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THE INDUCTION, BY FUNGAL INOCULATION, OF AYAPIN AND SCOPOLETIN BIOSYNTHESIS IN *HELIANTHUS ANNUUS*

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Key Word Index—*Helianthus annuus*; Compositae; sunflower; *Helminthosporium carbonum*; *Alternaria helianthi*; phytoalexin; non-host resistance; coumarins; ayapin; scopoletin.

Abstract—Inoculation of stem or leaf tissue of sunflower with the fungus *Helminthosporium carbonum* induced the biosynthesis of antifungal coumarins. Two were identified as ayapin and scopoletin.

INTRODUCTION

Although phytoalexins have now been described from 21 plant families [1, 2], with the exception of the Leguminosae [3] the number of species investigated has been small, if not minimal [4], and some crop plants of major economic importance have apparently been largely neglected in this respect. For example, in the Compositae phytoalexins have been reported, to date, in only one species namely *Carthamus tinctorius* L. (safflower) [4]. *Helianthus annuus* L. (sunflower), globally a much more important crop plant than safflower, was reported to accumulate "scopoletin-like fluorescent materials" following infection with the biotrophic pathogen *Plasmopara halstedii* [5] and quantitative data were given only in a relative manner. The coumarin derivative scopoletin has been reported to accumulate in tissues of sweet potato [*Ipomoea batatas* (L.) Lam., Convolvulaceae] [6] and tobacco [*Nicotiana tabacum* L., Solanaceae] [7] following infection. In certain plant species phytoalexins are known to be degraded rapidly [8] or elicited more slowly and to a lesser extent [9] by microorganisms pathogenic to these species as compared with non-pathogens. Therefore we initiated an investigation of *H. annuus* for the production of antimicrobial compounds in response to inoculation with the fungus *H. carbonum* Ullstrup which is a non-pathogen of sunflower, but pathogenic to maize (*Zea mays* L., Gramineae). Some of the results of this investigation are reported herein.

RESULTS AND DISCUSSION

Longitudinal sections of stem segments or excised leaves of *H. annuus* were inoculated with aqueous spore suspensions of *H. carbonum* (*ca* 10^5 conidia/ml). Following incubation for 72 hr at 25° the inoculated tissue was extracted by vacuum infiltration with ethanol. TLC analysis of diffusate and extracts of inoculated tissue (silica gel, ether) revealed two compounds which exhibited intense blue fluorescence under long wavelength UV light. On chromatograms of uninoculated control tissue the band at higher R_f (0.80) was not detected while the lower band (R_f 0.49) was barely detectable. When eluted into ethanolic solution and examined under UV, the upper and lower bands gave deep-blue and blue-green fluorescence, respectively. The compound at lower R_f possessed the same TLC mobility and HPLC retention time as scopoletin. Its identity with scopoletin (**1**) was confirmed by ^{1}H NMR and MS comparison with authentic material.

The second compound was further purified by HPLC on a C_{18} reversed phase column (H_2O–MeCN, 9:5; R_t 6.5 min) prior to spectral analysis. The neutral UV spectrum in methanol, which was reminiscent of that of scopoletin (see Experimental), remained unchanged however upon the addition of NaOH. HRMS afforded the $[M]^+$ as 190.0296 (calculated for $C_{10}H_6O_4$ 190.0266). From a consideration of the number of double bond equivalents and the similarity of the UV spectrum to that of scopoletin, the presence of three rings in the compound was indicated. A Labat test for a methylenedioxy group gave a positive result. When the ^{1}H NMR spectrum was compared with that of scopoletin (**1**) it was noted that the three proton singlet in the spectrum of **1** at δ3.96 (OMe) was absent; instead the two proton singlet at δ6.07 was assigned to the methylenedioxy protons of ayapin (**2**). Confirmation of the structure of the second coumarin as ayapin (6,7-methylenedioxycoumarin) was achieved by its synthesis from esculetin (6,7-dihydroxycoumarin) [10]. Synthetic ayapin proved to be chromatographically (TLC, HPLC) and spectroscopically (UV, ^{1}H NMR, MS) identical to the sunflower metabolite.

