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Phytochemistry 65 (2004) 2763–2774
PHYTOCHEMISTRY
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Hydrophobic metabolites of 2,4-dichlorophenoxyacetic acid (2,4-D) in cultured coconut tissue

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Received 5 April 2004; received in revised form 27 July 2004; accepted 12 August 2004

Abstract

Cultures of inflorescence and plumular tissues of coconut palm (*Cocos nucifera* L.) were maintained in the presence of the auxin, [^{14}C]2,4-dichlorophenoxyacetic acid (2,4-D), so that its metabolic fate could be studied. Thin layer chromatography of methanol extracts of the plumular tissue showed that four classes of metabolites, as well as the unchanged acid, were recovered in the extract. In inflorescence tissue, only the unchanged acid and the most polar class of metabolites (metabolite I) were recovered. Metabolite I was shown to consist mostly of a mixture of sugar conjugates and metabolite II (the next most polar) was an unidentified basic metabolite. Metabolites III and IV were both novel triacylglycerol analogues in which one of the natural fatty acids was replaced with a chain-elongated form of 2,4-D. Reversed-phase thin layer chromatography was used to identify the 2,4-D-derived acids and it was found that metabolite III contained the 2,4-dichlorophenoxy-moiety attached to a chain-length of between 2 and 12 carbons, whereas metabolite IV contained 12, 14 and 16 carbon chain lengths. In inflorescence tissue, and in plumular tissue at low sucrose or 2,4-D concentrations and after short periods in culture, metabolite I predominated. The other metabolites increased as a percentage when plumular culture was prolonged or when sucrose or 2,4-D concentrations were raised. These changes correlated with better development of the explant.

Keywords: Coconut; *Cocos nucifera* L; Tissue culture; 2,4-Dichlorophenoxyacetic acid; Xenobiotic metabolism; Chain-elongation; Triacylglycerol; Xenobiotic lipid

1. Introduction

There is a strong desire to develop a reliable method for the clonal propagation of the coconut palm (*Cocos nucifera* L.) for reasons of improvement of the farmed phenotypes, disease avoidance and ease of production at high quality (Blake, 1995; Buffard-Morel et al., 1995). Both plumular and inflorescence tissue can be propagated in vitro but the rate of conversion, in spite of recent advances, into viable plantlets is still very low (Chan et al., 1998; Verdeil et al., 1994).

One of the factors that is important in the induction of callus formation, an important stage in coconut somatic embryogenesis, is the presence of an auxin: low concentrations of the herbicide, 2,4-dichlorophenoxyacetic acid (2,4-D), are routinely added to culture media to serve this function (Blake and Hornung, 1995; Buffard-Morel et al., 1995). The effectiveness of 2,4-D depends upon the concentration of 2,4-D in the culture medium, its availability to the plant (i.e. the concentration in free solution rather than bound to activated charcoal), the rate of uptake and its rate of metabolism by the plant to form metabolites that are either inactive

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doi:10.1016/j.phytochem.2004.08.034

or are stored away from the site of action. After callus formation is complete, it is necessary to reduce substantially the concentration of 2,4-D which, otherwise, can act as a mutagen and interfere with further ontogenesis (Blake, 1995; LoSchiavo et al., 1989). Its removal from the culture medium is straightforward but reductions in intracellular concentrations depend upon conversion into inactive metabolites.

Studies of 2,4-D metabolism in a number of plants have revealed many routes of metabolism (Roberts et al., 1998) including ester conjugation with sugars, amino acid conjugation, formation of conjugates of hydroxyl metabolites, chain oxidation and even chain elongation (to form "xenobiotic lipid" metabolites). Here we report the results of studying metabolism of 2,4-D by plumular and inflorescence tissues in vitro and the effects of sucrose concentration, 2,4-D concentration and stage of development upon that metabolism. We present evidence for the biosynthesis of chain elongated 2,4-D acids, esterified to form analogues of triacylglycerols (TAG) which have not, hitherto, been reported.

2. Results and discussion

2.1. Uptake of 2,4-D by cultured tissues

Early stages of somatic embryogenesis were achieved as described earlier (Blake and Hornung, 1995) in all three tissues used (inflorescences at stages −4 and −5 and plumular tissue). This statement is based on visual assessment and histological studies.

All tissues, cultured in the presence of [^{14}C]2,4-D, took 2,4-D up from the medium throughout the course of the experiments. Fig. 1 (a)–(c) shows that the total uptake of 2,4-D increased as a function of time in culture, of the concentrations of 2,4-D and sucrose in the culture medium. The 2,4-D concentrations (0–0.6 mM) used are those used in previous reports (Chan et al., 1998; Hornung and Verdeil, 1999). Uptake was greater in plumular tissue than in inflorescence tissue. In contrast, control incubations (using tissue that had been killed by freezing and thawing) took up only insignificant quantities of radioactivity. When these results were expressed "per mass of tissue" (not shown) the 2,4-D content of the tissue showed relatively little response to sucrose concentration but increased with increasing 2,4-D concentration in the medium. The increase in total uptake in the presence of increasing sucrose concentrations can, therefore, be interpreted as being secondary to the effects of sucrose on tissue growth.

