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

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ABSTRACT: The isoflavonoid profile of soybean was altered in different ways by stimulation of defense response upon germination. The combination of simultaneous germination and induction by *Rhizopus oryzae* increased the total isoflavonoid content of soybeans over 2-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 3-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 2-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.

KEYWORDS: *phytoalexins, pterocarpan, glyceollin, glyceollidin, liquid chromatography, mass spectrometry*

INTRODUCTION

Isoflavonoids are a class of phenolic compounds mainly found in Leguminosae, comprising among 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 antiestrogenic 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 defense response,[6,13−15] the latter of which has the largest potential. Such defense 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 defense 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.[14,5] 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; Easy-Green, San Diego, CA, USA), which could accommodate 300 g of 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,

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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 homogeneously 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 day), germination (2 days), and elicitation (7 days) stages. Prior to the soaking step, soybeans were surface-sterilized by immersing them in a 1% (w/v) hypochlorite solution (5 L/kg of beans) for 1 h at room temperature and subsequently rinsed 4 times with Milli-Q water (3 L/kg of 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 days at 25 °C and 100% RH. Next, a spore suspension (0.2 mL/g of beans) was added to the soybeans, and the soybeans were incubated for 7 days 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, U.K.). The sporangia were scraped off from the agar plate and suspended in 0.85% (w/v) NaCl solutions (7×10^7 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 W 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/day (Table 1). All the experiments were performed in triplicate. All treated soybeans were collected after 10 days of treatment and directly stored at −20 °C.

Table 1. Summary of Different Soybean Treatments

	stage			
treatments	soaking (1 day)	wounding	germination (2 days)	elicitation (7 days)
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 of solvent and with a flow of nitrogen for 300 s. The extract was collected in a 150 mL glass vial. The extract was evaporated under reduced pressure. The dried extracts were resolubilized 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 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. Sample (1 μL) was injected onto an Acquity UPLC BEH shield RP18 column (2.1 mm i.d. × 150 mm, 1.7 μm particle size; Waters, Milford, MA, USA) with an Acquity UPLC BEH shield RP18 VanGuard precolumn (2.1 mm i.d. × 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% to 25% (v/v) B; 2−9 min, linear gradient from 25% to 50% (v/v) B; 9−12 min, isocratic on 50% B; 12−22 min, linear gradient from 50% to 100% (v/v) B; 22−24 min, isocratic on 100% B; 24−25 min, linear gradient from 100% to 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 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 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.

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 glyceollidin 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]