Original assays for antifungal compounds were performed qualitatively by direct bioautography on TLC using *Cladosporium herbarum* as test organism [11]. This assay revealed the presence of an antifungal zone which corresponded to scopoletin. The above saprophyte is, however, apparently insensitive to ayapin. Subsequent quantitative bioassays were performed with *H. carbonum* by measuring mycelial growth of single spore inocula on solid medium into which the compound under test was incorporated. Ayapin, at concentrations ranging from 0.25–1.0 mM, was more inhibitory to *H. carbonum* than was scopoletin (Table 1). Mycelial growth of *H. carbonum* was inhibited *ca* 95% by 0.5 mM ayapin (= 95 μg/ml) and by extrapolation the ED_{90} value was determined to be

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Table 1. Inhibition of growth of *H. carbonum* by ayapin and scopoletin*

	Ayapin		Scopoletin	
Conc (mM)	24 hr	48 hr	24 hr	48 hr
Control	0	0	0	0
0.25	60	15	13	12
0.5	95	95	13	12
1	100	95	66	62

* Expressed as percentage of the control which grew to the perimeter of the medium by 48 hr.

cv. Sunbird) following inoculation with *H. carbonum*, although the concentrations found were in general much 91 μg/ml. Ayapin was also inhibitory to a member of the Sphaeropsidales, growth of which was decreased by 87%, as compared with controls, at a level of 0.5 mM.

A time-course study of phytoalexin accumulation was performed utilising sunflower stem tissue (cv. Mammoth Grey Stripe) and *H. carbonum* as inducing agent, in which levels of ayapin and scopoletin were determined separately in both the medulla and the cortex. Trace amounts of scopoletin (< 20 μg/g) were detected in uninoculated control tissue at time zero. Inoculation with *H. carbonum* induced the rapid biosynthesis of scopoletin within 48 hr such that its concentration in the medulla attained the level of 1.62 mg/g dry wt of tissue. If we assume a water content in the fresh tissue of 90% the average level of scopoletin in *H. carbonum*-inoculated medullary tissue analysed is equivalent to a concentration of *ca* 0.85 mM.

Ayapin was not detected in uninoculated control tissue nor in inoculated stem segments until incubation for 48 hr. Concentrations of ayapin found to accumulate in the medulla and the cortex 2 and 3 days after inoculation were, in comparison, considerably lower than those of scopoletin (Table 2). The concentrations of both coumarins in the cortex 48 hr after inoculation with *H. carbonum* were significantly below those in medullary tissue of the same stem sections (Table 2). This result is consistent with reports of the preferential accumulation of the phytoalexin phaseollin in dead tissue of the hypocotyls of *Phaseolus vulgaris* L. [2].

Both ayapin and scopoletin were also induced in detached sunflower leaves (cv. Mammoth Grey Stripe and lower than those in inoculated stem tissue. This could conceivably be due primarily to the cuticle of the leaf acting as an effective barrier between plant cells and the fungus, whereas the cells of stem segments were exposed, due to sectioning, prior to inoculation. During a preliminary study of elicitation of 1 and 2 by other agents, inoculation of sunflower stem tissue with the necrotrophic sunflower pathogen *Alternaria helianthi* (leaf spot and seedling blight) [12] was found to induce the production of both compounds, as did exposure of excised leaves to short wavelength UV light (325 μW/cm²) for 45 min. Levels of 1 and 2 induced by these agents were lower than those induced by *H. carbonum*. Ayapin was also induced, by UV irradiation, in leaves of the wild *Helianthus* species *H. argophyllus* Torr. and Gray but not in *H. petiolaris*, nor was it detected in stem sections of *H. praecox* inoculated with *H. carbonum*. Trace amounts of 1 and 2 were previously reported as components of *H. annuus* treated with 2,4-dichlorophenoxyacetic acid [13].

Table 2. Concn of scopoletin (1) and ayapin (2) in corticular and medullary tissue of inoculated sunflower stem sections*

	Concn (μg/g dry wt)			
	Medulla		Cortex	
Time (hr)	1	2	1	2
0	< 20	—	< 20	—
24	71	—	21	—
48	1622	114	264	30
72	333	113	150	31

* The upper 1 mm of tissue was analysed (see text).

—, Not detected.

Scopoletin is a fairly commonly encountered secondary metabolite with a broad taxonomic distribution [14, 15]. Ayapin, however, which is presumably biosynthesised directly from scopoletin, has been described previously in only two other species, namely *Eupatorium ayapana* Vent. [16] and *Alomia fastigiata* Benth. [17], both of which are members of the Compositae, and moreover, belong to the same tribe (Eupatorieae) [18]. Assuming that ayapin plays some form of ecological role in plants which produce it, its biosynthesis from scopoletin may be regarded as an evolutionarily advanced trait. Interestingly, ayapin possesses potent haemostatic activity [16] as opposed to the haemorrhagic dicoumarol which is derived from coumarin itself [19]. No other biological activity was known to be associated with ayapin until the present investigation. Indeed, although scopoletin was shown to be fungitoxic to a *Pythium* sp., ayapin was previously reported to completely lack antifungal activity against the same organism [20]. However, *Pythium*, as a member of the Oomycetes, is fundamentally different from the fungi used in our present study as manifest by its coenocytic mycelium, and the major structural material of its hyphal walls is cellulose-glucan instead of chitin-glucan [21]. Coumarin itself, however, which is reported to inhibit cellulose biosynthesis in higher plants [22], was significantly more toxic to three oomycetous species than to those members of the higher fungi tested [20]. When liquid medium containing ayapin was inoculated with a mixture of conidia and mycelial fragments of *H. carbonum*, growth in shake culture was atypical in that the mycelium became fused in a single stroma and total biomass was greatly decreased [unpublished results]. It is tempting to speculate that this result is related to the haemocoagulant activity of ayapin [16].

Since scopoletin and ayapin are both antimicrobial and are synthesized by and accumulated in sunflower tissues after exposure to certain microorganisms they qualify as phytoalexins according to the recently revised definition [23]. Prior to this investigation the only compounds described as phytoalexins in the Compositae were the acetylenic compounds safynol and dehydrosafynol [4]. The Compositae in general, and the tribe Heliantheae in particular, represents an especially rich source of acetylenic secondary metabolites [24] and the production of acetylenic phytoalexins by *H. annuus* in addition to the coumarins ayapin and scopoletin is possible. Analogous situations exist in two leguminous spp., which produce

acetylenic as well as the more commonly encountered isoflavonoid phytoalexins [25, 26], and in *Lycopersicon esculentum* L. (Solanaceae) [27]. When phytoalexin induction was performed in the dark, using sunflower stem sections inoculated with *H. carbonum*, additional zone(s) of inhibition other than those corresponding to ayapin and scopoletin were observed on thin layer chromatograms assayed directly using *H. carbonum*. The component(s) responsible for this fungitoxic activity are under active investigation.

EXPERIMENTAL

Plant material and fungal cultures. Seed of *Helianthus* accessions was obtained from the Regional Plant Introduction Station, Iowa State University, Ames, Iowa, U.S.A. *Helianthus annuus* cv. Mammoth Grey Stripe or cv. Sunbird and wild *Helianthus* spp. were grown singly in 20 cm pots under controlled environmental conditions of 25°/20° with 14 hr photoperiods for 5–7 weeks prior to harvest for induction experiments. A voucher specimen of *H. argophyllus* (accession no. 413171) has been deposited at the herbarium of the University of California, Berkeley. *Helminthosporium carbonum* was maintained on V-8 agar and inoculum was taken from 2–3 week cultures grown at 28° on the above medium.

