Pergamon

PII: S0031-9422(97)00122-2

Phytochemistry, Vol. 45, No. 6, pp. 1109–1114, 1997

0031-9422/97 $17.00 + 0.00

CHARACTERIZATION AND INDUCIBILITY OF A SCOPOLETIN-DEGRADING ENZYME FROM SUNFLOWER

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(*Received in revised form* 9 *December* 1996)

Key Word Index—*Helianthus annuus*; Compositae; coumarins; peroxidase; phytoalexins; scopoletin; stress-inducible.

Abstract—The role of an enzyme, tentatively identified as a peroxidase, in the metabolism of the coumarin phytoalexin scopoletin has been investigated in sunflower. When sunflower leaf discs were fed with 0.05 mM scopoletin the coumarin was initially slowly metabolized by glycosylation. After a delay of 24 hr scopoletin then rapidly disappeared and this could not be accounted for by the accumulation of extractable metabolites. Instead, the disappearance of scopoletin was associated with the increased activity of a peroxidase which metabolized the coumarin to a coloured insoluble metabolite. The scopoletin-peroxidase was purified 222-fold and had a M_r of 46 000 and was associated with peroxidase activity toward esculetin and guaiacol, but not ayapin. In leaves, the activity of the scopoletin-peroxidase was increased locally around wound sites and its activity could be elevated at more distant sites by treatment with $CuCl_2$ or salicylic acid. The scopoletin-peroxidase activity was also identified in peas, tobacco and potato. © 1997 Elsevier Science Ltd. All rights reserved

INTRODUCTION

The accumulation of antifungal phytoalexins is a key defence response of many plants to infection by fungal pathogens [1–3]. Phytoalexins may be of a variety of structural types including sesquiterpenes, isoflavonoids, stilbenes, polyacetylenes and coumarins, but all share similar characteristics. All are synthesized *de novo* and rapidly accumulate at the site of infection up to millimolar concentrations, where they act as non-specific biocides [1]. The biosynthetic routes leading to the isoflavonoid [2] and sesquiterpene [3] phytoalexins are well established and their respective regulation the subject of intense study. In contrast, far less is known regarding the degradation of phytoalexins and the influence this has on determining their activity. The rates of disappearance of phytoalexins frequently mirrors their rates of accumulation, and although the invading fungi can account for some of this degradation, it is also clear that plants treated with abiotic elicitors are able to actively detoxify their own phytoalexins [1].

When damaged by insects [4], challenged with fungal pathogens [5, 6], or treated with abiotic elicitors such as sucrose or $CuCl_2$ [7], sunflower (*Helianthus annuus* L.) accumulates the coumarin phytoalexins scopoletin and ayapin in an organ specific manner [8]. In studies with both pathogenic and non-pathogenic fungi [5, 6] and abiotic elicitors [7], initially scopoletin accumulated as the major phytoalexin, but then declined to be replaced with ayapin. Following treatment with abiotic elicitors an inducible enzyme, tentatively identified as a peroxidase, degraded scopoletin, but not ayapin, to an insoluble blue metabolite [7]. We now report on the further characterization of this enzyme and its role in degrading coumarin phytoalexins in sunflower.

RESULTS AND DISCUSSION

Uptake and metabolism of scopoletin in sunflower leaf discs

Discs cut from freshly-detached leaves were incubated in either water or 0.05 mM scopoletin for 96 hr and the changes in concentration of fluorescent coumarins in the leaf disc and medium determined by fluorometry after their purification by TLC. Glycosylated coumarins were quantified following hydrolysis with cellulase. In the control incubations no fluorescent metabolites accumulated in either the discs or the medium either as aglycones or conjugates.

