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Enhanced biosynthesis of the natural antimicrobial glyceollins in soybean seedlings by priming and elicitation

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Highlights

- Priming prior to *Rhizopus*-elicitation enhances the production of glyceollins.
- Priming with reactive oxygen species was superior to wounding in glyceollin production.
- Reactive oxygen species effectively primed glyceollin production in two cultivars.
- Priming prior to *Rhizopus*-elicitation was specific in inducing glyceollins.

Abstract

Glyceollins are a class of antimicrobial prenylated pterocarpans produced in soybean seedlings upon fungus elicitation. Priming with reactive oxygen species (ROS) prior to elicitation with *Rhizopus oligosporus/oryzae* (R) was investigated for its potential to enhance glyceollin production. ROS-priming prior to R-elicitation (ROS+R) increased glyceollin production (8.6±0.9 μmol/g dry weight (DW)) more than 4-fold compared to elicitation without priming (1.9±0.4 μmol/g DW). Furthermore, ROS-priming was superior to two physical primers which were used as benchmark primers, namely slicing (5.0±0.6 μmol glyceollins/g DW) and sonication (4.8±1.0 μmol glyceollins/g DW). Subsequently, the robustness of ROS+R was assessed by applying it to another soybean cultivar, where it also resulted in a significantly higher glyceollin content than R-elicitation without priming. ROS-priming prior to elicitation provides opportunities for improving the yield in large-scale production of natural antimicrobials due to the ease of application and the robustness of the effect across cultivars.

Keywords

Reactive oxygen species; priming; elicitation; fungus; glyceollins; soybeans;

1. Introduction

Leguminosae is one of the largest plant families and many of its species are known to be excellent sources of potent antimicrobial compounds. Soybeans (*Glycine max*) are able to produce isoflavonoids. A family of C5-isoprenoid (prenyl)-substituted isoflavonoids with a pterocarpan backbone, collectively known as glyceollins, is accumulated in stressed soybeans (Aisyah, Gruppen, Madzora, & Vincken, 2013; Simons, Vincken, Bohin, Kuijpers, Verbruggen, & Gruppen, 2011; Van De Schans, Vincken, De Waard, Hamers, Bovee, & Gruppen, 2016). The production of antimicrobial glyceollins is one of soybeans' main defence responses to counteract stress. Glyceollins have been well documented to exert bioactivities, including antibacterial and antifungal properties (Araya-Cloutier et al., 2018; Lee et al., 2010; Nwachukwu, Luciano, & Udenigwe, 2013). Due to their antimicrobial properties, glyceollins can serve as promising candidates for natural food preservation.

Glyceollins exist predominantly as a mixture of three isomers: the pyran-ring prenylated isomers, glyceollins I and II, and the furan-ring prenylated isomer, glyceollin III. Glyceollin I is a *C*4-prenylated pterocarpan, whereas glyceollins II and III are *C*2-prenylated pterocarpans. Glyceollins derive from daidzein, the simplest, non-prenylated isoflavone which is further transformed into the pterocarpan, glycinol (**Fig. 1A**). In some soybean cultivars, glyceollin induction is accompanied by a minor induction of prenylated isoflavones, which are also acknowledged for their high antimicrobial potency (Araya-Cloutier et al., 2018; Simons et al., 2011).

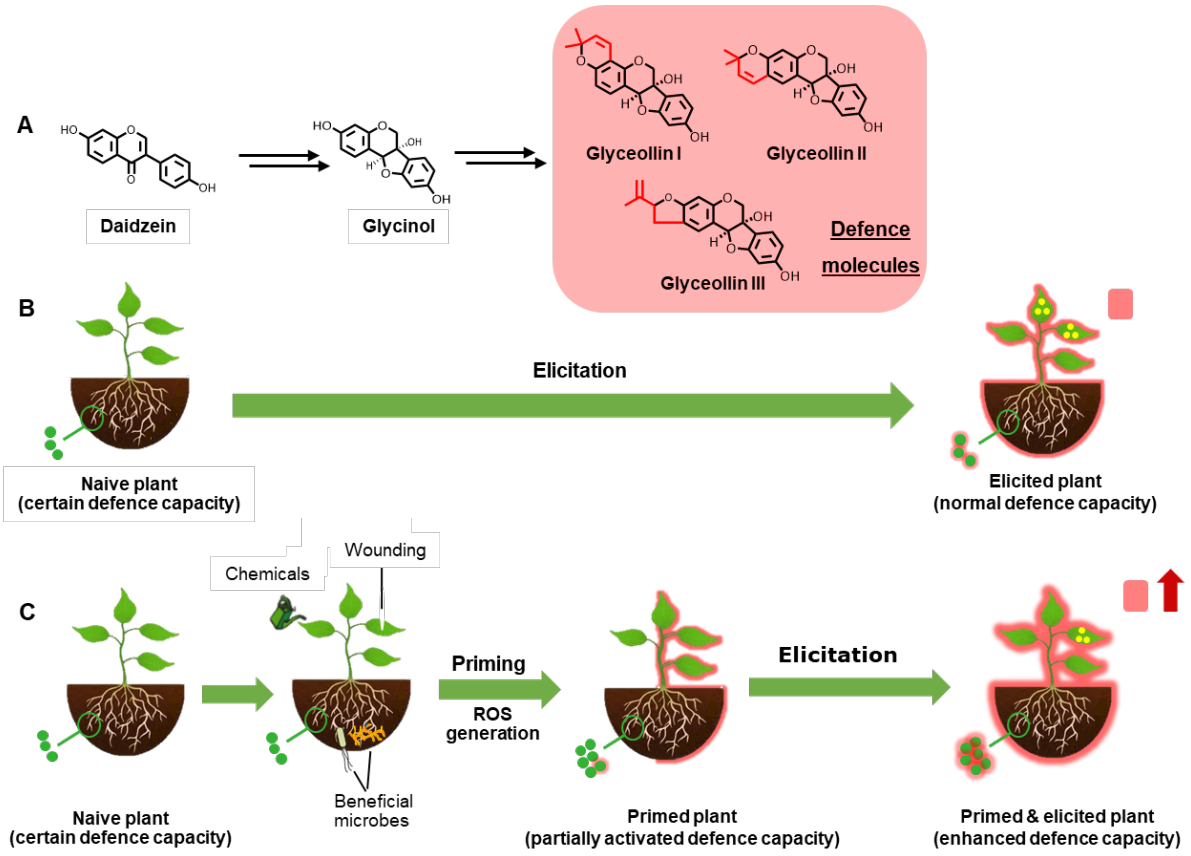


Fig. 1. Defensive prenylated isoflavonoids produced in soybeans to counteract stress. These defensive molecules in soybeans derive from the isoflavone, daidzein and the pterocarpan, glycinol (Simons et al., 2011) **(A).** Effect of direct elicitation on plant's defence responses (in the form of antimicrobial compound production) and overall fitness of the plant **(B).** Effect of plant-priming prior to elicitaion on plant's defence responses (in the form of antimicrobial compound production) and overall fitness of the plant **(C)**. Priming can be induced by chemicals, by physical damage, or by beneficial microorganisms. The intensity of the red glow surrounding the plant refers to the extent to which its defence mechanism is activated. The red boxes represent the production of antimicrobials. Green dots represent inactive signal amplifiers that become activated (green dots with red glow) upon elicitation, while yellow dots show disease symptoms of the plant. Adapted from Conrath and co-workers (2009).

It has been widely reported that plants transiently sensitized prior to fungus infection (i.e. elicitation, **Fig. 1B**), demonstrate enhanced expression of their defence responses, including the production of antimicrobial compounds. This sensitization process, known as priming, enables the plant to mount a faster and/or stronger activation of defence responses to a subsequent

elicitation (Graham & Graham, 1999). In this way, plants minimize their energy expenditure to respond to elicitation and are able to induce stronger responses upon subsequent stress (van Hulten, Pelser, Van Loon, Pieterse, & Ton, 2006). It is hypothesized that inactive signal amplifiers are accumulated in the plant during priming, which are activated upon subsequent elicitation (**Fig. 1C**) (Beckers et al., 2009; Conrath et al., 2006).

Priming can be achieved by external application of chemicals, colonization of the roots by beneficial microbes, or by physical damage to the plant (e.g. wounding) (Martinez-Medina et al., 2016). Chemical priming usually involves the external application of endogenous signalling molecules (Savvides, Ali, Tester, & Fotopoulos, 2016). Reactive oxygen species (ROS), for example, serve a multifaceted role in plants' defence mechanism. They are produced upon successful microbial infection (Degousee, Triantaphylidès, & Montillet, 1994) as they are rapidly and transiently produced in an "oxidative burst". In this way, they act as intracellular signalling molecules that regulate defence gene activation. ROS is also the main regulator of whether the infected plant will enter the cell death phase due to a prolonged oxidative phase, or whether it will enter the "primed" state upon adequate redox scavenging (Conrath, 2009).

The main distinctive characteristics of the processes of priming and elicitation are summarized in **Table 1**. Priming always takes place prior to elicitation, as it activates dormant defence mechanisms in anticipation of a major stress response, which is then triggered by elicitation (Mauch-Mani, Baccelli, Luna, & Flors, 2017). The intensity by which the two processes stimulate the defence mechanisms is another major difference. The short-term and transient nature of priming results in weak and local activation of the signal transduction pathway, whereas the more prolonged nature of elicitation leads to strong and more systemic activation of defence signal cascades. Furthermore, it has been shown that primed and elicited plants demonstrated lower fitness costs, i.e. increased growth rates, (van Hulten et al., 2006), leading

to increased secondary metabolism compared to directly elicited plants. The main similarity of the two concepts is that agents that are used for priming can be used for elicitation and vice and versa. One molecule that acts as a direct elicitor in one species, can act as a primer in others (Conrath et al., 2006).

Table 1. Main distinctive characteristics of priming and elicitation in plants (Baenas, Villaño, García-Viguera, & Moreno, 2016; Conceiçao, Ferreres, Tavares, & Dias, 2006; Conrath et al., 2006; Conrath, Beckers, Langenbach, & Jaskiewicz, 2015; van Hulten et al., 2006).

Process	**Duration of event**	**Way of application**	**Activation of defence responses (incl. production of antimicrobials)**	**Plant's fitness costs**
Priming	Short (h)	Imbibition	+	Low
Elicitation	Long (d)	Spraying	++	High
Priming + elicitation	Short (h), Long (d)	Imbibition + Spraying	+++	Moderate

The multifaceted, central role of ROS in signal transduction pathway prompted us to use ROS as a primer of soybean seedlings prior to *Rhizopus*-elicitation. The priming effect of ROS is compared with that of wounding, which is considered a traditional primer (Galis, Gaquerel, Pandey, & Baldwin, 2009). We hypothesize that priming prior to elicitation enhances the diversity and the quantities of glyceollins produced by soybean seedlings. Additionally, we expect that ROS-priming is more robust and less laborious than wounding. The robustness of ROS-priming prior to *Rhizopus*-elicitation was examined across two soybean cultivars. To our knowledge, this is the first report on the use of reactive oxygen species prior to fungus elicitation to enhance the biosynthesis of potent natural antimicrobials, glyceollins.

2. Materials and Methods

2.1 Materials

Soybeans from Cultivar I (CvI) were a mixture of different soybean lines of unknown origin, kindly provided by FRANK Food Products (Twello, The Netherlands). Soybeans from Cultivar II (CvII), *Glycine max,* were purchased from Vreeken's Zaden (Dordrecht, the Netherlands). Tempeh starter culture (a mixture of *Rhizopus oligosporus* and *Rhizopus oryzae*) was purchased from TopCultures (Zoersel, Belgium). H_2O_2 (30 % (*w/w*) and standards of daidzein (≥ 98 %) and genistein (≥ 98 %) were purchased from Sigma Aldrich Chemie B.V. (Zwijndrecht, The Netherlands). ULC-MS grade acetonitrile (ACN) with 0.1 % (*v/v*) formic acid (FA), water with 0.1 % (*v/v*) FA, methanol (MeOH) were purchased from Biosolve BV (Valkenswaard, The Netherlands). Iron (II) sulfate heptahydrate, sodium hypochlorite 14% (v/v) n-Hexane and ethanol 96% (v/v) were purchased from VWR International B.V. (Amsterdam, The Netherlands) and NaCl was purchased from Fisher Scientific (Landsmeer, The Netherlands). Peptone physiological salt solution (PPS) was purchased from Tritium Microbiologie (Eindhoven, The Netherlands).

2.2 Germination, priming, and elicitation

Soybeans were treated in a modified sprouting machine (MikroFarm^{TM} EQMM; Easy-Green, San Diego, CA, USA). The machine was modified to provide appropriate experimental conditions for both the soybeans and the fungus. The temperature was maintained at 30 ˚C by a heating mat with a thermostat and by a styrofoam box covering and insulating the machine. Humid air was generated by a fog generator (minifogger; Conrad, Hirschau, Germany) placed in the equipment's water compartment. Fog was generated every 3 h, with a duration of 15 min. In addition, a fan attached to the machine homogeneously distributed the fog for 4 s per 20 s.

In all treatments, seeds were subjected to three phases: soaking (1 day), germination (2 days i.e. "early", unless stated otherwise), elicitation (1-5 days) **(Fig. 2)**. Prior to the soaking step, the seeds (50 g) were surface sanitized with 4 % (*w/v*) NaOCl for 5 min at room temperature and then thoroughly rinsed with demineralized water. The surface-sterilized seeds were soaked for 24 h at 25 ˚C in demineralized water. The soaked seeds were placed in sterilized cartridges, which were then placed in the sprouting machine. Before this, the machine was sterilized according to the cleaning protocol provided by the manufacturer. After soaking, seeds were drained and germinated for 2 days at 30 ˚C and 100 % RH.

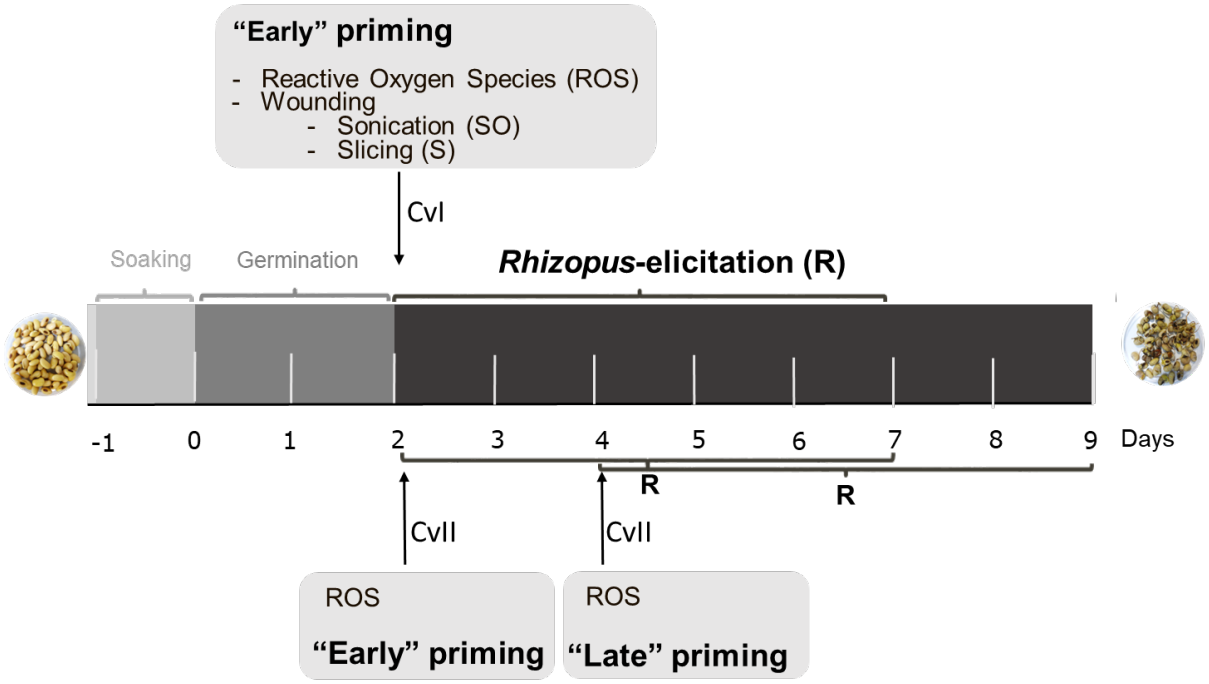


Fig. 2. Timeline of priming and elicitation experiments in soybean seedlings of two cultivars; Cultivar I (CvI) and Cultivar II (CvII).

In "Early" priming experiments, priming and elicitation treatments were sequentially applied on 2-day old germinated seeds (seedlings) **(Fig. 2).** In "Late" priming experiments, the treatments were performed on the 4-day old germinated seedlings. "Late" priming experiments were performed only in CvII. For wounding-based priming, two wounding methods were used:

slicing (S) and sonication (SO). Slicing was performed by cutting each cotyledon individually with a sterilized knife. First, the opposite side of the hilum was longitudinally cut to separate the cotyledon. Next, the newly-exposed cotyledon surface was cut in two parallel slices to form approximately 2-mm thick slices (Aisyah et al., 2013). For SO-priming, seeds were immersed in demineralized water and were sonicated for 10 seconds. Wounding-based priming was performed only on Cultivar I (CvI).

For ROS-priming of the seedlings, seedlings were immersed in 1 mM $FeSO_4$ solution (10 mL/g dry seed) for 30 min and continuously swirled. Then, the metal solution was drained and the seedlings were immersed in 1 M H_2O_2 solution (10 mL/g dry seed) for another 30 min and continuously swirled to generate ROS (Degousee et al., 1994)according to **Eq. 1**. Subsequently, the H_2O_2 solution was drained and the seedlings were thoroughly rinsed with water.

$$Fe\ (II) + H_2O_2 \rightarrow Fe\ (III) + HO^- + HO^{\cdot} \quad \textbf{(Eq.1)}$$

Wounded or ROS-treated seedlings were then immediately inoculated with a suspension of fungal sporangia (*Rhizopus*-elicitation) of approximately 10^7 CFU/ml in PPS (0.4 mL/g dry seed). All treatments were performed in the dark and in three independent replicates.

2.3 Soybean seedling extraction

Seedlings were freeze-dried and subsequently milled in a bead mill (Cryomill MM440; Retsch GmbH, Haan, Germany) into fine powder with large beads of 20 mm at a frequency of 30 s^{-1}, for 30 s. Seedling extraction was performed using a speed extractor (E-916; Büchi, Flawil, Switzerland). Approximately 300 mg of the powder was mixed with fat-free quartz sand (0.3-0.9 mm, Büchi) and filled into the extraction cells. Samples were defatted in a two-cycle programme (55 min, 40 °C, 100 atm) with 25.5 mL 96 % (*v/v*) *n*-hexane per cycle, and (iso)flavonoids were extracted in a three-cycle programme (75 min, 40 °C, 100 atm) with 25.5

mL 96 % (*v/v*) EtOH per cycle. The ethanol extract was evaporated under reduced pressure in a Syncore evaporator at 40 °C. The dried extract was re-dissolved to a concentration of5 mg/mL in MeOH and stored at −20 °C. All samples were centrifuged (15,000*g*, 5 min; room temperature) prior to analysis. The hexane extract was found to be isoflavonoid-free and was not considered further.

