Glyceofuran[a]		*Glycine max*	0.10±0.01
Lupinisoflavone B[b]		*Lupinus luteus*	0.05±0.01
Kievitol[a]		*Phaseolus coccineus* *P. vulgaris*	0.07±0.02 0.03±0.01

[a] The quantity of the compound was expressed as mg daidzein eq./g DW.
[b] The quantity of the compound was expressed as mg genistein eq./g DW.

In **Chapter 5** it was also observed that the type of fungus matters with respect to modifying the composition of phytoalexins in elicited peanut seedlings. In order to verify whether this also accounts for soybeans, an experiment additional to those described in **Chapter 2** was conducted. In this experiment, the soybean seedlings were elicited by *Aspergillus niger*, using the same experimental conditions as those in **Chapter 2**. Soybeans elicited by *Aspergillus niger* or *Rhizopus oryzae* after 2 d of germination showed no difference in phytoalexin composition (**Figure 1**). It is known that *Aspergilli* have the biochemical machinery to oxidize phenolic compounds with a catechol moiety. As isoflavonoids found in this study do not possess this moiety, this typical modification by *Aspergilli* was not observed.

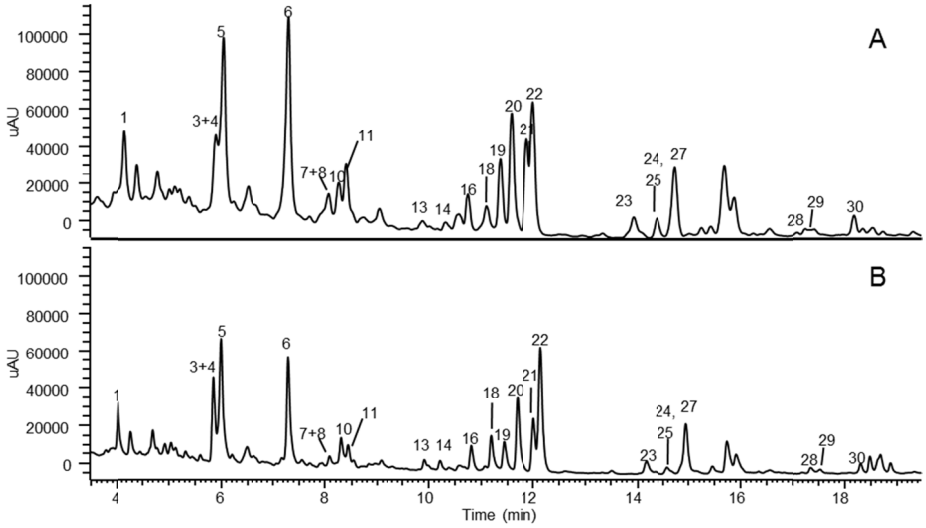


Figure 1. RP-UHPLC-UV profile of a 70% (v/v) aqueous EtOH extract at 280 nm of soybean seedlings elicited by *Aspergillus niger* (**A**) or *Rhizopus oryzae* (**B**). Peak numbers refer to compounds in **Table 3**.

INDUCTION METHODS OF LEGUMINOCEOUS SEEDS

Efficiency of different machines in induction of phytoalexins compared

In previous research,[10] the simultaneous germination and elicitation by *Rhizopus microsporus* of soybeans was performed up to 4.0 kg scale in a micro-malting machine (system unit 90-102; Joe White, Perth, Australia) used in the brewing industry. In the current research, we employed an EQMM sprouting machine (EasyGreen, San Diego, CA, USA). This sprouting machine could accommodate 20-300 g of dry beans, which makes it more attractive for screening purposes than the micro-malting machine. As the sprouting machine was designed for sprouting only, it was necessary to adjust the conditions in the machine, so that it not only would be appropriate for germination, but also for growing the fungus. The adjusted variables were temperature and relative humidity (RH), as described in **Chapter 2**.[11]

Prior to the experiments described in **Chapter 2**, the performance of the sprouting machine in modifying isoflavonoid composition of fungus-elicited soybean seedlings was compared to that of the Joe White micro-malting machine, using the same soybean batch.[10] After treatment, the soybean seedlings obtained with both machines were extracted and analysed using the same methods as described in **Chapter 2**.[11] Both machines produced a similar

set of compounds with respect to isoflavonoid subclasses obtained, although differences in accumulation of specific isoflavonoids were observed (**Figure 2, Table 3**).

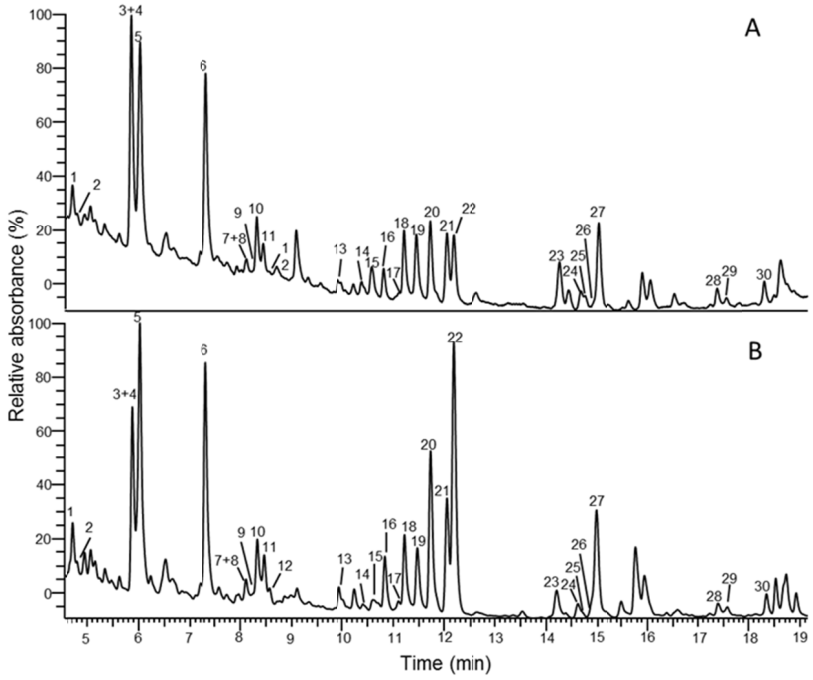


Figure 2. RP-UHPLC-UV profile at 280 nm of a 70% (v/v) aqueous EtOH extract of fungus-treated soybean seedlings using micro-malting machine (**A**) or sprouting machine (**B**). Peak numbers refer to compounds in **Table 3**.

The total quantity of isoflavonoids obtained with the two machines was comparable, 2.71 and 2.40 mg DE/g DW in the sprouting and the micro-malting machine, respectively (**Table 3**). Differences in specific compounds mainly related to pterocarpans. In the micro-malting machine, glyceollin I (**22**) was produced to the same extent as glyceollin II (**21**) and III (**20**), around 0.10 mg DE/g DW each, whereas the sprouting machine generated glyceollin I as the predominant prenylated pterocarpan (0.41 mg DE/g DW), and glyceollin II and III at lower levels, i.e. 0.15 and 0.23 mg DE/g DW, respectively (**Table 3**). Glycinol (**3**) was accumulated 1.5-fold more in the micro-malting machine than in the sprouting machine. The higher accumulation of glycinol, a precursor for prenylated pterocarpans, in the micro-malting machine compared to that in the sprouting machine indicated that the induction process in the micro-malting machine did not progress to the full extent. This was

in line with the observation that soybeans treated in the sprouting machine produced a higher amount of prenylated isoflavonoids, the most downstream set of compounds in the biosynthesis of pterocarpans, than those in the micro malting machine.