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Table 1. Uptake and metabolism of 0.05 mM scopoletin by sunflower leaf discs and associated increased activity of scopoletin-peroxidase

Time (hr)	Scopoletin (nmol) Medium	Discs	Scopolin* (nmol)	Total† (nmol) scopoletin	Peroxidase activity‡
0	1000±80	ND	ND	1000±80	0.2±0.0
12	790±0	75±15	41±2	906±10	0.3±0.1
24	810±95	70±21	99±23	979±73	0.5±0.3
48	550±65	46±12	112±19	708±50	6.0±0.5
72	240±10	20±6	133±14	393±8	10.7±1.4
96	235±35	25±8	114±31	374±22	11.1±0.8

Values refer to the mean of triplicate determinations ± s.d.
ND: not detected.
*Scopolin detected in the leaf discs only.
† Mean total scopoletin calculated as the sum of scopoletin in the medium and discs and scopolin.
‡ Peroxidase activity in units mg^{-1} protein.

Therefore, all the changes in coumarin content in the leaf discs fed with scopoletin were due to the metabolism of the exogenously-fed compound rather than due to the endogenous phytoalexin response.

In both the medium and leaf extracts, following treatment with scopoletin, the only fluorescent coumarin metabolite which moved off the origin of the TLC plate co-chromatographed with scopoletin. Appreciable additional quantities of scopoletin, but not other coumarins, could be released by treatment of the leaf extracts with cellulase (Table 1). Although the nature of the conjugated scopoletin was not investigated it was probably scopolin (scopoletin-7-*O*-glucoside), a well characterized metabolite of scopoletin in sunflower stems [6], and was quantified as such (Table 1). In contrast to the leaf discs, scopolin did not accumulate in the medium. In the unelicited leaf discs we did not observe the conversion of scopoletin to ayapin which had been reported in sunflower stems infected with *Helminthosporium carbonum* [6].

Between 0 and 24 hr uptake and conjugation of scopoletin by the leaf discs was relatively modest, but between 24 and 72 hr the scopoletin rapidly disappeared from the medium (Table 1). During this period the concentrations of scopoletin in the leaf discs actually declined, and conjugation could only account for a minor proportion of the missing scopoletin. TLC analysis failed to identify any extractable fluorescent or UV-absorbing metabolites which accumulated during the disappearance of scopoletin which could correspond to metabolites. Similarly, treatment of the insoluble fraction from the leaf discs with cellulase failed to release any coumarin.

Although these analyses were not exhaustive it was concluded that scopoletin had either been metabolized to an insoluble residue or degraded to metabolites which did not absorb UV light. In the absence of radiolabelled scopoletin no definitive explanation could be obtained. However, during the period of scopoletin disappearance there was a major increase in the activity of the scopoletin-degrading peroxidase (Table 1) which had been previously described [7]. Since this enzyme was known to metabolise scopoletin to an insoluble coloured precipitate, its appearance could account for the disappearance of the coumarin. In support of this suggestion, when scopoletin was fed to bisected sunflower stems, which had been incubated in water for 72 hr, the cut surfaces turned bright blue, confirming this reaction occurred *in vivo* (data not shown). Therefore, the enzyme was studied in greater detail.

Characterization of an enzyme with peroxidase activity toward scopoletin

With scopoletin as substrate the activities of the peroxidase were scarcely detectable in freshly-detached leaves from healthy sunflower plants grown in an environmental chamber. In contrast, unwounded leaves contained measurable peroxidase activities toward guaiacol (0.8 units mg^{-1} protein) and esculetin (0.5 units mg^{-1}). Previous studies had shown that the scopoletin-peroxidase activity was increased by detaching leaves [7]. Therefore, to induce the enzyme, young leaves (average axial length 70 cm) were detached and fed with water through the petiole for 3 days prior to assay. When crude extracts were incubated with scopoletin and hydrogen peroxide, a blue reaction product was formed which became progressively more insoluble with increasing time. Over a 2 min period the formation of the blue-coloured product was strictly dependent upon protein content in the range 0–0.3 mg total leaf protein per assay and could be abolished by boiling the leaf extract. No coloured product was formed in the absence of hydrogen peroxide, suggesting that the enzyme was not a laccase or polyphenol oxidase. The enzyme activity showed a defined pH optimum of pH 6.5 and was maximal when assayed at 30° (6.8 units mg^{-1} protein) with the activity declining to 5.7 units mg^{-1} at 40° and 2.6 units mg^{-1} at 50°. In crude extracts the enzyme activity was stable on thawing, following freezing at −20°.