2.4 Compositional analysis by RP-UHPLC-PDA-ESI-MS

Samples were analysed by RP-UHPLC-MS on an Accela UHPLC system (Thermo Scientific, San Jose, CA, USA) equipped with a pump, autosampler, PDA detector, and ESI-ion trap-MS. Injection volume was 1 µL and the extract was separated on an Acquity UPLC BEH C18 column (2.1 mm i.d. × 150 mm, 1.7 µm particle size; Waters, Milford, MA, USA) with a VanGuard (5mm × 2.1mm i.d., 1.7 µm) guard column of the same material (Waters, Milford, MA). Water acidified with 0.1 % (*v/v*) formic acid and 1 % (*v/v*) ACN, eluent A, and ACN acidified with 0.1 % (*v/v*) formic acid, eluent B, were used as solvents at a flow rate of 300 µL/min. The following elution gradient was used: 0-1 min, isocratic at 9% B; 1-3 min, linear gradient from 9-25% B; 3-10 min, linear gradient from 25-50% B; 10-13 min, isocratic at 50% B; 13-23 min, linear gradient from 50-100% B; 23-28 min, isocratic at 100% B; 28-29 min, linear gradient from 100-9% B; 29-34 min, isocratic at 9% B. Column oven and autosampler temperatures were set at 45 °C and 15 °C, respectively. The PDA detector was set to measure from 200-600 nm.

Mass spectrometric analysis was performed on a Velos Pro (Thermo Scientific) equipped with a heated ESI-MS probe coupled to *in-line* to the RP-UHPLC system. Nitrogen was used as a sheath and an auxiliary gas. Spectra were acquired over an *m/z* range of 150 – 1,500 Da in both positive ionization (PI) and negative ionization (NI) modes. Data-dependent MS^2 analysis was performed on the most intense ion by collision-induced dissociation (CID) with normalized

collision energy of 40%. For both modes, the ion transfer tube (ITT) temperature was 350 ˚C. The S-lens RF level 55 and the source voltage was 3.5 kV for NI and 4.0 kV for PI mode. A dynamic mass exclusion approach was used, in which a compound detected 3 times as most intense was subsequently excluded for the following 5 s, allowing data-dependent MS^2 of less intense co-eluting compounds. The system was tuned with genistein in both PI and NI mode via automatic tuning using Tune Plus (Xcalibur 2.1, Thermo Scientific).

2.5 Tentative annotation and quantification of phytochemicals

Isoflavonoids were tentatively annotated based on UV_{max} and MS spectral data, obtained by Xcalibur (v.2.2, Thermo Scientific). The identities of the peaks were previously determined in our laboratory (Aisyah et al., 2013; Araya-Cloutier, den Besten, Aisyah, Gruppen, & Vincken, 2017; Van De Schans et al., 2016). In brief, full MS and MS^2 scans provided information regarding the molecular weight of the isoflavonoids and substitutions of the phenolic skeleton by means of characteristic neutral losses. The configuration of the prenyl group (chain or ring-closed) attached to the skeleton was determined by typical neutral losses in MS^2 positive mode: a neutral loss of 56 Da (C_4H_8) was used to distinguish a prenyl chain, whereas a ring-closed prenyl typically showed neutral losses of 42 Da (C_3H_6), 56 Da (C_4H_6), 70 Da ($C_3H_6 + H_2O$) and 15 Da (CH_3).

The quantification of (iso)flavonoids was based on the ultraviolet (UV) absorbance at 280 nm. A standardized six-point (1-100 µg/mL) calibration curve based on an external standard of daidzein ($R^2 = 0.995$) was used for the quantification of (iso)flavonoids. Compounds were first converted to mg daidzein equivalents per g of dry weight of the seedling (mg DE /g dry weight (DW)). Then, the quantities of each compound were corrected for the differences in molar extinction coefficients between the standards and the compounds of interest, using Lambert-Beer's law (**Eq.2**), see **Table S1** (**supplementary information**) for an overview of the molar

extinction coefficients used (Araya-Cloutier et al., 2017; Murphy, Barua, & Hauck, 2002; Van De Schans et al., 2016).

$$\varepsilon_A C_A = \varepsilon_B C_B \quad \textbf{(Eq.2)}$$

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

2.6 Statistical analysis

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

3. Results and Discussion

3.1 Chromatographic profile of germinated and treated soybean seedlings

The composition of the extracts of germinated (control), *Rhizopus*-elicited (R), ROS-primed (ROS) and ROS-primed before *Rhizopus*-elicited (ROS+R) soybeans of Cultivar I (CvI) is shown in **Fig. 3**. UHPLC-PDA-MS analysis of the extracts from germinated and treated soybeans showed that (iso)flavonoid profiles were modulated upon priming and/or elicitation, as expected (Aisyah et al., 2013; Namdeo, 2007). The diversity of compounds increased when germinated seedlings were *Rhizopus*-elicited or primed with ROS. Peaks **2** and **10-12** (**Fig. 3**) were mainly induced upon the treatments. The diversity of the compounds increased further when seedlings were treated with ROS+R (peaks **6, 7** and **9**).

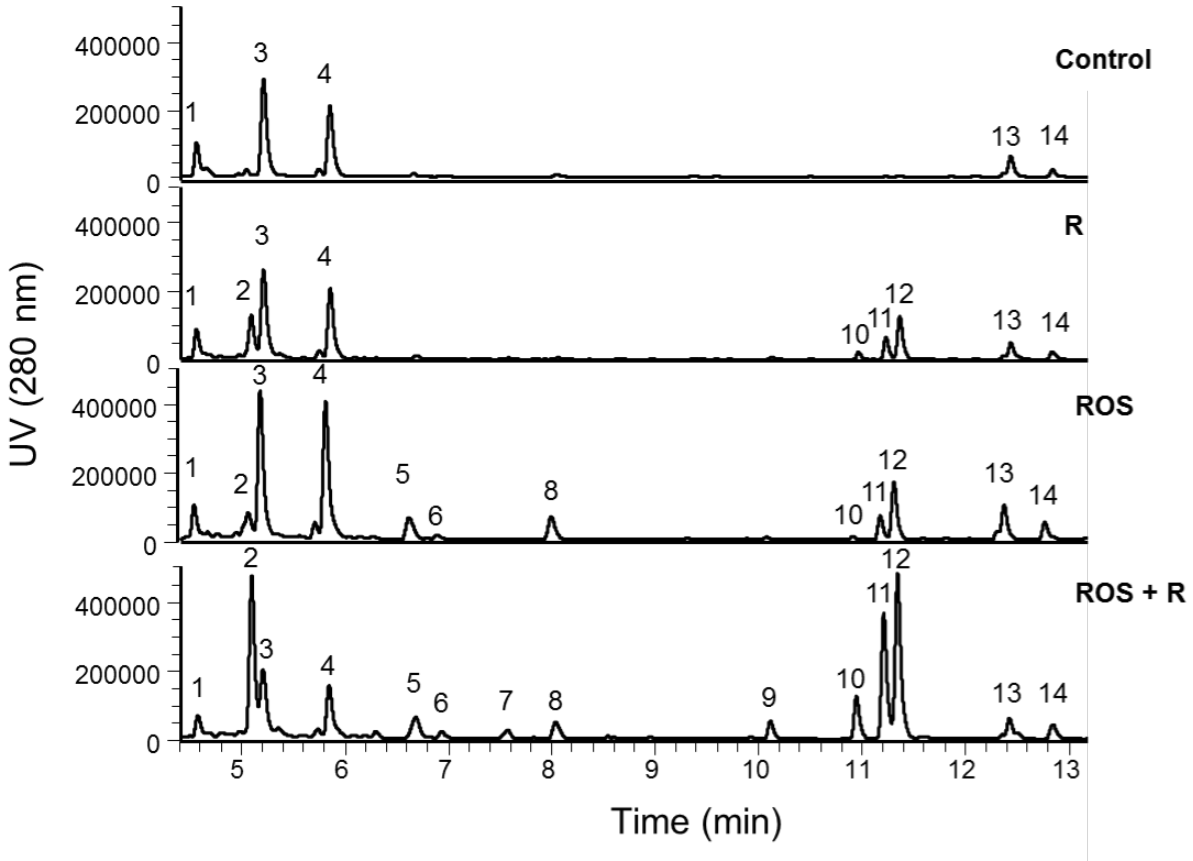


Fig. 3. RP-UHPLC-PDA (UV 280 nm) profiles of ethanol extracts of germinated (control), *Rhizopus* (R)-elicited, reactive oxygen species (ROS)-primed, and ROS-primed prior

to *Rhizopus* elicitation, ROS + R-seedlings of soybean cultivar I (CvI). The chromatograms refer to seedlings three days after priming and/or elicitation. Peak numbers refer to compounds in Table S2 (Supplementary information).

3.2 Annotation of isoflavonoids in treated soybean seedlings

In total, 12 isoflavonoids, belonging to the subclasses of pterocarpans and isoflavones, were annotated in CvI seedlings. Saponins (peak **13** and **14**, **Table S2, supplementary information**) were also present in the extracts but due to their weak antimicrobial potential (Araya-Cloutier et al., 2017), they were not considered further.

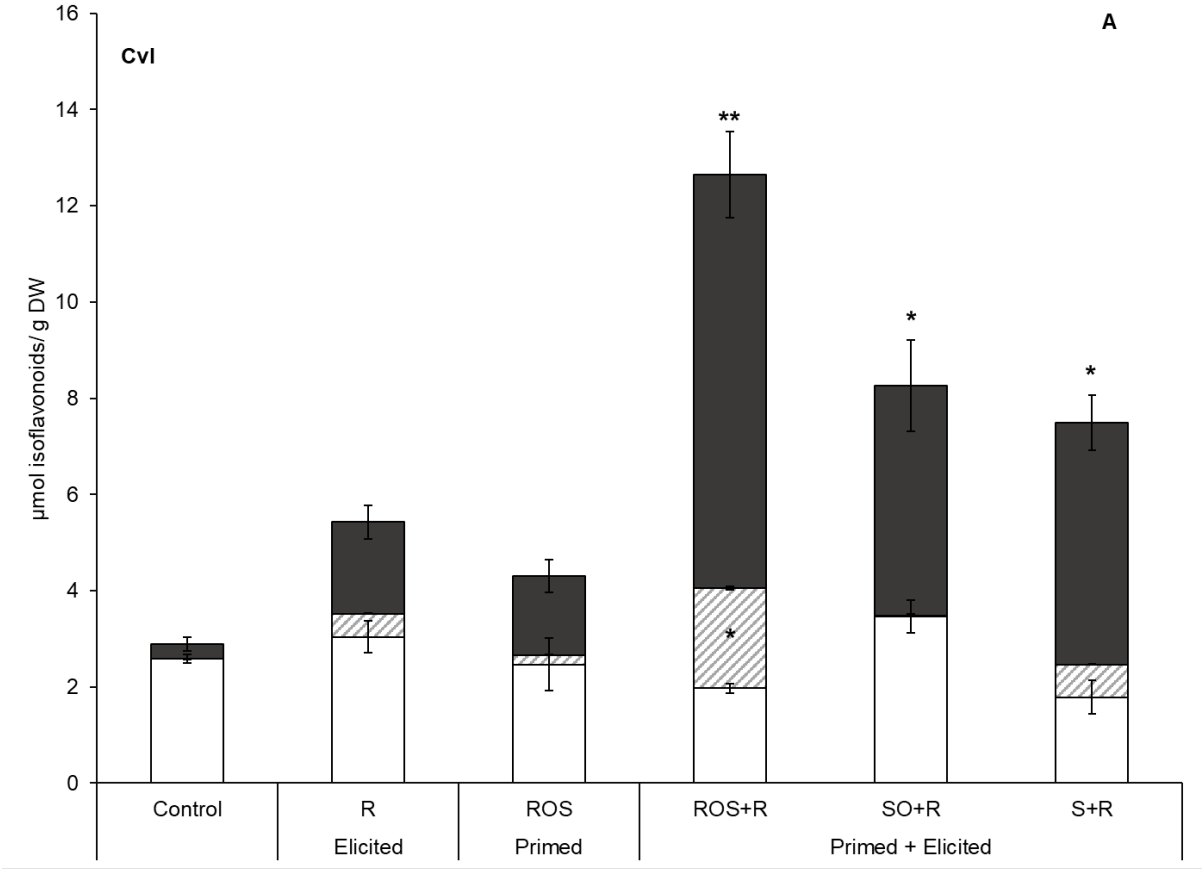
The control soybeans predominantly contained the glycoside daidzin (**1**) and malonylated glycosides of daidzein (**3**) and genistein (**4**), the two major isoflavones present in soybean. Upon R, soybeans of CvI accumulated glycinol (**2**), the non-prenylated pterocarpan precursor of all glyceollins (**Fig. 1**) and the three main glyceollin isomers (i.e. glyceollin I-III) (**10**-**12**). In ROS, the aglycones daidzein (**5**) and genistein (**8**) were also annotated. ROS+R led to accumulation of glycitein (**6**), a methoxy-isoflavone, glyceofuran (**7**), the detoxification product of glyceollin II (Aisyah et al., 2013) and glyceollidin II (**9**), the chain-prenylated glycinol, which is an intermediate to glyceollins II and III. Prenylated isoflavones and prenylated coumestans were not detected in CvI, contrary to what was anticipated (Simons et al., 2011).

