Phytoalexin Accumulation in *Ornithopus sativus* as a Response to Elicitor Treatment

K. Seifert
Lehrstuhl für Organische Chemie I/2 NW II, Universität Bayreuth, Universitätsstraße 30, D-95447 Bayreuth, Bundesrepublik Deutschland

S. Härtling, A. Porzel, S. Johne
Institut für Pflanzenbiochemie Halle, Weinberg 3, D-06120 Halle/Saale, Bundesrepublik Deutschland

G. Krauß
Umweltforschungszentrum Leipzig-Halle GmbH, Sektion Hydrogeologie, Hallesche Straße 44, D-06246 Bad Lauchstädt

Z. Naturforsch. **48 c,** 550–555 (1993); received January 11/May 5, 1993

Ornithopus sativus, Fabaceae, *Colletotrichum trifolii,* Phytoalexin, Glabridin

An isoflavanoid derivative has been isolated as a phytoalexin from intact plants and cell suspension cultures of *Ornithopus sativus* after treatment with $CuSO_4$, yeast elicitor, or a spore suspension of *Colletotrichum trifolii.* Based on its physical and spectroscopic properties the substance was identified as glabridin. After induction, the transient increase in the content of this phytoalexin was preceded by a transient increase in the activities of PAL and CHS.

Introduction

Disease resistance in plants depends on multiple defense mechanisms for which the concept of coordinated defense has been proposed [1, 2]. One common type of inducible defense response detected in plants is their ability to form low-molecular-weight antifungal compounds (phytoalexins) when brought into contact with a potentially pathogenic microorganism or an abiotic elicitor (UV irradiation, heavy metal salts etc.).

Ornithopus sativus Brot. (Fabaceae) is used as fodder plant in Central Europe. Anthracnose of *O. sativus,* caused by *Colletotrichum trifolii* Bain & Ess. (Deuteromycetes) is a highly destructive disease responsible for yield and quality reductions. No studies have been directed to biochemical aspects of the interaction between *O. sativus* and *C. trifolii.* This report describes the response of plants and cell suspension cultures of *O. sativus* to elicitor treatment with $CuSO_4$, yeast polysaccharides, or spores of *C. trifolii.* We also report on the isolation and characterization of the phytoalexin glabridin (**1 a**) and its rates of accumulation in relation to the activities of PAL and CHS.

Abbreviations: PAL, phenylalanine ammonia-lyase; CHS, chalcone synthase; TLC, thin layer chromatography; HPLC, high performance liquid chromatography; NOE, nuclear Overhauser effect.

Reprint requests to Prof. K. Seifert.

Verlag der Zeitschrift für Naturforschung, D-72072 Tübingen
0939–5075/93/0700–0550 $ 01.30/0

Materials and Methods

Biological material

Colletotrichum trifolii isolates were obtained from Centralbureau voor Schimmelcultures, Baarn, The Netherlands, and maintained on malt-extract-agar (Serva) at 27 °C. The cultures were transferred at intervals of 4 weeks. The spores were isolated from 3-week old cultures by flooding with distilled H_2O. For the suspension cultures, a medium (pH 5–6) supplemented with 15 g sucrose, 5 g casein hydrolysate, 1 g KH_2PO_4, 250 mg $MgSO_4$, and 27 mg $FeCl_3$, per litre was used.

For aerial tissue assays, seeds of *Ornithopus sativus* (a voucher specimen of *O. sativus* was deposited in the herbarium of the Institute for Plant Biochemistry Halle) were sown in soil and seedlings grown for 40–50 days in a greenhouse at 15–20 °C. The plants were sprayed with 0.05 M $CuSO_4$ solution (10 ml/60 g plant material) or a spore suspension of *C. trifolii* (15 ml/60 g plant material; 1 ml contains 1×10^7 spores).

