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ISOFLAVONE GLUCOSIDE STRESS METABOLITES OF SOYBEAN LEAVES

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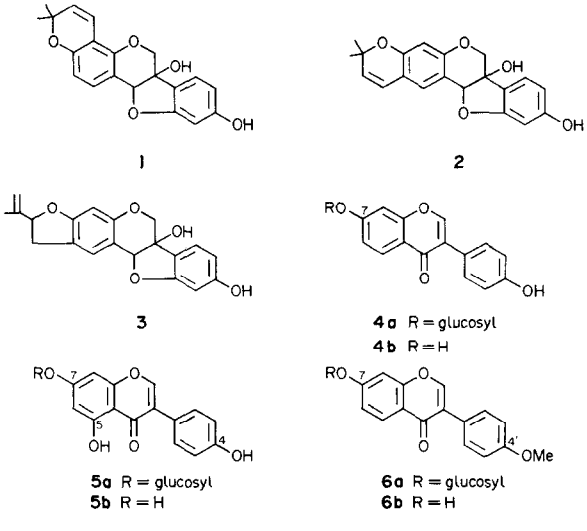
Key Word Index—*Glycine max*; Leguminosae; soybean; bacteria; isoflavone glucosides; daidzin; genistin; ononin; phytoalexins; formononetin.

Abstract—The isoflavone glucosides daidzin, genistin and ononin, the isoflavones daidzein and formononetin, and glyceollins I–III accumulated in soybean leaves inoculated with phytopathogenic bacteria. Treatment of leaves with sodium iodoacetate or yeast extract also led to isoflavonoid accumulation. Various other stress-inducing treatments were not effective. Bacterially-induced accumulation of isoflavone glucosides and the occurrence of ononin and formononetin in soybean are reported for the first time.

INTRODUCTION

Soybean [*Glycine max*. (L.) Merr.] cotyledons, hypocotyls and leaves accumulate various isoflavonoids [e.g. coumestrol, daidzein (**4b**) and glyceollin isomers I–III (**1**–**3**)] when subjected to biotic (fungal or bacterial) or abiotic (e.g. exposure to chemicals or UV light) stress [1–7]. The induced isoflavonoids and, in particular, the glyceollin isomers have been proposed as factors responsible for resistance of soybean to several microorganisms [4–8]. The accumulation of isoflavone glucosides in stressed soybean has not been previously reported even though soybean seed contains high constitutive levels of daidzin (**4a**) and genistin (**5a**) [10].

In an investigation of the interaction of compatible (disease-inducing) and incompatible (resistant-response inducing) phytopathogenic bacteria with soybean leaves, we have observed the accumulation of three isoflavone glucosides: daidzin, genistin and ononin (**6a**). In this report, we characterize these stress metabolites and the ability of various stress-inducing treatments to cause their accumulation. The role of these compounds in resistance of soybean to bacteria will be assessed in another publication.



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RESULTS AND DISCUSSION

In contrast to soybean seeds [9], untreated leaf tissue contains low levels of the isoflavone glucosides daidzin (**4a**) and genistin (**5a**). Infiltration of leaves with either compatible or incompatible strains of *Pseudomonas syringae* pv *glycinea* (Psg) (a pathogen of soybean) or incompatible strains of *P. syringae* pv *phaseolicola* (Psp) (a pathogen of French bean, *Phaseolus vulgaris* L., but not of soybean) led in most instances to accumulation of **4a** and **5a** (Table 1). Concomitant with accumulation of **4a** and **5a**, another glycoside, not detectable or present in low amounts in untreated leaves, was observed to accumulate. The compound was shown to be ononin (**6a**), a substance that has not been observed, prior to this report, as either a stress metabolite, or a constitutive isoflavone glucoside of soybean. However, ononin is found constitutively in other Leguminosae [10]. As demonstrated by Si gel TLC, the only compounds in ethanol fractions from bacterially-infiltrated leaves that appeared to be present in increased amounts compared to levels in comparable extracts from water infiltrated leaves were **4a**, **5a** and **6a**.

