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ABIOTIC ELICITATION OF COUMARIN PHYTOALEXINS IN SUNFLOWER

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Key Word Index—*Helianthus annuus*; Compositae; coumarins; phytoalexins; abiotic elicitors.

Abstract—Detached leaves, leaf disks and cut stems and hypocotyls, but not roots of sunflower (*Helianthus annuus*) plants accumulated the coumarin phytoalexins, scopoletin and ayapin, when treated with solutions of $CuCl_2$ or sucrose. Irradiation with shortwave UV and exposure to Triton X-100 was also effective, while no effect was observed with longwave UV, salicylic acid, dichloroisonicotinic acid or glutathione. The elicitation of the coumarins was dependent on the type of elicitor used and the age of the plant tissues. Treatment with sucrose elicited similar amounts of scopoletin and ayapin while treatment with $CuCl_2$ favoured the formation of ayapin with low concentrations of scopoletin and none of the scopoletin 7-*O*-glucoside being observed. The selective induction of ayapin by $CuCl_2$ was associated with an inhibition in the activity of the *O*-glucosyltransferase responsible for glycosylating scopoletin, and an induction in a peroxidase which metabolized scopoletin but not ayapin. The abiotic elicitation of the phytoalexins increased in the leaves of sunflower as a function of age, with seedlings and immature plants being unable to synthesize scopoletin or ayapin. Inhibitor and precursor feeding studies were unable to elucidate the biosynthetic route to scopoletin although an elicitor-inducible methyltransferase activity which could methylate esculetin to produce scopoletin was identified.

INTRODUCTION

Sunflower (*Helianthus annuus*) is a major oil seed bearing crop which is prone to severe damage by a range of fungal and insect pests [1]. The observation that sunflower cultivars vary in their susceptibility to these pathogens has led to an interest in determining the basis of resistance to biotic stress with the intention of improving tolerance through plant breeding or genetic engineering techniques [1]. Sunflower contains a diverse range of secondary products including terpenes [2], flavonoids [3] and simple phenolic compounds [4–7] which have been implicated in the resistance of sunflower to both insect pests [8, 9] and fungal pathogens [6, 7]. The majority of these compounds are present as constitutive metabolites in healthy plants. However, the coumarins scopoletin and ayapin (Fig. 1) have been observed to accumulate in sunflower tissues following mechanical wounding or insect feeding damage [8, 9] and following infection with pathogenic and non-pathogenic fungi [6, 7]. The coumarins accumulate around the site of mechanical injury [8] or invading fungal hyphae [10] and have been shown to have activities as both insecticide feeding deterrents [8] and as fungicides [7]. Furthermore, somaclonal variants of sunflower containing elevated levels of ayapin showed improved resistance to leaf feeding insects as compared with normal plants [9]. The relationship between coumarins and resistance to fungi has not been similarly tested in sunflower. However, Goy *et al.* [11] have reported that a diploid hybrid of *Nicotiana* which contained elevated levels of scopoletin was highly resistant to a range of microbial pathogens. It, therefore, seems probable that scopoletin and ayapin contribute to the resistance of sunflower to biotic stress and it is of interest to study both the factors which control their accumulation in various tissues and to determine their routes of synthesis. To clarify the study we have elicited the formation of the coumarin phytoalexins by exposing detached plant tissues to abiotic elicitors, namely $CuCl_2$ and sucrose. Although such elicitors have a number of limitations [12] their use eliminates the effects of metabolism of the phytoalexins by the pathogen. This was considered important as previous studies have demonstrated that microbial pathogens rapidly degrade coumarin phytoalexins from sunflower [6].

