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QUANTIFICATION OF GLYCEOLLINS IN NON-ELICITED SEEDLINGS OF *GLYCINE MAX* BY GAS CHROMATOGRAPHY–MASS SPECTROMETRY

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Key Word Index—*Glycine max*: Fabaceae; phytoalexins; glyceollins; canescacarpin; quantification; GC-MS.

Abstract—A sensitive gas chromatography–mass spectrometry method is described for detection and quantification of small amounts of phytoalexins, especially glyceollins. This method allowed identification of canescacarpin (glyceollin V) besides appreciable amounts of glyceollin isomers I–III and glyceofuran in unchallenged parts of soybean seedlings. Based on this quantification method the glyceollin content of soybean roots, either untreated, wounded or incubated with a glucan elicitor from *Phytophthora sojae*, was compared. The proportions of glyceollin isomers in the mixture of compounds were determined in different soybean tissues. Single-ion monitoring revealed the presence of four additional glyceollin isomers with benzofuranoid structure.

INTRODUCTION

Glyceollins, phytoalexins from *Glycine max* (L.) Merr. and other species of the genus *Glycine* are produced in high concentrations in all tissues after treatment with a variety of biotic or abiotic elicitors [1–4]. Only traces of these phytoalexins have been identified so far in untreated or wounded plant tissue [5].

Only the glyceollin isomers I–III (**1**–**3**) have been isolated from *G. max* (L.) Merr., whereas glyceollin V (canescacarpin) (**4**) has been found in *G. canescens* F. J. Herm. and some cultivars of *G. clandestina* Wendl. and *G. tomentella* Hayata [6]. In these plants, **3** is missing [7]. Thus, the occurrence of both **3** and **4** in the same species has not been reported so far. Glyceofuran (**5**), a glyceollin derivative with low antimicrobial activity, has been isolated from leaves of soybean. It has been assumed to originate from **3** [8] (Fig. 1).

2-Dimethylallylglycinol (glyceocarpin) (**6**) is the biosynthetic precursor of the isomers **2** and **3**; **1** is formed from 4-dimethylallylglycinol (**7**) [9]. Benzopyranoid glyceollins (compounds **1** and **2**) were shown to be generated oxidatively by action of a microsomal cyclase [9]. Another cyclase obviously produces **3** with a benzofuranoid structure. Therefore, we expected that the same enzyme might catalyse also the formation of an analogous glyceollin with a benzofuran ring (**8**) which is derived from **7** (Fig. 2).

The developed sensitive GC–mass spectrometry (MS) method enabled us to detect and quantify phytoalexins in all parts of untreated soybean seedlings. Thus, we found **4** besides considerable amounts of **1**–**3** and **5**. However, unexpectedly, no **8** was detected.

RESULTS AND DISCUSSION

Ethyl acetate extracts of seven-day-old soybean seedlings [*G. max* (L.) Merr. cv Kalmit] neither treated with an elicitor nor wounded before work-up were separated by TLC (silica gel; chloroform–acetone–ammonium hydroxide, 50:50:1) according to the procedure described by Ingham *et al.* [8]. Glyceollins were detected on thin-layer chromatograms by their fluorescence-quenching properties. The corresponding zone was eluted, and the compounds were converted into their trimethylsilyl derivatives and investigated by GC and GC-MS. Mass spectrometry indicated the presence of four isomers of glyceollin.

Glyceollins with benzofuranoid and benzopyranoid structure can be distinguished by their mass spectra. Those with a benzopyranoid structure are identified by a base peak at mass 467 $[M\text{-}15]^+$ since a stable pyrilium ion is produced by expulsion of one of the two methyl groups in α-position to the pyrene oxygen. Reference compound measurements provided a distinction between **1** (R_I = 2755) and **2** (R_I = 2805). In contrast, the spectra of the other two isomers showed $M^+ = 482$ as a base peak whereas the ion of $[M\text{-}15]^+$ was of only minor intensity: the loss of a methyl group from the isomers with benzofuranoid structure is less favoured. Compound **3** (R_I = 2878) could be identified with a reference

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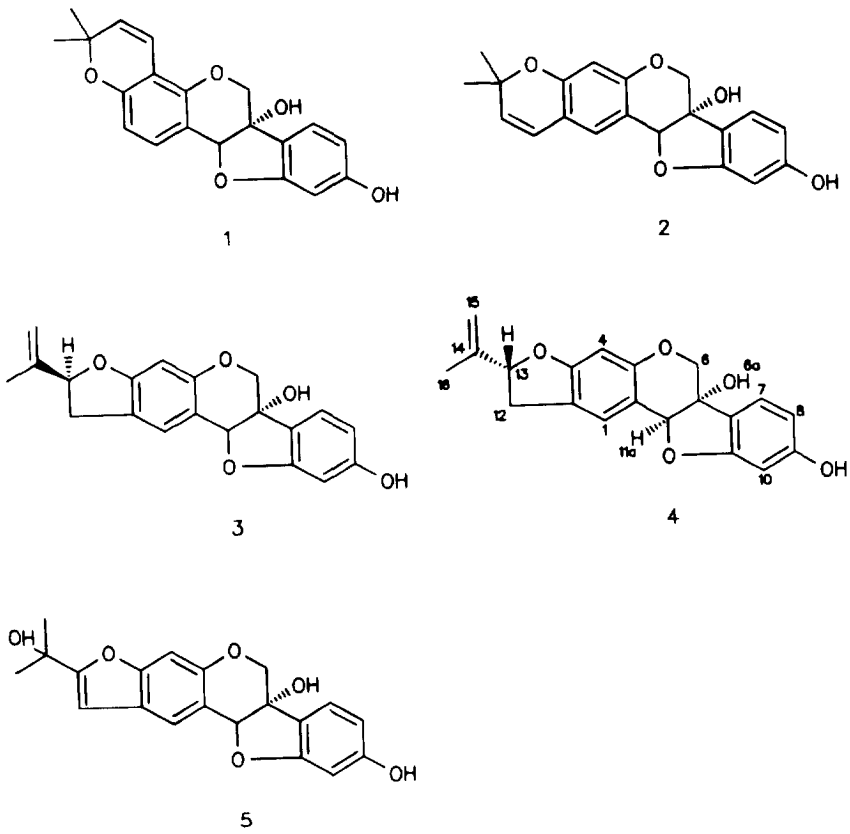

Fig. 1. Glyceollin isomers from *Glycine*.

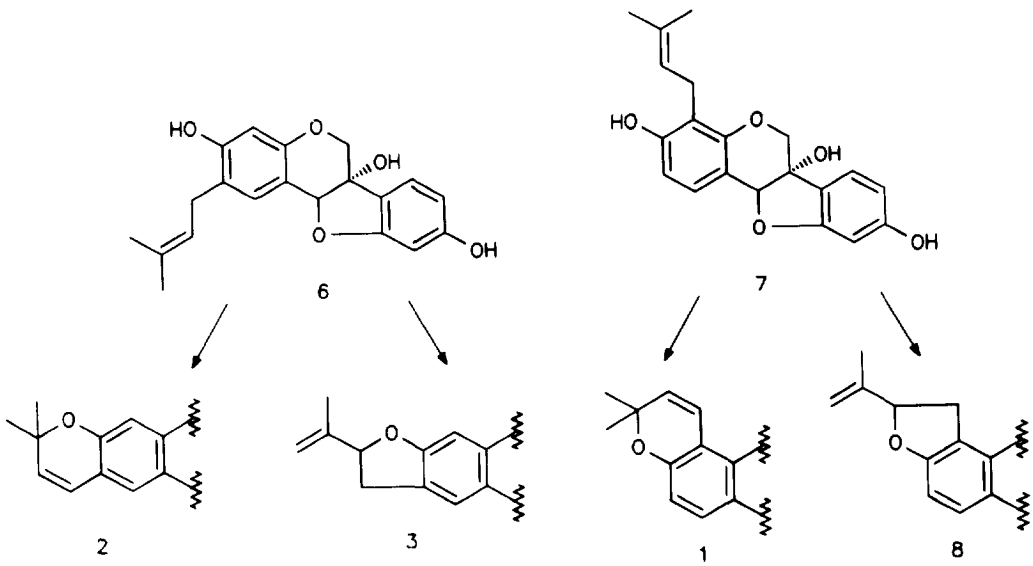

Fig. 2. Last step in glyceollin biosynthesis.

sample. The mass spectrum of the fourth isomer ($R_f = 2896$) indicated the presence of either **4** or the isomer **8**.

Distinction between the two possible structures, **4** and **8**, was achieved by ^{1}H NMR. The glyceollin mixture obtained by TLC was separated by reversed phase HPLC using gradient elution with water–acetonitrile. The unknown isomer was detected as a shoulder of the **2** peak in the HPLC chromatogram between the peaks of **2** and **3**. It was isolated after repeated preparative HPLC.

The data of the ^{1}H NMR spectrum (Table 1) obtained from the purified compound were in accordance with those partly published for **4** [6].

Enzymic cyclization of prenylated pterocarpans has been shown to result in the formation of **1**–**3** [9]. It is not yet known whether formation of **8** would require an additional cyclase isoenzyme which is not expressed in soybean.

Compound **5**, glycinol (**9**) and **6** were also identified by GC-MS of their trimethylsilyl derivatives (See Experimental). The total content of glyceollin isomers and **5** was determined by GC using 4-hydroxydiphenyl ether as an internal standard with comparable polarity.

Surprisingly the glyceollin content of one-week-old untreated seedlings of *G. max* cv Kalmit (22.5 $\mu g\,g^{-1}$ fr. wt) proved to be much higher than described in the literature [10] using another highly sensitive method based on polyclonal antibodies. Therefore, the content of phytoalexins in various tissues of the soybean seedlings was reinvestigated.

While the content of glyceollin isomers was nearly constant in all tissues of cv 9007 (roots 6.0 $\mu g\,g^{-1}$ fr. wt, hypocotyls 9.0 $\mu g\,g^{-1}$ fr. wt, cotyledons 4.6 $\mu g\,g^{-1}$ fr. wt, leaves 2.6 $\mu g\,g^{-1}$ fr. wt) and comparable to other results [10], in cultivar Kalmit similar glyceollin contents were only found in roots (4.5 $\mu g\,g^{-1}$ fr. wt), hypocotyls (2.9 $\mu g\,g^{-1}$ fr. wt) and leaves (1.0 $\mu g\,g^{-1}$ fr. wt). In contrast a rather high content of glyceollins was detected in untreated cotyledons of cv Kalmit (55.8 $\mu g\,g^{-1}$ fr. wt). This exceptional result could indicate that the cotyledons of the investigated batch of cultivar Kalmit were in a prestressed state. The reason(s) for this preinduction of phytoalexins is (are) unknown.

The possibility that the rather high glyceollin levels might have resulted during the extraction procedure of the seedlings was excluded. Mixing and extraction of the plant material in either hot or cold methanol led to the same results. This showed that the comparatively high glyceollin levels pre-existed in cotyledons of soybean cv Kalmit.

The proportions of glyceollin isomers within the whole plant, as well as in different tissues of the plant were also determined by GC (Table 2). Thus, both quantification of glyceollins and determination of their proportions can be done within the same measurement. An additional HPLC step for separation is not necessary.

Our investigations on the proportions of glyceollin isomers in untreated plants of the cultivar 9007 and the cultivar Kalmit (Table 2), respectively, led to similar results as already published by Keen *et al.* [7] for elicited soybean seedlings. Compound **1** accumulated in roots and hypocotyls, whereas in cotyledons the amounts of **1** and **3** were comparable. In leaves of soybean, **3** was the dominating compound. A similar distribution was observed for **4**.

Compound **5** was found in all parts of the investigated seedlings. However, in cv 9007 its content was remarkable only in cotyledons. The comparable increase in the content of **5** and **3** from roots to leaves corroborates the assumption of ref. [8] that **5** is a metabolite of **3**.

In order to compare the glyceollin content in elicited and non-elicited tissues, roots of non-injured plants, wounded roots from which the tips were cut off, and roots treated with a crude glucan elicitor fraction were investigated (Table 3). While wounding alone had a minor effect on the level of the glyceollin isomers, treatment with a glucan elicitor fraction resulted in an enhanced production of glyceollins. Again, the proportions of the glyceollin isomers followed the typical distribution for root tissue (see Table 2).

Applying single-ion monitoring—a GC-MS method that allows the identification of traces of compounds in a crude extract by scanning only single fragment ions in a spectrum—besides the isomers **1**–**3** and **4**, four additional isomers were detected in soybean plants. They showed mass spectra identical to those of the known glyceollins with a benzofuranoid structure. The trace amounts excluded their isolation. These isomers prob-

Table 1. ^{1}H NMR data for glyceollin III (**3**) and glyceollin V (**4**) measured in Me_2CO-d_6

	Glyceollin V	Glyceollin III
Proton	δ J (Hz)	δ J (Hz)
H-1	7.24 *s*	7.21 *s*
H-4	6.23 *s*	6.23 *s*
H-6	4.01 *d* 11.3	4.01 *d* 11.3
H-6′	4.10 *d* 11.3	4.10 *d* 11.3
H-7	7.19 *d* 8.1	7.19 *d* 8.1
H-8	6.41 *dd* 2.1, 8.1	6.41 *dd* 2.1, 8.1
H-10	6.23 *d* 2.1	6.23 *d* 2.1
H-11a	5.26 *s*	5.26 *s*
H-12	2.98 *m*	2.98 *m*
H-12′	3.35 *m*	3.35 *m*
H-13	5.21 *t* 8.6	5.21 *t* 8.6
H-15	4.88 *m*	4.83 *m*
H-15′	5.05 *m*	5.03 *m*
H-16	1.75 *m*	1.73 *m*

Table 2. Proportions of glyceollin isomers in various tissues of *G. max*

	Glyceollin isomers %			
Plant part	I	II	III	V
Kalmit				
Roots	91.4	1.8	6.8	tr*
Hypocotyls	79.5	5.1	15.4	tr*
Cotyledons	50.5	10.0	38.8	0.7
Leaves	32.9	10.6	56.5	tr*
9007				
Roots	86.3	7.0	6.7	tr*
Hypocotyls	86.6	7.0	6.4	tr*
Cotyledons	42.3	14.2	43.3	0.2
Leaves	32.3	13.0	54.5	0.2

*Traces (< 0.1%).

Table 3. Accumulation of glyceollin isomers in roots of *G. max* cv Kalmit and cv 9007 in response to a glucan elicitor or wounding

	Glyceollin isomers $\mu g\,g^{-1}$ fr. wt				
	I	II	III	V	Total
9007 (g.e.)*	40.00	1.00	1.50	0.50	43.00
9007 (w)†	6.18	0.13	0.15	tr‡	6.46
9007 (H_2O)§	0.59	0.01	tr‡	tr‡	0.60
Kalmit (g.e.)*	6.00	0.80	1.20	tr‡	7.20
Kalmit (w)†	2.80	0.03	0.07	tr‡	2.90
Kalmit (H_2O)§	0.55	0.01	0.04	tr‡	0.60

*Root tips were cut off and treated with glucan elicitor.
†Root tips were cut off and treated with sterile water.
‡Traces, less than 0.01 $\mu g\,g^{-1}$.
§Unwounded roots were treated with sterile water.

ably differ from **3** and **4** in the steric configuration at the positions 6a and 11a. The R_I values of these compounds are higher (3041, 3078, 3233 and 3329, respectively) than those of glyceollin III. That means that the molecules are less volatile in GC. A structure with 6a*S*, 11a*R* or 6a*R*, 11a*S* configuration would be more planar than those with the 6a*S*, 11a*S* configuration of **1–3** and **4**. This would cause lower volatility and higher R_I values. A glyceollin isomer with structure **8** would give a similar mass spectrum, as well as isomers with 6a*R*, 11a*R* configuration.

EXPERIMENTAL

GC-MS. Measurements were performed on a DB-1 fused silica capillary column (length: 30 m; i.d.: 0.32 mm; film thickness: 0.1 μm; carrier gas: H_2, 2 ml min^{-1}; temp. programme: 80–280° at 3° min^{-1}. The GC was coupled to a double focusing mass spectrometer running under EI conditions at 70 eV. For single-ion monitoring of glyceollin isomers the ions of mass 482 $[M]^+$ and 467 $[M - Me]^+$ were selected.

GC. The column conditions for analyt. GC were the same. Detector: FID; injector temp.: 270°; detector temp.: 290°; split ratio 1:30. Retention indices were calculated according to Kováts [11] with *n*-alkanes C_{10}–C_{30} as ref. compounds.

Trimethylsilylation. A 0.3 mg sample was dissolved in 10 μl THF (purified and dried) and 20 μl MSTFA added. The mixt. was allowed to stand at room temp. for 12 hr. 1 μl of the mixt. was then subjected to GC and GC-MS.

TLC. This was carried out using 0.75 mm layers of Silica gel 60 PF_{254} ($CHCl_3$–Me_2CO–NH_4OH, 50:50:1 [8]). TLC zones were eluted with EtOAc. Samples of 10 mg were sepd.

HPLC. This was performed on a Spherisorb ODS II 5 μm column (250 × 8 mm) with a solvent flow rate of 2 ml min^{-1} using UV detection at 254 nm.

Plant material. Soybean seeds were obtained from KWS, Einbeck, Germany (cv Kalmit) and from Pioneer, Buxtehude, Germany (cv 9007). They were surface sterilized by washing with 5% NaOCl for 5 min and grown on moist vermiculite at 25°. Seedlings were harvested after 7 days. For analysis of the leaves, plants were grown for 12 days.

Induction of phytoalexins. Prepn of glucan elicitor from cell walls of *Phytophthora sojae* was done as described [12]. Etiolated soybean plants, grown for 3 days at 25° in the dark, were incubated in sterile H_2O or 200 μg glucan elicitor per ml sterile H_2O. For wounded or glucan treated plants 1 cm of the root tips were cut off before incubation. For glyceollin isolation, roots were harvested after standing for 24 hr at 25° in the dark.

Isolation of glyceollin isomer mixture. Complete plants (100 g fr. wt) were homogenized in 1 l EtOH with a commercial Waring Blendor at room temp., allowed to stand for 4 hr, centrifuged and the residue re-extracted twice with 300 ml MeOH. The combined supernatants were evapd to dryness, resuspended in 100 ml MeOH–H_2O (1:1) and extracted with 2 × 80 ml cyclohexane to remove non-polar compounds. The MeOH–H_2O layer was evapd to dryness, resuspended with 100 ml H_2O and extracted with 3 × 80 ml EtOAc. The organic layer was washed with 2 × 50 ml H_2O, dried over Na_2SO_4 and concd *in vacuo*. TLC (silica gel; $CHCl_3$–Me_2CO–NH_4OH, 50:50:1 [8]) provided 10 mg of a crude mixt. of 4 glyceollin isomers at R_f = 0.69–0.75. The sample was trimethylsilylated and investigated by GC and GC-MS.

Glyceollin I (**1**). GC: R_I = 2755; GC-MS 70 eV, *m/z* (rel. int.): 482 $[M]^+$ (36), 467 $[M - Me]^+$ (100), 393 (7), 392 (15), 378 (2), 377 (19).

Glyceollin II (**2**). GC: R_I = 2805; GC-MS 70 eV, *m/z* (rel. int.): 482 $[M]^+$ (39), 467 $[M - Me]^+$ (100), 393 (6), 392 (11), 378 (7), 377 (21).

Glyceollin III (**3**). GC: R_I = 2878; GC-MS 70 eV, *m/z* (rel. int.): 482 $[M]^+$ (100), 467 $[M - Me]^+$ (12), 393 (11), 392 (22), 378 (4), 377 (13).

Glyceollin V (**4**). GC: R_I = 2896; GC-MS 70 eV, *m/z* (rel. int.): 482 $[M]^+$ (100), 467 $[M - Me]^+$ (13), 393 (13), 392 (26), 378 (5), 377 (15).

Isolation of **4**. Isolation of **4** was achieved by sepn of the isomers by HPLC using H_2O–acetonitrile gradient

elution from 40 to 60% acetonitrile in 16 min. Individual glyceollins were collected (R_t values for **1**–**4**: 12.5–12.8, 12.2–12.5, 11.6–12.0, 12.0–12.2 min, respectively). The fr. containing **4** was rechromatographed twice to yield 200 μg of **4**. ^{1}H NMR (500 MHz, Me_2CO–d_6, TMS): δ = 1.75 (3H, *m*, $-Me_{16}$), 2.98 (1H, *m*, H_{12}), 3.35 (1H, *m*, $H_{12'}$), 4.01 (1H, *d*, $J_{6,6'}$ = 11.3 Hz, H_6), 4.10 (1H, *d*, $J_{6',6}$ = 11.3 Hz, $H_{6'}$), 4.88 (1H, *m*, H_{15}), 5.05 (1H, *m*, $H_{15'}$), 5.21 (1H, *t*, $J_{13,12}$ = 8.6 Hz, H_{13}), 5.26 (1H, *s*, H_{11}), 6.23 (1H, *d*, $J_{10,8}$ = 2.1 Hz, H_{10}), 6.23 (1H, *s*, H_4), 6.41 (1H, *dd*, $J_{8,7}$ = 8.1 Hz, $J_{8,10}$ = 2.1 Hz, H_8), 7.19 (1H, *d*, $J_{7,8}$ = 8.1 Hz, H_7), 7.24 (1H, *s*, H_1).

MS data for TMSi derivatives of **5**, **9** *and* **6**. These were obtained from GC-MS measurements of the EtOAc extracts.

Glyceofuran (**5**). GC: R_I = 3073; GC-MS 70 eV, *m/z* (rel. int.): 570 $[M]^+$ (100), 555 $[M - Me]^+$ (75), 481 (33), 480 (21), 466 (13), 391 (13), 390 (14).

Glycinol (**9**). GC: R_I = 2598; GC-MS 70 eV, *m/z* (rel. int.): 488 $[M]^+$ (100), 487 (12), 473 $[M - Me]^+$ (7), 398 (18).

Glyceocarpin (**6**). GC: R_I = 2873; GC-MS 70 eV, *m/z* (rel. int.): 556 $[M]^+$ (100), 541 $[M - Me]^+$ (7), 487 (5), 468 (9), 467 (11), 466 (20), 375 (10).

Quantifications. The absolute content of the glyceollin isomers in various tissues of the plants was determined by GC of the TMSi derivatives of EtOAc extracts. 4-Hydroxydiphenyl ether (R_I = 1786) was added as int. standard to the homogenate before work-up. Peak areas were integrated. The response factor of glyceollin and standard was 0.87. Quantifications were performed at least 3 × to ensure reproducibility. The proportions of the glyceollin isomers were determined by GC of the TMSi derivatives of the same EtOAc extracts. Peak areas were integrated and the concns of the compounds expressed as percentage of the total glyceollin peak area.

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