Infiltration with bacteria also caused accumulation of the aglycones daidzein (**4b**) and formononetin (**6b**) along with glyceollins I–III (**1**–**3**) (isomeric composition *ca* 6:3:1) (Table 1). Their identity was confirmed by direct comparison of chromatographic (TLC, HPLC) behaviour, UV spectra and anti-fungal activity [5, 7, 11, 12] with commercial standards, or with glyceollin isolated from soybean seed inoculated with *Cladosporium* sp. as previously described [13]. Like its glucoside, **6b** has not been previously reported in healthy or stressed soybean. However, isoformononetin was previously reported to

Table 1. Levels of stress metabolites in soybean leaves after stress-inducing treatments

Treatment	Strain (interaction)*	Time (hr)	μg/g fr. wt leaf tissue					
			Daidzin	Daidzein	Ononin	Formononetin	Genistin	Glyceollins
Chippewa 64								
Water	—	72	2†	—	7	—	8	—
Psg	K1 (I)	72	63	15	49	27	46	17
Psp	1134 (I)	72	72	8	14	14	43	4
Untreated	—	24	1	—	TR	—	4	—
Sodium iodoacctate (0.5 mM)	—	24	2	—	3	—	6	—
Freezing	—	24	TR	—	TR	—	4	—
Flambeau								
Water	—	72	10	—	TR	TR	18	—
Psg	K1 (C)	72	238	5	33	TR	69	57
	J3-20-4A (C)	72	212	1	6	2	74	53
	J3-17-2 (I)	72	44	4	4	3	23	101
Psp	1134 (I)	72	43	9	3	2	17	28
	1137 (I)	72	52	7	13	1	12	50
Harosoy								
Water	—	72	6	—	TR	TR	7	—
Psg	K1 (I)	72	92	7	12	17	32	119
	2159 (I)	72	205	9	18	26	70	105
	J3-17-2 (C)	72	91	1	40	5	37	66
Psp	1134 (I)	72	76	6	18	21	11	25
	1137 (I)	72	78	3	52	19	34	39
Untreated	—	24	3	—	—	—	3	—
Sodium iodoacetate (1.0 mM)	—	72	14	3	1	TR	14	TR
Cupric chloride (1.0 mM)	—	24	1	—	—	—	2	—
Yeast extract	—	72	64	TR	16	7	2	13
Spray damage	—	24	1	—	TR	—	5	—
Sliced	—	24	TR	—	TR	—	4	—

*(I) Incompatible interaction—resistance; (C) compatible interaction—disease.

†Data represent averages of values from at least two experiments. Values are uncorrected for extraction efficiencies of *ca* 60%.

—, None detected; Psg; *Pseudomonas syringae* pv glycinea; Psp, *P. syringae* pv *phaseolicola*; TR, trace (< 1.0 μg/g fr. wt).

accumulate in soybean leaves inoculated with incompatible phytopathogenic bacteria [2].

Of the various chemical treatments (sodium iodoacetate at 0.5 or 1.0 mM, yeast extract at 5 g/l., and cupric chloride at 1.0 mM) only infiltration with sodium iodoacetate (1.0 mM) and yeast extract led to increased levels of glucosides, aglycones and/or glyceollins (Table 1). Severe necrosis of the infiltrated leaf tissue was evident 3 days after infiltration with sodium iodoacetate (1.0 mM), while yeast extract caused only slight browning of the leaf tissue. After 24 hr infiltration with sodium iodoacetate (0.5 mM) leaf tissue had a 'greasy' appearance, but was not necrotic. Previously, sodium iodoacetate [2, 3] and extracts from yeast [14, 15] were reported to be elicitors of glyceollin production by soybean.

No increase in glucosides, aglycones or glyceollins I–III occurred after leaf tissue was stressed by physical means even though much necrosis was evident after freezing or infiltration of water at elevated pressure. The results indicate that increased levels of glucosides, aglycones and glyceollins are not due solely to tissue necrosis.

The contribution of host plant or bacterial β-glucosidase activity in aglycone accumulation is unknown, but we determined that the bacterial strains used in this study do not produce β-glucosidase when tested under a variety of *in vitro* conditions [16–18] using aesculin or arbutin as inducers.

The accumulation of glucosides after microbial attack of plants has been observed previously. Daidzin and ononin increase upon infection of alfalfa leaves by the fungal pathogen *Ascochyta imperfecta* Pk. [19]. The coumarin glucosides skimmin and/or scopelin accumulate upon fungal [20, 21] or bacterial [22] attack of Solanaceous hosts. However, a significant role for increased levels of glucosides in plant disease resistance has not yet been demonstrated.

EXPERIMENTAL

Induction and isolation of compounds **1–6b** *from leaves.* Bacteria used were Psg strains K1 (from B. W. Kennedy, University of Minnesota, U.S.A.) J3-17-2, J3-20-4A [23] and 2159 (from the National Collection of Plant Pathogenic Bacteria (NCPPB), Harpenden, U.K.) and Psp strains 1134 and 1137 [24] (also from the NCPPB).