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RESULTS

Elicitation of coumarin phytoalexins in sunflower

Coumarin phytoalexins which had been partialy purified by TLC were identified and quantified by HPLC using a UV detector to monitor the eluant. Previous studies [7, 8] had used TLC combined with fluorescence detection by HPLC to quantify scopoletin and ayapin and although the use of UV absorbance detection in place

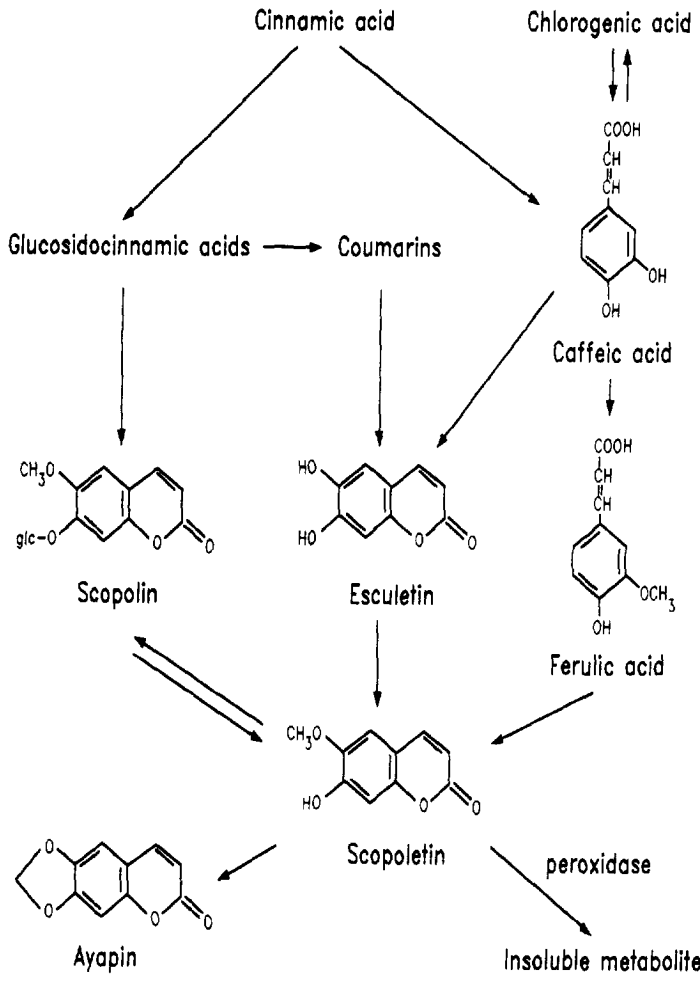

Fig. 1. Potential routes of biosynthesis and metabolism of scopoletin in sunflower.

of fluorescence reduced the sensitivity of determination, the limit of detection of the assay remained high (50–100 pmol coumarin g^{-1} leaf fr. wt). Using spiked stem and leaf samples, recoveries of scopoletin and ayapin by this method were 39 ± 7 and 28 ± 6%, respectively. It was also possible to determine the concentrations of the TLC-purified coumarins directly by spectrofluorimetry (excitation and emission wavelengths 340 and 430 nm, respectively) though it was necessary to use an internal standard of authentic coumarin to correct for the quenching of the fluorescence by co-chromatographing pigments. All of the results presented were obtained by a combination of HPLC–TLC and have been corrected for recoveries. Initial experiments concentrated on the development of a reliable and simple abiotic elicitation system using detached plant parts or cell cultures. Detached roots, bisected stem sections and detached leaves or leaf disks from mature plants (45-days-old) were exposed for three days to a range of abiotic elicitor treatments including UV irradiation at 254 or 345 nm, 10 mM–1 M sucrose, 1 mM $CuCl_2$, 0.1% Triton X-100, $CHCl_3$ vapour, 1 mM salicylic acid, 1 mM glutathione and 0.1 mM 2,6-dichloro-isonicotinic acid. As determined qualitatively by monitoring the fluorescence of scopoletin and ayapin by TLC, the latter four treatments and the UV_{345} did not induce detectable quantities of coumarin phytoalexins in any of the plant parts. Similarly none of the abiotic elicitors induced the accumulation of scopoletin or ayapin in detached roots. In the cut stems ayapin, but not scopoletin, was observed in the controls suggesting that it was accumulating in response to wounding. $CuCl_2$, sucrose and UV_{254} elicited the accumulation of scopoletin in the stems in addition to the wound-induction of ayapin with sucrose being the most effective treatment. Using detached leaves, treatment with $CuCl_2$ was found to be the most effective elicitor with Triton X-100 and irradiation with UV_{254} causing rather less accumulation of scopoletin and ayapin and sucrose having no effect. In contrast sucrose was the most effective elicitor in leaf disks with the other elicitors causing severe phytotoxic damage. Similar effects of abiotic elicitation were observed with plant parts from other sunflower cultivars confirming that cv Peredovick was not atypical. Suspension cultured cells of Peredovick did not prove to