Table 3. Contents of isoflavonoids in treated soybean (expressed in mg daidzein equivalent (DE) per gram dry weight (DW) of soybean) using the sprouting or micro-malting machine.

No[a]	Compound	Sprouting machine	Micro-malting machine
1	Daidzin	0.07	0.04
2	Glycitin	0.02	0.01
4	Genistin	0.04	0.06
5	6''-*O*-Malonyldaidzin	0.41	0.39
6	6''-*O*-Malonylgenistin	0.32	0.46
7	7-*O*-(6''-*O*-Malonyl-Glc) demethyltexasin	0.02	0.03
8	6''-*O*-Malonylononin	0.01	<0.01
9	Glycitein	<0.01	<0.01
11	Daidzein	0.08	0.04
12	2'-OH-Genistein	0.02	<0.01
14	Prunetin	0.01	0.01
16	Genistein	0.07	0.05
17	Formononetin	0.01	0.01
24	A-prenyl-daidzein	0.02	0.02
25	A-prenyl-2'-OH daidzein	0.01	0.02
26	B-prenyl-daidzein	0.01	0.01
28	A-prenyl-genistein	0.02	0.03
29	B-prenyl-genistein	0.01	0.01
Total isoflavones		1.16	1.18
3	Glycinol	0.17	0.27
10	Glyceofuran	0.09	0.09
18	Glyceollidin I/II	0.11	0.10
20	Glyceollin III	0.23	0.13
21	Glyceollin II	0.15	0.10
22	Glyceollin I	0.41	0.10
23	Glyceollin VI	0.05	0.09
27	Glyceollin IV	0.17	0.16
Total pterocarpans		1.40	1.04
15	Isotrifoliol	0.03	0.05
19	Coumestrol	0.10	0.10
30	Phaseol	0.03	0.04
Total coumestans		0.16	0.19
Total prenylated isoflavonoids		1.31	0.90
Total non-prenylated isoflavonoids		1.40	1.50
Total isoflavonoids		2.71	2.40

[a] Numbers refer to peaks in **Figure 2**.

Efficiency of biotic elicitors

As described in the *General Introduction*, two types of elicitors can be used to trigger the defence response in seedlings, i.e. biotic and abiotic elicitors.[12] In our studies, we have used edible fungi, non-pathogenic to human, for induction of legume seedlings. The main

observation is that *Rhizopus oryzae* was able to induce phytoalexins in almost all legume species studied (**Chapters 2-5**).

To investigate the effect of different types of edible fungi, apart from *Rhizopus oryzae*, *Aspergillus oryzae* was also used to elicit peanut seedlings (**Chapter 5**). As an additional experiment to those in **Chapter 5**, three more edible fungi: *Rhizopus microsporus*, *Aspergillus niger* and *Neurospora* species were used to elicit peanut seedlings (**Figure 3**). In this experiment, the peanut seedlings were elicited after 2 d of germination, similar as described in **Chapter 5**. The main conclusion of using different types of food-grade fungi to elicit peanut seedlings is that they seem to induce the same set of phytoalexins in approximately similar quantities (at least when the fungus is applied at day 3 of the induction process). Nevertheless, depending on the type of fungus used, these sets can be modified by enzymes from the fungus. The accumulation of glycosylated arachidin-1 (**1**) and stilbene-1 (**2**) solely in peanut seedlings elicited with *Rhizopus* and *Aspergillus*, respectively, was the main difference in stilbenoid profiles of peanut seedlings elicited by different types of fungi (**Figure 3**). Moreover, the distinctive abilities of fungi to modify phytoalexins have to be considered. This might hamper efficient production of bioactive compounds when employing the process of combined germination and elicitation by fungus.

The absence of stilbene-1, a prenylated stilbenoid with a muconolactone ring, in peanuts elicited with *Neurospora* is interesting, as both *Aspergillus* and *Neurospora* have been reported to perform the β-ketoadipate pathway, a pathway for aromatic compound degradation suggested to be responsible for converting arachidin-1 into stilbene-1.[8, 13, 14] The β-ketoadipate pathway of fungi has two branches, the so-called catechol and protocatechuate branches, which require different substrates: catechol (1,2-dihydroxybenzene) and protocatechuate (3,4-dihydroxybenzoate), respectively. The catechol branch is present in some fungi.[13] The ability of *Aspergilli* to perform the β-ketoadipate pathway via both branches has been reported before,[15, 16] whereas only the protocatechuate branch has been reported for *Neurospora*.[17] In line with this, our results suggest that the catechol branch of the β-ketoadipate pathway is present in *Aspergillus*, but not in *Neurospora*, causing the differences in stilbenoid composition observed in elicited peanut seedlings. Moreover, the β-ketoadipate pathway has not been reported in *Rhizopus*.

Glycosylation is another mechanism by which many fungi can detoxify phytoalexins. Glycosides of prenylated stilbenoids were accumulated in peanut seedlings elicited with *R. oryzae* (**Chapter 5**), *R. microspores* and *Neurospora* spp. (**Figure 3**), but not with *Aspergilli*. This indicates genus-dependent detoxification strategies for prenylated stilbenoids. The absence of glycosylated products in peanut seedlings elicited by *Aspergilli* might find its origin in poor expression of the genes encoding the required glycosyl

transferases, or in an inappropriate acceptor substrate specificity of the glycosyl transferases.[18]

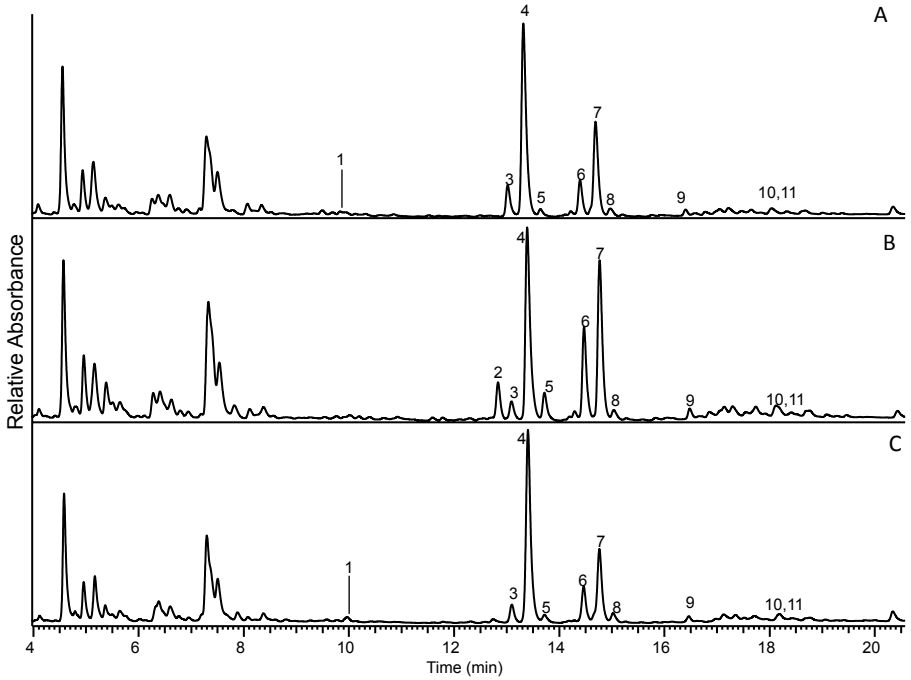


Figure 3. RP-UHPLC-UV profile at 310 nm of a 80% (v/v) aqueous MeOH extract of peanut seedlings elicited by *Rhizopus microsporus* (**A**), *Aspergillus niger* (**B**) and *Neurospora* spp. (**C**). Peak numbers refer to main stilbenoid compounds as follows:

1. arachidin-1 *O*-hexoside	4. *trans*-arachidin-1	7. IPD	10. arahypin-6
2. stilbene-1	5. *trans*-arachidin-2	8. IPP	11. arahypin-7
3. arachidin-1 isomer	6. *trans*-arachidin-3	9. arahypin-5	

Efficiency of abiotic elicitors

Wounding as an elicitor. Abiotic elicitation can be induced by light or wounding.[19-21] Wounding, a mechanical stress, triggers the plant to release signal substances, including oligosaccharides, jasmonates, abscisic acid and ethylene.[22] These signal molecules play a role in activating downstream defence response, such as the synthesis of secondary metabolites.[12] Our results showed that wounding alone can induce glyceollins in soybean seedlings (**Chapter 2**), although wounding appeared to be a weaker elicitor than fungus.[11]

The accumulation of phytoalexins after wounding has also been reported in other legume seeds, like peanuts and kidney beans.[23-26]

Often, the wounding treatment was combined with other elicitors, such as fungi. The sites of wounding are easily penetrated, facilitating the infection of seeds/seedlings by fungi.[22] Nevertheless, we observed that the ultimate effect of this combination was not always the sum of the two stress factors individually. Wounding has been reported to increase the glyceollin production of soybean elicited by *Aspergillus*, but the wounded soybean seedlings elicited by *Rhizopus* accumulated the same quantity of glyceollins (**Chapter 2**).[11, 27] To conclude, the effect of wounding (prior to application of fungus) on the accumulation of phytoalexins can be different amongst experiments, but it remains to be established to which extent other factors, such as variety of the legume species, fungal genotype employed, incubation conditions or time point of application of the fungus, play a role.

Light as an elicitor. Our research also identified the use of light as an important factor for enhancing the isoflavonoid content of soybean seedlings, in line with previous reports.[11, 28, 29] The ratio between prenylated and unprenylated isoflavonoids remained more or less constant, when comparing treatments of soybean seedlings in presence and absence of light, whereas the total isoflavonoid content increased by approximately 1.5 fold (**Chapter 2**). It is known that light can affect the formation of secondary metabolites, by acting as a signal to activate enzymes involved in biosynthetic pathways of secondary metabolites.[12] The effect varies, depending on the light parameters (radiation intensity, wavelength and duration) and legume variety.[11, 28-30] In general, seedlings grown in light showed higher isoflavonoid content compared to ones grown in the dark.[11, 28, 29] However, the opposite result was reported for some soybean cultivars.[29]

An interesting observation in this PhD research was that the use of light in combination with fungus only increased the amount of certain phytoalexins in soybean seedlings, i.e. the content of 2-prenylated pterocarpans, but not that of 4-prenylated pterocarpans (**Chapter 2**).[11] The use of light together with elicitation by fungus also seems to be a factor of importance in other bean species. A more than 6-fold increase of the phaseollin content has been observed upon elicitation of *Phaseolus vulgaris* seedlings by fungus in light, compared to those grown in the dark, whereas no increase was detected for other phytoalexins such as phaseollidin and kievitone.[31] Based on all these results, it can be concluded that it is attractive to grow elicited seedlings in light, when the objective is to enhance the accumulation of isoflavonoids in legume seedlings.

Time point of application of fungus to seedlings

In our studies, the fungus was mostly applied onto 2 d old legume seedlings,[11, 32, 33] whereas in many other studies the fungus was applied directly after soaking.[34-36] Early and

late application of fungus were compared with respect to accumulation of phytoalexins in peanut seedlings (**Chapter 5**).[37] Interestingly, the impact of the time point of application of the fungus on the stilbenoid content of peanut seedlings depended on the fungus, as early inoculation with *Aspergillus* led to higher stilbenoid content, up to 3-fold, compared to that after two days, whereas inoculation with *Rhizopus* showed the contrary (**Chapter 5**). The reason behind this is not clear. It might be speculated that *Aspergillus* is more aggressive than *Rhizopus*, which is corroborated by the occurrence of brown lesions, a parameter often used as an indicator of virulence, in *Aspergillus*-elicited peanut seedlings. The brown lesions were not observed in those elicited by *Rhizopus*.[38] So, the time point of application of fungus seems an important factor when optimizing the production of phytoalexins. The optimum time for applying the fungus seems to be legume species-dependent as well.[39] Thus, further investigation of the optimal time point for application of fungus is necessary, and it is not unlikely that such optimization needs to be done for each combination of legume and fungus.

QUANTIFICATION OF PHENOLIC COMPOUNDS IN CRUDE EXTRACTS

UHPLC with PDA allows in-line quantification of compounds in the samples analysed.[40] In this PhD research, where complex mixtures of over 30 compounds were no exception, we had the dilemma on the best way of quantification of the individual compounds. As only few standard compounds were commercially available, direct quantitative analysis by using the peak area of a compound and extrapolating its amount from a calibration curve with the respective compound was in most cases impossible. It is thus common practice to use other reference compounds.[41, 42] An alternative would be to use a single reference compound to make a calibration curve, calculate the amounts of other compounds of interest by extrapolation of their peak areas, and account for the ratio of the molar extinction coefficient of the reference and that of the compound of interest. As only few molar extinction coefficients have been published, this was not considered a good alternative. Because of this, it was decided to keep the quantification of crude extracts as transparent as possible, and use the aglycone of a main phenolic as the calibrant, i.e. daidzein (for soybean and Phaseoleae), genistein (for lupine) or *trans*-resveratrol (for peanut). For compounds eluting at the same retention time, the ratio of the relative intensity of molecular ions in the mass spectrum at that retention time was used to calculate the contribution of each molecule to the UV-Vis peak area. The quantification was performed at one wavelength, representing the maximum absorbance of the compound in question. The quantity of compounds in the crude extract was expressed as milligram of external standard per gram dry weight, without correcting for the difference in molecular weight between the compound of interest and reference compound.