On their own, these results do not allow conclusions about whether the uptake is by passive diffusion (Koens et al., 1995; Shvetson and Gamburg, 1984) or by an active transport (or facilitated diffusion) mechanism (Delbarre et al., 1996; Minocha and Nissen, 1985; Rubery, 1987; Sabater and Rubery, 1987) to be drawn. The effect of 2,4-D concentrations may have been direct upon an active transport system or indirect by stimulating an intracellular transport or metabolism system, both of which would have the effect of displacing the equilibrium of trans-membrane movement towards uptake.

2.2. Identification and quantification of 2,4-D metabolites

Fig. 2 shows a typical radio-scan of a silica gel thin layer chromatography (TLC) plate used to separate the 2,4-D metabolites extracted from plumular explants. Five clear "peaks" of radioactivity can be observed, one of which co-chromatographed with the (unchanged) free acid form of 2,4-D. Even this preliminary analysis suggested that the classes may contain more than one component each. Metabolite I, which did not migrate from the origin, and metabolite II were more polar than the 2,4-D precursor whereas metabolites III and IV were hydrophobic and migrated close to the solvent front (SF) in positions where neutral lipids might be expected. Control calli, killed by freezing and thawing, contained radioactivity corresponding only to unmodified, non-esterified, 2,4-D.

2.2.1. Metabolite I

After recovery from the plate, metabolite I was acetylated and the products were subjected once more to TLC. A fraction, representing about 17% of metabolite I, co-chromatographed with the chemically synthesised tetra-acetyl-glucose ester of 2,4-D (Fig. 3). The other components of metabolite I all migrated further on the plate than the non-acetylated ones. Incubation of metabolite I with the enzyme β-glucosidase, liberated about 14% of the metabolite as 2,4-D. Controls indicated that about half of this conversion could be accounted for by chemical decomposition, a finding that agrees with earlier observations of the instability of chemically synthesised 2,4-D glucose ester (see Section 4). Incubation of metabolite I with protease also released about 18% of the radioactivity in the form of 2,4-D indicating that a proportion of metabolite I is a peptide-bonded conjugate of 2,4-D. In summary, the evidence that suggests that metabolite I comprises a mixture of carbohydrate conjugates is as follows: (i) TLC mobility (normal phase) is consistent with a very polar metabolite; (ii) treatment with β-glucosidase released a proportion of the radioactivity as 2,4-D (non-esterified form); (iii) acetylation of metabolite I reduced the polarity of all components, indicating the presence of free hydroxyl groups; (iv) one component, after acetylation, co-chromatographed with synthetic tetra-acetyl-2,4-D-glucose ester.

The possibility that some components were ether-conjugates of sugars with a hydroxylated metabolite of 2,4-D cannot be totally excluded. Work with young wheat plants has shown that 2,4-D can also form conju-