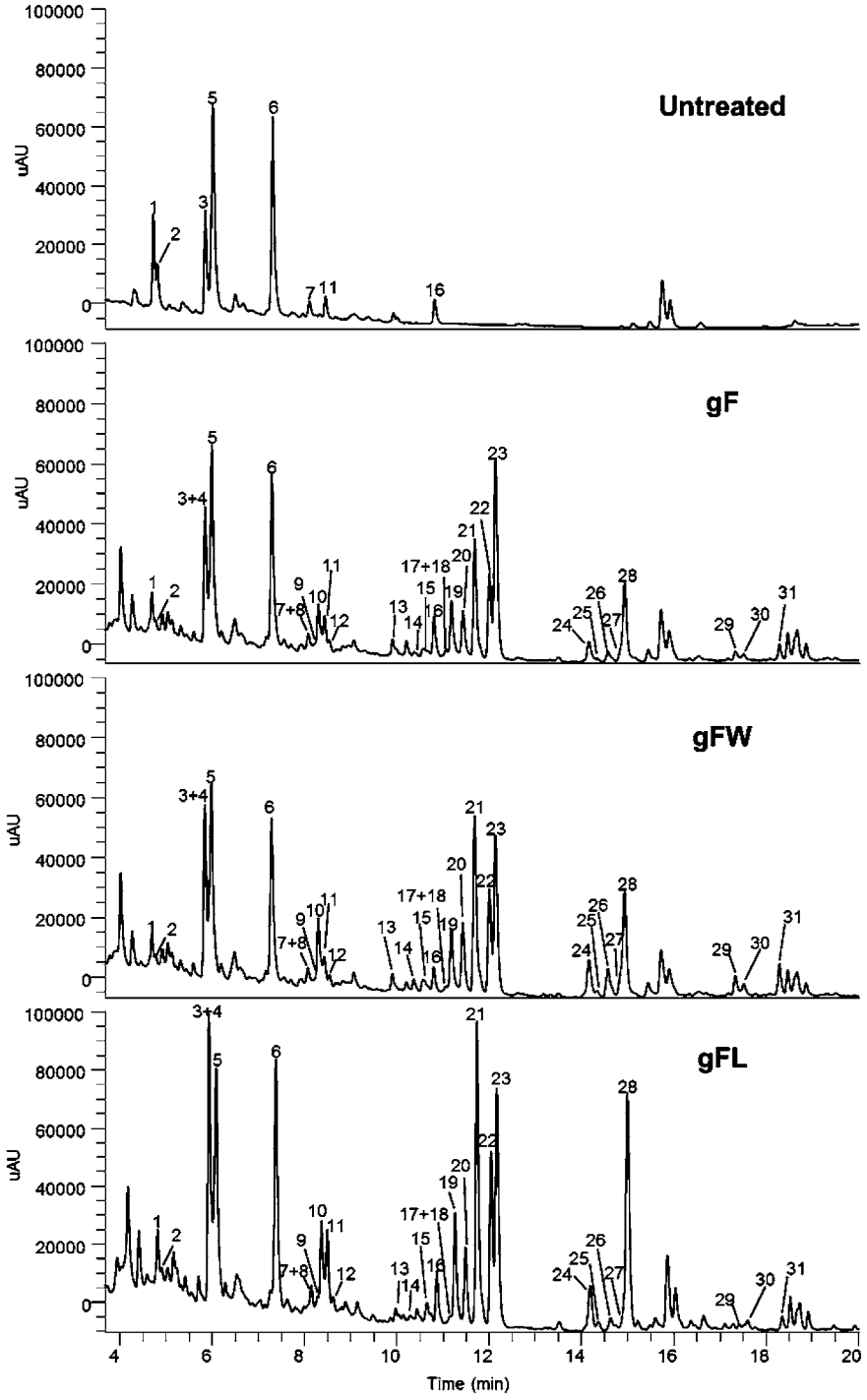

Figure 1. RP-UHPLC−UV profile of 70% aqueous EtOH extracts of untreated and fungus-elicited soybean. Codes (Untreated, gF, gFW, gFL) refer to the treatment in Table 1, and peak numbers refer to compounds in Table 2.

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 aglycon **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

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Table 2. Compounds Tentatively Identified by RP-UHPLC-PDA-MS in Elicited Soybean Extracts

peak no.	t_R (min)	compounds[a]	UV max (nm)	$[M - H]^-$	MS^2 NI product ion[b] (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[c]	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[c]	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[c]	267 (100)
19	11.19	glyceollidin II	284	339	324 (44), 161 (100)	323[c]	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[c]	306 (73), 279 (100), 251 (64)
22	12.01	glyceollin II	283	337	319 (100), 149 (40)	321[c]	306 (55), 279 (100), 251 (53)
23	12.15	glyceollin I	283	337	319 (100), 149 (86)	321[c]	306 (80), 303 (100), 293 (37)
24	14.17	glyceollin VI	278, 317	335	317 (100), 149 (39)	319[c]	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[c]	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]Standard three letter code for Glc (glucoside) is used. [b]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. [c]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.

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 aglycon 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 from genistin, which eluted earlier.

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 nonprenylated precursor of all prenylated pterocarpans in soybean. The prenyl group can be attached to the 4-position (glyceollidin I and glyceollin I and VI) or the 2-position (glyceollidin II, glyceollin II, III, and 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

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Table 3. MS^2 Product Ions Obtained from the $[M - H]^-$ Precursor Ion of Pterocarpans

	nonprenylated	4-prenylated pterocarpans		2-prenylated pterocarpans					
product ion	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).