be a reliable system for further study. The accumulation of scopoletin and ayapin was only observed constitutively in cells which had undergone at least 10 rounds of subculturing with small amounts of the coumarins being determined in cells in late stationary phase. Treatment of these older cultures with yeast extract caused a modest accumulation of both coumarins in cells in mid-logarithmic growth phase. Cultures which had not undergone 10 rounds of subculture did not produce coumarins on elicitation though three UV-absorbing metabolites with R_f values of 0.31, 0.36 and 0.44 accumulated transiently in the medium 5–22 hr after elicitor treatment. The accumulation of these inducible metabolites was associated with a 10-fold increase in the specific activity of phenylalanine ammonia lyase (data not shown).

Based on these results two elicitation systems were chosen for further study. For studies with whole leaves $CuCl_2$ was chosen as the optimal elicitor. Sucrose elicitation of leaf disks and cut stems was chosen as the second system, as sucrose was less toxic than the other treatments and has been partially characterized as an elicitor in previous studies [13].

When detached leaves from mature plants were treated for 72 hr with 1 mM $CuCl_2$ or leaf disks were treated with sucrose, as determined by TLC, two fluorescent metabolites accumulated which were identified as scopoletin (R_f 0.25) and ayapin (R_f 0.57). In addition to the two coumarins a UV-absorbing, but non-fluorescent uncharacterized metabolite with an R_f 0.65 (UN1) accumulated in the elicited leaf tissue but not in the controls. It has been reported that during biotic elicitation sunflower plants accumulate large amounts of scopolin, the 7-*O*-glucoside of scopoletin [6]. To determine whether this was also the case with abiotic elicitation, extracts from 45-day-old leaves treated with either water or $CuCl_2$ and leaf disks exposed to 0.1 M sucrose were acid hydrolysed. No scopoletin was released from any of the samples, suggesting that scopolin did not accumulate in either healthy leaves or in response to abiotic elicitation. To test whether scopoletin was being sequestered into insoluble cell wall components the residue remaining after solvent extraction of sunflower leaves was treated with acid but no scopoletin was released.

To determine the rates of accumulation of the phytoalexins, leaves fed with $CuCl_2$ and leaf disks treated with 10 mM sucrose were analysed for scopoletin and ayapin at intervals (Table 1.) In the $CuCl_2$ treated leaves ayapin was the dominant phytoalexin at all time points, being first observed 24 hr after copper treatment and accumulating 20-fold by 96 hr.

Scopoletin was first determined 48 hr after treatment with $CuCl_2$ and relative to ayapin was 10 times less abundant, with levels increasing only 2-fold between 48 and 96 hr. In contrast to the results with $CuCl_2$ more scopoletin than ayapin accumulated in leaf disks treated for 72 hr with sucrose, though by 120 hr ayapin was predominant. Ayapin was also the dominant phytoalexin when detached leaves were fed for 72 hr with Triton X-100 with the concentrations of scopoletin and ayapin being 4 nmol g^{-1} and 13 nmol g^{-1}, respectively. However, when detached leaves were irradiated with UV_{254} the concentrations of scopoletin and ayapin were similar at 5 and 8 nmol g^{-1}, respectively. To determine whether the accumulation of the coumarins was due to *de novo* synthesis from phenylalanine as proposed for other coumarin phytoalexins [14] the leaves were fed with $CuCl_2$ in the presence of L-2-aminoxy-3-phenylpropionic acid (L-AOPP). L-AOPP is a potent inhibitor of phenylalanine ammonia lyase and at the concentration used (0.3 mM) has been shown to inhibit the formation of phytoalexins derived from phenylpropanoids in similar studies in legumes [12].