The peroxidase activity was sequentially purified by hydrophobic interaction chromatography, gel fil-

Table 2. Summary of the purification of scopoletin-peroxidase from detached sunflower leaves fed with water for 72 hr

Purification stage	Protein (mg)	Enzyme activity	Purification (-fold)	Total activity	Recovery (%)
Crude extract	627	1.0	1	1190	100
Ammonium sulphate precipitate	480	4.6	2	2210	185
Phenyl-Sepharose	160	22.8	12	3650	306
Sephacryl S-200	17	56.2	30	955	80
DEAE-Sepharose	1	422.0	222	422	36

Enzyme activity in units mg^{-1} protein.

tration chromatography and anion exchange chromatography. A summary of the purification obtained is given in Table 2. On the phenyl-Sepharose column all the peroxidase activity toward scopoletin eluted as a broad peak in the presence of 0.5 M ammonium sulphate. Following this purification step there was a three-fold increase in the recovery of total enzyme activity, suggesting that the enzyme had been resolved from an endogenous inhibitor (Table 2). On Sephacryl S-200 the peroxidase eluted as a single peak with a similar retention to ovalbumin, with a molecular mass of 46 000 being calculated [Fig. 1(A)]. The active fractions were then applied to a DEAE column at pH 7.

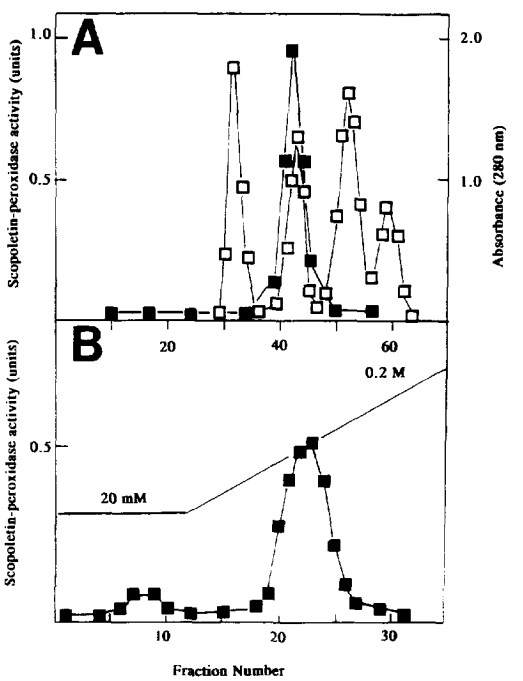

Fig. 1. Purification of scopoletin-peroxidase activity from detached sunflower leaves. Extracts previously purified by hydrophobic interaction chromatography were applied to (A) a Sephacryl S-200 column and fractions assayed for activity (■) with the sequential elution of the UV-absorbing calibration proteins bovine serum albumin (66 000), ovalbumin (45 000), chymotrypsin (25 000) and RNAse (14 000) shown (□). Active fractions were then applied to (B) a DEAE-Sepharose column and fractions assayed for activity (■) following elution with an increasing concentration of KPi.

The majority of the activity was retained on the column and was recovered using an increasing concentration of KPi, indicating that the enzyme was acidic [Fig. 1(B)].