To give an indication of the experimental error made by our quantification method, a recalculation of the amount of a few representative compounds was performed (**Table 4**). The glyceollin contents were recalculated using the reported ε values, which were measured at λ_{max}, and the molecular weight correction factor.[43] It can be seen that the amounts of the different pterocarpans produced by elicited soybean seedlings are actually up to 1.4- to 2.8-fold underestimated by our quantification method (**Table 4**). Although it should be kept in mind that one will find differences in absolute amounts of isoflavonoids between the calculation methods, our approach provides reliable data for making comparisons between various treatments.

Table 4. Comparison of the content of glyceollin based on a calibration curve of external standard expressed in mg daidzein equivalent (DE)/g DW and that of corrected amounts using molar extinction coefficients and molecular weight expressed as mg/g DW.

Compounds	ε ($M^{-1}cm^{-1}$)	mg DE/g DW	mg/g DW
Glyceollin I	(λ_{285}) 10,300	0.44	0.76
Glyceollin II	(λ_{286}) 8,700	0.23	0.48
Glyceollin III	(λ_{292}) 9,600	0.25	0.35
Glycinol	(λ_{287}) 5,870	0.15	0.41

INDUCIBILITY OF PHENOLICS CORRELATED TO PHYLOGENY OF LEGUMINOSAE

The ability of all legume species studied in this PhD research (**Chapters 2-5**) to induce (iso)flavonoids/stilbenoids during germination and/or fungal elicitation is summarized in **Figure 4**. In general, isoflavonoids and stilbenoids were more inducible than flavonoids during germination and fungal elicitation. The isoflavonoid and stilbenoid content is best boosted by germination in presence of fungus. The phylogenetic relatedness between the species is discussed in terms of inducibility of isoflavonoids/stilbenoids, more in particular their content and molecular diversity.

The phylogenetic tree was built as described in **Chapter 4**, with an addition of two species: *Arachis hypogaea* and *Lupinus luteus*. The *Matk* encoding regions of these species were extracted from the following NCBI nucleotide sequences: *Arachis hypogaea*: gi|166919340 (Full) and *Lupinus luteus*: gi|568244796 (2052-3572). The *Matk* encoding regions of two other *Lupinus* species (*L. angustifolius* and *L. albus*) were not available. It can be seen that the extra two species incorporated in the phylogenetic tree corroborate our conclusion in **Chapter 4**, in that the extent of inducibility is not necessarily linked to phylogenetic relatedness. The closely related species, such as *Phaseolus*, *Vigna* and *Lablab*, showed different inducibilities during germination and/or fungal elicitation.

The structures of the predominant isoflavonoids/stilbenoids in elicited seedlings were compared amongst all species studied (**Figure 4**). The main observation is that the species of *Vigna*, *Phaseolus*, *Lablab* and *Lupinus* produced relatively simple isoflavonoid moieties,

such as isoflavone and isoflavanone. In contrast, *Glycine* and *Psophocarpus* produced skeletons more downstream the isoflavonoid pathway, with an extra D-ring, such as pterocarpan. *Arachis* was the only legume species studied that produced stilbenoids.

Our results suggest that the type of skeleton induced upon elicitation might be deduced from the phylogenetic relationship of the species, whereas this is less clear for the extent and position of prenylation. Isoflavanones (dalbergioidin derivatives) were the main isoflavonoids induced in the closely related genera of *Vigna*, *Phaseolus*, and *Lablab*. The main induced isoflavonoids of *Psophocarpus* were demethylmedicarpin derivatives, belonging to the subclass of 6a-H-pterocarpans, different from those in *Glycine*, that accumulated glycinol derivatives, 6a-OH-pterocarpans, as major isoflavonoid induced.[11] The other two genera, *Lupinus* and *Arachis*, located in a different cluster compared to previous genera, accumulated isoflavones (genistein and 2'-OH-genistein derivatives) and stilbenoids (resveratrol and piceatannol derivatives), respectively. Some of the compounds induced were found to be prenylated, but the preferred position of prenylation amongst the subclasses was different. Prenylation at the A-ring was favored for dalbergioidin, 2'-OH-genistein, and glycinol. In contrast, prenylation of demethylcarpin was mainly performed at the B-ring. Prenylation of stilbenoids mainly occurred at the resorcinol moiety that biosynthetically originated from the three units of malonyl Co-A, similar to the A-ring in isoflavonoids (**Figure 4**).

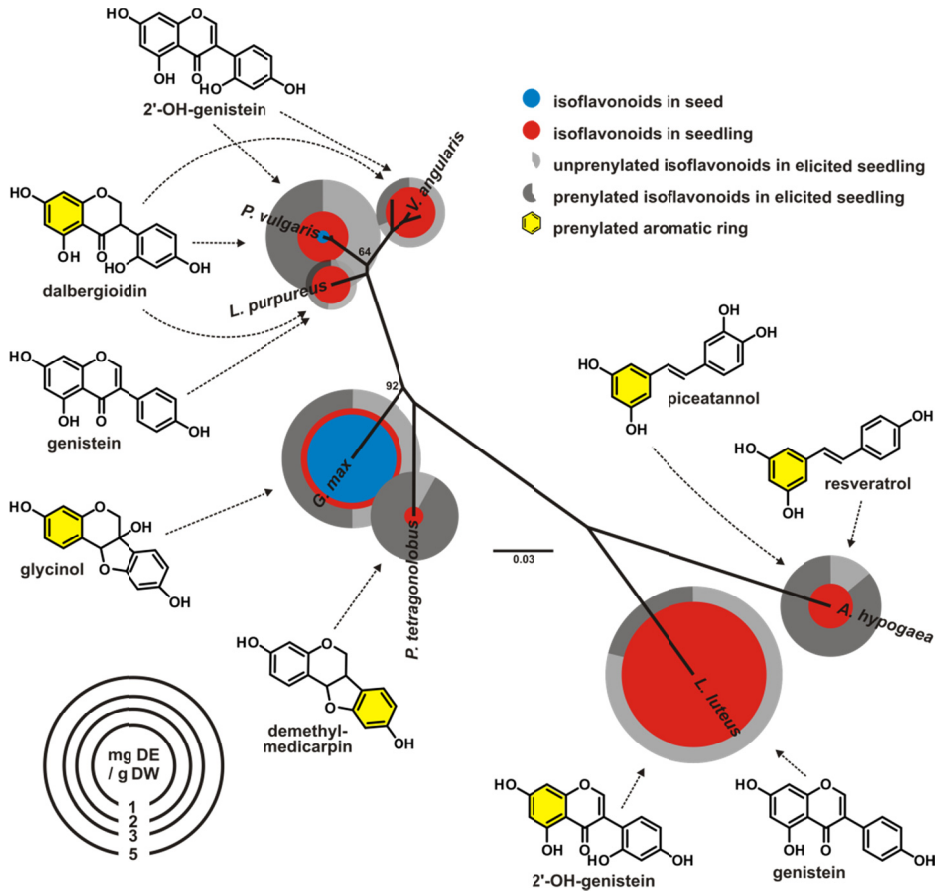


Figure 4. Overview of the inducibility of isoflavonoids/stilbenoids in the species studied during various treatments. The phylogenetic relationship amongst species is described by the un-rooted tree that was constructed using maximum likelihood method. Number at branch point in the tree represents deviating bootstrap support value (%), the other bootstrap support values were 100%. The scale bar (0.03) indicates branch length. The range of total isoflavonoid content of the respective seeds, seedlings and *Rhizopus*-elicited seedlings are indicated by colored circles. The structures indicate the major isoflavonoid/stilbenoid induced, when the sum of all compounds with that skeleton represented more than 30% (w/w) of the total isoflavonoid/stilbenoid content in *Rhizopus*-elicited seedlings. The colored aromatic rings represent the most favored ring for prenylation.

FUTURE PERSPECTIVES

The aim of this study was to optimize the production of prenylated phenolics using an induction process. In this respect, optimal means that high quantity of and large variety (skeleton and decorations) in molecules can be directed by selection of legume species and stress factors. Fungus was the most effective stress factor compared to light and wounding. Moreover, combination of fungus and light was more promising than combination of fungus and wounding. The inducibility of the legume seeds studied during the induction process varied. In terms of total isoflavonoid/stilbenoid quantity, *Glycine max*, *Phaseolus* spp., *Lupinus* spp. and *Arachis hypogaea* were more promising than *Vigna* spp., *Lablab purpureus* and *Psophocarpus tetragonolobus*. In terms of composition of prenylated compounds that offer promising bioactivity, *Glycine max*, *Arachis hypogaea* and *Psophocarpus tetragonolobus* are recommended.

REFERENCES

[1] D. A. Smith, P. J. Kuhn, J. A. Bailey, R. S. Burden, Detoxification of phaseollidin by *Fusarium solani* f.sp. *phaseoli*, *Phytochemistry* **1980**, *19*, 1673-1675.

[2] S. Tahara, E. Misumi, J. Mizutani, J. L. Ingham, Fungal metabolism of the prenylated isoflavone 2,3-dehydrokievitone, *Zeitschrift für Naturforschung C, Tübingen* **1987**, *42*, 1055-1062.

[3] S. Tahara, S. Nakahara, J. Mizutani, J. L. Ingham, Fungal transformation of the antifungal isoflavone luteone, *Agricultural and Biological Chemistry* **1984**, *48*, 1471-1477.

[4] T. P. Denny, H. D. VanEtten, Metabolism of the phytoalexins medicarpin and maackiain by *Fusarium solani*, *Phytochemistry* **1982**, *21*, 1023-1028.

[5] M. S. C. Pedras, P. W. K. Ahiahonu, Metabolism and detoxification of phytoalexins and analogs by phytopathogenic fungi, *Phytochemistry* **2005**, *66*, 391-411.

[6] C. S. Turbek, D. A. Smith, C. L. Schardl, An extracellular enzyme from *Fusarium solani* f. sp. *phaseoli* which catalyses hydration of the isoflavonoid phytoalexin, phaseollidin, *FEMS Microbiology Letters* **1992**, *94*, 187-190.

[7] M. Slana, D. Žigon, T. Makovec, H. Lenasi, The response of filamentous fungus *Rhizopus nigricans* to flavonoids, *Journal of Basic Microbiology* **2011**, *51*, 433-441.

[8] A. Hammerbacher, A. Schmidt, N. Wadke, L. P. Wright, B. Schneider, J. Bohlmann, W. A. Brand, T. M. Fenning, J. Gershenzon, C. Paetz, A common fungal associate of the spruce bark beetle metabolizes the stilbene defenses of Norway spruce, *Plant Physiology* **2013**, *162*, 1324-1336.

[9] S. Tahara, R. K. Ibrahim, Prenylated isoflavonoids: An update, *Phytochemistry* **1995**, *38*, 1073-1094.

[10] R. Simons, J.-P. Vincken, N. Roidos, T. F. H. Bovee, M. Van Iersel, M. A. Verbruggen, H. Gruppen, Increasing soy isoflavonoid content and diversity by simultaneous malting and challenging by a fungus to modulate estrogenicity, *Journal of Agricultural and Food Chemistry* **2011**, *59*, 6748-6758.

[11] S. Aisyah, H. Gruppen, B. Madzora, J.-P. Vincken, Modulation of isoflavonoid composition of *Rhizopus oryzae* elicited soybean (*Glycine max*) seedlings by light and wounding, *Journal of Agricultural and Food Chemistry* **2013**, *61*, 8657-8667.

[12] J. W. Wang, J. Y. Wu, Effective elicitors and process strategies for enhancement of secondary metabolite production in hairy root cultures, *Advances in Biochemical Engineering/Biotechnology* **2013**, *134*, 55-89.

[13] C. S. Harwood, R. E. Parales, The β-ketoadipate pathway and the biology of self-identity, *Annual Reviews in Microbiology* **1996**, *50*, 553-590.

[14] V. S. Sobolev, S. T. Deyrup, J. B. Gloer, New peanut (*Arachis hypogaea*) phytoalexin with prenylated benzenoid and but-2-enolide moieties, *Journal of Agricultural and Food Chemistry* **2006**, *54*, 2111-2115.

[15] Kuswandi, C. F. Roberts, Genetic control of the protocatechuic acid pathway in *Aspergillus nidulans*, *Journal of General Microbiology* **1992**, *138*, 817-823.

[16] K. H. Jones, P. W. Trudgill, D. J. Hopper, Metabolism of p-cresol by the fungus *Aspergillus fumigatus*, *Applied and Environmental Microbiology* **1993**, *59*, 1125-1130.