Surprisingly, following a 72 hr treatment the concentrations of scopoletin and ayapin were lower in the leaves treated with copper alone (10.1 + 2.1 nmol g^{-1} and 129.8 ± 8.3 nmol g^{-1}, respectively) than in the leaves elicited in the presence of L-AOPP (12.7 and 157.9 ± 15.1 nmol g^{-1}). Significantly L-AOPP treatment completely inhib-

Table 1. The elicitation of coumarin phytoalexins in detached leaves fed with 1 mM $CuCl_2$ and leaf disks treated with 10 mM sucrose (in both cases leaves were from 45-day-old sunflower plants)

	$CuCl_2$-fed leaves		Sucrose-treated disks	
Treatment time (hr)	Scopoletin	Ayapin	Scopoletin	Ayapin
	(nmol g^{-1} fr. wt)			
0	ND	ND	ND	ND
8	ND	ND	—	—
24	ND	3 ± 0	ND	ND
48	18 ± 1	70 ± 6	—	—
72	16 ± 3	148 ± 16	71 ± 16	46 ± 10
96	31 ± 4	228 ± 36	—	—
120	—	—	71 ± 13	119 ± 2

Values refer to the means of triplicate determinations ± S.D.
ND: Not detected.
—: not determined.

ited the accumulation of UN2 suggesting that it was capable of disrupting inducible secondary metabolism in sunflower.

Initial experiments suggested that the abiotic elicitation of coumarin phytoalexins in detached plant parts was influenced by the age of the plants used. To investigate this further, leaves were detached from plants of various ages and treated with $CuCl_2$ to elicit the formation of scopoletin and ayapin (Table 2). Three-day-old seedlings were unable to synthesize scopoletin or ayapin when treated with $CuCl_2$, Triton X-100 or UV irradiation. Similarly, leaves from 28-day-old plants did not accumulate scopoletin or ayapin when elicited with $CuCl_2$, though scopoletin (14.5 ± 0.7 nmol g^{-1}) could be determined when the leaves were irradiated with UV_{254}. In older leaves $CuCl_2$ was an effective elicitor of both scopoletin and ayapin with the accumulation of total coumarins being maximal in the leaves of 70-day-old plants. As determined in 45-day-old leaves (Table 1), the predominant phytoalexin in all cases was ayapin, though the ratio of scopoletin: ayapin did vary in leaves of different ages (Table 2). The effect of plant age on the phytoalexin response in sunflower was further confirmed in leaf disks from 16- and 45-day-old plants using 10 mM sucrose as the elicitor (Table 3). The elicitation of the coumarin phytoalexins was dependent upon the concentration of sucrose used and the age dependent effect could be overridden if the younger leaf disks were treated with the higher concentration of sucrose.

Table 2. Effect of plant age on the elicitation of coumarin phytoalexins, in detached sunflower leaves, treated with 1 mM $CuCl_2$ for 72 hr

Plant age (day)	Scopoletin (nmol g^{-1} fr. wt)	Ayapin (nmol g^{-1} fr. wt)
3	ND	ND
28	ND	ND
38	0.7 ± 0	5.7 ± 2.4
52	26.3 ± 4.2	32.4 ± 3.9
70	15.7 ± 2.9	147.5 ± 16.1
91	2.1 ± 0.6	25.4 ± 6.8

Values refer to the means of triplicate determinations ± S.D.
ND: Not detected.

Enzymes involved in the synthesis and metabolism of coumarin phytoalexins in sunflower

It was of interest to determine whether any of the biosynthetic steps implicated in the synthesis of scopoletin could be detected *in vitro* in control and elicited sunflower. Labelling studies with potential precursors in tobacco have suggested that scopoletin is formed directly from ferulic acid (Fig. 1) following an undefined *trans–cis* isomerization and oxidative cyclization step, possibly involving the formation of 2-hydroxyferulic acid [15]. Crude protein extracts from control and $CuCl_2$ treated leaves were incubated with ferulic acid in the presence of a variety of co-substrates required for the activity of monoxygenases, dioxygenases and peroxidases but no 2-hydroxyferulic acid or scopoletin could be determined in any of the incubations. Since the formation of scopoletin from ferulic acid by *in vitro* preparations has not been described in other systems it was, therefore, not possible to further examine this route of synthesis. It also seemed unlikely that scopoletin was formed from scopolin (Fig. 1) as this glucoside could not be detected in abiotically elicited leaves which were accumulating scopoletin. In an alternative route of synthesis esculetin is the immediate precursor of scopoletin (Fig. 1). In tobacco the methylation of esculetin to yield scopoletin could not be demonstrated *in vitro*, though the activity of the 3-*O*-methyltransferase with activity toward caffeic acid was observed [15]. This observation, together with precursor feeding studies suggested that esculetin was not a precursor of scopoletin in tobacco [14, 15]. To test whether this was also the case in sunflower esculetin and caffeic acid were incubated with desalted protein preparations from leaves treated with water or $CuCl_2$ in the presence of *S*-adenosyl-L-[^{14}C-methyl]methionine and the incorporated radioactivity determined after partitioning into ethyl acetate (Table 4). Using extracts from elicited leaves the incorporation of radioactivity was shown to be both time and protein dependent with the activity being optimal at pH 8.5. Methyltransferase activities toward caffeic acid and esculetin were detected in both control and $CuCl_2$