Cell suspension cultures were obtained from stem-derived callus cultures of sterile *O. sativus* seedlings. They were grown in 250 ml conical flasks containing 30 ml of a modified Murashige and

Skoog medium [3] supplemented with sucrose (3%), Ca-pantothenate (5 mg/l), inositol (80 mg/l), 2,4-dichlorophenoxyacetic acid (1 mg/l) and kinetin (0.2 mg/l) on rotary shakers (80 rpm) at 27°C either in the dark or under illumination (2000 lux provided by white fluorescent tubes). The pH of the medium was adjusted to 5.6–5.8. Transfer of cells (10 ml) to fresh medium (30 ml) was performed every 10–11 days.

Induction experiments

Inoculation of the cell suspension cultures of *O. sativus* was carried out with 0.5 ml spore suspension (1 ml contains 10^6 spores) of *C. trifolii* or yeast polysaccharide [4] (5 g of 6-day old cells were transferred to 30 ml of fresh medium and incubated with 20 mg of yeast elicitor from *Saccharomyces* cerevisiae). Cells were harvested 3–48 h after induction and were frozen in liquid nitrogen.

The preparation of the yeast glucan elicitor was performed according to Schumacher *et al.* [4]. The final preparation was lyophilized and stored as a dry powder until used. For elicitation, this powder was dissolved in H_2O, autoclaved for 20 min and applied under sterile conditions to the cell cultures.

Antifungal bioassay

The glabridin containing fractions were subjected to TLC in $CHCl_3$–MeOH (9:1), the plates then sprayed with a spore suspension of *Cladosporium cucumerinum* (obtained from the firm Fahlberg-List Magdeburg), and incubated at 25 °C in the dark for 2 days. The fungitoxic compounds on the TLC plates were visible as white spots bare of mycelium in a dark grey layer of the mycelium covering the plates [5].

Tissue extraction and enzyme assays

Crude cell extracts were obtained by stirring frozen cells of *O. sativus* (2 g) for 5 min on ice with 4 ml of 0.1 M Tris-HCl buffer pH 7.5 containing 10% glycerol, 14 mM 2-mercaptoethanol in the presence of 200 mg Dowex 1×2, which had been equilibrated with 0.1 M Tris-HCl buffer pH 7.5. The slurry was centrifuged for 15 min at 28 000×*g* and 4 °C. The supernatant was used as crude enzyme extract to measure the activities of PAL [6], CHS [7] and protein content [8].

Product analysis

General

Mps: corr. ^1H NMR spectra were recorded at 300 MHz and 500 MHz, and ^{13}C spectra at 75.5 MHz and 125 MHz. The mass spectra were measured at 16 eV and 70 eV. Column chromatography was carried out on silica gel 60 (0.063–0.2 mm), TLC on silica gel sheets (1 mm, PF_{254}) and HPLC on LiChrosorb (100×4) RP 18 or LiChrospher (100×4) RP 18. Methylation was done with CH_2N_2 and acetylation with pyridine–Ac_2O.

Isolation of glabridin (**1a**)

Aerial parts of *O. sativus,* harvested at various times (untreated or after treatment with 0.05 M $CuSO_4$ solution or with a spore suspension of *C. trifolii*) were homogenized (Ultra Turrax) in MeOH. After filtering, the solvent was evaporated to dryness at 40 °C under reduced pressure. The residue was suspended in H_2O and extracted (×4) with $CHCl_3$. The $CHCl_3$ phases were combined, evaporated and the residue chromatographed on a silica gel column in $CHCl_3$ and $CHCl_3$–MeOH (49:1–9:1), or on prep. TLC in $CHCl_3$–MeOH (9:1), Rf 0.40. HPLC LiChrosorb (250×4.6) RP 18 MeOH–H_2O (3:1), 1 ml×min^{-1}, R_T 7.32. HPLC LiChrospher (250×4.6) RP 8 MeOH–H_2O (7:3), 1 ml×min^{-1}, R_T 9.66. Prep. HPLC LiChrosorb (100×4) RP 18 or LiChrospher (100×4) RP 8, MeOH–H_2O (3:1), UV-detection at 280 nm yielded pure glabridin (**1a**). The quantitative determination of **1a** was performed by HPLC under the same conditions. The isolation of **1a** from cell suspension cultures was carried out in the same way.

Glabridin (**1a**)

Gibbs test on TLC: + violet.

M.p. 155 °C; $[\alpha]_D^{23}$ + 10° ($CHCl_3$, c = 0.25); ref. [9]. M.p. 154–155 °C, $[\alpha]_D^{20}$ + 8.2° ($CHCl_3$); UV λ_{max}^{MeOH} nm (log ε): 281 (4.07), 287 (4.04), 312 (3.45); IR $\nu_{max}^{CHCl_3}$ cm^{-1}: 3270, 2980, 2940, 1625, 1611, 1587, 1526, 1480, 1375, 1195, 1175; MS 70 eV *m/z* (rel. int.): 324 $[M]^+$ (28), 309 (100), 187 (18), 173 (50).

Glabridin dimethylether (**1b**)

M.p. 110 °C; ref. [9]. M.p. 109–111 °C; MS 70 eV *m/z* (rel. int.): 352 $[M]^+$ (42), 337 (100), 173 (57), 164 (16), 151 (28).

Glabridin diacetate (**1c**)

M.p. 164 °C; ref. [9]. M.p. 164–166 °C; MS 70 eV *m/z* (rel. int.): 408.1572 [(calcd. for $C_{24}H_{24}O_6$ 408.1573 $[M]^+$ (10)], 393 (100), 173 (14), 43 (9).

Isolation of phaseollinisoflavan (**2a**)

Seeds (800 g) of *Phaseolus vulgaris* L. were imbibed in distilled H_2O for 2 h in the dark. Subsequently the seeds were soaked in 1 mM $AgNO_3$ solution for 2 h, rinsed with distilled H_2O and incubated in the dark at 25 °C and 90% rel. humidity for 5 days [10]. Germinating seeds were homogenized (Ultra Turrax) in EtOH and extracted with EtOH. The crude extract was evaporated to dryness under reduced pressure and chromatographed on a silica gel column in $CHCl_3$ and $CHCl_3$–MeOH (19:1–17:3). Further purification was carried out by prep. TLC in toluene –$CHCl_3$– Me_2CO (9:5:7), R_f 0.52 and gave 230 mg **2a;** TLC in $CHCl_3$–MeOH (9:1), R_f 0.46.

Phaseollinisoflavan (**2a**)

Gibbs test on TLC: + deep blue.

UV λ_{max}^{EtOH} nm (log ∈): 281 (4.04), 310 sh (3.25); ref. [11] UV λ_{max}^{EtOH} nm (log ∈): 280 (4.01), 310 sh (3.27); MS 70 eV *m/z* (rel. int.): 324.1371 [calcd. for $C_{20}H_{20}O_4$ 324.1370 $[M]^+$ (17)], 309 (50), 187 (22).

Phaseollinisoflavan dimethylether (**2b**)

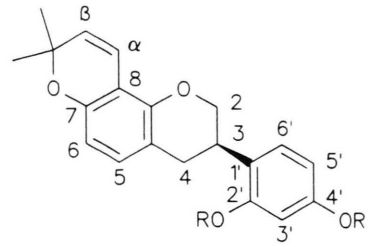
MS 16 eV *m/z* (rel. int.): 352 $[M]^+$ (68), 337 (90), 216 (60), 201 (100), 185 (28).

Phaseollinisoflavan diacetate (**2c**)

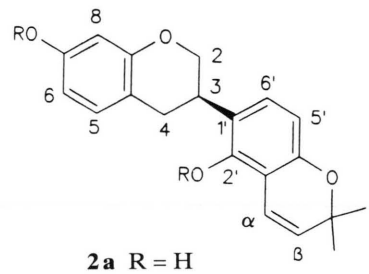
MS 70 eV *m/z* (rel. int.): 408 $[M]^+$ (14), 393 (26), 352 (12), 351 (100), 309 (8), 187 (20), 185 (10).

Results and Discussion

Bioassay-directed fractionation of the methanolic extracts from *O. sativus* plants which had been treated with $CuSO_4$ solution or inoculated with a spore suspension of *C. trifolii,* resulted in the isolation of an antifungal compound **1a,** which completely inhibited the germination of *Cladosporium cucumerinum* conidia. The same compound was also obtained after elicitation of cell suspension cultures of *O. sativus* with yeast polysaccharide or spores of *C. trifolii.*



1a R = H
1b R = Me
1c R = Ac



2a R = H
2b R = Me
2c R = Ac

Due to the fact that the unequivocal discrimination between glabridin (**1a**) and phaseollinisoflavan (**2a**) based on IR-, UV- and ^1H NMR-spectra (Table I) was difficult, and since detailed ^{13}C NMR data have not yet been published, **2a** was isolated from germinated seeds of *Phaseolus vulgaris* L. elicited with $AgNO_3$ solution. The UV-, along with the mass spectra of **1a** and **2a** suggest that the compounds should be isoflavans [12, 13].

The mass spectra of isoflavans such as glabridin (**1a**) and phaseollinisoflavan (**2a**) are characterized by loss of a geminal methyl group (*m/z* 309). The retro Diels-Alder fragmentation of the ion at *m/z* 309 leads to *m/z* 173 (**1a**) and *m/z* 187 (**2a**). Striking differences in the mass spectra of glabridin diacetate (**1c**) and phaseollinisoflavan diacetate (**2c**) were observed. The mass spectrum of **1c** showed the ion *m/z* 393 $[M-Me]^+$ and the spectrum of **2c** the ion *m/z* 351 $[M-Me-CH_2{=}C{=}O]^+$ as base peak.

The assignments of the ^1H NMR signals for compounds **1a, 1b** and **2a–2c** (Table I) were based on

Table I. ^{1}H NMR spectral data of compounds **1a***, **1b**$^+$, **2a***, **2b***, and **2c***, δ [ppm], *J* [Hz].

H	**1a** (DMSO-D_6)	**1b** ($CDCl_3$)	**2a** (DMSO-D_6)	**2b** ($CDCl_3$)	**2c** ($CDCl_3$)
2_{ax}	3.92 dd (10.2/10.2)	3.98 dd (10.2/10.2)	3.89 dd (10.2/10.2)	3.96 dd (10.5/10.5)	3.92 dd (10.6/10.6)
2_{eq}	4.22 ddd (10.2/3.3/1.9)	4.34 m	4.13 br d (10.2)	4.28 ddd (10.5/3.5/1.7)	4.25 ddd (10.6/3.5/1.8)
3		3.56 m	3.43 m	3.52 m	3.16 m
4_{ax}	2.88 dd (15.8/11.7)	2.96 dd (15.6/10.9)	2.82 dd (15.3/11.1)	ca. 2.9	ca. 2.9
4_{eq}	2.68 ddd (16.0/3.8/1.9)	2.83 ddd (15.6/5.0/1.7)	2.71 dd (15.3/4.3)	ca. 2.9	ca. 2.9
5	6.82 d (8.1)	6.82 d (8.1)	6.85 d (8.2)	6.98 d (8.4)	7.06 d (8.9)
6	6.27 d (8.1)	6.36 d (8.2)	6.28 dd (8.1/2.4)	6.48 dd (8.6/2.6)	6.61 dd (8.9/2.3)
8	–	–	6.18 d (2.3)	6.44 d (2.5)	6.59 d (2.3)
3'	6.31 d (2.4)	6.49 d (2.4)	–	–	–
5'	6.17 dd (8.3/2.4)	6.46 dd (8.2/2.4)	6.26 d (8.3)	6.58 d (8.3)	6.71 d (8.5)
6'	6.85 d (8.6)	7.03 d (8.3)	6.84 d (8.3)	6.87 d (8.3)	6.93 d (8.5)
α	6.52 d (9.9)	6.65 d (9.9)	6.72 d (10.0)	6.58 d (10.0)	6.24 d (10.0)
β	5.64 d (9.9)	5.56 d (9.9)	5.66 d (10.0)	5.66 d (10.0)	5.67 d (10.0)
Me	1.33 s	1.43 s	1.32 s	1.43 s	1.43 s
Me	1.32 s	1.41 s	1.32 s	1.43 s	1.43 s
7-OR	–	–	9.14 s	3.77 s	2.29 s
2'-OR	9.38 s	3.81[a]s	8.91 s	3.75 s	2.35 s
4'-OR	9.11 s	3.80[a]s	–	–	–

* 300 MHz. $^+$ 500 MHz. [a] Assignments may be reversed.

the coupling patterns and chemical shift data. The position of the two hydroxy groups of glabridin (**1a**) and phaseollinisoflavan (**2a**) could be estimated using NOE difference spectra. Irradiation of the hydroxy signal of **1a** at 9.11 ppm caused an enhancement of the doublet at 6.31 ppm (J = 2.4 Hz, H-3') and of the double doublet at 6.17 ppm (J = 8.3/2.4 Hz, H-5'). Irradiation of the other hydroxy group at 9.38 ppm also gave an NOE for the doublet at 6.31 ppm.

In DMSO-D_6 solution the ^{1}H NMR spectrum of **2a** contained two hydroxy signals at 9.14 and 8.91 ppm. Irradiation at 9.14 ppm enhanced the double doublet at 6.28 ppm (J = 8.1/2.4 Hz, H-6) and the doublet at 6.18 ppm (J = 2.3 Hz, H-8), whereas irradiation at 8.91 ppm resulted in a positive NOE for the doublet at 6.72 ppm (J = 10 Hz, H-α). Thus the hydroxy groups have to be at C-7 and C-2', respectively. In a similar manner 7-OMe and 2'-OMe for **2b** and 7-OAc and 2'-OAc for **2c** were distinguished by NOE differences experiments.

The known assignments of the proton signals of **1a** and **2a** were transferred to carbons with attached protons using a one-bond heteronuclear chemical shift correlation 2D NMR experiment. By a proton detected multiple-bond ^{1}H ^{13}C correlation spectrum (HMBC) all quaternary carbon signals could be assigned. Thus, the unequivocal assignment of ^{13}C resonances in **1a** and **2a** was achieved (Table II). All the data for **1a** are consistent with the structure being that of glabridin, previously isolated from the roots of *Glycyrrhiza glabra* L. [9] and *Glycyrrhiza glabra* L. var typica [14]. The correlations for phaseollinisoflavan (**2a**) between the two hydroxy proton signals and C-6/C-7/C-8 and C-1'/C-2'/C-3' respectively, are in agreement with the results of the above mentioned NOE difference spectra.

Table II. ^{13}C chemical shifts of compounds **1a*** and **2a**$^+$ in $CDCl_3$ δ [ppm].

C	**1a**	**2a**
2	69.7	69.3
3	30.9	31.0
4	29.9	30.1
4a	114.7	112.8
5	129.3	130.1
6	108.0	107.9
7	151.2	156.5
8	109.0	102.5
8a	149.2	154.6
1'	117.4	121.2
2'	155.8	150.2
3'	102.5	110.1
4'	156.8	151.8
5'	106.2	107.9
6'	127.5	126.8
α	116.4	117.2
β	129.1	129.0
Me_2C	75.2	74.9
Me	27.3	27.3
Me	27.2	27.3

* 75.5 MHz $^+$ 125 MHz

A time course study of glabridin (**1a**) accumulation was performed using *O. sativus* plants. The re-

sponses towards treatment with $CuSO_4$ solution or inoculation with spores of *C. trifolii* were significantly different. After treatment with the abiotic elicitor, glabridin accumulation reached a maximum 24 h after application. In obvious contrast, **1a** was increased 10-fold above the control 8 days after inoculation with spores of *C. trifolii* (Fig. 1). After that no further increase was recorded.