3.3 ROS-priming is superior to physical priming

The glyceollin content of the seedlings was monitored daily for five days after applying the different treatments, to determine the optimal end-time with respect to maximum glyceollin induction. The content of glyceollins together with the levels of their biosynthetic precursors in the treated CvI-soybeans over five days are shown in **Fig. S1 and S2 (supplementary information)**. The ROS+R CvI samples (**Fig. S2)** showed a gradual increase in the glyceollin accumulation over time, reaching a clear maximum of 8.6±0.9 μmol/g DW on the 3rd day after

fungus inoculation. This finding is in line with a study where maximal glyceollin levels were observed 40 h after elicitation of 3d-old soybean seedlings with hydroperoxides (Degousee et al., 1994). A maximum after 3 days was also observed for the glyceollin content of the seedlings upon other treatments (**Fig. S2)**, thus henceforth all treatments were compared based on the samples taken three days after fungus inoculation.

In **Fig. 4A** the glyceollin content of the seedlings upon different treatments is compared. Glyceollin levels in ROS+R-seedlings were more than four times higher (8.6±0.9 µmol/g DW) than those in the ROS-seedlings (1.6±0.3 µmol/g DW) and the R-seedlings (1.9±0.3 µmol/g DW). ROS+R stimulated the accumulation of both glyceollin subclasses, i.e. *C*2- and *C*4-prenylated, compared to R-seedlings (**Fig. 5**). The extent of increase was larger for the *C*2-glyceollins (five-fold increase) than for *C*4-glyceollins (three-fold increase). ROS+R also accumulated significantly more glycinol on the 3rd day after fungus inoculation (**Fig. 4A**). The induction of glycinol in the ROS+R-seedlings was thrice more than that of the ROS-seedlings (0.2±0.01 µmol glycinol/g DW) and the R-seedlings (0.5±0.01 µmol glycinol/g DW).


A
CvI
µmol isoflavonoids/ g DW
16
14
12
10
8
6
4
2
0
**
*
*
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Control
R
Elicited
ROS
Primed
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SO+R
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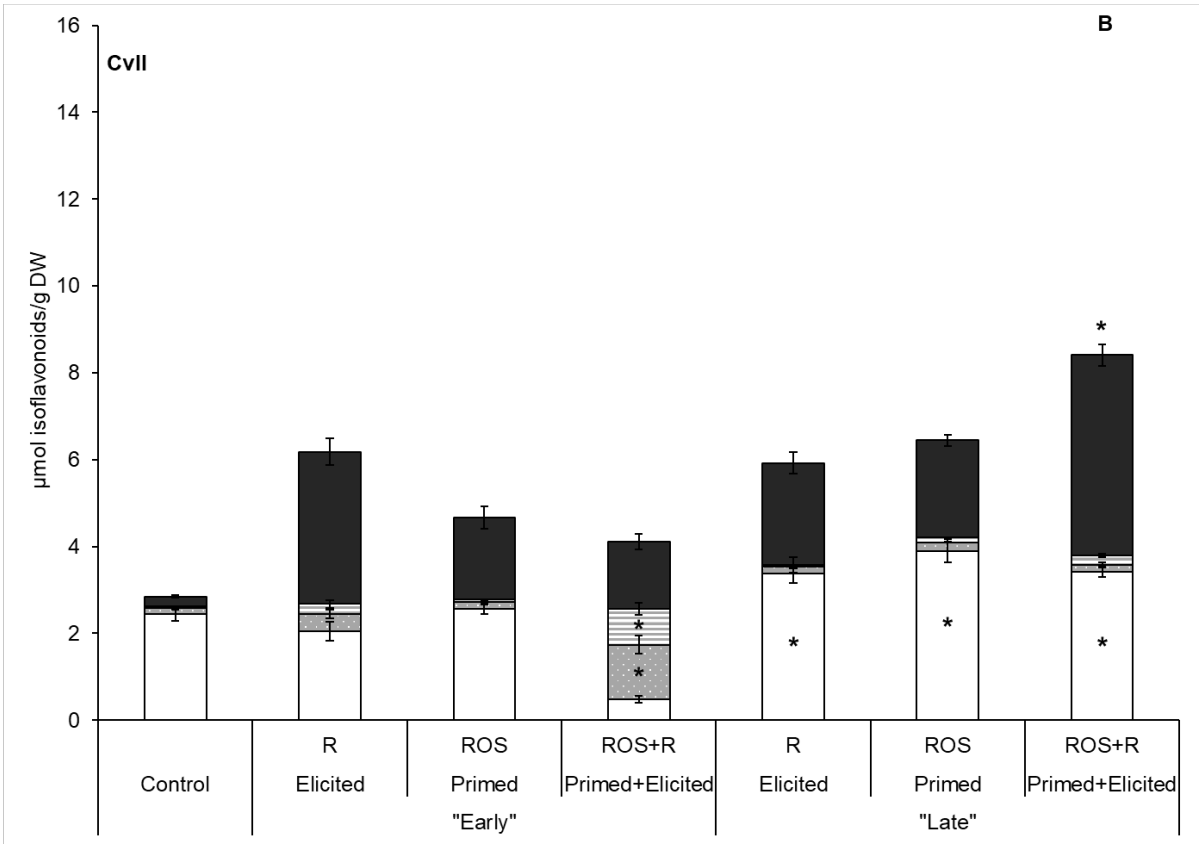
Primed + Elicited


B
CvII
µmol isoflavonoids/g DW
16
14
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10
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6
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2
0
*
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Control
R
Elicited
ROS
Primed
"Early"
ROS+R
Primed+Elicited
R
Elicited
ROS
Primed
"Late"
ROS+R
Primed+Elicited

Fig.4. Content (µmol/g DW) of glycosylated isoflavonoids (white bars) and glyceollins (black bars) in seedlings of the two soybean cultivars, CvI and CvII. Vertically striped grey bars represent the non-prenylated pterocarpan precursor of glyceollins, glycinol, in CvI (**A**), grey dotted bars represent the non-prenylated aglycone, daidzein and horizontally striped grey bars represent daidzein's hydroxylated derivative, genistein in CvII. For "Early" treatments, the metabolite content on 4th day after priming and/or elicitation is shown, whereas for "Late" treatments that on the 3rd after priming and/or elicitation is shown (**B**). Asterisks signify the statistical increase between the treatments and the benchmark *Rhizopus*-elicited sample on the corresponding isoflavonoid subclass in each cultivar (p <0.05). Error bars indicate the standard deviation of the three biological replicates. The germination controls are presented for comparison.