Table 3. Effect of a 72 hr sucrose treatment (10 and 100 mM) on the accumulation of coumarin phytoalexins in leaf disks prepared from 16- and 45-day-old sunflower plants

	10 mM Sucrose		100 mM Sucrose	
Plant age (day)	Scopoletin	Ayapin (nmol g^{-1} fr. wt)	Scopoletin	Ayapin
16	ND	ND	359 ± 73	157 ± 57
45	71 ± 21	46 ± 9	360 ± 13	77 ± 12

Values refer to the means of triplicate determinations ± S.D.
ND: Not detected.

Table 4. *O*-Methyltransferase and *O*-glucosyltransferase activities toward scopoletin, and potential scopoletin precursors, in detached leaves treated with water or elicited with 1 mM $CuCl_2$ for 72 hr

	O-Methyltransferase		*O*-Glucosyltransferase	
Substrate	Control	Elicited	Control	Elicited
	(nkat kg^{-1} protein)			
Methanol	13.5 ± 0.5	21.5 ± 0.5	2.1 ± 0.0	1.2 ± 0.2
Caffeic acid	37.5 ± 0.5	151.0 ± 0.0	3.5 ± 0.1	1.2 ± 0.1
Esculetin	26.0 ± 1.0	179.0 ± 13.0	6.6 ± 0.2	2.2 ± 0.0
Scopoletin	13.5 ± 0.5	39.0 ± 1.5	3.4 ± 0.3	1.0 ± 0.3

Values refer to the means of duplicate determinations ± the variation between the mean and the individual replicates.

treated leaves with the specific activities of the enzymes towards the two substrates increasing 4 and 7-fold, respectively, on elicitation. To confirm that the radioactivity was incorporated into the authentic compounds the reaction products were analysed by TLC. Using caffeic acid and esculetin as the substrates it was confirmed that 82 and 88% of the radioactivity was incorporated into ferulic acid and scopoletin, respectively. The position (6 or 7) of methyl substitution of the reaction products from the incubations with scopoletin was not investigated but the 7-hydroxy group of scopoletin was a poor substrate for the methylase in the elicited leaves (Table 4).