To confirm that the 222-fold purified enzyme was a peroxidase, when 1 mg of catalase was added to standard incubations the formation of coloured product from scopoletin was reduced by 97%, showing that the reaction was dependent on hydrogen peroxide. To determine how many peroxidase isoenzymes showed activity toward scopoletin, preparations at various stages of purification were analysed by non-denaturing gel electrophoresis and enzyme activity located by incubating the gel with scopoletin and hydrogen peroxide. In all cases a single stained band was observed (data not shown). The formation of coloured product was prevented if hydrogen peroxide was omitted from the incubation, further confirming that the activity was due to a peroxidase, rather than a laccase. The final 222-fold purified peroxidase preparation showed no activity toward ayapin, but when analysed for enzyme activity using the *in situ* gel assay the same band which stained for activity with scopoletin, also stained for activity with guaiacol and esculetin. This demonstrated that either there was a single peroxidase present with activities toward all three substrates, or that isoenzymes with distinct substrate specificities but identical M_r had co-purified with the scopoletin peroxidase activity. Although peroxidases with activity toward scopoletin have been described in plants [9] their specificity toward other substrates have not been well defined. Since unwounded leaves contain appreciable peroxidase activity toward esculetin and guaiacol, but not scopoletin, it is clear that sunflower must contain multiple peroxidases, some which have activity toward scopoletin and some which do not.

Inducibility of scopoletin-peroxidase

Our preliminary studies showed that the activity of scopoletin-peroxidase was increased in detached sunflower leaves fed with abiotic elicitors, with $CuCl_2$ being more effective than sucrose [7]. The leaf disc metabolism studies also suggested that the peroxidase could be induced by wounding alone (Table 1). To extend these studies, detached sunflower leaves of differing axial lengths were placed in water and per-

Table 3. Effect of detachment and treatment with 10 μM $CuCl_2$ or 100 μM salicylic acid on scopoletin-peroxidase activity in sunflower leaves of differing axial length

Leaf length* (mm)	Feeding solution	Peroxidase activity (units mg^{-1} protein) 24 hr	48 hr	72 hr	96 hr
75	Water	0.1±0.0	1.0±0.4	6.1±0.2	14.6±0.2
107	Water	0.2±0.0	0.4±0.3	2.0±0.0	5.1±0.2
123	Water	0.1±0.1	0.3±0.1	1.2±0.1	3.2±0.2
110	Water	0.1±0.1	0.2±0.0	1.3±0.3	3.6±0.3
110	$CuCl_2$	0.2±0.0	1.2±0.0	3.3±0.5	2.0±0.1
110	Salicylic acid	1.2±0.3	3.7±0.4	4.6±0.2	2.2±0.1

Values represent the mean of results obtained with two pairs of leaves ($n = 4$) ±standard deviations.
* Leaf lengths refer to mean lengths with the variation being less than 10%.

oxidase activity determined at timed intervals (Table 3). In all the leaves the maximal increase in scopoletin-peroxidase activity occurred between 48 and 72 hr after detaching the leaves, with the enhancement being greatest in the smaller, younger leaves. A set of detached leaves of intermediate size were then incubated with either water, $CuCl_2$ or salicylic acid. $CuCl_2$ was selected as it had been previously shown to increase scopoletin-peroxidase activity in sunflower leaves [7]. In addition, salicylic acid is an effective inducer of anionic peroxidases in tobacco [10] and its derivative aspirin causes the accumulation of extracellular pathogenesis-related proteins in sunflower [11]. Both treatments gave a more rapid increase in peroxidase activity than was determined with wounding alone, though overall, the maximal activities observed after wounding were comparable to those obtained with the chemical treatments (Table 3). The treated leaves were then sectioned to determine where the peroxidase activity was being elevated (Table 4). In the case of water-fed leaves the peroxidase activity was highest at the base of the leaf near the wounded petiole and then declined towards the tip of the leaf. With both $CuCl_2$ and salicylic acid treatment this gradation of enzyme activity was less pronounced, demonstrating that the abiotic elicitors were exerting their effect over a greater proportion of the leaf than wounding alone.

Table 4. Activity of scopoletin-peroxidase in sections cut across the width of 11 cm-long detached leaves treated for 72 hr with either water, 10 μM $CuCl_2$ or 0.1 mM salicylic acid

Section* (mm)	Peroxidase activity (units mg^{-1} protein) Water	$CuCl_2$	Salicylic acid
0–22	2.8±0.0	2.3±0.4	2.7±0.6
22–44	1.4±0.3	2.8±0.0	3.3±0.3
44–66	0.7±0.2	2.4±0.3	4.0±1.0
66–88	0.8±0.1	1.8±0.3	3.3±0.2
88–110	0.9±0.0	1.7±0.3	3.0±0.7

Mean results obtained from two pairs of leaves±s.d. ($n = 4$).
* Lateral sections cut at right angles to the axis of the leaf, where 0 mm was the base of the leaf nearest the wound site.