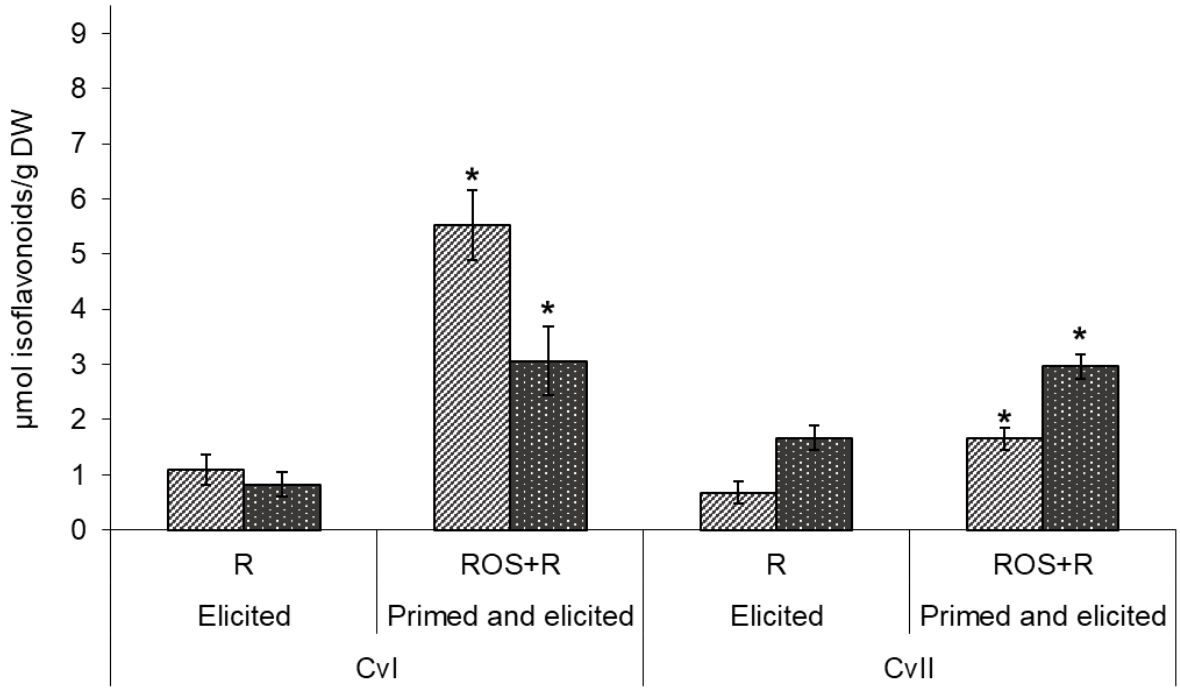

Fig.5. Influence of ROS-priming prior to R-elicitation (ROS+R) on the synthesis of the *C*2- (striped bars) and *C*4- (dotted bars) glyceollins in both soybean cultivars employed in this study. For CvII, the "Late" ROS+R is being shown. Error bars indicate the standard deviation of three

biological replicates. Asterisks signify the statistical difference in each glyceollin subclass between R and ROS+R (p <0.05).

Priming by sonication or slicing prior to *Rhizopus* elicitation (SO+R or S+R) yielded similar amounts of glyceollins, i.e. 4.8±0.9 μmol/g DW for SO+R and 5.0±0.6 μmol/g DW for S+R (**Fig. 4A**). Overall, all three types of priming prior to *Rhizopus*-elicitation significantly increased the glyceollin levels of elicited seedlings, compared to R-elicitation without priming. In CvI, ROS-priming was almost twice as effective as physical priming (i.e. sonication or slicing) prior to *Rhizopus*-elicitation in stimulating glyceollin production (**Fig. 4A**).

Additionally, ROS+R yielded more reproducible inductions than physical priming (SO+R or S+R) in time (**Fig. S2**). This might be attributed to the more systemic and homogeneous effect that imbibition of the seedlings in the ROS solution, compared to the more localized effect of slicing.

3.4 ROS+R protocol for induction of glyceollins in CvI is not directly extrapolatable to CvII

To assess the robustness of ROS+R, the treatment was also applied to a second soybean cultivar, CvII. This soybean cultivar possessed the capacity to produce a wider variety of prenylated (iso)flavonoids (**Fig. S3**, **Table S2, supplementary information**).

Overall, the isoflavonoid profile of seedlings of CvII was similar to that of CvI (**Fig. S3)**. The main differences between CvII and CvI were that CvII constitutively contained phaseol (**15**), a 3,9-dihydroxy-4-prenylcoumestan and that it accumulated four prenylated isoflavones (**17**-**20**) upon priming and/or elicitation. These prenylated isoflavones were tentatively annotated as B-prenyl daidzein (**17**), A-prenyl daidzein (**18**), A-prenyl genistein (**19**), and B-prenyl genistein (**20**) (Aisyah et al., 2013) (**Table S2)**. Due to the absence of treatment effects on phaseol and

the inconsistent treatment effects on prenylated isoflavones (**Fig. S3)**, these compounds are not further discussed in this study.

Remarkably, the primed and/or elicited seedlings of CvII did not accumulate glycinol, as opposed to the findings for CvI. ROS+R on CvII led to maximal glyceollin accumulation of 1.5±0.3 µmol/g DW on the 4th day after the fungus inoculation (**Fig. S4, supplementary information**), which was lower than those in the unprimed R-seedlings and similar to those of the ROS-seedlings (**Fig. 4B**, **"Early"**). However, ROS+R synergistically induced non-prenylated aglycones (2.2±0.3 µmol/g DW) compared to *Rhizopus*-elicited (0.7±0.1 µmol/g DW) and ROS-seedlings (0.2±0.01 µmol/g DW) (**Fig. 4B**, **"Early"**). This synergistic accumulation of non-prenylated aglycones, mainly comprised of daidzein and genistein, was observed in all the time points of ROS+R (**Fig. S4)**. It seemed as if the ROS+R-seedlings were not sufficiently prepared to make use of the large biosynthetic pool of aglycone isoflavonoids for the production of prenylated derivatives.