[17] P. Mazur, W. J. Henzel, S. Mattoo, J. W. Kozarich, 3-Carboxy-cis,cis-muconate lactonizing enzyme from *Neurospora crassa*: An alternate cycloisomerase motif, *J Bacteriol* **1994**, *176*, 1718-28.
[18] O. Yu, B. McGonigle, Metabolic engineering of isoflavone biosynthesis, *Advances in Agronomy* **2005**, *86*, 147-190.
[19] T. L. Graham, M. Y. Graham, Signaling in soybean phenylpropanoid responses: Dissection of primary, secondary, and conditioning effects of light, wounding, and elicitor treatments, *Plant Physiology* **1996**, *110*, 1123-1133.
[20] J. Ebel, Phytoalexin synthesis: The biochemical analysis of the induction process, *Annual Review of Phytopathology* **1986**, *24*, 235-264.
[21] A. Ramakrishna, G. A. Ravishankar, Influence of abiotic stress signals on secondary metabolites in plants, *Plant Signaling and Behavior* **2011**, *6*, 1720-1731.
[22] J. León, E. Rojo, J. J. Sánchez-Serrano, Wound signalling in plants, *Journal of Experimental Botany* **2001**, *52*, 1-9.
[23] S. M. Boué, F. F. Shih, B. Y. Shih, K. W. Daigle, C. H. Carter-Wientjes, T. E. Cleveland, Effect of biotic elicitors on enrichment of antioxidant properties and induced isoflavones in soybean, *Journal of Food Science* **2008**, *73*, H43-H49.
[24] V. S. Sobolev, Production of phytoalexins in peanut (*Arachis hypogaea*) seed elicited by selected microorganisms, *Journal of Agricultural and Food Chemistry* **2013**, *61*, 1850-1858.
[25] C. J. Cooksey, P. J. Garratt, S. E. Richards, R. N. Strange, A dienyl stilbene phytoalexin from *Arachis hypogaea*, *Phytochemistry* **1988**, *27*, 1015-1016.
[26] I. M. Whitehead, P. M. Dey, R. A. Dixon, Differential patterns of phytoalexin accumulation and enzyme induction in wounded and elicitor-treated tissues of *Phaseolus vulgaris*, *Planta* **1982**, *154*, 156-164.
[27] H. J. Kim, H. J. Suh, J. H. Kim, S. C. Kang, S. Park, C. H. Lee, J. S. Kim, Estrogenic activity of glyceollins isolated from soybean elicited with *Aspergillus sojae*, *Journal of Medicinal Food* **2010**, *13*, 382-390.
[28] E. H. Kim, S. H. Kim, J. I. Chung, H. Y. Chi, J. A. Kim, I. M. Chung, Analysis of phenolic compounds and isoflavones in soybean seeds (*Glycine max* (L.) Merill) and sprouts grown under different conditions, *European Food Research and Technology* **2006**, *222*, 201-208.
[29] A. Kirakosyan, P. Kaufman, R. L. Nelson, M. J. Kasperbauer, J. A. Duke, E. Seymour, S. C. Chang, S. Warber, S. Bolling, Isoflavone levels in five soybean (*Glycine max*) genotypes are altered by phytochrome-mediated light treatments, *Journal of Agricultural and Food Chemistry* **2006**, *54*, 54-58.
[30] Y. Jiao, O. S. Lau, X. W. Deng, Light-regulated transcriptional networks in higher plants, *Nature Reviews Genetics* **2007**, *8*, 217-230.
[31] P. M. Rowell, J. A. Bailey, Influence of light and excision of organs on accumulation of phytoalexins in virus-infected hypocotyls of bean (*Phaseolus vulgaris*), *Physiological Plant Pathology* **1982**, *21*, 75-84.
[32] S. Aisyah, J. P. Vincken, S. Andini, M. Bettonvil, H. Gruppen, Variation in accumulation of isoflavonoids between Phaseoleae seedlings elicited by *Rhizopus*, *Submitted* **2015**.
[33] S. Aisyah, H. Gruppen, S. Andini, Z. Mardiah, J.-P. Vincken, Compositional changes in (iso)flavonoids and estrogenic activity of three edible *Lupinus* species by germination and *Rhizopus*-elicitation, *Submitted* **2015**.
[34] S. M. Boué, M. E. Burow, T. E. Wiese, B. Y. Shih, S. Elliott, C. H. Carter-Wientjes, J. A. McLachlan, D. Bhatnagar, Estrogenic and antiestrogenic activities of phytoalexins from red kidney bean (*Phaseolus vulgaris* L.), *Journal of Agricultural and Food Chemistry* **2011**, *59*, 112-120.
[35] S. M. Boué, C. H. Carter, K. C. Ehrlich, T. E. Cleveland, Induction of the soybean phytoalexins coumestrol and glyceollin by *Aspergillus*, *Journal of Agricultural and Food Chemistry* **2000**, *48*, 2167-2172.
[36] H. J. Kim, H. J. Suh, J. H. Kim, S. Park, Y. C. Joo, J. S. Kim, Antioxidant activity of glyceollins derived from soybean elicited with *Aspergillus sojae*, *Journal of Agricultural and Food Chemistry* **2010**, *58*, 11633-11638.
[37] S. Aisyah, H. Gruppen, M. Slager, B. Helmink, J. P. Vincken, Modification of prenylated stilbenoids in peanut seedlings by the same fungi that elicited them: The fungus strikes back, *Submitted* **2015**.
[38] H. D. VanEtten, P. S. Matthews, K. J. Tegtmeier, M. F. Dietert, J. I. Stein, The association of pisatin tolerance and demethylation with virulence on pea in *Nectria haematococca*, *Physiological Plant Pathology* **1980**, *16*, 257-268.
[39] P. A. Abbasi, T. L. Graham, Age-related regulation of induced isoflavonoid responses in soybean lines differing in inherent elicitation competency, *Physiological and Molecular Plant Pathology* **2001**, *59*, 143-152.

[40] A. Marston, K. Hostettmann, Separation and quantification of flavonoids, in *Flavonoids: Chemistry, biochemistry and applications* (Eds.: O. M. Andersen, K. R. Markham), CRC Press Boca Raton, FL, USA, **2006**, pp. 1-36.
[41] J. Oszmiański, J. Kolniak-Ostek, A. Wojdyło, Application of ultra performance liquid chromatography-photodiode detector-quadrupole/time of flight-mass spectrometry (UPLC-PDA-Q/TOF-MS) method for the characterization of phenolic compounds of *Lepidium sativum* L. sprouts, *European Food Research and Technology* **2013**, 1-8.
[42] D. Guajardo-Flores, M. García-Patiño, D. Serna-Guerrero, J. A. Gutiérrez-Uribe, S. O. Serna-Saldívar, Characterization and quantification of saponins and flavonoids in sprouts, seed coats and cotyledons of germinated black beans, *Food Chemistry* **2012**, *134*, 1312-1319.
[43] P. Moesta, M. G. Hahn, H. Grisebach, Development of a radioimmunoassay for the soybean phytoalexin glyceollin I, *Plant Physiology* **1983**, *73*, 233-237.

Summary

The Leguminosae constitute a plant family that has been domesticated by human worldwide for many purposes, including food. In the last decade the consumption of legumes, such as soybeans, has been linked to several health-promoting effects, including reduced risk on various cancers, cardiovascular diseases and risks associated with hormone replacement therapy. The health benefits associated with legume consumption have been linked to the action of secondary metabolites, e.g. isoflavonoids. Nevertheless, the production of bioactive compounds in legumes as such is often inadequate, both from a qualitative and a quantitative point of view. It is known that the production of bioactive compounds in legume seeds can be increased during germination. It can be enhanced even further by performing the germination under stress. The latter process, the so-called induction process, results in accumulation of defense molecules, phytoalexins, many of which contain 5-carbon prenyl substituents. These prenylated molecules in particular appear to have potential as health-promoting compounds. To optimize the production of phytoalexins in legumes, particularly the prenylated ones, it is important to correlate various legume species and stress factors employed in the induction process to compositional changes in the seedlings. In this thesis we aimed to: (i) extrapolate the induction process established previously for soybean to a number of other legume species, with respect to enhancing the content and molecular diversity of prenylated compounds; (ii) investigate whether a change in biotic and abiotic stress factors, in particular light, wounding, time point of application of biotic stress, different kinds of fungus, can enhance the efficiency of the induction process. In this respect, efficiency relates to both quantity of phytoalexins and variety (skeleton and decorations) of the phytoalexins produced.

Chapter 1 provides an overview of the different factors, which are known to affect the accumulation of phytoalexins. A number of economically important legume species is described and the selection of legume species studied in this thesis was motivated. An overview of flavonoid, isoflavonoid and stilbenoid classes is presented in this chapter, mainly focused on their structural classification and biosynthesis. The identification strategies for distinguishing the various (iso)flavonoids and stilbenoids with different types of substitution by mass spectrometry and UV-vis spectrophotometry is summarized.