Soybean cvs Chippewa 64, Flambeau and Harosoy were

grown as described previously [25] except soil was substituted for vermiculite. First or second trifoliolate leaves were used for all treatments. Leaves were infiltrated [23] with bacterial suspensions in sterile H_2O containing *ca* 1×10^8 colony forming units/ml. Sterile H_2O alone was used as a control treatment. Other treatments included infiltration with NaIOAc at 0.5 or 1.0 mM (pH 7.0), $CuCl_2$ (1.0 mM) and yeast extract (Difco) at 5 g/l H_2O. Stressing of leaves by physical means was accomplished by: (1) infiltration with sterile H_2O at elevated pressure; (2) freezing by touching the end (0.9 cm diam.) of a cooled (dry ice) metal rod to the leaf surface at four sites per leaflet; and (3) slicing leaves along each lateral vein from the midrib to the leaf edge with a razor blade.

At 24 hr [for NaIOAc (0.5 mM), $CuCl_2$ and physically-induced stress treatments] or 3 days [for bacterial, H_2O alone, NaIOAc (1.0 mM) and yeast extract treatments] after treatment leaves were rapidly detached, weighed and plunged into boiling 80% aq. EtOH for 10 min. Leaf residue was removed by filtration and filtrates were evaporated to dryness under a stream of N_2. Each sample was partitioned between Et_2O–30% aq. EtOH (2:1). Under these conditions it was determined, using known compounds, that the glycosides partitioned quantitatively into the EtOH, and the aglycones, except for **4b**, partitioned into the Et_2O layer; **4b** partitioned equally into both layers. Si gel TLC (Analtech, GF-254) (mobile phase: $CHCl_3$–Me_2CO–MeOH, 20:6:5) of aliquots of EtOH fractions gave fluorescence-quenching zones at R_f 0.41, 0.45 and 0.51 corresponding to **4a**, **5a** and **6a**. The area of the plate containing **4a**, **5a** and **6a** was scraped and the glycosides eluted with MeOH. The MeOH extract was analysed by HPLC on a C_{18} μBondapak (10 μm) column eluting with 30% aq. MeOH. *A* at 260 nm was monitored. Compounds **4a**, **5a** and **6a** had elution times of *ca* 8, 14 and 31 min, respectively. Quantification was done by comparison of peak areas for standards and leaf samples.

For comparison, **4a** and **5a** were isolated from soybean meal [9]. Compound **6a** was prepared by addition of ethereal CH_2N_2 to a methanolic soln of **4a**. Aglycones were prepared from the corresponding glycoside by hydrolysis in 1 N H_2SO_4 at 100°. TMS derivatives of the aglycones were prepared using Tri-Sil Z (Applied Sciences).

Si gel TLC (Analtech, GF-254) (mobile phase: cyclohexane–EtOAc, 1:1) of aliquots of Et_2O fractions gave a fluorescence-quenching zone at R_f 0.75 corresponding to **1–3** and yellow fluorescent zones at R_f 0.53 and 0.67 corresponding to **4b** and **6b**, respectively. Leaf isoflavonoids **1–3**, **4b** and **6b** were indistinguishable from authentic standards when compared by 2D-Si gel TLC (cyclohexane–EtOAc, 1:1, × MeOH–CH_2Cl_2, 1:49).

Zones corresponding to **1–3**, **4b** or **6b** obtained after 1D-Si gel TLC (mobile phase: cyclohexane–EtOAc, 1:1) were scraped from the plate and the compounds eluted with MeOH. The MeOH extracts were analysed by HPLC on a μPorasil (10 μm) column eluting with *iso*-PrOH in *n*-hexane (7:93). *A* at 285 nm (for **1–3**) or 260 nm (for **4b** and **6b**) was monitored. Elution times for **6b**, **1**, **2**, **3** and **4b** were *ca* 4.8, 5.1, 5.4, 5.7 and 8.6 min, respectively. Quantification was done by comparison of peak areas for standards and leaf samples.

Glucoside characterization. The glucosides were tentatively identified by TLC co-chromatography with authentic samples of **4a**, **5a** and **6a**. Individual glucosides were then isolated by prep. TLC and further characterized. Compound **4a**: R_f = daidzin, UV λ_{max}^{EtOH} nm: 250; aglycone R_f = daidzein. Compound **5a**: R_f = genistin, UV λ_{max}^{EtOH} nm: 262; aglycone: R_f = genistein, GC/MS (TMS derivative) *m/z* 471 $[M-15]^+$, 414 $[B]^+$, 339, 192. Compound **6a**: R_f = ononin, UV λ_{max}^{EtOH} nm: 250; aglycone: R_f = formononetin, GC/MS (TMS derivative), *m/z* 340 $[B]^+$, 325 $[M-15]^+$, 268. Glucose was characterized by GC as the aldononitrile [26].

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