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Biosynthesis of scopoletin and scopolin in cassava roots during post-harvest physiological deterioration: The *E*-*Z*-isomerisation stage

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ABSTRACT

Two to three days after harvesting, cassava (*Manihot esculenta* Crantz) roots suffer from post-harvest physiological deterioration (PPD) when secondary metabolites are accumulated. Amongst these are hydroxycoumarins (e.g. scopoletin and its glucoside scopolin) which play roles in plant defence and have pharmacological activities. Some steps in the biosynthesis of these molecules are still unknown in cassava and in other plants. We exploit the accumulation of these coumarins during PPD to investigate the *E*-*Z*-isomerisation step in their biosynthesis. Feeding cubed cassava roots with *E*-cinnamic-3,2′,3′,4′,5′,6′-d_5 acid gave scopoletin-d_2. However, feeding with *E*-cinnamic-3,2′,3′,4′,5′,6′-d_6 and *E*-cinnamic-2,3,2′,3′,4′,5′,6′-d_7 acids, both gave scopoletin-d_3, the latter not affording the expected scopoletin-d_4. We therefore synthesised and fed with *E*-cinnamic-2-d_1 when unlabelled scopoletin was biosynthesised. Solely the hydrogen (or deuterium) at C2 of cinnamic acid is exchanged in the biosynthesis of hydroxycoumarins. If the mechanism of *E*-*Z*-cinnamic acid isomerisation were photochemical, we would not expect to see the loss of deuterium which we observed. Therefore, a possible mechanism is an enzyme catalysed 1,4-Michael addition, followed by σ-bond rotation and hydrogen (or deuterium) elimination to yield the *Z*-isomer. Feeding the roots under light and dark conditions with *E*-cinnamic-2,3,2′,3′,4′,5′,6′-d_7 acid gave scopoletin-d_3 with no significant difference in the yields. We conclude that the *E*-*Z*-isomerisation stage in the biosynthesis of scopoletin and scopolin, in cassava roots during PPD, is not photochemical, but could be catalysed by an isomerase which is independent of light.

1. Introduction

Cassava (*Manihot esculenta* Crantz Family Euphorbiaceae) is an important economical and nutritional crop, the fourth most important food source in tropical countries due to its high root starch content. Although cassava is relatively easy to grow, even in poor soils and under drought conditions, its roots have a short shelf-life of only one to three days due to post-harvest physiological deterioration (PPD) which can cause significant wastage and economic losses. Within 2–3 days of harvesting, the roots show blue to black vascular streaking and are unpalatable and therefore unmarketable, significantly affecting the crop's economic value. PPD has been explained as a physiological process not due to microorganisms, (Averre, 1967; Noon and Booth, 1977) and on a molecular basis as an oxidative burst which initiates within 15 min of the root being injured, (Reilly et al., 2003, 2004) followed by altered gene expression. This genetic change is predicted to play roles in cellular processes including: reactive oxygen species turnover, cell wall repair, programmed cell death, ion, water or metabolite transport, signal transduction or perception, stress response, metabolism and biosynthesis, activation of protein synthesis (Reilly et al., 2007) and the accumulation of secondary metabolites (Tanaka et al., 1983; Buschmann et al., 2000). Amongst these secondary metabolites are hydroxycoumarins (e.g. scopoletin and its glucoside scopolin) which show antioxidant properties and which may by oxidation and polymerisation give rise to the blue/black discolouration. These hydroxycoumarins are important in plant defence as phytoalexins due to the induction of their biosynthesis following various stress events (wounding, bacterial and fungal infections) (Giesemann et al., 1986; Gutierrez et al., 1995). Additionally, they display a wide range of pharmacological activities, including anti-coagulant (Mueller, 2004), anti-inflammatory (Silvan et al., 1996), antimicrobial (Valle et al., 1997; Cespedes et al., 2006) and anticancer (Kawaii et al., 2001; Kawase et al., 2003; Lacy and O'Kennedy, 2004) activities. However, their biosynthesis in cassava is not known and neither is it clearly understood in other plants (Petersen et al., 1999).

Indeed, the biosynthesis of coumarins in plants is not well understood, although these metabolic pathways are often found in the plant kingdom. From biosynthetic studies in *Arabidopsis thaliana* ecotype Columbia, Kai et al. (2006) recently reported in this

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Journal on the occurrence of high levels of the coumarins scopoletin and its β-D-glucopyranoside, scopolin, found in the wild-type roots 180-fold higher than in aerial parts. Their studies with mutants led to the identification of 3′-hydroxylation of *p*-coumarate catalysed by CYP98A3, one of the few enzymes to be identified unambiguously along this complex phenylpropanoid pathway. More recently in *A. thaliana*, the same research group showed that a Fe(II)- and 2-oxoglutarate-dependent dioxygenase, rather than a cytochrome P450 enzyme, catalyses the *o*-hydroxylation of feruloyl-CoA in scopoletin biosynthesis (Kai et al., 2008).

As part of on-going studies into these hydroxycoumarins (Buschmann et al., 2000; Reilly et al., 2003, 2004), we investigated the incorporation of cinnamic acid-d_7 into cassava roots under PPD, in order to prove that it was a potential precursor of scopoletin and scopolin on the phenylpropanoid pathway (Fig. 1), from phenylalanine following the action of phenylalanine ammonia lyase (PAL).

The unexpected experimental result that scopoletin-d_3 was produced, and not scopoletin-d_4 (Fig. 2), has led us to study in detail the *E*-*Z*-isomerisation step in the biosynthesis of scopoletin and scopolin. This isomerisation is not resolved in plants, and has not been previously reported in cassava under PPD. Here, we exploit the increase in hydroxycoumarin accumulation in cassava roots post-harvest to investigate this isomerisation step in the biosynthesis of scopoletin and scopolin.