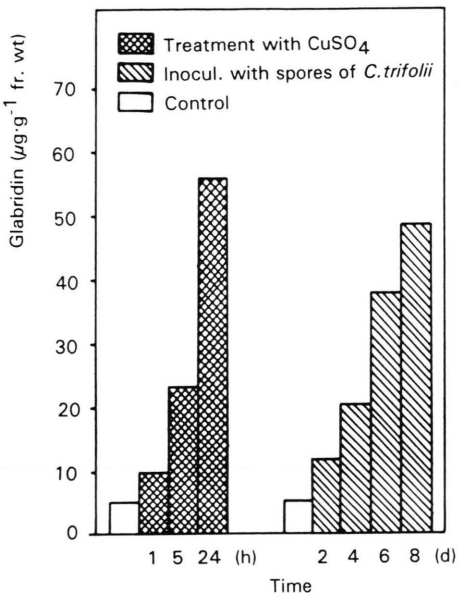


Fig. 1. Accumulation of glabridin (**1a**) in the aerial parts of *Ornithopus sativus*.

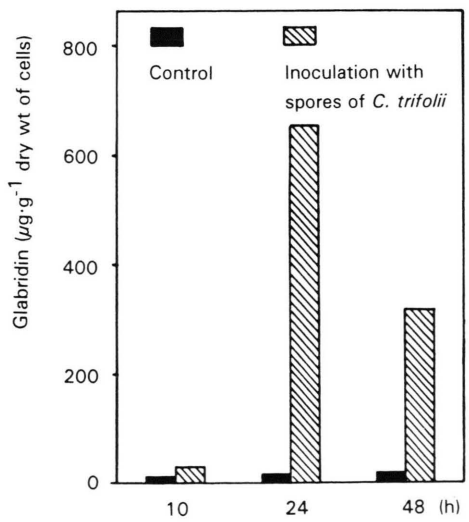


Fig. 2. Accumulation of glabridin (**1a**) in the cell suspension culture of *Ornithopus sativus*.

The application of spores of *C. trifolii* to a cell suspension culture of *O. sativus* led to a considerable accumulation of **1a** during the first 24 h. Decline in the level of **1a** after 48 h implies the possibility of metabolism of the phytoalexin by the microorganism and/or the plant tissue and/or excretion of **1a** into medium (Fig. 2).

The analysis of biosynthetic pathways leading to phytoalexin formation requires a detailed knowledge of enzymes involved. It has been demonstrated that isoflavanoid phytoalexin formation is associated with large transient increases in the activities of the enzymes of general phenylpropanoid metabolism. The results indicate that the activities of most