The effects of wounding and light on (iso)flavonoid content and composition of *Rhizopus*-elicited soybean seedlings is described in **Chapter 2**. The combination of simultaneous germination and induction by *Rhizopus oryzae* increased the total isoflavonoid content of soybean seedlings over two-fold, dominated by pterocarpans (up to 50% (w/w) of total isoflavonoids). The total isoflavonoid content could be increased further by growing

fungus-elicited soybean seedlings in light, whereas wounding combined with *Rhizopus* was less effective. Apart from increasing the total isoflavonoid content, light altered the composition of prenylated pterocarpans of *Rhizopus*-elicited soybean seedlings by mediating the position of prenylation. The level of 2-prenylated pterocarpans increased two-fold, whereas that of 4-prenylated pterocarpans remained similar. Taken together, fungus was the most effective elicitor to alter the isoflavonoid content and composition of soybean seedlings, the impact of which can be further enhanced and mediated by light.

In **Chapter 3**, the changes in (iso)flavonoid content and composition of three edible lupine species during *Rhizopus*-elicitation is described. The total (iso)flavonoid content of lupine increased over 10-fold upon germination, with the total content and composition of isoflavonoids being more affected than those of flavonoids. Elicitation with *Rhizopus oryzae*, in addition to germination, raised the content of isoflavonoids further. Interestingly, elicitation with *Rhizopus* increased the total content of 2'-hydroxygenistein derivatives considerably, without increasing that of genistein derivatives. Nevertheless, the composition of genistein derivatives changed due to deglycosylation and prenylation. A tool to characterize the position of the prenyl group of prenylated isoflavones was developed and applied on extracts of elicited lupine seedlings. This revealed that the preferred position of prenylation of (2'-hydroxy)genistein derivatives differed among the three lupine species. The changes in isoflavone composition increased the agonistic activity of the extracts towards the human estrogen receptors, whereas no antagonistic activity was observed.

Application of *Rhizopus* to induce prenylated (iso)flavonoids was extended to seeds from seven species of the tribe Phaseoleae, i.e. *Phaseolus* (2 species), *Vigna* (3 species), *Lablab* and *Psophocarpus* (**Chapter 4**). Germination alone poorly induced isoflavonoid production in Phaseoleae, whereas application of *Rhizopus* onto the seedlings increased the isoflavonoid content considerably. The inducibility of different subclasses of isoflavonoids in seedlings with *Rhizopus* varied per species. *Phaseolus*, *Vigna* and *Lablab* species accumulated mainly isoflavones and isoflavanones, whereas *Psophocarpus* accumulated mainly pterocarpans. Isoflavones were mainly found as non-prenylated aglycones or glycosides, whereas isoflavanones and pterocarpans were primarily accumulated in their prenylated form. Moreover, for all species, prenylation of the main isoflavonoids predominantly occurred on the A-ring, except for *Psophocarpus* for which B-ring prenylation was predominant. Thus, despite their phylogenetic relatedness, the seeds of various species within the Phaseoleae tribe appeared to respond differently towards elicitation by *Rhizopus* during germination.

In **Chapter 5**, two food-grade fungi, *Rhizopus oryzae* and *Aspergillus oryzae*, were compared for inducing the production of prenylated molecules in peanut (*Arachis hypogaea*) seedlings. Contrary to all other legume species studied, stilbenoids instead of

isoflavonoids were induced in peanut seedlings. The *Aspergillus*- and *Rhizopus*-elicited peanut seedlings accumulated an array of prenylated stilbenoids, with overlap in compounds induced, but also with compounds specific to the fungal treatment. The differences were confirmed to be due to modification of prenylated stilbenoids by the fungus themselves. Each fungus appeared to deploy a different strategy for modification, i.e. glycosylation by *Rhizopus* and oxidative cleavage by *Aspergillus*. Apart from the type of fungus, the time point of inoculation appeared to be an important parameter for optimizing the accumulation of prenylated stilbenoids in peanut seedlings. With respect to production of pharmaceuticals or ingredients for functional foods, conversion of potentially bioactive prenylated stilbenoids in elicited peanut seedlings to modified prenylated stilbenoids has to be considered when fungal elicitation is used.

In **Chapter 6**, key factors that affect the production of phytoalexins as found in this PhD research are discussed, including the modification of phytoalexins by fungus during elicitation. The inducibility of particular isoflavonoid and stilbenoid skeletons, and that of prenylation, was elaborated in this chapter, and correlated with the phylogenetic relationships of Leguminosae. The limitations of the methodology used, i.e. the efficiency of the modified sprouting machine (as opposed to the micromalting system used in previous research), the use of different stress factors, the time point of application of the fungus to seeds or seedlings, and the quantification of phenolic compounds are discussed. Taken together, it is concluded that the seeds of various species appeared to respond differently towards elicitation by *Rhizopus* during germination. The kind of molecules induced followed the phylogenetic relationship of the various species, but their amounts induced during germination, alone or combined with elicitation, did not. In terms of total isoflavonoid/stilbenoid quantity, *Glycine max*, *Phaseolus* spp., *Lupinus* spp. and *Arachis hypogaea* were more promising than *Vigna* spp., *Lablab purpureus* and *Psophocarpus tetragonolobus*. Nevertheless, in terms of composition of prenylated compounds that offer promising bioactivity, *Glycine max*, *Arachis hypogaea* and *Psophocarpus tetragonolobus* are recommended.

Acknowledgment

Many people directly and indirectly contributed in making the accomplishment of this thesis possible. Therefore, I would like to express my sincere gratitude to all of them. First and foremost, I wish to express my utmost gratitude to my supervisors, Harry and Jean-Paul, for the continuous support of my PhD study. Harry, I would like to thank for providing me with an excellent atmosphere for doing research, for your insightful comments, as well as for the hard questions. I do benefit greatly from your immense knowledge in food chemistry. Jean-Paul, I could not have imagined having a better supervisor and mentor for my PhD study. Thank you for all your patience, motivation, encouragement and profound knowledge, as well as for the glasses of tea and a bunch of tissue that you offered me during my hard times. Your guidance helped me in all the time of research and writing of this thesis. I am really indebted to you.

Special thanks are also delivered for Henk, Peter W., Mirjam, Julia, Stefano, Mark, Gerard, Margaret, Edwin, Rene, and Peter, for all the helps and expertise. I sincerely thank Judith and Ingrid from Laboratory of Food Microbiology for hands-on and expertise, and Edouard from Plant Research International for helping me with phylogenetic analysis. My sincere thank also goes to Jolanda for all the helps in administrational issues. Jolanda, you made my life much easier. I want to take this opportunity to thank Marian for the hospitality during my staying in Wageningen, and for Jacqueline from IA&S for taking care of my immigration matters.

My appreciation goes to phytonutrient fellows: Milou, Carla, Anne, Silvia, Renske, Annewieke, Ya, and Wouter. Also for the former phytonutrient buddies: Carlos, Rudy, Tomas, Maxime, Yannick, Wibke, and Nicos. Thank you for all the helps, for the stimulating and fruitfull discussions, and for fun that we had. In particular, I am grateful to Milou for being my paranymph, Carla and Wouter for critically reading my thesis, and Silvia for being my (former) awesome student, my dear colleague, and my paranymph.