1.1. The E-Z-cinnamic acid isomerisation stage in different plants

In order to interpret this unexpected loss of deuterium at position 2 when *E*-cinnamic-d_7 acid was fed to cassava root under PPD, we undertook a detailed literature search on previous studies of the *E*-*Z*-isomerisation step in coumarin biosynthesis. These report that the mechanism of this step varies between genera and even between species. It has been reported (Edwards and Stoker, 1967, 1968) that the isomerisation may be induced by UV light in vivo, as has been demonstrated in vitro (Koenigs et al., 1993; Zheng et al., 1999). Photoisomerisation of the *E*-double bond, in *p*-coumarate, has been studied using yellow protein that ultimately mediates a phototactic response to blue light in certain purple bacteria *Ectothiorhodospira* (Ryan et al., 2002; Dugave and Demange, 2003). Feeding *Melilotus officinalis* shoots with *E*-*o*-coumaric acid-2-^{14}C in both dark and light conditions showed much more radioactivity in the coumarins isolated from shoots exposed to light than in the coumarins isolated from shoots kept in the dark. Edwards and Stoker therefore concluded that an isomerase enzyme is not involved in the isomerisation of *o*-coumaric acid in *M. officinalis* shoots (Edwards and Stoker, 1967). In lavender (*Lavandula officinalis* and *L. spica*), the biosynthesis of herniarin (7-methoxycoumarin) has been shown to be non-enzymatic, and the reaction is light catalysed. Therefore, based on the available evidence in 1967, Edwards and Stoker concluded that "it is probable therefore that the isomerisation step in the biosynthesis of all plant coumarins is entirely photochemical" (Edwards and Stoker, 1968). More recently, in this *Journal*, the isomerisation of cinnamic acid derivatives in barley and wheat was "directly attributed to the effect of light, and not apparently modulated by any enzymic reactions" (Turner et al., 1993), and in *A. thaliana* "sunlight was able to isomerise both cinnamic acid isomers" (Wong et al., 2005).

M. alba (sweet clover) plants fed with *E*-cinnamic acid-3-^{14}C in the dark produced ^{14}C-labelled coumarin. The amount of radioactivity in the coumarin was less than when plants were exposed

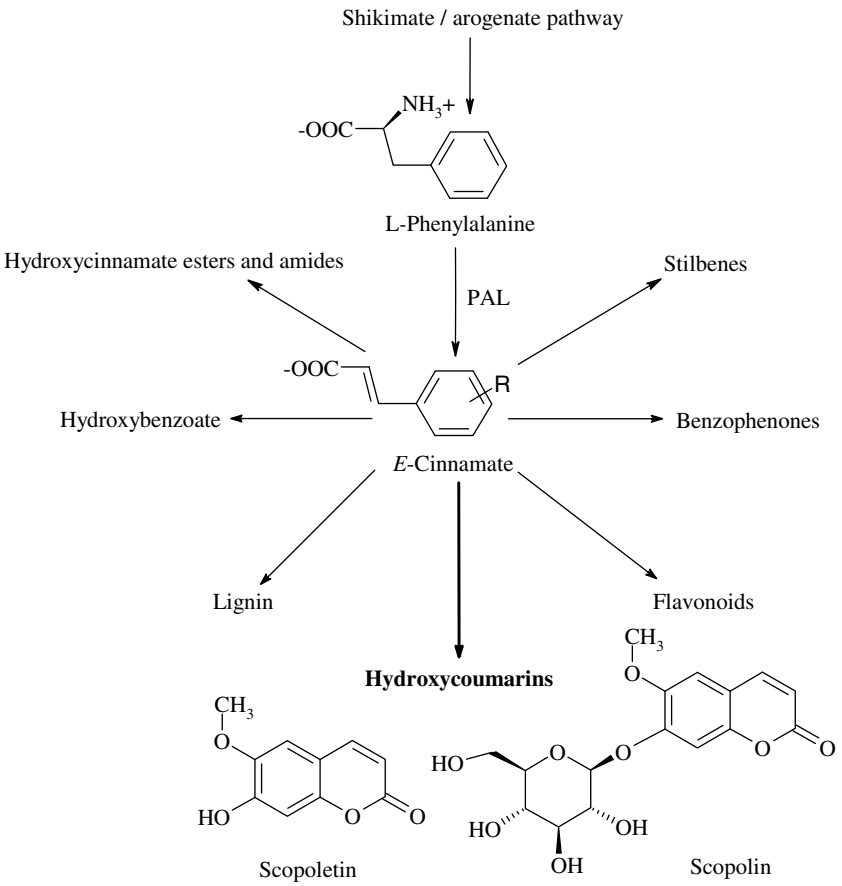

Fig. 1. Biosynthetic pathways of phenylpropanoids.