In view of the activity of salicylic acid in systemic acquired resistance in tobacco [10] it was of interest to determine whether salicylic acid could increase scopoletin-peroxidase activity systemically as well as locally in sunflower plants. To test this possibility 0.1 millimolar salicylic acid was applied to the lower leaves of sunflower and, after 72 hr, scopoletin-peroxidase activity was determined in the upper leaves and compared with the activities determined in leaves of comparable size from untreated plants. Salicylic acid increased scopoletin-peroxidase activity in the upper leaves from a control value of 0.16±0.04 units mg^{-1} protein (mean±s.d. $n = 3$ plants), to 0.35±0.08 units mg^{-1} protein.

It was also of interest to determine whether scopoletin-peroxidase could be determined in plants other than sunflower, and if so whether or not such an activity was similarly inducible. Peroxidase activity was determined in detached leaves of tobacco (*Nicotiana tabaccum* L. cv. Samsun), potato (*Solanum tuberosum* L. cv. Desiree) and pea (*Pisum sativum* L. Feltham's First) fed with water for 48 hr. These species were selected, as potato [12] and tobacco [13] are known to synthesize scopoletin, while pea does not. The peroxidase activity in potato, tobacco and pea leaves were, respectively, 2.3±0.1 units mg^{-1} protein (mean±s.d. $n = 4$), 2.3±0.3 units mg^{-1} and 1.3±0.2 units mg^{-1}. Treatment of potato or pea leaves with either $CuCl_2$ or salicylic acid did not further increase scopoletin-peroxidase activity. In tobacco $CuCl_2$ was similarly ineffective, but salicylic acid increased the activity of scopoletin-peroxidase to 4.2±0.4 units mg^{-1} protein.

The potential role of the inducible scopoletin-peroxidase in the phytoalexin response of sunflower and other species, such as tobacco and potato, which produce this coumarin is intriguing. When sunflower leaves are challenged with fungal pathogens [5, 6], or treated with $CuCl_2$ [7] scopoletin accumulates rapidly and then declines, while ayapin is longer lived. Our current studies would suggest that this differential disappearance of the two phytoalexins can be accounted

for by the induction of the peroxidase which can degrade scopoletin, but not ayapin. the peroxidative metabolism of scopoletin may have a direct defensive function in sunflower, as suggested in tobacco [13]. Alternatively, the function of the peroxidase may be to protect plants from the potential phytotoxic effects of scopoletin.

EXPERIMENTAL

Treatment of plants. Sunflower (*Helianthus annuus* L. cv. Peredovick) plants were grown in Levingtons compost for 8–10 weeks as described previously [7]. For metabolism studies healthy leaves were removed from sunflower plants and 1 cm leaf discs prepd by cutting the leaves with a cork borer under water. Leaf discs (3 g fr. wt) were placed in a glass Petri dish containing 20 ml of either 0.5% EtOH or 0.05 mM scopoletin which had been prepared by diluting a 10 mM soln of scopoletin in EtOH 200-fold in H_2O and incubated in a growth chamber at 25° under a 16 hr photoperiod at a light intensity of 510 μmol m^{-2} s^{-1}. Leaf discs were separated from the media at timed intervals prior to storage at −20°.

For enzyme studies leaves were detached from the plant and their petioles re-cut under H_2O prior to transferring to the feeding solns which consisted of either sterile H_2O, 10 μM $CuCl_2$ or 100 μM salicylic acid, the latter adjusted to pH 7 with 0.1 M NaOH. The leaves were then incubated under the conditions described for the leaf discs and harvested at timed intervals for analysis.

Analysis of coumarin metabolites. Samples of the medium (1 ml) from the leaf disc study were incubated for 16 hr at 30° with either 0.2 ml 0.15 M Pi–citrate pH 5.2 or Pi–citrate containing 2 mg ml^{-1} cellulase from *Trichoderma viride* (Bohringer). The reaction mixt. was partitioned twice with 1 (v/v) EtOAc and the organic phase evapd to dryness before dissolving in MeOH (50 μl). The leaf discs (3 g) were extracted as described previously [7] and the concentrate redissolved in MeOH (1 ml). Duplicate 0.1 ml samples were then either incubated in 900 μl of 0.15 M Pi–citrate pH 5.2 with or without cellulase and the samples processed as described for the medium. Samples (10 μl) of the concd medium and leaf extracts were applied to TLC plates (silica gel 60 F_{254}, Merck) and the plate developed in Et_2O. Fluorescent compounds co-chromatographing with authentic coumarin metabolites [7] were carefully scraped off the plate and the silica suspended in MeOH (1 ml). After standing for 30 min the silica was sedimented by centrifugation 10 000 ***g***, 5 min and 0.8 ml of the supernatant transferred to a fluorometer cuvette and made up to 3 ml with MeOH. The amount of coumarin was then quantified from the emission at 430 nm after excitation at 340 nm using a Shimadzu RF-5001 PC spectrofluorophotometer with reference to a standard curve prepared with scopoletin. Quenching of fluorescence due to co-chromatographing pigments was corrected for by re-measuring the fluorescence after the addition of 500 ng scopoletin. By spiking control medium and control leaf discs each with 1 μmol of scopoletin the final recoveries by this method were 68% ± 12% (mean ± s.d. $n = 3$) for the medium and 73% ± 8% for the leaf discs.

Purification of peroxidase. All purification steps were carried out at 4° using a Pharmacia GradiFrac apparatus. Leaves were extracted in 4 v/w ice-cold 0.1 M KPi pH 6.5 using a pestle and mortar with acid-washed sand as an abrasive. After straining through 4 layers of muslin the filtrate was centrifuged (17 000 ***g***, 20 min). The peroxidase activity was routinely assayed in the supernatant as this stage. For further purification $(NH_4)_2SO_4$ was added to the supernatant to 80% satn and the protein pellet collected by centrifugation (17 000 ***g***, 30 min) prior to re-suspending in 20 mM KPi pH 7 containing 2 M $(NH_4)_2SO_4$ (loading buffer). After clarifying the suspension the soluble protein was applied to a pre-equilibrated column (10 ml) of phenyl-Sepharose. The column was washed with loading buffer at 0.5 ml min^{-1} and the eluent monitored for *A* at 280 nm until the chart recorder indicated that no further unbound protein was present. The column was then sequentially washed at 0.5 ml min^{-1} with 20 mM K–Pi pH 7.0 containing firstly 1.0 M $(NH_4)_2SO_4$ and then 0.5 M $(NH_4)_2SO_4$. Frs (2.5 ml) were collected and assayed for activity and the wash continued until the *A* had returned to zero. The active frs were pooled and the proteins ppd with 80% $(NH_4)_2SO_4$. After re-suspension in 0.1 M KPi pH 7 (10 ml) the sample was applied in 5 ml lots to a Sephacryl S-200 HR column (Pharmacia 46 cm by 2.5 cm i.d.) at a flow rate of 0.3 ml min^{-1} and frs (3 ml) collected and assayed for peroxidase activity. The column had previously been calibrated with bovine serum albumin (66 000), ovalbumin (45 000), chymotrypsin (25 000) and RNAse (14 000). Active frs were pooled and dialysed against 20 mM KPi pH 7 before application to a 3 ml column of DEAE-Sepharose pre-equilibrated in 20 mM KPi. After washing at 0.5 ml min^{-1} with 10 ml of 20 mM KPi, the peroxidase was covered with a 23 ml linear gradient of KPi pH 7.0 up to a final concn of 0.2 M.

Peroxidase assays. Either 10 μl of 10 mM scopoletin, 10 mM esculetin or 10 mM guaiacol was added to a 1 ml plastic cuvette containing 10 μl of 0.1 M H_2O_2 and 0.88 ml of KPi pH 6.5. After mixing the reaction mixture was incubated at 30° for 5 min and the reaction then initiated by the addition of 100 μl enzyme. The formation of coloured product was then determined by monitoring the change in *A* at 595 nm for scopoletin, at 450 nm for esculetin and at 470 nm for guaiacol. Enzyme activity was expressed in arbitrary units, with 1 unit corresponding to an *A* change of 1 min^{-1}. Protein content was determined using a dye-binding assay with gamma-globulin as the reference protein as recommended by the manufacturer (Biorad).

Polyacrylamide gel electrophoresis. Protein extracts

were dialysed against 0.5 M Tris–HCl pH 6.8 and then 0.1 v/v glycerol and 0.1 v/v bromophenol blue (0.2%) added, prior to application on a 7.5% polyacrylamide minigel. A non-denaturing discontinuous buffer system was used of pH 6.8 in the stacking gel and pH 8.8 in the resolving gel [14]. After electrophoresis at 200 V, the gel was incubated in 0.1 M KPi containing 1 mM H_2O_2 and either 0.1 mM scopoletin, 0.1 mM guaiacol or 0.1 mM esculetin, until a stained band appeared. To confirm that the coloured band was due to peroxidase activity, H_2O_2 was omitted from the incubation.

Acknowledgements—The authors acknowledge the financial assistance of the Royal Society and the Acciones Integradas scheme jointly run by the British Council and Spanish Ministry of Education and Science.

REFERENCES

1. Barz, W., Bless, W., Börger-Papendorf, G., Gunia, W., Mackenbrock, U., Meier, D., Otto, C. and Süper, E., in *Bioactive Compounds from Plants, Ciba Foundation Symposium*. Wiley, Chichester, 1990, p. 140.
2. Dixon, R. A., Harrison, M. J. and Paiva, N. L., *Physiologia Plantarum*, 1995, **93**, 385.
3. Weissenborn, D. L., Denbow, C. J., Laine, M., Lång, S. S., Yang, Z., Yu, X. and Cramer, C. L., *Physiologia Plantarum*, 1995, **93**, 393.
4. Olson, M. M. and Roseland, C. R., *Environmental Entomology*, 1991, **20**, 1166.
5. Tal, B. and Robeson, D. J., *Phytochemistry*, 1986, **25**, 77.
6. Tal, B. and Robeson, D. J., *Plant Physiology*, 1986, **82**, 167.
7. Gutiérrez, M.-C., Parry, A. D., Tena, M., Jorrin, J. and Edwards, R., *Phytochemistry*, 1995, **38**, 1185.
8. Gutiérrez-Mellado, M.-C., Edwards, R., Tena, M., Cabello, F., Serghini, K. and Jorrin, J., *Journal of Plant Physiology*, 1996, **149**, 261.
9. Ros Barcelo, A. and Muñoz, R., *Phytochemistry*, 1989, **28**, 1331.
10. Ward, E. R., Uknes, S. J., Williams, S. C., Dincher, S. S., Wiederhold, D. L., Alexander, D. C., Ahl-Goy, P., Métraux, J.-P. and Ryals, J. A., *Plant Cell*, 1991, **3**, 1085.
11. Jung, J.-L., Fritig, B. and Hahne, G., *Plant Physiology*, 1993, **101**, 873.
12. Clarke, D. D., *Plant Pathology*, 1973, **3**, 347.
13. Goy, P. A., Signer, H., Reist, R., Aichholz, R., Blum, W., Schmidt, E. and Kessmann, H., *Planta*, 1993, **191**, 200.
14. Hames, B. D., in *Gel Electrophoresis of Proteins, a Practical Approach*, ed. B. D. Hames and D. Rickwood. IRL Press, Oxford, 1988, p. 1.