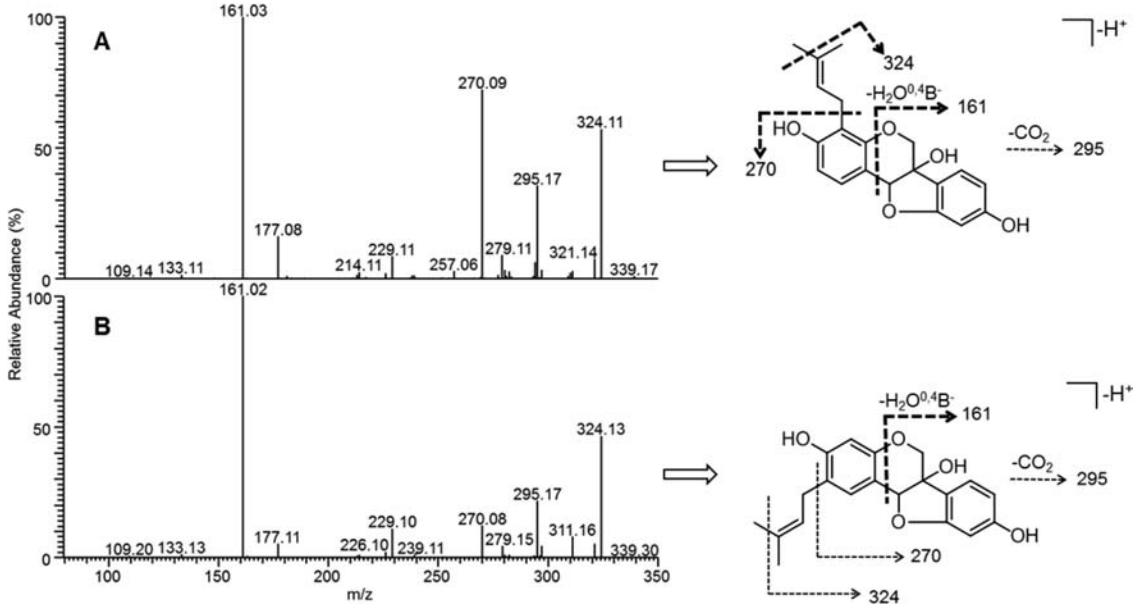

Figure 2. MS^2 spectra of *m/z* 339 in NI mode and proposed cleavage of glyceollidin I eluted at t_R 11.08 (A) and of glyceollidin II eluted at t_R 11.19 (B). Bold dashed arrows indicate cleavage yielding product ions with relative abundance over 50%.

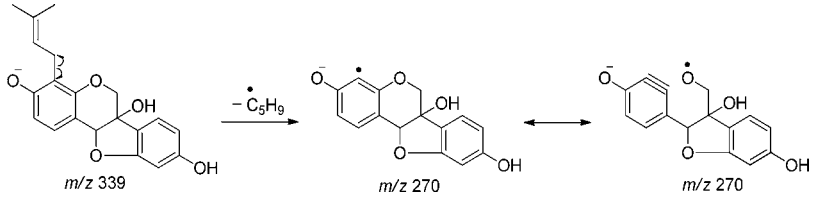

Figure 3. Proposed route for the formation of radical fragment ion *m/z* 270 of glyceollidin I, which is resonance-stabilized.

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 glyceollidin isomers. The main fragment ions of *m/z* 339

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Table 4. Quantitative Analysis of Isoflavonoids in Extracts from Untreated and Variously Treated Soybeans

		mg DE/g DW[a]						
peak no.	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]Data are the means ± SD of experiments performed in triplicate. Values within the same row with different letters show significant differences (Tukey's test, $P < 0.05$).

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 $[M - H]^-$ 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 glyceollidin 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, and 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 among 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-

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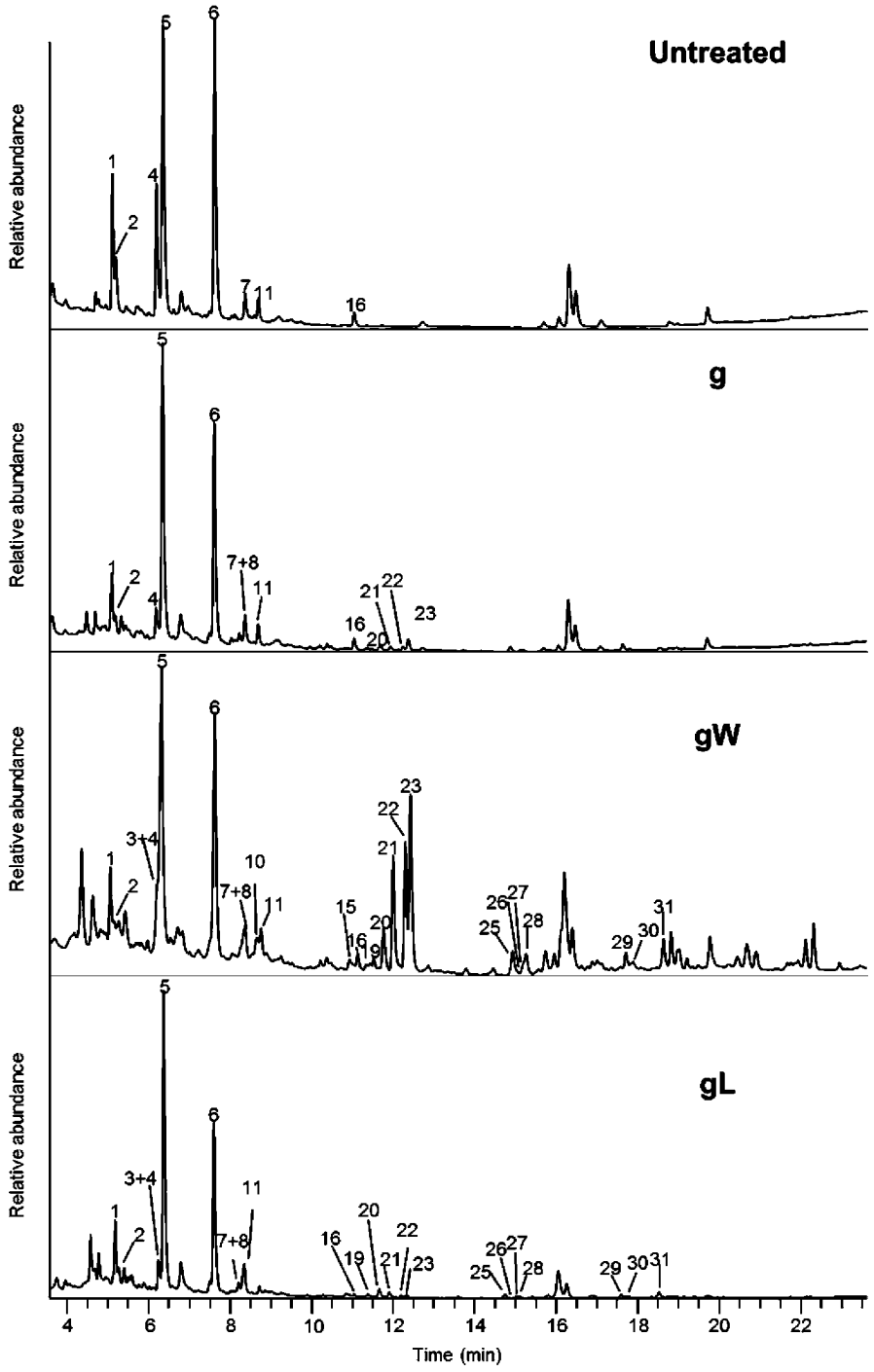

Figure 4. RP-UHPLC−UV profile of 70% aqueous EtOH extracts of untreated and germinated soybean in the absence of fungus. Codes (Untreated, g, gW, gL) refer to the treatment in Table 1, and peak numbers refer to compounds in Table 2.

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.

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. Among 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).

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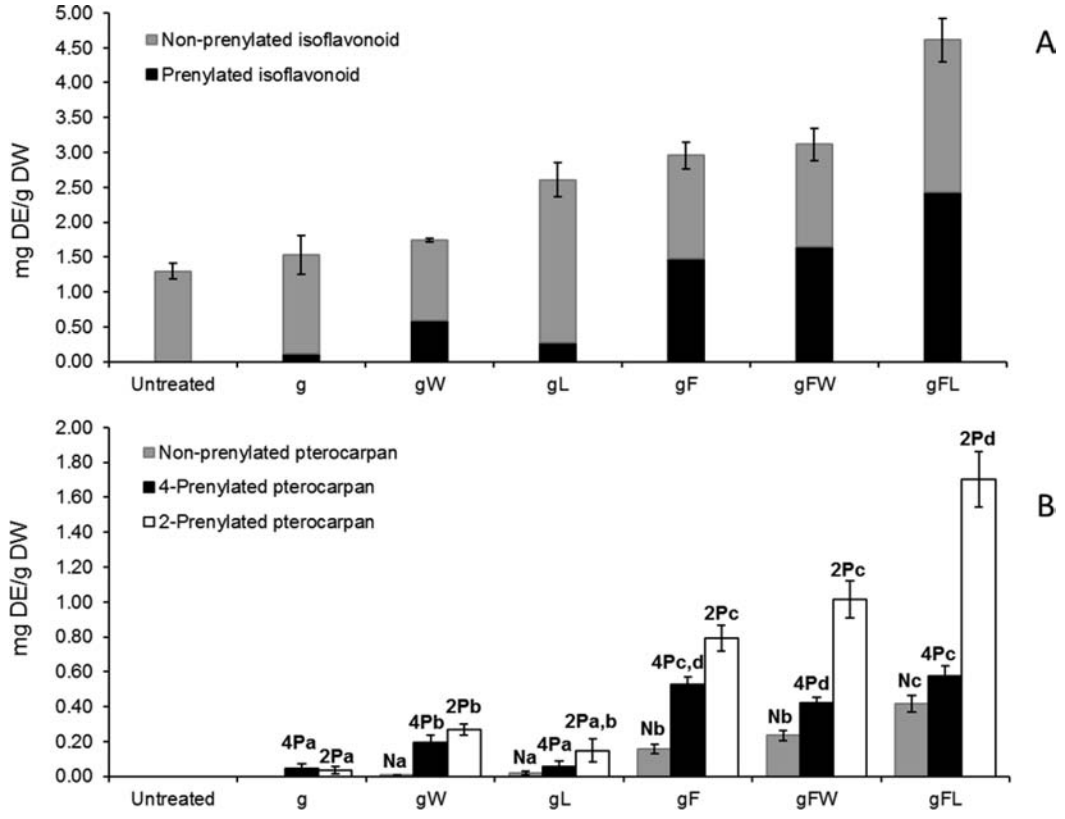