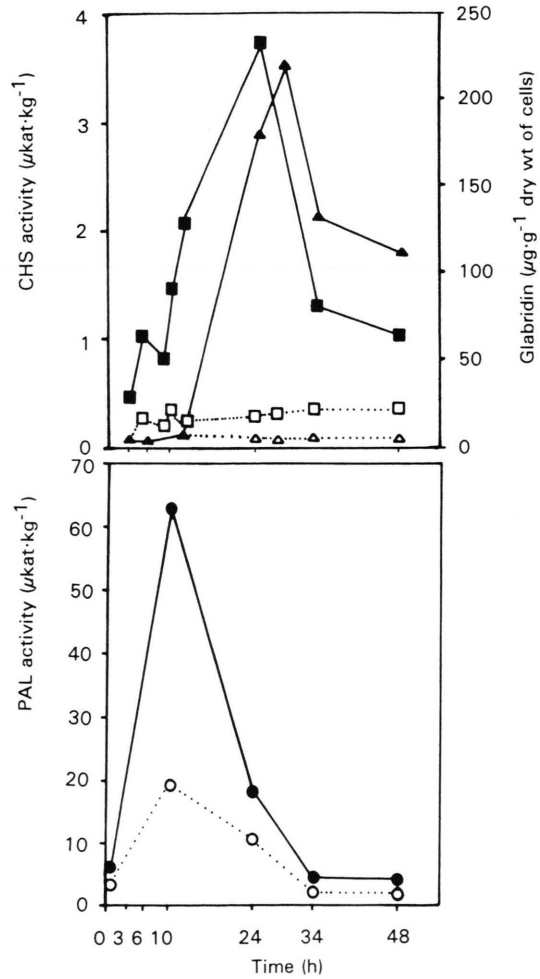


Fig. 3. Time course of elicitor-induced changes in the activity of PAL (●), CHS (■) and accumulation of glabridin (**1a**) (▲) in the cell suspension culture of *Ornithopus sativus*. Open symbols are controls (○), (□), (△).

of the enzymes related to phytoalexin biosynthesis are probably enhanced after elicitor treatment.

The biosynthesis of glabridin (**1a**) includes a number of reactions catalyzed by PAL, CHS, prenyltransferase etc. The assays of PAL and CHS activities during the accumulation of **1a** after elicitation with a polysaccharide from yeast [4] (Fig. 3) or spores of *C. trifolii* demonstrated a maximal increase of the PAL activity 10 h after inoculation. The highest CHS activity was detected after 24 h. The accumulation of **1a** was also monitored and the results indicated that the highest concentration of glabridin occurred 28 h after induction (Fig. 3). Thus there was a time shift between the increase in PAL and CHS and the appearance of glabridin. The results suggest that the regulation of glabridin biosynthesis in *O. sativus* is directly associated with changes in the of activities of PAL and CHS.

Acknowledgements

We thank Dr. G. Schneider for the HPLC-measurements, Mrs. Dr. B. Schumann for the cultivation of the fungus and Prof. Dr. J. Ebel for 4-coumaroyl-CoA and for constructive discussions.

[1] J. Ebel and H. Grisebach, Trends Biochem. Sci. **13,** 23 (1988).
[2] J. C. Horsfall and E. B. Cowling, eds. Plant Disease **Vol. 5,** Academic Press, New York 1980.
[3] T. Murashige and F. Skoog, Physiol. Plant. **15,** 473 (1962).
[4] H. M. Schumacher, H. Gundlach, M. Fiedler, and H. Zenk, Plant Cell Reports **6,** 410 (1987).
[5] D. Gottstein, D. Gross, and H. Lehmann, Arch. Phytopathol. Pflanzenschutz **20,** 111 (1984).
[6] A. Zimmermann and K. Hahlbrock, Arch. Biochem. Biophys. **166,** 54 (1975).
[7] J. Schröder, W. Heller, and K. Hahlbrock, Plant Sci. Lett. **14,** 281 (1979).
[8] M. M. Bradford, Anal. Biochem. **72,** 248 (1976).
[9] T. Saitoh, T. Kinoshita, and S. Shibata, Chem. Pharm. Bull. **24,** 752 (1976).
[10] P. Stössel and D. Magnolato, Experientia **39,** 153 (1983).
[11] R. S. Burden, J. A. Bailey, and G. W. Dawson, Tetrahedron Letters 1972, 4175.
[12] T. J. Mabry and K. R. Markham, in: The Flavonoids (J. B. Harborne, T. J. Mabry, and H. Mabry, eds.), p. 78, Chapman & Hall, London 1975.
[13] P. M. Dewick, in: The Flavonoids (J. B. Harborne and T. J. Mabry, eds.), p. 535, Chapman & Hall, London 1982.
[14] L. A. Mitscher, Y. H. Park, and D. Clark, J. Nat. Prod. **43,** 259 (1980).