It was also of interest to identify the enzyme activities responsible for the further metabolism of scopoletin in sunflower. Tal and Robeson [6] reported that biotically elicited sunflower stems metabolized [^{14}C]scopoletin to its 7-*O*-glucoside scopolin. Although scopolin was not observed in leaves exposed to abiotic elicitors, crude extracts from leaf tissue treated with water, $CuCl_2$ and sucrose were assayed for *O*-glucosyltransferase activities with scopoletin and potential precursors as substrates (Table 4). A protein dependent activity with a pH optimum of pH 7 which catalysed the glucosylation of esculetin and scopoletin was identified in control leaves with esculetin being the preferred substrate. The specific activity of the *O*-glucosyltransferase toward all substrates was reduced 3-fold in extracts from leaves treated with $CuCl_2$ such that no activity could be determined with scopoletin as substrate as compared with controls. Similarly no *O*-glucosyltransferase activity could be determined in sucrose treated leaf disks (data not shown). Since scopoletin does not accumulate to appreciable concentrations in the $CuCl_2$ treated leaves and was not glycosylated, it was of interest to determine whether this coumarin could be metabolized by alternative mechanisms. Scopoletin is known to be metabolized to ayapin [6]. However, when 0.2 mM scopoletin was fed to leaves elicited with $CuCl_2$ no additional ayapin accumulated even though scopoletin rapidly disappeared, suggesting the existence of an alternative route of metabolism. Scopoletin is a substrate for peroxidases in a number of plant species [16] and it was of interest to determine whether sunflower plants contained peroxidases capable of metabolizing their own coumarin phytoalexins. When scopoletin was incubated in the presence of H_2O_2 with extracts from 45-day-old detached leaves which had been fed with water for three days a blue metabolite accumulated. As the reaction progressed the product became increasingly insoluble indicating that it was a polymer. The compound was not formed in the absence of H_2O_2 and its rate of formation was dependent on the amount of protein present. As determined from the rate of formation of coloured product, where 1 unit = 1 A.U. min^{-1} mg^{-1} protein, the activity of this enzyme was greater in extracts from leaves treated for three days with $CuCl_2$ (56 units) and in leaf disks treated with 0.1 M sucrose (9 units) than in the corresponding water treated controls (2 units). No activity was observed in unwounded healthy mature leaves. When ayapin was incubated with extracts from either elicited or non-elicited leaves the blue metabolite or other coloured oxidation products were not observed. However, a yellow reaction product was formed when esculetin was incubated with H_2O_2 with extracts from healthy leaves (1 unit) and leaves treated with sucrose (5 units) and $CuCl_2$ (7 units). The presence of this latter activity in unwounded leaves and its lower induction in response to abiotic elicitation suggests that it is a distinct peroxidase from the enzyme responsible for scopoletin degradation.

DISCUSSION

Our results demonstrate that a variety of abiotic elicitors can induce the accumulation of coumarin phytoalexins in sunflower in a similar manner to that observed when plants are infected with fungi [6, 7] or damaged by insects [8]. The phytoalexin response was dependent on the tissue type, the elicitor used and the age of the plants.

Elicited leaf tissue accumulated the highest concentrations of coumarin phytoalexins followed by stems and hypocotyls with detached roots being unable to synthesize scopoletin or ayapin. Elicitors such as $CuCl_2$, which are believed to work through the release of endogenous elicitors following cellular damage [12] preferen-

tially induced the accumulation of ayapin while sucrose elicited more scopoletin. In both cases the phytoalexins only began to accumulate 24 hr after elicitor treatment. When stems were inoculated with fungal conidia of *Helminthosporium carbonum* or *Alteraria helianthi* scopoletin was rapidly induced being the predominant phytoalexin with ayapin accumulating more slowly [6]. It, therefore, appears that the use of sucrose as an elicitor more accurately mimics the phytoalexin response to biotic elicitation in sunflower. The reasons for the difference in the relative accumulation of scopoletin and ayapin in response to differing elicitors may be explained in two ways. Either $CuCl_2$ treatment selectively increases the metabolism of scopoletin to form ayapin [6] or this elicitor stimulates the selective metabolism and degradation of scopoletin. We have no evidence for the stimulation of ayapin synthesis and the enzyme responsible for its formation has not yet been reported *in vitro*. Studies with fungal pathogens of sunflower, such as *Phoma macdonaldii*, have demonstrated that ayapin is more resistant to degradation by the fungi than scopoletin and can selectively accumulate in pathogen infected stems [6]. Our results would suggest that scopoletin may also be selectively metabolized by an elicitor-inducible peroxidase which is more strongly induced by $CuCl_2$, than by sucrose treatment. As ayapin is not a substrate for this enzyme the effect of the peroxidase would be to reduce the levels of scopoletin while having no effect on the accumulating ayapin. The role of this enzyme in regulating the phytoalexin response in sunflower during interactions with fungi and its further characterization are currently under investigation. Another difference in the phytoalexin response of sunflower to biotic and abiotic treatments was the accumulation of scopolin. Scopolin was the major coumarin metabolite in sunflower stems and leaves tissues infected with *H. carbonum* or *A. helianthi* [6]. In contrast scopolin was not observed when these tissues were treated with $CuCl_2$ or sucrose. The results of the *O*-glucosyltransferase assays suggest that scopolin may not be accumulating in response to abiotic elicitation due to the inhibition of the respective scopoletin 7-*O*-glucosyltransferase. Similarly, when alfalfa is elicited with $CuCl_2$, isoflavonoids accumulate while the induction of the respective glucosides is inhibited owing to the inactivation of the respective *O*-glucosyltransferase [12].