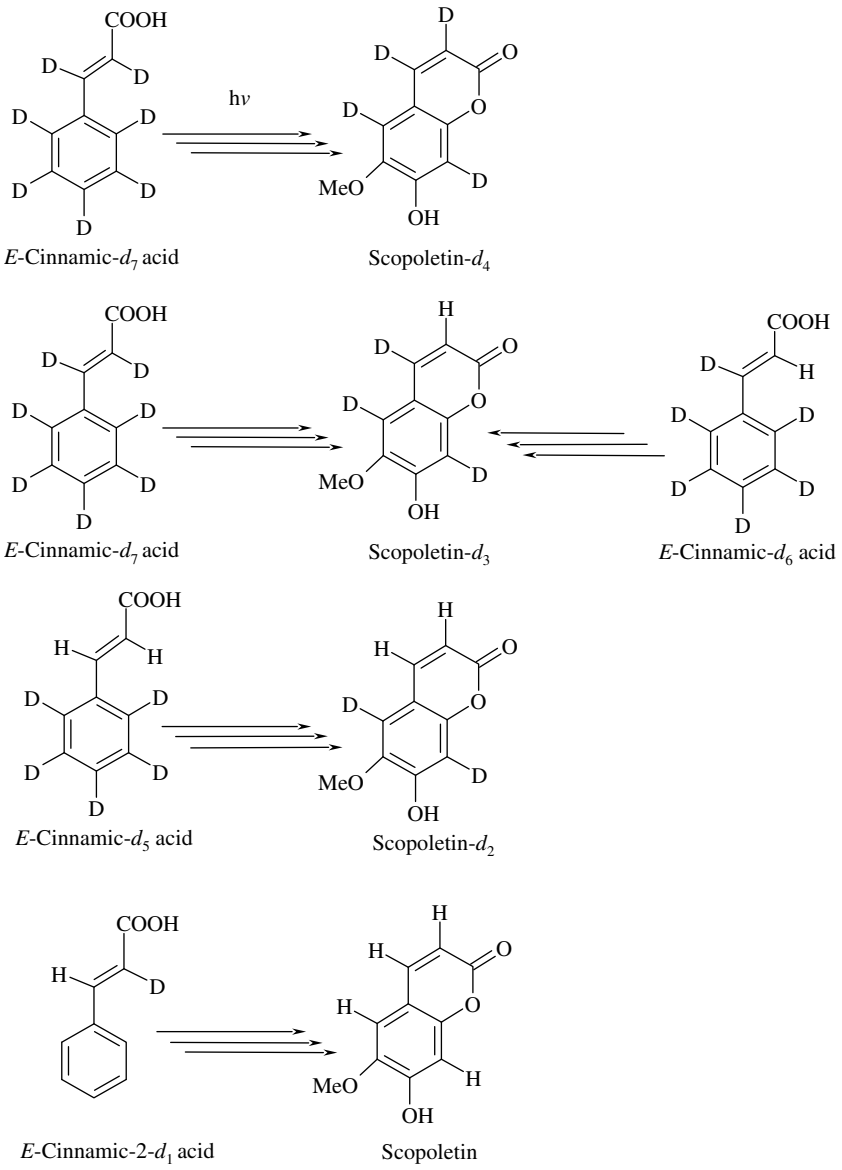

Fig. 2. The expected result of feeding *E*-cinnamic-d_7 acid is scopoletin-d_4 (first line). In cassava under PPD, the actual results of feeding experiments with *E*-cinnamic-2,3,2′,3′,4′,5′,6′-d_7 acid and *E*-cinnamic-3,2′,3′,4′,5′,6′-d_6 acid are that both precursors gave scopoletin-d_3 (second line). Experimental results from feeding *E*-cinnamic-2′,3′,4′,5′,6′-d_5 acid and *E*-cinnamic-2-d_1 acid show the isotopic labelling patterns of scopoletin-d_2 and unlabelled scopoletin respectively (bottom two lines). The biosynthetic steps represented by the multiple arrows include: *p*-, *m*-, and *o*-hydroxylation, *E*-*Z*-isomerisation, lactonisation and *O*-methylation (in a sequence to be defined).

to sunlight during the feeding experiments. Homogenates of *M. alba* leaves were also found to convert the 2′-β-glucoside of *o*-coumaric acid-2-^{14}C into coumarin in the dark. These results are indicative of the presence of an isomerase enzyme system in *M. alba* (Stoker, 1964). Moreover, there is evidence for light-independent isomerisation in the vacuole (Strack, 1997). *E*-*Z*-Isomerisation of *o*-coumaric acid glucoside was studied in *M. alba* mespophyll cell protoplasts (Rataboul et al., 1985). Protoplasts were isolated and incubated in the presence of ^{14}C-labelled phenylalanine. After 4 h incubation in the light, ^{14}C-labelled *o*-coumaric acid glucoside was biosynthesised and transported to the vacuole. Sixty percent of the synthesised *o*-coumaric acid glucoside was the *E*-isomer and 40% the *Z*-isomer. After longer incubation, 10.5 h in light, there was no significant change in the proportion of the two isomers. In contrast, if the protoplasts were incubated in light for 4 h then transferred to the dark for 6.5 h, the amount of synthesised *o*-coumaric acid glucoside was decreased while the percentage of the *E*-isomer was 28% and the percentage of the *Z*-isomer was 72% which indicates that isomerisation increased. These experiments indicate that *E*-*Z*-isomerisation of *o*-coumaric acid glucoside is not light dependent (Rataboul et al., 1985).

This review of the literature revealed conflicting views on the *E*-*Z*-isomerisation mechanism in coumarin biosynthesis which varies from one species to another, but the isomerisation stage has not been reported previously for scopoletin and scopolin biosynthesis in cassava or in other plants. In our study on cassava roots, if the isomerisation step in scopoletin and scopolin biosynthesis is photochemical, scopoletin-d_4 and scopolin-d_4 should be biosynthesised in cassava roots during PPD when fed *E*-cinnamic-d_7 acid. If the isomerisation is enzymatic, scopoletin-d_4 and scopolin-d_4 or scopoletin-d_3 and scopolin-d_3 could be biosynthesised depending on the enzyme mechanism. This specific loss of a proton has not been discussed before in the biosynthesis of any coumarins neither in cassava nor in other plants.

2. Results and discussion

Hydroxycinnamates are utilized in various pathways in the formation of different phenylpropanoids (Parveen et al., 2008) (Fig. 1) including hydroxycoumarins. *E*-Cinnamic acid is one of the early precursors in the biosynthesis of scopoletin under PPD (Fig. 1). A preliminary experiment was carried out by feeding cubed cassava root with deuterium labelled *E*-cinnamic-d_7 acid, to determine the level of incorporation of label observed under these simple conditions, in order to investigate intermediates along the hydroxycoumarin biosynthetic pathway. However, the intriguing result (Fig. 2) that scopoletin-d_3 and its *O*-glycoside scopolin-d_3 were obtained and not the (expected) corresponding d_4 analogues caused us to investigate the isomerisation stage in detail.

2.1. Feeding experiments with deuteriated E-cinnamic acids

In order to investigate the biosynthesis of scopoletin and scopolin during PPD, a feeding experiment was developed. Freshly harvested cassava roots were fed with different deuteriated *E*-cinnamic acid intermediates, at pH 7.5 adjusted to be compatible with the physiological state of the roots. A commercial authentic sample of scopoletin was used to identify its HPLC peak in the root extracts, however, scopolin is not commercially available. Scopolin and scopoletin were collected and unambiguously identified by NMR and HR MS, and therefore our isolated scopolin was used as a reference sample. HR ESI MS spectra (Fig. 3) showed the presence of the natural abundance peaks of scopolin and scopoletin in addition to peaks of scopolin-d_3 and scopoletin-d_3. There were no MS peaks corresponding to scopolin-d_4 or scopoletin-d_4 which we expected from the feeding experiment of *E*-cinnamic-d_7 acid. This loss of one deuterium atom more than expected during the biosynthesis requires a reconsideration of the mechanisms along the pathway. In order to determine which additional deuterium atom had been lost, a further feeding experiment was carried out using *E*-cinnamic-3,2′,3′,4′,5′,6′-d_6 acid, i.e. with ^{1}H isotope at C-2. This afforded the same two products as judged by both HPLC and HR MS. Feeding with *E*-cinnamic-d_6 acid did not result in an MS peak at 195 (scopoletin-d_2, $C_{10}H_7D_2O_4$ $[M+H]^+$) which was to be expected if the deuterium loss was from any other position than C-2.

In order to confirm this loss of proton or deuterium from C-2, we decided to feed with the deuterium isotope located at C-2. We therefore synthesized *E*-cinnamic-2-d_1 acid by a Knoevenagel reaction (Robbins and Schmidt, 2004; Ji et al., 2005), the nucleophilic addition of malonic acid-d_4 to the carbonyl group of benzaldehyde, as in an aldol condensation. This condensation reaction as well as losing a molecule of water (HOD) undergoes decarboxylation in a subsequent step. The catalyst was a basic mixture of piperidine (0.00011 eq.) pK_a 11 and pyridine (as solvent) pK_a 5.5 (Clayden et al., 2001) at 68 °C for 24 h, and it afforded a white crystalline compound (59%, mp 127–130 °C). It has been synthesised previously from the corresponding α-trimethylamino acid via Hofmann elimination in deuteriated water (Manitto et al., 1973), but no characterisation details were reported in that communication. HR MS gave *m/z* 148.0516 $[M-H]^-$, and comparison of the peak heights at *m/z* 148 (*E*-cinnamic-2-d_1 acid) and *m/z* 147 (unlabelled *E*-cinnamic acid) showed a deuterium label incorporation of 88%. Correspondingly, the ^{13}C NMR spectra showed two key signals corresponding to C-2 for labelled and unlabelled cinnamic acid respectively: δ 119.1 (*t*, 1:1:1, $^1J_{CD}$ = 25 Hz), shifted upfield by 0.2 ppm by α-substitution with deuterium, typically 0.25 ppm per α-deuterium atom (Tulloch and Mazurek, 1973; Wehrli and Wirthlin, 1983; Hardick et al., 1996), and a small singlet at 119.3 for C-2 attached to proton, the residual isotopomer. In the ^{1}H NMR spectrum, H-3 signals (integrating for 1) appear at 7.66 ppm as a slightly broadened singlet (with a small $^3J_{HD}$ 2 Hz, integral 0.88) (Williams and Fleming, 2008), superimposed on 7.67 (*d*, *J* = 16 Hz, integral 0.12) due to ^3J H-2–H-3 *E*-coupling. The small signal at δ 6.48 corresponding to H-2 (*d*, *J* = 16 Hz) due to ^3J H-2-H-3 *E*-coupling likewise integrated for 0.12, confirming the HR MS labelling result of 88%. The presence of the 12% of unlabelled cinnamic acid could be due to exchange of malonic acid-d_4 deuterium at position 2 with protons of piperidine. Finally, ^{2}H NMR observation confirmed these results by the signal at δ 6.51 (*d*, *J* = 2 Hz) which fits within the published range of 1–3 Hz for D-2-H-3 coupling as $J_{HH} = 6.5J_{HD}$ (Williams and Fleming, 2008).

Feeding the cassava cubes with this synthetic (88% enriched) *E*-cinnamic-2-d_1 acid gave HPLC peaks and corresponding HR MS data entirely consistent with unlabelled scopolin (R_t = 7.1 min, Table 1) and scopoletin (R_t = 24.7 min, Table 2). Finally, in this series, feeding with *E*-cinnamic-2′,3′,4′,5′,6′-d_5 acid gave the same HPLC peaks, but HR MS data consistent with scopolin-d_2 and scopoletin-d_2 and therefore, apart from the 3 sites of oxygenation (2′,4′,5′), there was no further loss of deuterium from the aromatic ring. We therefore compared the percentage incorporation between the labelled and unlabelled hydroxycoumarins, scopolin (Table 1) and scopoletin (Table 2) from these four experiments feeding with *E*-cinnamic-2-d_1, *E*-cinnamic-2′,3′,4′,5′,6′-d_5, *E*-cinnamic-3,2′,3′,4′,5′,6′-d_6 and *E*-cinnamic-2,3,2′,3′,4′,5′,6′-d_7 acids.

Isotope peaks monitored are shown in Tables 1 and 2. The accurate mass of the [M+1] isotope peaks for scopoletin are (found) 194.0532 and 194.0527, both values agreeing with the natural abundance calculated for $C_9{}^{13}CH_9O_4$ (194.0529) and the percentage of the isotope peak is similar (Table 2). The [M+1] peak is not due to the presence of 1 × D isotope at natural abundance which is calculated as 195.0562 for $C_{10}H_8DO_4$ and thus falls outside the 5 ppm limit of acceptable variation in HR MS. Thus, although at a superficial level adding a neutron to make ^{13}C or ^{2}H (D) looks like adding any other neutron, it is not the same mass gain, and this small, but measurable difference is called the mass defect. For scopolin (Table 1), the corresponding isotope peaks both show the same percentage (13.9%) and are within 5 ppm of the natural abundance calculated for $C_{15}{}^{13}CH_{19}O_9$ (356.1057).

HPLC separation of the peak at 7.1 min is consistent with scopolin and HR ESI MS for this peak in each experiment are summarized (Table 1). HPLC separation of the peak at 24.7 min is consistent with an authentic standard of scopoletin and HR ESI MS for this peak in each experiment are also summarized (Table 2). The percentages of isotopically labelled scopolin and scopoletin were calculated and are shown in Tables 1 and 2 respectively. Our results (summarised above in Fig. 2) confirm that the *E*-*Z*-isomerisation of the C=C double bond in the biosynthesis of scopoletin and scopolin in cassava roots during PPD involves the specific exchange of the hydrogen atom at position 2 of *E*-cinnamic acid and thus is likely to be enzyme catalysed. If this biosynthetic isomerisation step along the pathway to scopoletin was a non-enzymatic photochemical reaction, it would be expected to proceed through a diradical arising by light mediated homolytic fission (π to π^* excitation), σ-bond rotation of the diradical which then closes the C=C double bond to yield the Z-isomer. Therefore, there would be no loss from C-2 of deuterium (or proton) during the photochemical isomerisation of *E*-cinnamic acid-d_7 which would be converted into scopoletin-d_4 and this was not observed. Feeding experiments with *E*-cinnamic acid-d_7 in light or in dark conditions were therefore carried out to determine whether or not the enzymatic reaction required light. The percentage of scopoletin-d_3 recovered in light was 9.8% while in dark was 9.1%. As there is no significant difference in the amount of scopoletin-d_3 biosynthesised in light or in dark conditions, this demonstrates that the enzymatic step is light independent.

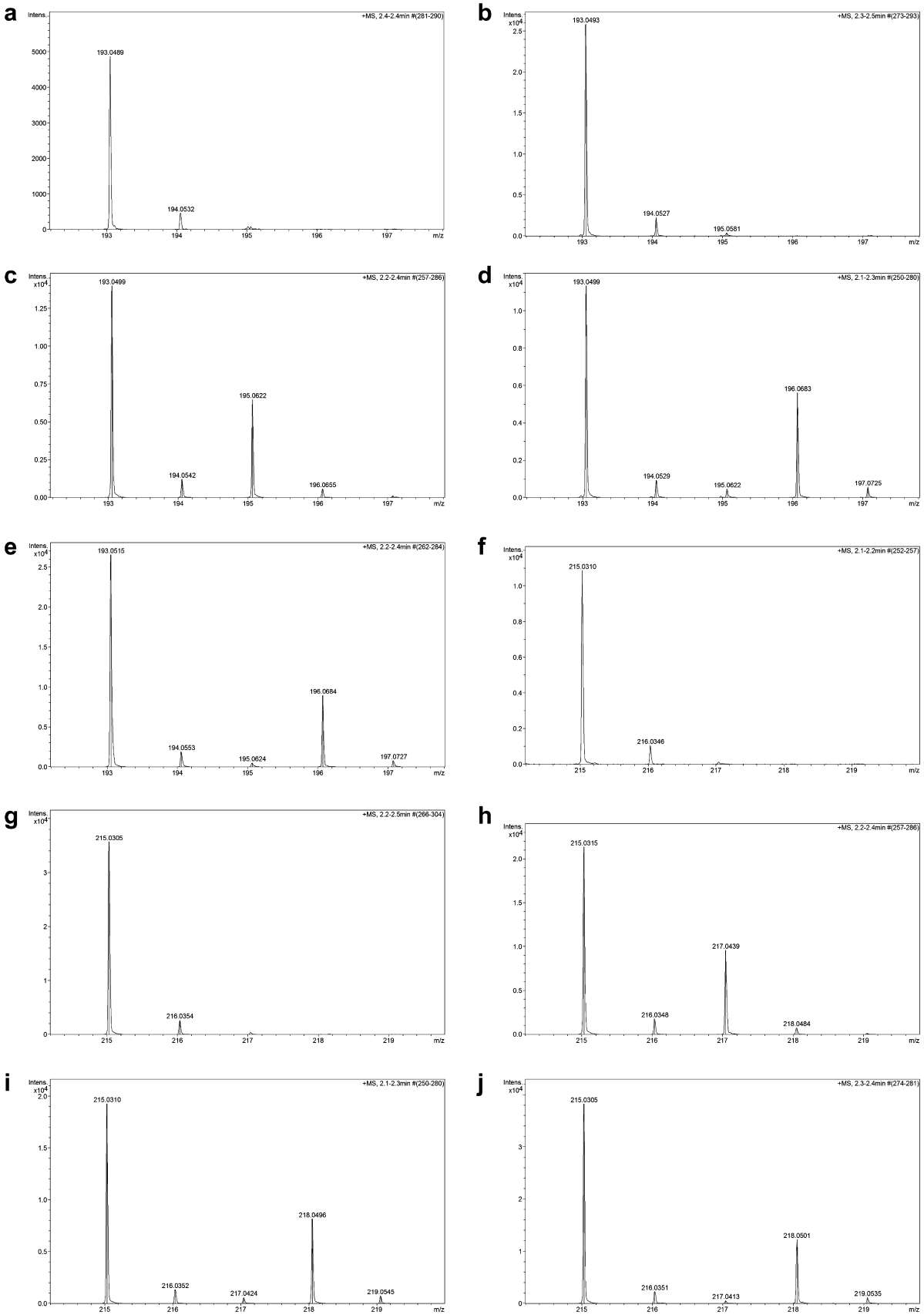

Fig. 3. Mass spectra of scopoletin after feeding experiments with (a) nothing, (b) *E*-cinnamic-2-d_1, (c) *E*-cinnamic-2′,3′,4′,5′,6′-d_5 acid, (d) *E*-cinnamic-3,2′,3′,4′,5′,6′-d_6 acid, (e) *E*-cinnamic-2,3,2′,3′,4′,5′,6′-d_7 acid showing the isotopic labelling patterns found in the product scopoletin ($M+^1H$) and ($M+^{23}Na$) (f–j, respectively).

From studies with maleylacetone *Z*-*E*-isomerase, it was proposed that glutathione transferase could catalyse the addition of glutathione (GSH) acting as cellular nucleophile in the enzyme-catalyzed *Z*-*E*-isomerisation. The proposed intermediate is a dienediol

Table 1
HR MS data of the HPLC peak at 7.1 min

Feeding cassava cv MCOL 22 with	*m/z* found of scopolin	Isotope peak monitored	*m/z* found of scopolin-d_{1-3} (calc.)	% of isotope peak/*m/z* 355
None (natural abundance ^{13}C)	355.1033	$C_{15}{}^{13}CH_{19}O_9$	356.1075 (356.1057)	13.9
Cinnamic-d_1 acid	355.1038	$C_{16}H_{18}D_1O_9$	356.1075 (356.1086)	13.9
Cinnamic-d_5 acid	355.1059	$C_{16}H_{17}D_2O_9$	357.1186 (357.1149)	32.3
Cinnamic-d_6 acid	355.1108	$C_{16}H_{16}D_3O_9$	358.1245 (358.1212)	34.0
Cinnamic-d_7 acid	355.1148	$C_{16}H_{16}D_3O_9$	358.1214 (358.1212)	26.1

Scopolin, $C_{16}H_{19}O_9$ $[M+H]^+$ (calc. 355.1024).

Table 2
HR MS data of the HPLC peak at 24.7 min

Feeding cassava cv MCOL 22 with peak/*m/z* 193	*m/z* found of scopoletin	Isotope peak monitored	*m/z* found of scopoletin-d_{1-3}, (calc.)	% of isotope
None (natural abundance)	193.0489	$C_9{}^{13}CH_9O_4$	194.0532 (194.0529)	7.5
Cinnamic-d_1 acid	193.0493	$C_{10}H_8D_1O_4$	194.0527 (194.0558)	8.3
Cinnamic-d_5 acid	193.0499	$C_{10}H_7D_2O_4$	195.0622 (195.0621)	31.8
Cinnamic-d_6 acid	193.0499	$C_{10}H_6D_3O_4$	196.0683 (196.0684)	33.1
Cinnamic-d_7 acid	193.0515	$C_{10}H_6D_3O_4$	196.0684 (196.0684)	25.0

Scopoletin, $C_{10}H_9O_4$ $[M+H]^+$, (calc. 193.0495).

formed by sulfur attack at C-2 (Fig. 4) and this intermediate undergoes σ-bond rotation and then elimination (possibly even before protonation at C-3 can take place) (Seltzer and Lin, 1979; Dixon et al., 2000). Alternatively, a nucleophilic addition of water across the C=C double bond may occur to yield the β-hydroxyacid, as in the second step of the well-known β-oxidation of saturated fatty acids, followed by σ-bond rotation and elimination of water to effect the isomerisation. In the fermentation of sweet clover (*M. alba*), microbial attack on *o*-coumaric acid leads to 4-hydroxycoumarin and ultimately to dicoumarol (from a crossed-aldol reaction with microbial formaldehyde) (Bellis et al., 1967; Bye and King, 1970) and the well-known warfarin story (Last, 2002). Such a reaction would lead to loss of H (or D) from the C-3 position of cinnamic acid and thus we do not consider micro-organisms to be involved in coumarin biosynthesis in cassava.

Water is similarly added to *E*-cinnamic acid during first step of benzaldehyde, vanillin, benzoic, salicylic and vanilloic acid biosynthesis; enoyl-SCoA hydratase in plants and bacteria catalyses the addition of water across the double bond of the α,β-unsaturated thiol ester (e.g. cinnamoyl SCoA or feruloyl SCoA) (Gasson et al., 1998; Abd El-Mawla and Beerhues, 2002; Bahnson et al., 2002; Walton et al., 2003). The intermediate from such a nucleophilic addition at the β-position could undergo σ-bond rotation and then antiperiplanar-(β-) elimination leading to the formation of the geometrical isomer.

We therefore conclude that as scopoletin-d_3 and scopolin-d_3 (not d_4) were biosynthesised when cassava cubes were fed with *E*-cinnamic-d_7 acid, and as we have established that the isomerisation stage in their biosynthesis in cassava roots during PPD is not photochemical, rather it is enzymatic, it could be catalysed by an isomerase that is not dependent on light.

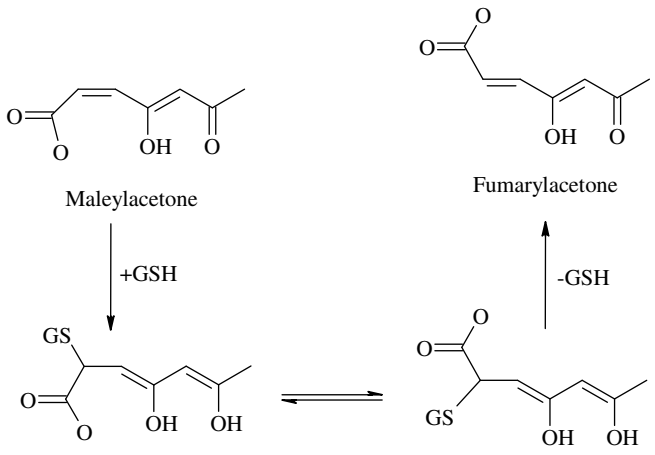

Fig. 4. Isomerisation of maleylacetone to fumarylacetone via formation of the GSH intermediate (though a regiospecific 1,4- or 1,6-Michael addition to the enone, not to the unsaturated carboxylate), (Seltzer and Lin, 1979; Dixon et al., 2000) σ-bond rotation and GSH elimination (retro Michael addition).

3. Experimental

3.1. Plant material

Root tubers of different cultivars (MCOL 22, MNGA 19, MNGA 2) were harvested from cassava plants growing in the tropical glass house at the University of Bath under the following condition: 22–28 °C, relative humidity (R.H.) (40–80%) and a light period of 14 h per d.

3.2. General methods

Chemicals were obtained routinely from Sigma–Aldrich Chemical Co. Ltd, UK. *E*-Cinnamic-2′,3′,4′,5′,6′-d_5 acid was obtained from CDN Isotopes, Canada. The HPLC instrument consisted of a solvent delivery system equipped with Jasco PU-980 pump and monitored at 360 nm with a Jasco UV-975 detector, using 16% acetonitrile in water containing 0.1% formic acid, flow rate 4 ml/min at 20 °C. The chromatograms were recorded on a Goerz Metrawatt Servogor 120 recorder. HPLC columns were purchased from Phenomenex Inc.: Phenomenex Gemini 10 μ C18 110A 250 × 10 mm with guard column Phenomenex Gemini 5 μ C18 10 × 10 mm. Samples were injected using a 100 μl loop. HR ESI MS was carried out on a Bruker micrOTOF mass spectrometer in the department of Pharmacy and Pharmacology, University of Bath or on a Micromass Quattro II in EPSRC National Mass Spectrometry Service Centre, University of Wales, Swansea. NMR spectra were obtained on a Varian Mercury Spectrometer at 400 MHz (^1H) and 100 MHz (^{13}C) in CD_3OD, all chemical shifts are reported in parts per million (ppm) relative to internal tetramethylsilane, and coupling constants (*J*) are absolute values in Hz.

3.3. Synthesis of E-cinnamic-2-d_1 acid

Benzaldehyde (127 mg, 1.2 mmol) and malonic-d_4 acid (99 at.% d) (276 mg, 2.63 mmol) were dissolved in pyridine (660 μl). Piper-

idine (13 μl, 131 nmol, 0.00011 eq.) was added, the reaction was heated to 68 °C (oil bath) for 24 h. Water (7.5 ml) and then conc. HCl (0.4 ml) were added dropwise until a precipitate appeared which was collected and recrystallized (water) to afford a product, homogenous by TLC, R_f = 0.46, *n*-hexane-ethyl acetate–acetic acid (1:1:0.01, v/v/v), as white crystals 127-130 °C (Lit. 133 °C for unlabelled *E*-cinnamic acid, Merck Index), yield 59% relative to benzaldehyde used. $C_9H_6D_1O_2$ requires 148.0514, HR MS gave *m/z* 148.0516 $[M-H]^-$. ^{1}H NMR: δ 7.39 (3H, *m*, H-3′,4′,5′), 7.58 (2H, *m*, H-2′,6′), 7.66 (0.88H, *br s*, H-3), as well as 6.48 (0.12H, *d*, *J* = 16) from the residual isotopomer at H-2 and 7.67 (0.12H, *d*, *J* = 16, H-3). ^{13}C NMR: δ 119.1 (C-2-D, 1:1:1 *t*, $^1J_{CD}$ = 25), 129.2 (C-3′,5′), 130.0 (C-2′,6′), 131.4 (C-4′), 135.8 (C-1′), 146.2 (C-3), 170.3 (C-1), as well as a small singlet at 119.3 from the residual isotopomer at C-2. ^{2}H NMR (61.41 MHz): δ 6.51 (2-D, *d*, *J* = 2).

3.4. Extraction, purification and characterisation of scopolin and scopoletin

Cassava roots (1.3 kg, various cultivars) were peeled (1.1 kg) and then cut into approximately 1 cm^3 cubes, allowed to deteriorate for up to 6 d under controlled conditions (20 °C, 80–90% R.H.) until crushing and extraction. Daily from day 3, approximately 270 g of the sliced roots were crushed and macerated (EtOH 200 ml). The EtOH extracts of these four samples were analysed by TLC ($CHCl_3$:EtOAc:MeOH 2:2:1,visualised by UV at 365 nm) and there was no significant difference between the first two samples, days 3 and 4, where it is known that the highest amount of scopoletin and scopolin accumulation occurs (Buschmann et al., 2000). The combined ethanolic extracts were filtered and evaporated under reduced pressure at 35–40 °C. The residue was fractionated between water (see below) and $CHCl_3$. The combined $CHCl_3$ layers were concentrated to yield a pale yellow viscous oil (240 mg) which was then further purified by solid-phase extraction. C_{18} (1 g cartridge) was washed (MeOH, 5 ml) and conditioned (50% aq. MeOH, 5 ml) and 8 ml of the extract (1 mg/ml in MeOH with sufficient water added to the solution until it became opalescent) was loaded and eluted with 50% aq. MeOH. The eluant was concentrated under reduced pressure which afforded scopoletin, identical to a commercial sample. The residue was dissolved in MeOH (0.2 g/ml) and purified by HPLC. A 24.7 min peak was detected, collected, concentrated and dissolved in MeOH and identified by high field NMR spectroscopy and high resolution electrospray ionisation mass spectrometry (HR-ESI-MS) (Hirata et al., 2000), in order to identify the unlabelled coumarin, scopoletin (4 mg). ^{1}H NMR: δ 3.90 (3H, *s*, 6-OCH_3), 6.19 (1H, *d*, *J* = 9.4, H-3), 6.75 (1H, *s*, H-8), 7.09 (1H, *s*, H-5), 7.84 (1H, *d*, *J* = 9.4, H-4). HR MS: Naturally occurring scopoletin $C_{10}H_9O_4$ requires 193.0495, HR MS found *m/z* 193.0489 $[M+H]^+$, $C_{10}H_8O_4Na$ requires 215.0315, found 215.0309 $[M+Na]^+$.

The concentrated aq. fraction (7.6 g) was separated using the above HPLC conditions. A 7.1 (and a trace of a 27.4) min peak was detected, collected, concentrated and dissolved in MeOH and identified by high field NMR spectroscopy and HR MS (Fliniaux et al., 1997), in order to identify the unlabelled coumarin, scopolin (7 mg). ^{1}H NMR: δ *3.41–3.55* (4H overlapping, H-2′,3′,4′,5′), 3.71 (1H, *br s, H-6′a),* 3.84 (1H, *br s, H-6′b),* 3.91 (3H, *s*, 6-OCH_3), 5.09 (1H, *d*, *J* = 8.2, H-1′), 6.31 (1H, *d*, *J* = 9.4, H-3), 7.18 (1H, *s*, H-8), 7.22 (1H, *s*, H-5), 7.91 (1H, *d*, *J* = 9.4, H-4). ^{13}C NMR: δ 55.8 (6-OCH_3), 61.2 (C-6′), 70.0 (C-4′), 73.5 (C-2′), 76.6 (C-5′), 77.2 (C-3′), 100.8 (C-1′), 104.0 (C-8), 109.5 (C-5), 113.3 (C-3, C-4a), 144.5 (C-4), 147.0 (C-6), 149.5 (C-8a), 150.5 (C-7), 162.4 (C-2). HR MS: Naturally occurring scopolin $C_{16}H_{19}O_9$ requires 355.1024, HR MS found *m/z* 355.1033 $[M+H]^+$, $C_{16}H_{18}O_9Na$ requires 377.0843, found 377.0860 $[M+Na]^+$.

3.5. General feeding procedure

Cassava roots (typically 1 kg) were peeled, then cut into approximately 1 cm^3 cubes and divided into groups (typically 100 g). One group was immediately crushed and extracted with EtOH (fresh cassava extract) and another (control group) was stored under controlled conditions (20 °C, 80–90% R.H.) until crushing and extraction. Where possible biosynthetic precursors were introduced, this was by spraying a group with *E*-cinnamic acid (typically 30 mg dissolved in aq. 4% Na_2CO_3 (3 ml) then adjusted to pH 7.5 with 1 M HCl) with a simple hand-pumped aerosoliser. As the highest amount of scopoletin and scopolin accumulation occurs between days 3 and 4 (this is cultivar dependent (Buschmann et al., 2000)), after 3 d, half the group was crushed and macerated (EtOH 200 ml, 3 d), and the other half was crushed after 4 d and then macerated (EtOH 200 ml, 2 d). The combined ethanolic extracts were filtered. A second round of maceration (EtOH, 400 ml, 2 d) was performed. The combined EtOH extracts (after 8 d), were filtered and evaporated under reduced pressure at 35–40 °C. The residue was dissolved in MeOH (0.2 g/ml) and purified by HPLC. From repeat injections (*n* = 12, each of 100 μl) two peaks of retention time 7.1 min and 24.7 min (scopolin and scopoletin respectively, the latter by comparison with a commercial authentic sample) were detected, collected, concentrated and dissolved in MeOH and identified by high resolution electrospray ionisation mass spectrometry (HR-ESI-MS), in order to separate isotopes and to identify the percentage of labelled scopoletin and scopolin in relation to the unlabelled coumarins.

3.6. Feeding with E-cinnamic-d_7 acid

Using the general feeding procedure, cassava roots (1.4 kg, cv MNGA 2) were peeled (1.2 kg) and fed with *E*-cinnamic-2,3,2′,3′,4′,5′,6′-d_7 acid (150 mg) dissolved in aq. 4% Na_2CO_3 (10 ml). A representative sample of the combined EtOH extract (32.9 g) was then purified by HPLC. The HPLC peak at 7.1 min, consistent with scopolin, naturally occurring scopolin $C_{16}H_{19}O_9$ requires 355.1024, HR MS found *m/z* 355.1041 $[M+H]^+$, $C_{16}H_{18}O_9Na$ requires 377.0843, found 377.0847 $[M+Na]^+$, also $C_{16}H_{16}D_3O_9$ requires 358.1212, found 358.1229 $[M+H]^+$, and $C_{16}H_{15}D_3O_9Na$ requires 380.1031, found 380.1017 $[M+Na]^+$.

The HPLC peak at 24.7 min, scopoletin, naturally occurring scopoletin $C_{10}H_9O_4$ requires 193.0495, HR MS found *m/z* 193.0497 $[M+H]^+$, $C_{10}H_8O_4Na$ requires 215.0315, found 215.0309 $[M+Na]^+$, also $C_{10}H_6D_3O_4$ requires 196.0684, found 196.0687 $[M+H]^+$, $C_{10}H_5D_3O_4Na$ requires 218.0503, found 218.0506 $[M+Na]^+$, $C_{20}H_{16}O_8Na$ requires 407.0737, found 407.0729 $[2M+Na]^+$, and $C_{20}H_{13}D_3O_8Na$ requires 410.0921, found 410.0926 $[2M+Na]^+$.

3.7. Feeding with E-cinnamic-d_6 acid

Using the General Feeding procedure, cassava roots (1.23 kg, cv MCOL 22) were peeled (1.0 kg) and a group (195 g) was fed with *E*-cinnamic-3,2′,3′,4′,5′,6′-d_6 acid (30 mg) dissolved in aq. 4% Na_2CO_3 (3 ml). A representative sample of the combined EtOH extract (~4 g) was then purified by HPLC. The HPLC peak at 7.1 min scopolin, $C_{16}H_{19}O_9$ requires 355.1024, HR MS *m/z* found 355.1037 $[M+H]^+$, $C_{16}H_{18}O_9Na$ requires 377.0843, found 377.0852 $[M+Na]^+$, also $C_{16}H_{16}D_3O_9$ requires 358.1212, found 358.1215 $[M+H]^+$ and $C_{16}H_{15}D_3O_9Na$ requires 380.1031, found 380.1029 $[M+Na]^+$.

The HPLC peak at 24.7 min, scopoletin, naturally occurring scopoletin $C_{10}H_9O_4$ requires 193.0495, HR MS *m/z* found 193.0488 $[M+H]^+$, $C_{10}H_8O_4Na$ requires 215.0315, found 215.0314 $[M+Na]^+$, also $C_{10}H_6D_3O_4$ requires 196.0684, found 196.0689 $[M+H]^+$.

3.8. Feeding with E-cinnamic-2-d_1 acid, E-cinnamic-2′,3′,4′,5′,6′-d_5 acid, E-cinnamic-3,2′,3′,4′,5′,6′-d_6 acid and E-cinnamic-2,3,2′,3′,4′,5′,6′-d_7 acid

Using the General Feeding procedure, cassava roots (800 g, cv MCOL 22) were peeled (640 g) and divided into four groups (85 g) which were fed with *E*-cinnamic-2-d_1 acid, *E*-cinnamic-2′,3′,4′,5′,6′-d_5 acid, *E*-cinnamic-3,2′,3′,4′,5′,6′-d_6 acid and *E*-cinnamic-2,3,2′,3′,4′,5′,6′-d_7 acid (20 mg of each acid) dissolved in aq. 4% Na_2CO_3 (2.0 ml). A representative sample of the combined EtOH extract (~2 g) was then purified by HPLC. The HPLC peak at 7.1 min scopolin, $C_{16}H_{19}O_9$ requires 355.1024, HR MS *m/z* found 355.1033 $[M+H]^+$, and the corresponding HR MS data are listed in Table 1. The HPLC peak at 24.7 min, scopoletin, naturally occurring scopoletin $C_{10}H_9O_4$ requires 193.0495, HR MS *m/z* found 193.0489 $[M+H]^+$, and the corresponding HR MS data are listed in Table 2.

3.9. Feeding with E-cinnamic-2,3,2′,3′,4′,5′,6′-d_7 acid in light and in dark

Using the General Feeding procedure, cassava roots (690 g, cv MNGA 19) were peeled (490 g) and divided into two groups (85 g) which were fed with *E*-cinnamic-2,3,2′,3′,4′,5′,6′-d_7 acid (20 mg) dissolved in aq. 4% Na_2CO_3 (2.3 ml). One group was treated as above and then stored in the dark (in a box double wrapped with aluminium foil) while the other was treated as usual. Representative samples of the EtOH extract (~2 g) were then purified by HPLC. In the light conditions, the HPLC peak at 7.1 min, consistent with scopolin, naturally occurring scopolin $C_{16}H_{19}O_9$ requires 355.1024, HR MS found *m/z* 355.1068 $[M+H]^+$, $C_{16}H_{18}O_9Na$ requires 377.0843, found 377.0812 $[M+Na]^+$, also $C_{16}H_{16}D_3O_9$ requires 358.1212, found 358.1243 $[M+H]^+$, and $C_{16}H_{15}D_3O_9Na$ requires 380.1031, found 380.1021 $[M+Na]^+$. The percentage of scopolin-d_3 was 18.7%. In the dark conditions, naturally occurring scopolin $C_{16}H_{19}O_9$ requires 355.1024, HR MS found *m/z* 355.1049 $[M+H]^+$, $C_{16}H_{18}O_9Na$ requires 377.0843, found 377.0850 $[M+Na]^+$, also $C_{16}H_{16}D_3O_9$ requires 358.1212, found 358.1197 $[M+H]^+$, and $C_{16}H_{15}D_3O_9Na$ requires 380.1031, found 380.1069 $[M+Na]^+$. The percentage of scopolin-d_3 was 15.7%.

In the light conditions, the HPLC peak 24.7 min, scopoletin, naturally occurring scopoletin $C_{10}H_9O_4$ requires 193.0495, HR MS *m/z* found 193.0498 $[M+H]^+$, $C_{10}H_8O_4Na$ requires 215.0315, found 215.0324 $[M+Na]^+$, also $C_{10}H_6D_3O_4$ requires 196.0684, found 196.0703 $[M+H]^+$ and $C_{10}H_5D_3O_4Na$ requires 218.0503, found 218.0512 $[M+Na]^+$. The percentage of scopoletin-d_3 was 9.8%. In the dark conditions, the HPLC peak 24.7 min, scopoletin, $C_{10}H_9O_4$ requires 193.0495, HR MS *m/z* found 193.0494 $[M+H]^+$, $C_{10}H_8O_4Na$ requires 215.0315, found 215.0324 $[M+Na]^+$, also $C_{10}H_6D_3O_4$ requires 196.0684, found 196.0697 $[M+H]^+$ and $C_{10}H_5D_3O_4Na$ requires 218.0503, found 218.0527 $[M+Na]^+$. The percentage of scopoletin-d_3 was 9.1%.

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