Figure 5. (A) Total nonprenylated and prenylated isoflavonoid content of untreated and treated soybean. (B) Total nonprenylated, 4-prenylated, and 2-prenylated pterocarpan content of untreated and treated soybean. Codes (Untreated, g, gW, gL, gF, gFW, and gFL) refer to the treatment in Table 1. All contents are expressed in mg daidzein equivalents (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, nonprenylated; 4P, 4-prenylated; 2P, 2-prenylated)).

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 2-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 characterized 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

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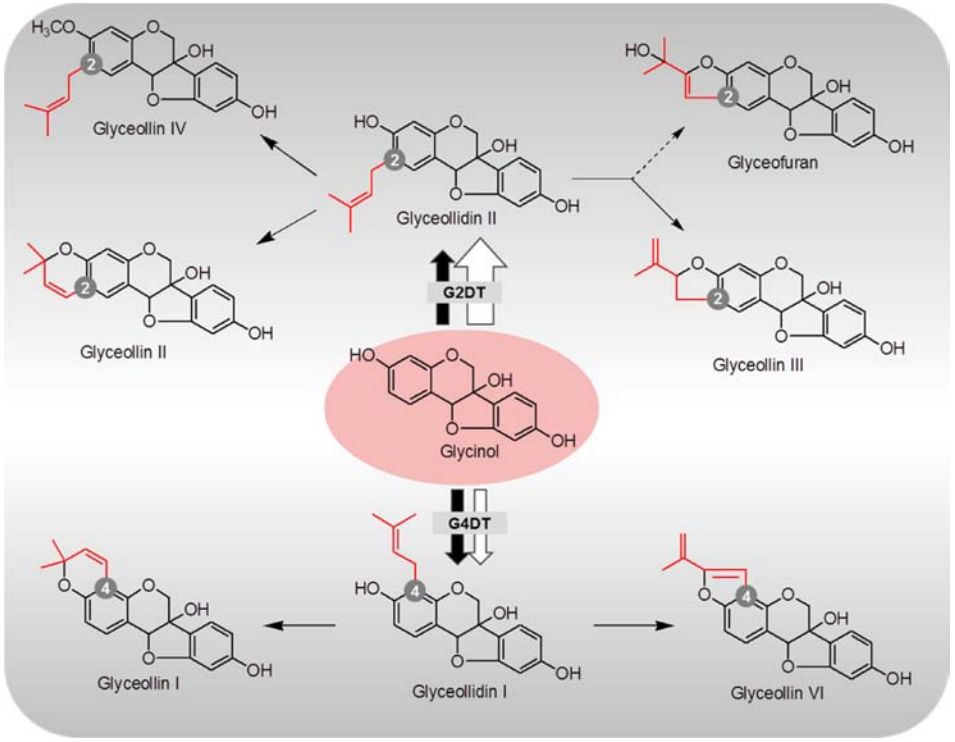

Figure 6. Schematic illustration summarizing the influence of light on the position of prenylation of glycinol in fungus-elicited soybeans. The block arrows with black solid fill and white solid fill represent the flux of prenylated pterocarpan production obtained in dark and light, respectively. The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

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 characterized 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.

DISCUSSION

Six different treatments with soybean seedlings were performed to investigate whether the accumulation of phytoalexins can be directed toward larger amounts of these molecules and toward 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).

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

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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*.[24] 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 antiestrogenic 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 toward a higher proportion of 2-prenylated pterocarpans.

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