The effect of plant age on the abiotic elicitation of the phytoalexin response in sunflower was very pronounced with young seedlings being unable to synthesize either ayapin or scopoletin. The effect of plant age on the phytoalexin response of sunflower to fungal infection and wounding has not been reported though the effects of ageing on constitutive phenolic accumulation are well characterized [5]. It would be of interest to determine whether this inability to accumulate coumarin phytoalexins was also shown when young plants were challenged with biotic agents. The results of the L-AOPP inhibitor studies suggested that the synthesis of scopoletin and ayapin synthesis was not dependent upon *de novo* synthesis from phenylalanine. Similarly in $CuCl_2$ treated leaves feeding studies with 0.2 mM esculetin, caffeic acid, *para*- and *ortho*-coumaric acid and ferulic acid all failed to stimulate coumarin phytoalexin production (data not shown). These results are consistent with scopoletin and ayapin being derived, at least in part, from a large pool of pre-formed precursor. Significantly sunflower leaves contain large amounts of chlorogenic acid (1–5 mg g^{-1}) and related compounds [5] and it is interesting to speculate that these pools can be mobilized to release caffeic acid [17] to serve as a precursor for coumarin synthesis as has been proposed for lignin synthesis in sunflower [5]. The results of the enzyme assays were also of interest as they demonstrated the presence of an elicitor-inducible methyltransferase which methylated esculetin to give scopoletin. However, the caffeic acid *O*-methyltransferase activity responsible for ferulic acid synthesis was also elicited and the presence of the esculetin methyltransferase cannot be considered strong evidence for the synthesis of scopoletin from esculetin. In tobacco, esculetin was not a substrate for methylation and this together with studies with radiolabelled precursor studies suggested that scopoletin was synthesized from ferulic acid rather than esculetin.

Future studies will utilize the abiotic elicitation systems to elucidate the route of biosynthesis of scopoletin in sunflower and determine the relative importance of *de novo* synthesis from phenylalanine as compared with the utilization of preformed precursors.

EXPERIMENTAL

Chemicals and plant material. Coumarins and cinnamic acids were obtained from Sigma and ayapin synthesized from escaletin as described [18]. L-AOPP was obtained from Cambridge Research Biochemicals Ltd (Northwich, Cheshire, U.K.) and 2,6-dichloro-isonicotinic acid was donated by the Plant Protection Division, Ciba Ltd (Basle, Switzerland). Seeds of sunflower cultivar Peredovick were provided by Eurosemillas S.A. (Cordoba, Spain) and after surface sterilizing in Chloros (0.4% available chlorine) germinated in Perlite. Seedlings were grown under a 16 hr photoperiod (light intensity 300 μE m^{-2} s^{-1}) in Perlite for up to 15 days and then transferred to Levington's multipurpose compost. Healthy leaves were detached and their petioles cut under water prior to feeding with the aqueous test solution through the petiole. For studies with L-AOPP the leaves were fed with the inhibitor (0.3 mM) for 24 hr prior to exposure to the elicitor treatment. Alternatively discs (1 cm) were cut from the detached leaves with a cork borer and floated on the test solution in a Petri dish. Stems, petioles or hypocotyls (length 5 cm) were cut longitudinally with a razor blade prior to floating the plant parts on the test soln. Washed roots were immersed directly in the test soln. For UV treatments detached plant parts were placed 3–4 cm from the source (Camag universal UV TL-900 lamp) and irradiated at a wavelength of 254/345 nm. Callus cultures were initiated from seed and maintained in suspension culture [18] and treated as required with a yeast extract (7 mg ml^{-1}, Bacto yeast extract, Difco Laboratories) to elicit a phytoalexin response. Cells were

harvested by filtration on nylon mesh. All plant material was weighed and frozen in liquid N_2 prior to storage at $-80°$.

