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Variation in accumulation of isoflavonoids in Phaseoleae seedlings elicited by *Rhizopus*

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ABSTRACT

Seeds from seven species of tribe Phaseoleae, i.e. *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 the range of 0.2–0.7 mg representative compound equivalents (RCE)/g DW), whereas application of *Rhizopus* onto the seedlings increased the isoflavonoid content considerably (in the range of 0.5–3.3 mg RCE/g DW). The inducibility of different isoflavonoid subclasses in seedlings with *Rhizopus* varied per species. Isoflavones and isoflavanones were mainly found in elicited seedlings of *Phaseolus*, *Vigna* and *Lablab*, whereas pterocarpans were mainly observed in those of *Psophocarpus*. Despite their phylogenetic relatedness, the seeds of various species within Phaseoleae 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.

1. Introduction

Flavonoids and isoflavonoids are major plant secondary metabolites that are synthesised *via* the phenylpropanoid pathway (Dixon et al., 2002). 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 (Ralston, Subramanian, Matsuno, & Yu, 2005). 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, particularly with prenyl substituents, might offer opportunities in therapies for hormone-dependent diseases (Simons, Gruppen, Bovee, Verbruggen, & Vincken, 2012; Vitale, Piazza, Melilli, Drago, & Salomone, 2012; Zimmermann et al., 2010).

Flavonoids are widely distributed in plants, whereas the majority of isoflavonoids were found in the Leguminosae family (Veitch, 2007). 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 (Kaufman, Duke, Brielmann, Boik, & Hoyt, 1997; Mazur, Duke, Wähälä, Rasku, & Adlercreutz, 1998). Our previous research on soybean has shown that the content of isoflavonoids can be enhanced by germinating the seeds in the presence of fungus (Aisyah, Gruppen, Madzora, & Vincken, 2013; Simons, Vincken, Roidos et al., 2011). In addition, the diversity in isoflavonoid skeletons increases, and many of the compounds induced are prenylated (Aisyah et al., 2013; Simons, Vincken, Roidos et al., 2011). The presence of the fungus seemed particularly important for boosting the content and altering the isoflavonoid composition (Aisyah et al., 2013; O'Neill, Adesanya, Roberts, & Pantry, 1986; Whitehead, Dey, & Dixon, 1982).

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*) (Ingham, 1990; Stefanović, Pfeil, Palmer, & Doyle, 2009). Species other than soybean within this tribe have been reported amenable to induction of isoflavonoids, using wounding, fungal elicitors, bacterial elicitors and chemicals (Doherty & Buescher, 1978; Ingham, 1990; O'Neill et al., 1986; Whitehead et al., 1982). However, the inducibility of isoflavonoids in these seeds during germination with concomitant elicitation by fungus has never been systematically compared. In this study, we investi-

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gated 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 the presence or absence of food grade *Rhizopus oryzae*. It was hypothesised that these closely related species would respond similar to the treatment of germination under stress.

2. Materials and methods

2.1. Materials

Seeds from seven edible Phaseoleae species (from four different genera) were purchased from Vreeken's Zaden (Dordrecht, The Netherlands): *P. vulgaris*, *Phaseolus coccineus*, *Lablab purpureus*, *Vigna angularis*, *Vigna unguiculata*, *V. radiata*, and *Psophocarpus tetragonolobus*. The authentic standards of daidzein and genistein were purchased from Sigma Aldrich (St. Louis, MO, USA). UHPLC–MS grade acidified acetonitrile (ACN), water, methanol and acetic acid (HOAc) were obtained from Biosolve BV (Valkenswaard, The Netherlands). The fungus, *R. 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).

2.2. Treatments with Phaseoleae seeds

The treatment of Phaseoleae seeds was performed in an EQMM sprouting machine (EasyGreen, San Diego, CA), which was modified as described previously (Aisyah et al., 2013). The seeds of all species were consecutively subjected to soaking (1 d) and germination (7 d) stages. Prior to the soaking step, seeds were surface-sterilised by immersing them in 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 sterilised seeds were soaked for 24 h at 25 °C in sterilised Milli-Q water. Subsequently, the soaked seeds were put into sterilised plastic cartridges (sterilised by soaking them in 1%hypochlorite 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 sterilised 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, 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.

2.3. Extraction of (iso)flavonoids from Phaseoleae seeds and seedlings

The extracts of untreated, germinated and elicited Phaseoleae seeds were prepared as described previously for soybean, with some modification (Aisyah et al., 2013). Briefly, 200 mg dried and milled Phaseoleae seed(ling)s were extracted using a speed extractor (E-916; Buchi, Flawil, Switzerland). Hexane and 80% (*v*/*v*) aqueous methanol (MeOH) were used for defatting and extraction of (iso)flavonoids, respectively. During extraction, the cell was filled with solvents until the pressure in the cell reached 100 atm, and heated (40 °C). For each solvent, the sample was extracted using two consecutive extraction cycles of 10 min, followed by flushing the cell with 40 mL solvent and with a flow of nitrogen for 300 s. Corresponding extracts were combined and evaporated under reduced pressure. The dried extracts were re-solubilised 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.

2.4. RP-UHPLC–MS 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. 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) 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 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 at 50% **B**; 12–22 min, linear gradient from 50% to 100% (*v*/*v*) **B**; 22–24 min, isocratic at 100% **B**; 24–25 min, linear gradient from 100% to 10% (*v*/*v*) **B**; 25–30 min, isocratic at 10% (*v*/*v*) **B**. Mass spectrometric analysis was performed on an 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 150–1500. Data-dependent MS^n analysis was performed with normalised 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 (iso)flavonoids 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. The amounts of (iso)flavonoid were expressed as mg representative compound equivalent per g dry weight (mg RCE/g DW). Isoflavones were quantified using daidzein as a generic standard to make a calibration curve with five data points (0.001–0.1 mg/mL, $r^2 = 0.996$). The content of compounds in (iso)flavonoid subclasses other than isoflavones was determined by relating their peak areas with the daidzein calibration curve and correcting for the differences in molar extinction coefficients (ε) between daidzein and the representative compound in that subclass. As the molar extinction coefficients of the representative compound from a subclass were provided in the literature at λ_{max} and not at 280 nm, the ε_{280} was calculated from the spectrum of the representative compound (Table S1 in Supporting Information). The difference in molecular weight between daidzein and the various compounds was accounted for as well.

2.5. Phylogenetic analysis of Leguminoceous species

The *Matk* encoding regions were extracted from the following NCBI nucleotide sequences: *L. purpureus*: gb|EU717408.1 (725-2239); *P. coccineus*: gb|DQ445964.1 (654-2198); *P. vulgaris*: gi|139387430 (4964-6505); *P. tetragonolobus*: gi|378757903 (full); *V. angularis* gi|501594910 (5024-6538); *V. radiata*: gi|289066804 (4996-6510); *V. unguiculata*: gb|AY589510.1 (692-2206); *G. max*: