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Determination of the variations in levels of phenolic compounds in soybean (*Glycine max* Merr.) sprouts infected by anthracnose (*Colletotrichum gloeosporioides*)

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Abstract

BACKGROUND: Soybean sprouts (Kongnamool) are one of the most popular and nutritive traditional vegetables in East Asia. Anthracnose caused by *Colletotrichum gloeosporioides* is one of the most serious diseases of soybean sprouts. In order to obtain basic information for breeding and/or selecting soybean genotypes with increased natural defense against anthracnose, phenolic compounds were profiled for healthy and infected soybean (*Glycine max* Merr.) sprouts by using high-performance liquid chromatography coupled with tandem mass spectrometry.

RESULTS: Tryptophan and eight phenolic compounds (daidzin, genistin, malonyldaidzin, malonylgenistin, daidzein, glycitein, genistein and coumestrol) were determined from healthy and inoculated sprouts. Total identified phenolic content was 40.02 ± 0.03 mg kg^{-1}, 99.4% of which was isoflavones.

CONCLUSION: The monitoring suggested that *de novo* induced glycitein appeared to act as a phytoalexin in the defence mechanism of the soybean sprouts against *C. gloeosporioides*, and constitutively formed seven phenolic components that functioned as phytoanticipins in the diseased soybean sprouts.

Keywords: *Colletotrichum gloeosporioides*; defence materials; phenolic compounds; soybean sprouts; tandem mass spectrometry

INTRODUCTION

During the last few decades, our knowledge of dietary impact on human health has greatly increased and is often related to specific functional foods. Increasing consumption of functional food has become a recent trend in the daily diet. Cereal and vegetable sprouts are good examples of functional foods that can lower the risk of various chronic diseases and/or exert beneficial health effects. They have a number of nutrients including amino acids, dietary fibre, trace elements and vitamins as well as phenolic compounds.[1] A number of the phenolic compounds play a role as defence materials against external stressors such as fungi, insects, drought and UV, and their concentration changes in response to such stressors.[2] Among the defence materials, phytoalexins are phytochemicals synthesised *de novo* by plants and accumulated in plants after exposure to stressors, whereas constitutive phytochemicals with a defence function are classified as phytoanticipins.[3]

Soybean sprouts (Kongnamool) are one of the most popular and nutritive traditional vegetables in East Asia and their current market is estimated to be 700 million dollars per annum in South Korea.[4] Several classes of substances have been characterised in soybean sprouts, including vitamins B_1, B_2, C, carotene and flavonoids.[5]

Soybean sprouts are grown using either an under-watering or upper-watering method, in which water is supplied for 10–20 min

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at 2–3 h intervals. It takes approximately 1 week for harvest. However, the sprouts are susceptible to infection by various pathogens including bacteria and fungi due to high density, humidity and CO_2 concentration and poor ventilation when using these methods.[6,7] The infection usually becomes an epidemic during the summer particularly in mass production systems using recycled water. Pathogens such as *Fusarium* spp., *Pseudomonas* spp., *Fusarium solani*, *F. oxysporum*, *Rhizoctonia solani*, *Macrophmia phaseoli* and *Colletotrichum gloeosporioides* can attack the sprouts, and among them, anthracnose caused by *C. gloeosporioides* is particularly serious. This pathogen results in dark-brown lesions on the cotyledons and hypocotyls of the sprouts leading to softening and rot of the whole tissue. Sprout rot results in a yield decrease and low quality product.[8,9]

Agrochemicals are applied to control pathogens and to avoid economic loss. However, such treatment causes significant problems due to agrochemical residues and the possible evolution of pathogenic strains resistant to the agrochemicals.[10] Accordingly, a strategy such as breeding and/or selecting soybean genotypes with increased natural defence may represent an effective alternative to control *C. gloeosporioides*. Accurate profiling of their plant defence compounds is essential to look for and/or breed soybean with increased natural defence against *C. gloeosporioides* attack.

We determined the phenolic compounds in healthy sprouts and monitored their levels in sprouts infected by *C. gloeosporioides* during progress of the disease by utilising high-performance liquid chromatography–tandem mass spectrometry (HPLC-MS/MS). As a number of phenolic compounds can play a role in the defence mechanism and may be toxic to pathogens but not mammals, they have attracted much research attention during recent years. Information for a phenolic compound metabolomics profile would be useful to control plant diseases in an environmentally friendly manner and improve programmes to breed more resistant crops.[11]

MATERIALS AND METHODS

Materials and chemicals

Seeds of *Glycine max* Merr. (S01) were obtained from the National Academy of Agricultural Science, Rural Development Administration, Republic of Korea. The seeds were planted in a field at Gyeongsang National University (GNU) during May 2010. The soybean seeds were harvested at maturity and air dried to provide a seed moisture content of about 8.0%. The seeds were authenticated as having a homozygous genetic background by Professor Jong-Il Jeong of the Research Institutes of Life Science at GNU. Voucher seeds were deposited in the herbarium of the institute. The seeds were washed with water and stored at 4 °C until extraction and inoculation. *C. gloeosporioides* was obtained from the Korean Agricultural Culture Collection. Tryptophan (98%), daidzein (98%), genistein (98%) and coumestrol (97.5%) were used as external standards and purchased from Sigma–Aldrich (St. Louis, MO, USA). Glycitein (98%) was purchased from LC Laboratories (Woburn, MA, USA). All solvents and pure water were purchased from Duksan Pure Chemical Co. Ltd. (Ansan, Republic of Korea).

Soybean inoculation and sprout cultivation

A *C. gloeosporioides* mycelial colony was cultured on a potato dextrose agar (PDA; Difco, Sparks, MD, USA) plate at 27 °C for 5 days in the dark. Large quantities of conidia were collected from the PDA by washing the colony surface with 10 mL of sterilised water. Then, spores and the mycelial mixture were filtered with cheesecloth. The spore suspension was centrifuged for 10 min at $3000 \times g$ and resuspended in sterilised water to about 10^4 conidia mL^{-1}. Soybean seeds were submerged in the fungal conidia suspension for 5 min and dried on a clean bench at room temperature for 3 h. The inoculated soybean sprouts were grown in the dark at 20 ± 2 °C for 9 days using sprouting equipment (Sinchang Inc., Seoul, Republic of Korea). Fresh water was used for irrigation for 10 min every 2 h and replaced every day. Disease lesions on the hypocotyls were periodically collected at 24 h intervals on days 5–9 after inoculation.

Sample preparation

Hypocotyls of infected soybean sprout and healthy sprout were sampled exclusive cotyledon and root. A hypocotyl sample (10 g) was ground in liquid nitrogen and extracted with 80% aqueous methanol (20 mL). The mixture was homogenised using a Polytron blender (Brinkman Instruments, Westbury, NY, USA) for 5 min, treated in a sonicator (Bransonic 3510R-DTH; Branson Ultrasonics Corporation, Danbury, CT, USA) for 10 min, and filtered through filter paper (Whatman No. 1; Whatman International Ltd. Maidstone, UK) under reduced pressure. The filtrate was centrifuged at $4000 \times g$ using a model SCT4B centrifuge (Hitachi, Ibaraki, Japan) for 15 min.

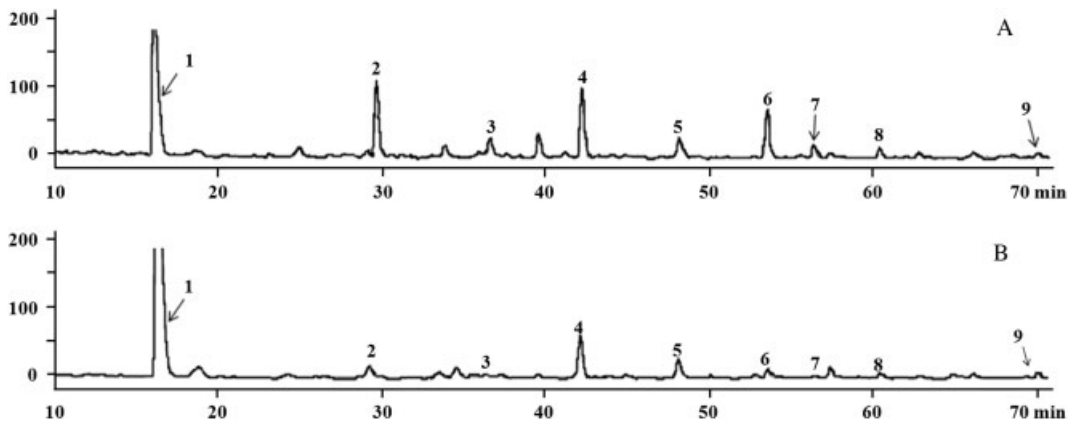
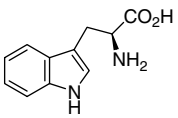


Figure 1. High-performance liquid chromatography (HPLC) chromatograms of the phenolic compounds isolated from soybean sprouts infected by *Colletotrichum gloeosporioides* 7 days post-infection (A) and healthy sprouts (B). **1**, tryptophan; **2**, daidzin; **3**, genistin; **4**, malonyldaidzin; **5**, malonylgenistin; **6**, daidzein; **7**, glycitein; **8**, genistein; **9**, coumestrol. Detection wavelength: 280 nm.

Table 1. Structures and high-performance liquid chromatography and mass spectral data of tryptophan and phenolic compounds isolated from sprouts infected with *Colletotrichum gloeosporioides*

Structures



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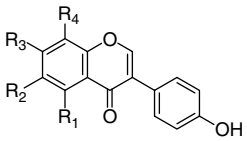
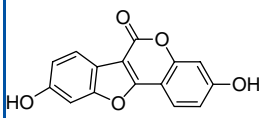


2: $R_1 = R_2 = R_4 = H$, R_3 = glucosyl
3: R_1 = OH, $R_2 = R_4 = H$, R_3 = glucosyl
4: R_1 = OH, $R_2 = R_4 = H$, R_3 = 6''-malonlyglucosyl
5: R_1 = OH, $R_2 = R_3 = H$, R_4 = 6''-malonylglucosyl
6: $R_1 = R_2 = R4 = H$, R_3 = OH
7: $R_1 = R_4 = H$, $R_2 = OCH_3$, R_3 = OH
8: $R_1 = R_3$ = OH, $R_2 = R_4 = H$



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Compounds	t_R	$[M+H]^+$	MS/MS
Tryptophan (**1**)	15.6	205	205, 188, 144
Daidzin (**2**)	29.7	417	255, 227, 137
Genistin (**3**)	37.0	431 ([M−H−])	431, 269
Malonyldaidzin (**4**)	43.0	503	255
Malonylgenistin (**5**)	49.0	519	271, 215, 197, 153, 149
Daidzein (**6**)	54.2	255	237, 199, 181, 171, 157, 153
Glycitein (**7**)	57.0	285	285, 270, 242, 229, 197
Genistein (**8**)	61.2	271	253, 243, 225, 215, 197, 187
Coumestrol (**9**)	70.6	269	269, 213, 197, 137

Table 2. Validation data for the representative standards

	Recovery ± RSD (%) ($n = 3$)					
Standard	10 mg L^{-1}	500 mg L^{-1}	1000 mg L^{-1}	r^2	LOD (mg L^{-1})	LOQ (mg L^{-1})
Tryptophan (**1**)	96.9 ± 0.1	99.2 ± 0.2	88.1 ± 0.1	0.999	0.038	0.115
Daidzein (**6**)	91.6 ± 0.9	83.9 ± 0.5	99.8 ± 0.1	0.999	0.012	0.037
Glycitein (**7**)	85.4 ± 0.2	91.7 ± 0.3	98.5 ± 0.1	0.998	0.016	0.048
Genistein (**8**)	89.7 ± 0.4	95.9 ± 0.1	90.6 ± 0.2	0.998	0.012	0.035
Coumestrol (**9**)	97.1 ± 0.2	95.1 ± 0.1	98.1 ± 0.1	0.999	0.018	0.055

LOD, limit of detection; LOQ, limit of quantification.

The solvent was removed with a rotary evaporator (Eyela NVC-2100; Tokyo Rikakikai Co. Ltd, Tokyo, Japan) and reconstituted in 80% aqueous methanol (1 mL). The mixture was filtered through a PTFE syringe filter (Titan, 0.45 µm; SMI-Lab Hut Co. Ltd., Maisemore, UK), transferred to silanised vials, and stored at −70 °C until analysis.

HPLC-MS/MS analysis

HPLC analysis was performed using an 1100 series LC system equipped with a G1322A degasser, G1312A pump, a G1313A auto-sampler and a G1316A oven (Agilent Technologies, Palo Alto, CA, USA). Chromatographic separation was carried out on a Zorbax Stable Bond Analytical SB-C18 column (4.6 × 250 mm, 5 µm; Agilent Technologies, Columbia, MD, USA). The flow rate was 0.5 mL min^{-1}, with a column temperature of 30 °C and an injection volume of 10 µL. The system consisted of 0.1% aqueous acetic acid (A) and methanol (B). The gradient conditions for the mobile phase were from 10% to 20% B for 5 min, increased to 70% B for 65 min, followed by isocratic elution for 10 min.

MS/MS experiments were performed using a 3200 Q TRAP LC/MS/MS system (Applied Biosystems, Forster, CA, USA) with a Turbo VTM source and a Turbo Ion Spray probe (500 °C). The mass spectrometer was operated in positive and negative ion mode. Nitrogen was used as a nebulising and as well as a drying gas. The flow rates in both cases were 45 psi. The capillary voltage was set at 5.5 kV and the source temperature at 500 °C. The resolutions of the first and third quadrupole were between 0.6 and 0.8 (unit resolution). Mass spectra were recorded between *m/z* 100 and 1000 with a step size of 0.1 amu.

Quantification

All phenolic compounds were quantified using chromatograms extracted at 280 nm. Tryptophan (**1**), daidzein (**6**), glycitein (**7**), genistein (**8**) and coumestrol (**9**) were calibrated with purchased standards. Commercially unavailable components were quantified using the calibration curves of the standards possessing the same retention time as aglycone. Thus, daidzin (**2**) and malonyldaidzin (**4**) were quantified as daidzein; genistin (**3**) and malonylgenistin

Table 3. Average contents of tryptophan (**1**) and eight phenolic compounds (**2–9**) during 5–9 days in healthy soybean sprouts (mg kg^{-1})

Compound	Mean ± SD
1	445.00 ± 0.47
2	4.97 ± 0.04
3	1.40 ± 0.02
4	18.04 ± 0.01
5	8.63 ± 0.06
6	4.45 ± 0.01
7	ND
8	2.29 ± 0.01
9	0.24 ± 0.01

ND, not detected.

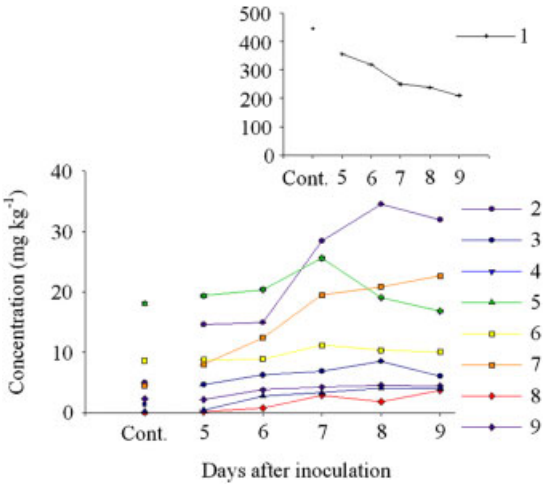

Figure 2. Content changes in tryptophan (**1**) and eight phenolic compounds (**2–9**) in soybean sprouts from 5–9 days after infection ($P < 0.001$). The control (Cont.) is the average value of the phenolic compounds in healthy soybean sprouts from days 5–9. Tryptophan (**1**) is depicted in the inset. Data are shown as mean ± standard deviation, $n = 3$.

(**5**) as genistein. Plant phenols for which standards are not available can be quantified using the standard curve of a related compound.[12] The calibration curves were prepared using seven different concentrations (0.1, 1, 10, 50, 100, 200, and 1000 mg L^{-1}) of each standard and plotting the standard concentration against the peak area.

Assay for antifungal activity against *Colletotrichum gloeosporioides*

Commercially available phenolic compounds **6–9** were used to investigate antifungal activity. PDA was used as the culture medium. The compounds, melted in dimethyl sulfoxide, were finally adjusted to PDA concentrations (25, 50 and 100 mg L^{-1}) in a six-well plate. *C. gloeosporioides* was inoculated at the centre of each plate. A mycelial block (5 mm) was prepared from the plate cultured for 7 days using a cork borer. The blocks were placed at the centre of individual six-well plates, and the inoculated plates were incubated at 27 °C for 5 days. A control (PDA) without extracts was also maintained. After incubation, the diameter of the fungal colony was measured. The % inhibition of mycelial growth of the test fungus was calculated by the formula $I = [(C - T)/C] \times 100$, where I is the % inhibition, C is the diameter of the fungal colony in the control, and T is the diameter of the fungal colony in the treatment.

Statistical analyses

All experiments were conducted in triplicate. All results are expressed as mean ± standard deviation. Significant differences among treatment means ($P < 0.001$) were determined via a one-way analysis of variance using SPSS version 12.0 (SPSS, Chicago, IL, USA).

RESULTS AND DISCUSSION

Separation and characterisation

The HPLC chromatograms of the extracts from soybean sprouts showed good specificity (peak separation), as shown in Fig. 1. Tryptophan and eight phenolic compounds that could be characterised from the healthy and diseased sprouts 7 days post-inoculation were labelled in the 10–70 min retention time segment of chromatograms recorded at 280 nm. The structures and HPLC-MS/MS data of the nine components are shown in Table 1. Tryptophan (**1**) and phenolic compounds (**2–9**) were previously reported from soybean germplasms.[13–15]

Quantification

The regression equation was constructed in the form of $y = ax + b$, where y and x represent the peak area and the concentration of each compound, respectively. Validation data are summarised in Table 2. Coefficients (r^2) were >0.998, indicating good linearity. Recovery was calculated as A/B where A is the peak area obtained for the analyte spiked pre-extraction, and B was that obtained for the analyte spiked post-extraction. Recovery of the analytes was determined by quality control samples at three different levels of 10 mg L^{-1}, 500 mg L^{-1}, and 1000 mg L^{-1} ($n = 3$).

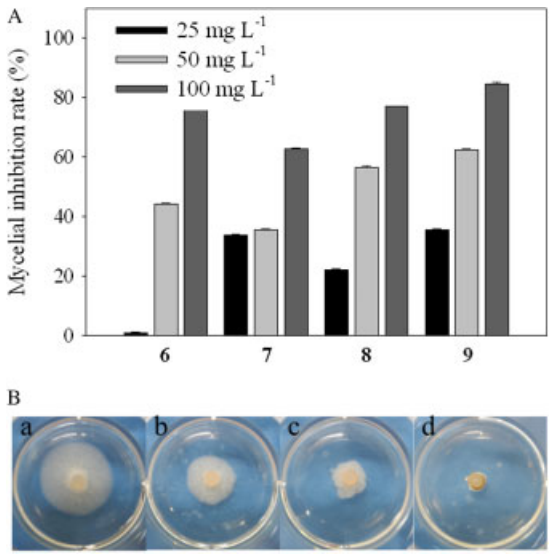

Figure 3. Antifungal activity assays for four commercially available daidzein (**6**), glycitein (**7**), genistein (**8**), and coumesterol (**9**) identified using high performance liquid chromatography–tandem mass spectroscopy (HPLC-MS/MS) analysis. (A) Mycelial inhibition rate for *Colletotrichum gloeosporioides*. (B) Inhibition of spore production at different concentrations of daidzein (**6**): a, control; b, 25 mg L^{-1}; c, 50 mg L^{-1}; d, 100 mg L^{-1}.

The recoveries were 85.4–97.1%, 83.9–99.2%, and 88.1–99.8% at 10 mg L^{-1}, 500 mg L^{-1} and 1000 mg L^{-1}, respectively. Precision was evaluated as the relative standard deviation (RSD). The RSD values were <0.9%, <0.5% and <0.2% at the three different levels, respectively. These results indicate that the present assay method was good. The performance limits were represented in terms of limit of detection (LOD) and limit of quantification (LOQ). The LOD and LOQ were calculated using signal-to-noise ratios of 3 and 10 on the chromatogram, respectively. LODs and LOQs were <0.038 mg L^{-1} and <0.115 mg L^{-1}, respectively, indicating acceptable levels.

Tryptophan and individual phenolic contents in the healthy soybean sprouts are listed in Table 3. The tryptophan concentration was 445.0 ± 0.47 mg kg^{-1} in healthy soybean sprouts. The total amount of identified phenolic compound was 40.02 ± 0.03 mg kg^{-1}, of which 99.4% was isoflavones (**2–8**). The content of phenolic compound **4** was highest, and that of **9** was lowest. Phenolic compound **7** was not detected in healthy sprouts.

Antifungal action of phenolic compounds in soybean sprouts against *Colletotrichum gloeosporioides*

To investigate the role of the phenolic compounds as defence materials against *C. gloeosporioides*, healthy soybean seeds were inoculated with the fungus, and their sprouts were cultivated for 9 days under conventional sprouting conditions.

Observable macroscopic disease symptoms started to develop as pale pink lesions 5 days after inoculation, and the sprouts became slightly sunken and tanned about 7 days after inoculation. The lesion flesh became pulpy at around 9 days. When the healthy sprouts were inoculated with *C. gloeosporioides*, the phenolic compound levels underwent significant changes ($P < 0.001$) (Fig. 2). Tryptophan (**1**), which showed a large change compared to that of the phenolic compounds, is depicted in the inset of Fig. 2. The concentration of tryptophan (**1**) decreased gradually with disease progression. The quantities of phenolic compounds **2** and **3** increased to peak concentrations of 34.51 ± 0.03 mg kg^{-1} and 8.47 ± 0.05 mg kg^{-1}, respectively, around 8 days after inoculation and then decreased. Phenolic compounds **5** and **6** increased to maximum concentrations of 25.61 ± 0.02 mg kg^{-1} and 11.19 ± 0.03 mg kg^{-1}, respectively, at 7 days after inoculation and then decreased. Phenolic compound **7**, which was not detected in healthy sprouts, began to be detected about 6 days after inoculation. Phenolic compounds **6–9** increased slowly up to 9 days after inoculation. These observations suggest that the *de novo* synthesised **7** may match the phytoalexin requirements and that the constitutively formed phenolic compounds **2–9** may function as phytoanticipins against *C. gloeosporioides*. The mycelial inhibition rates revealed that phenolic compounds **6–9** inhibited the growth of *C. gloeosporioides* dose-dependently (Fig. 3A). A typical mycelial inhibition rate was highest for component **9** (35.6%), followed by **7** (33.8%), **8** (22.1%), and **6** (0.9%) at 25 mg L^{-1}, compared with the growth observed in the corresponding controls. Additionally, inhibition of *C. gloeosporioides* spore production was observed in the presence of daidzein (**6**) (Fig. 3B) and **7–9** (data not shown). It is remarkable that all these compounds played an active role protecting soybean sprouts against *C. gloeosporioides* attack at a concentration as low as 0.0025% (25 mg L^{-1}).

Daidzein (**6**), an important phenolic compound in soybean tissues, is the precursor for the synthesis of the soybean phytoalexin coumestrol[16–18] but nothing is known about its role as a defence material until now. A glycetein conjugate, structurally related to **7**, accumulates in soybean cotyledons following stimulation with Lactofen, which is the active ingredient in a herbicide that induces disease resistance in soybeans[19] and inhibits the growth of the soil-borne fungus *Fusarium solani* f. sp. glycines in soybean hairy roots.[20] Genistein (**8**) also reduces the growth of the fungi *Fusarium solani* f. sp. glycines[21] and *Phytophthora sojae*[21] in soybean tissue. Coumestrol (**9**) functions as a phytoalexin in soybean inoculated with microbials such as *Aspergilli* spp.[22] and *Rhizobium leguminosarum* bv. *Phaseoli* bacteria.[23]

CONCLUSION

Tryptophan and eight phenolic compounds were characterised from healthy soybean sprouts and those infected by *C. gloeosporioides* using HPLC-MS/MS. Monitoring variations in phenolic compound levels in soybean sprouts infected by *C. gloeosporioides* demonstrated that the *de novo* induced glycitein (**7**) appeared to act as a phytoalexins and the constitutively formed daidzin (**2**), genistin (**3**) and malonyldaidzin, malonylgenistein, daidzein (**4–6**), genistein (**8**), and coumestrol (**9**) acted as phytoanticipins.

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