Extraction and analysis of plant material. Frozen tissue (1–3 g) was homogenized in 10 v/w Me_2CO pre-chilled to $-20°$ with sand in a pestle and mortar and after filtering off the solvent extract the residue was further sequentially extracted with a similar volume of Me_2CO and Me_2CO–MeOH (1:1). The combined solvent extract was concd under vacuum and then either analysed directly or cleaned up prior to TLC–HPLC. For direct analysis by TLC the concd extract was redissolved in MeOH (1 ml), clarified by centrifugation and a subsample (2–400 μl) dried under vacuum before redissolving the residue in 50 μl of EtOAc and applying 10 μl to a TLC plate (silica gel 60 F_{254}, Merck). The TLC plate was developed with Et_2O and the fluorescent coumarin metabolites visualized by illuminating the plate with UV light (254 nm). The fluorescent compounds co-chromatographing with scopoletin and ayapin were then scraped from the plate and eluted from the silica with 0.5 ml MeOH. After extraction at $-20°$ for 60 min the sample was centrifuged and 50 μl applied to a Spherisorb ODS HPLC column (250 × 4.4 mm, Fisons). The sample was eluted at 0.8 ml min^{-1} with a linear gradient (45 min) from 10 to 50% MeCN in 1% H_3PO_4, eluant monitored for *A* at 340 nm. Peaks corresponding to scopoletin (R_t 20 min) and ayapin (30 min) were quantified and identified using calibrated standards. Crude samples requiring clean up prior to TLC were dissolved in MeOH–0.05 M HCl (33:67) and partitioned against 0.75 vol. hexane–Et_2O (3:2). Under these conditions the majority of the pigments partitioned into the hexane: ether with the coumarins residing in the methanolic phase. To monitor for losses of the coumarin phytoalexins during clean-up, unelicited leaves were spiked with 5 μg of the authentic compounds g^{-1} fr. wt of tissue. 39 ± 7% (mean + s.d. n = 4) of the scopoletin and 28 ± 6% of the ayapin were recovered. Crude samples requiring hydrolysis were redissolved in MeOH–1 M HCl (1:1) containing 0.3% w/v ascorbic acid and incubated for 2 hr at 50°. The aglycones were quantified by TLC–HPLC after partitioning into EtOAc.

Enzyme assays. Leaves were homogenized in liquid N_2 and extracted with 3 v/w 0.1 M Tris–HCl pH 7.4 containing 0.3 w/w insoluble PVP. After straining the homogenate through muslin the extract was centrifuged at 12 000 *g*, 20 min and then desalted on a Sephadex G 25 column (Pharmacia PD 10) in 0.1 M Tris–HCl pH 7.4. For the *O*-methyltransferase (OMT) assays 50 μl of the enzyme was incubated with 5 μl of phenolic substrate (10 mM in methanol) 5 μl *S*-adenosyl-[^{14}C-methyl]-L-methionine (916 Bq, sp. act. 2.07 GBq $mmol^{-1}$, Amersham) and 40 μl of 1.0 M Tris pH 8.5 containing 2 mM DTT. After a 30 min incubation at 30° the reaction products were partitioned into an equal vol. of EtOAc and 50 μl quantified by liquid scintillation counting (LSC). The identity of the radiolabelled reaction products was verified by TLC using Et_2O as the developing solvent after overspotting the EtOAc extracts onto authentic compounds and quantifying the resolved compunds by LSC. *O*-Glucosyltransferase (OGT) assays were almost identical to the OMT assays except that 5 μl uridine diphospho-D-[U-^{14}C]glucose (940 Bq, sp. act. 12.0 GBq $mmol^{-1}$, Amersham) was used instead of the *S*-adenosylmethionine and the 1.0 M Tris–HCl was adjusted to pH 7.0. The reaction products were partitioned into water-saturated *n*-BuOH prior to quantification by LSC. For peroxidase assays 100 μl of the enzyme extract was incubated with 880 μl 0.1 M K-Pi buffer pH 6.5, 20 μl phenolic substrate (10 mM in MeOH) and 20 μl 1 mM H_2O_2. Samples were incubated at 30° and the *A* change monitored at 595 nm with scopoletin as substrate and 450 nm with esculetin as substrate.

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