Induction and isolation of **1** *and* **2**. Excised leaves or longitudinal stem sections were placed on moist blotters in plastic boxes. Fungal inoculum consisting of 10^5 conidia/ml in deionized H_2O was applied to adaxial leaf or cut stem surfaces with a Pasteur pipette. Sterile deionised H_2O was applied to control tissue. After incubation at 25° in the light (3000 lux) or in the dark, diffusate from leaf inoculations was collected, concd *in vacuo*, and filtered prior to chromatography. Leaf tissue underlying inoculum or the upper 1 mm of stem tissue was extracted by vacuum infiltration in EtOH. Leaves exposed to UV were incubated for 72 hr at 25° prior to extraction. Extracts were diluted with an equal volume of H_2O and partitioned against *n*-hexane to remove pigments. Compounds **1** and **2** were purified by TLC and HPLC.

Chromatography. TLC (silica gel, 0.2 mm; Et_2O) gave **1**, R_f 0.49 and **2**, R_f 0.80 as bright fluorescent bands, visualized under long λ (360 nm) UV. Compound **2** could also be detected by spraying with a solution of chromotropic acid in 50% aq. H_2SO_4 [28]. HPLC (Waters Z Module, Radial Pak column, 5 μm, UV absorption detector at 346 nm and Gilson Spectra/glo Filter Fluorometer detector equipped with 330–380 nm excitation filter and 410–560 nm emission filter, H_2O–MeCN, 9:5; 1.4 ml/min) furnished **1**, R_t 3.7 and **2**, R_t 6.5 min. Levels of **2** in inoculated tissue and of **1** in inoculated and control tissue were determined directly from peak areas using a Data Module integrator.

Synthesis of ayapin (**2**). Ayapin was synthesized from esculetin by a procedure based on the method of Fujita and Yamashita for the preparation of alkylenated ether derivatives of the corresponding vicinal dihydroxy coumarins [10]. Esculetin (1 g, ex Sigma Chem. Co.) in DMSO (10 ml) was refluxed with CH_2Cl_2 (4 ml) and K_2CO_3 (1.5 g) at 125° for 3 hr. H_2O was added to give a volume of 100 ml. Partition against Et_2O × 2 and evaporation of the organic phase afforded ayapin in high yield (> 90%) which behaved chromatographically (TLC, HPLC) as a single component and was essentially homogeneous (^{1}H NMR). Crystallization from MeOH–$CHCl_3$ gave needles, mp 221–222° (lit. [17] = 220–221°).

Ayapin (6,7-*methylenedioxycoumarin*; **2**). UV λ_{max}^{MeOH} nm (log ε) 233 (4.04), 260 sh (3.56), 293 (3.46), 344 (3.90); cf. scopoletin: λ_{max} nm (log ε) 230 (4.11), 260 (3.63), 298 (3.68), 346 (4.07). EIMS (probe) 70 Ev, m/z (rel. int.): 190 [M]$^+$ (85), 162 [M − CO]$^+$ (100), 161 [M − COH]$^+$ (68), 104 (11). ^{1}H NMR (300 MHz, $CDCl_3$): δ6.07 (2H, *s*, CH_2), 6.28 (1H, *d*, *J* = 10 Hz, H-4), 6.83 (2H, *s*, H-5 and H-8), 7.58 (1H, *d*, *J* = 10 Hz, H-3).

Bioassays. Compounds **1** and **2** in DMSO were incorporated into V-8 agar to give concentrations of 0–1 mM and a final DMSO concn of 2%. Media were poured into wells (*ca* 2 cm diameter) of serological wellracks (3 wells/concn). Single conidia of *H. carbonum*, which were previously plated out on V-8 agar and allowed to germinate overnight, were transferred to the centre of each well via a fine needle with the aid of a dissecting microscope. After incubation for 48 hr at 28°, inhibition was determined from areas of mycelial growth.

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