3.5 ROS+R was adaptable to elicit prenylated isoflavonoid production in both soybean cultivars

Unexpectedly, ROS+R, as such, was not successful in enhancing the glyceollin content more than R without priming in CvII, as opposed to its effect in CvI. However, the increase in the non-prenylated precursors in the CvII-soybean seedlings, urged us to apply the treatments at a later stage of seedling germination. Seedlings' age has been established to affect their competency to accumulate glyceollins (Abbasi and Graham, 2001). Therefore, the treatment was applied also on the 4th day of germination of CvII ("Late" application) in addition to the 2nd day ("Early" application) that has been shown so far for both cultivars (**Fig. 4B**). The aim was to investigate whether perhaps the ideal time point of elicitation was different for CvII than CvI and whether ROS+R could prove to be applicable in CvII for glyceollin enhancement.

In **Fig. 4B** the effect of later application of the treatments on the different isoflavonoid subclasses in CvII is compared. “Late” ROS+R had an enhancing effect on the accumulation of glyceollins compared to “Late” ROS or “Late” R. The treatment yielded the highest average of 4.6±0.3 µmol glyceollins/g DW on the 3rd day after fungus inoculation, even though there was no significant change in glyceollin levels over time (**Fig. S5, supplementary information**). This finding is in line with the optimal day for glyceollin accumulation found in ROS+R-seedlings of CvI.

In comparison to “Early” application, “Late” ROS+R-seedlings accumulated far less non-prenylated aglycone isoflavonoids (**Fig. 4B)**. It seems that the seedlings of CvII needed to be more developed to process the increased levels of non-prenylated biosynthetic precursors that accumulated upon the “Early” ROS+R-treatment. Moreover, glyceollin accumulation in “Late” R was lower compared to that in “Early” R, therefore “Early” R was used as a benchmark for assessing the effectiveness of the “Late” ROS+R-treatment (**Fig. 4B**). “Late” ROS+R was statistically better with respect to glyceollin induction than the R-benchmark. Similar to CvI, the treatment significantly enhanced the accumulation of both *C*2- and *C*4-glyceollins in CvII compared to R (**Fig. 5)**. It is interesting to note that the two cultivars intrinsically accumulated different ratios of *C*2:*C*4-glyceollins (**Fig. 5, R elicited)**. Thus, we propose that ROS+R enlarges the diversity of generated potent antimicrobials, despite the intrinsic tendency of soybean seedlings of different cultivars to favour the accumulation of a specific glyceollin subclass over the other.

Overall, “Late” ROS+R was the superior treatment in CvII, yielding the highest overall metabolite content and the highest glyceollin content. ROS+R outperformed R with respect to the production of potent antimicrobials (i.e. glyceollins) three days after fungus inoculation, in both soybean cultivars employed in this study. The non-laborious application and the robustness

of ROS across two soybean cultivars renders it promising as a priming candidate for large scale applications.

3.6 ROS+R stimulated accumulation of non-prenylated aglycone biosynthetic precursors of glyceollins

The similar effects of ROS+R in the two cultivars gives an insight on the possible effect of the treatment on the biosynthetic pathway of glyceollins. In both cultivars, "Early" ROS+R samples consistently accumulated less glycosylated isoflavonoids compared to the control and R samples (**Fig. 4 and 6**). The increased accumulation of glycosylated isoflavonoids in the "Late" ROS+R is most likely due to the older age of the seedlings, rather than the treatment itself, since all treatments applied later induced higher accumulation of glycosides (**Fig. 4 and Fig. S4 and S5, supplementary information**). Glycosylated isoflavonoids are the dormant isoflavonoid reserves in plants that become biologically active upon stress by splitting their sugar moiety (Miadoková, 2009). In both cultivars, the decreased levels of glycosylated isoflavonoids were accompanied with an increase in aglycone isoflavonoids upon "Early" ROS+R. In CvI, deglycosylation resulted in the synergistic accumulation of the aglycone glycinol, which was produced at three times higher quantities than the additive response in ROS and R (**Fig. 4A**). In CvII, daidzein was synergistically induced upon ROS+R treatment (two times more than the calculated additive response in ROS and R) (**Fig. 4B, "Early"**), whereas glycinol was not detected at all. Based on the consistent decrease in glycosides and the concomitant increase in non-prenylated aglycones upon ROS+R, it seems that the treatment promotes deglycosylation of isoflavones. Moreover, the unequal reduction in molar content of glycosides and the simultaneous increase in glycinol in CvI (**Fig. 4A**) as well as the absence of glycinol in CvII suggest that ROS+R may also stimulate *de novo* biosynthesis of daidzein in both cultivars. Similarly, increased accumulation of distant and more downstream precursors of defence-

related secondary metabolites has been also reported during the post-challenge primed stage of sorghum seedlings (Tugizimana, Steenkamp, Piater, Labuschagne, & Dubery, 2019). Given the increased accumulation of daidzein, we infer that conversion of daidzein to glycinol might be the rate-limiting step in CvII. Most likely, this is also the reason why CvII was able to accumulate prenylated isoflavones. Prenylated isoflavones and glyceollins share daidzein as their non-prenylated aglycone precursor (Yoneyama, Akashi, & Aoki, 2016).

4. Conclusions

In this work, we employed a key molecule in plant's defence response (ROS) as a priming agent prior to *Rhizopus*-elicitation to stimulate the synthesis of soybean's potent antimicrobials, glyceollins. Priming of soybean seedlings with reactive oxygen species (ROS+R) was almost twice as effective as physical priming (sonication or slicing) at stimulating the production of the natural potent antimicrobials, glyceollins. ROS+R enhanced the production of antimicrobial glyceollins 1.3 to 4-fold compared to unprimed *Rhizopus*-elicitation. The treatment had a stimulating effect on the content of non-prenylated aglycone precursors of glyceollins. Lastly, ROS+R stimulated prenylation at both positions (*C*2 and *C*4) of the glyceollin backbone, generating larger diversity of prenylated pterocarpans. This, together with the possibility for large-scale applicability and the robustness across cultivars of the ROS+R method, make ROS a promising primer for triggering the synthesis of antimicrobial compounds in soybean.

Credit authorship contribution statement

Sylvia Kalli: Conceptualization, Investigation, Methodology, Formal analysis, Writing - original draft. **Carla Araya-Cloutier**: Conceptualization, Supervision, Writing - review & editing. **Yiran Lin**: Conceptualization, Investigation. **Wouter J.C. de Bruijn**:

Conceptualization, Supervision, Writing - review & editing. **John Chapman**: Supervision, Writing - review & editing. **Jean-Paul Vincken**: Supervision, Writing - review & editing.

Declaration of Competing Interest

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

Appendix

The following are the Supplementary data to this article:

Supplementary.docx

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