I sincerely thank to my dedicated students: Betty, Monique, Alexia, Silvia, Mathijs, Zahara, and Bianca. I learnt so many things from all of you, and do really appreciate all the hard works and dedications during the thesis time. I am also very grateful to my officemates, Christiane and Lingmin, for the nice discussion, for the encouragement, for the tears that we shared, and for fun that we had. I give my sincere appreciation also to Claire for the unlimited supports and to Abhishek for always being there when I need a help.

I would like to thank to all Food Chemistry fellows: Yuxi, Bianca (thanks for taking care of mimie), Roy, Red, Emma, Maaike, Suzanne, Melliana, Hugo, Elisabetta, Ginny, Edita,

Matthias, Frederik, Thibaut, Patricia, Alexandra, Surrender, Uttara, Urmila, Connie, Marijn, Anja, Hans, Martine, Martijn, Stephanie, Stefan, Annemieke, Janine, Koos, Lia, Arjen, Rianne, Eline, Marit, Marlies, Hetty, and Hante. Thanks for all your helps and the fun during my PhD study.

I would like to thank the academic board of my home university, Universitas Pendidikan Indonesia (UPI), for giving me the opportunity to conduct my PhD study, and to Directorate General of Higher Education Ministry of National Education of Indonesia (DIKTI) for financial support. In particular, I would like to sincerely thank to faculty and staff of Department of Chemistry Education, FPMIPA, UPI, for always supporting me to finish my PhD thesis.

I would like to gratitude my Indonesian friends: kang Apri & teh Yayu (thanks for being lovely friends, and for the foods, hours, experiences shared with us), kang Maman & teh Ovi, teh Novi & kang Indra, bu Rina & pa Iis, Nizar's family, mba Atin, mba Nurmi, mba Diah, mba Vitri, mba Yessi, teh Ria, teh Eva, Tika, Zahara & bang Ary, mba Oni, Melliana & bang Audrie, mas Gusti, mas Awang, mas Firdaus, mba Wulan's family, mas Jimmy's family, and all Indonesian friends (I wish I can write down all of your name). I also would like to express my gratitude to Sunda-Netherlands members.

Special thank goes to my beloved husband, Asep Bayu Ekawijaya. Without your encouragement and understanding, it would have been impossible to finish this study, thanks my love!! My special gratitude goes to my beloved parents, Bapa dan Mamah, who always bring my name in each their prayer. I also would like to extent my gratitude to Apa & Mamah, Papah (alm) & Mamah Ai, and to all my brothers and sisters, especially, Wahyu (words cannot express how grateful I am to have you as my brother, thank you…).

Once again, thank you all!!

About the author

Siti Aisyah was born on September 30, 1975 in Bandung, Indonesia. After finishing secondary education, she started her study in Department of Chemistry Education at Universitas Pendidikan Indonesia (UPI) and graduated from her study in 1999. In 2001, she started to work as a lecturer at UPI. She followed her master study in Department of Chemistry at Institut Teknologi Bandung in 2003. Her MSc thesis was on the isolation and purification of oligomer resveratrol from steam bark of *Shorea platyclados* and awarded as the best MSc thesis in 2005 by Lembaga Penelitian Indonesia (LIPI). She conducted her MSc thesis under the supervision of Prof. Dr. Yana Maolana Syah. From 2010 to 2015 she conducted her PhD research on the induction of prenylated isoflavonoids and stilbenoids on legume seeds as described in this thesis. After her defense, she will recommence her work at UPI.

LIST OF PUBLICATIONS

S. Aisyah, H. Gruppen, B. Madzora, J.-P. Vincken, Modulation of isoflavonoid composition of *Rhizopus oryzae* elicited soybean (*Glycine max*) seedlings by light and wounding, *Journal of Agricultural and Food Chemistry* **2013**, *61*, 8657.

S. Aisyah, Y.M. Syah, E. Hakim, L.D. Juliawaty, J. Latip, Two new ketonic resveratrol tetramers from *Shorea platyclados*. *The Natural Products Journal* **2014,** *4*, 299-305.

S. Aisyah, H. Gruppen, S. Andini, M. Bettonvil, E. Severing, J.-P. Vincken, Variation in accumulation of isoflavonoids between Phaseoleae seedlings elicited by *Rhizopus*, *Submitted* **2015**.

S. Aisyah, J.-P. Vincken, S. Andini, Z. Mardiah, H. Gruppen, Compositional changes in (iso)flavonoids and estrogenic activity of three edible *Lupinus* species by germination and *Rhizopus*-elicitation, *Submitted* **2015**.

S. Aisyah, H. Gruppen, M. Slager, B. Helmink, J.-P. Vincken, Modification of prenylated stilbenoids in peanut seedlings by the same fungi that elicited them: The fungus strikes back, *Submitted* **2015**.

OVERVIEW OF COMPLETED TRAININGS ACTIVITIES

Discipline specific activities

Courses

Food and bio refinery enzymology, Wageningen, The Netherlands, 2011
Food fermentation, Wageningen, The Netherlands, 2012
International course chemical discovery & design, Nijmegen, The Netherlands, 2012
Advance food analysis, Wageningen, The Netherlands, 2013
Industrial food protein, Wageningen, The Netherlands, 2013

Conferences

Euro-Mediterranian symposium fruit and vegetables, Avignon, France, 2011
9th International conference on chemistry structure, The Netherlands, 2011
International conference on polyphenol, Florence, Italy, 2012
EPS theme 2 symposium, Utrecht, The Netherlands, 2014
EPS theme 3 symposium, Wageningen, the Netherlands, 2014

General courses

VLAG PhD week, Wageningen, The Netherlands, 2011
Project and time management, Wageningen, The Netherlands, 2011
Competence assessment, Wageningen, The Netherlands, 2011
Scientific writing, Wageningen, The Netherlands, 2012
Information literacy including EndNote introduction, Wageningen, The Netherlands, 2012
Scientific publishing, Wageningen, The Netherlands, 2013
Reviewing a scientific paper, Wageningen, The Netherlands, 2013
Data management course, Wageningen, The Netherlands, 2013
Technique writing & presenting scientific paper, Wageningen, The Netherlands, 2013
Interpersonal communication for PhD students, Wageningen, The Netherlands, 2013
PhD workshop carousel, Wageningen, The Netherlands, 2014

OPTIONALS

Preparing PhD research proposal, Wageningen, The Netherlands, 2010
PhD trip FCH 2010, Italy and Switzerland, 2010
PhD trip FCH 2012, Singapore and Malaysia, 2012
BSc/MSc student presentations, Wageningen, The Netherlands, 2010-2014
PhD presentations, Wageningen, The Netherlands, 2010-2014

Research presented in the Ph.D. dissertation was financially supported by Directorate General of Higher Education Ministry of National Education of Indonesia.

This thesis was printed by Gildeprint drukkerijen, Enschede, The Netherlands.

Cover design: Siti Aisyah and Asep Bayu Ekawijaya.