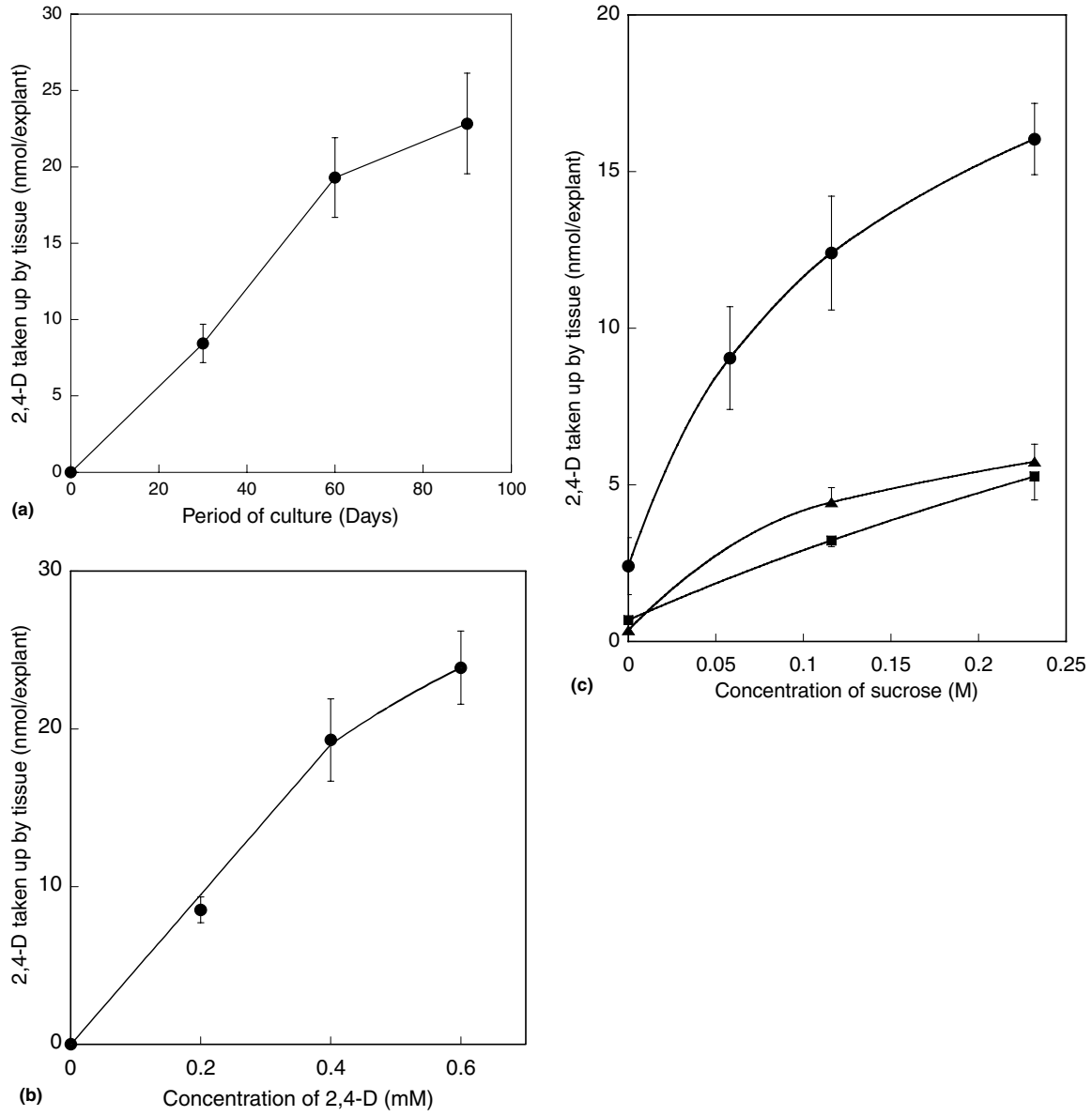

Fig. 1. Graphs to show the uptake of [^{14}C]2,4-D into methanolic extracts of plumules (a and b) or plumules and inflorescence tissue (c). The tissue was grown in culture and incubated with [^{14}C]2,4-D as described under Section 4. The standard conditions used, except as indicated on the horizontal axes of the individual graphs, were 60 days in culture, 0.4 mM 2,4-D and 0.116 M sucrose. For inflorescence tissue the 60-day results are the sum of the uptakes measured separately in the first 30 days period of culture and the second 30 days period of culture; plumular tissue was exposed to radioactive 2,4-D continuously over the whole 60 days. The effects of duration of incubation (a), 2,4-D concentration in the incubation medium (b), and sucrose concentration in the incubation medium (c) are shown. Symbols indicate plumular tissue (●), inflorescence tissue at developmental stage −5 (▲) or stage −4 (■). Error bars represent means ± standard errors of the mean from five individual cultures but are not shown where they are smaller than the point.

gates with a number of mono-, di- and poly-saccharides (Chkanikov et al., 1982). Conjugates of xenobiotic carboxylic acids with 6-*O*-malonylglucose, glucosylxylose, gentiobiose and triglucose have been noted in other plant species (Mikami et al., 1984, 1985).

The finding that about 18% of metabolite I was liberated as 2,4-D after proteolytic digestion suggests the presence of peptide bonded 2,4-D. The evidence is not consistent with the formation of simple amino acid conjugates because metabolite I is too polar on silica gel TLC so a conjugate with a bipeptide or a longer peptide chain is suggested. Conjugates of hydroxylated 2,4-D with peptides between 2 and 12 amino acids in length have been reported in wheat, barley and maize (Chkanikov et al., 1982). The same group, also working with young wheat and barley, had earlier reported the formation of peptide linked conjugates of 2,4-D, its hydroxylated metabolites and the glycosyl conjugates thereof

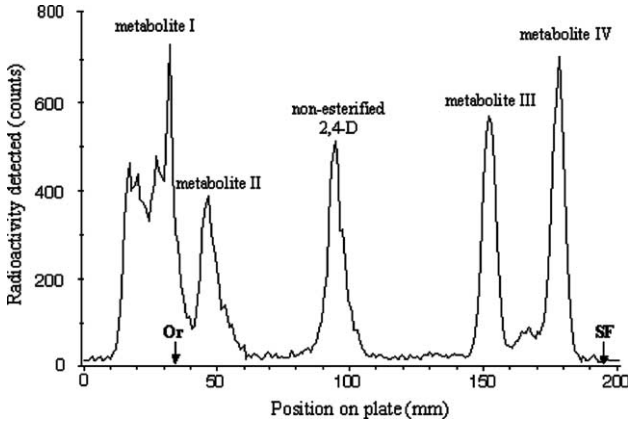

Fig. 2. Radiochromatogram of 2,4-D metabolites. Plumules were incubated with [^{14}C]2,4-D as described in the text. The tissue was extracted with methanol and the extract subjected to TLC on a silica gel plate developed in toluene:butanone:acetic acid (45:55:3, by volume). The plate was visualized for non-radioactive standards and radio-scanned for 30 min as described in Section 4. This figure represents a ''typical'' scan and was obtained from tissue cultured in 0.4 mM 2,4-D and 0.116 M sucrose for 90 days. The position of the origin (Or) and solvent front (SF) are indicated.

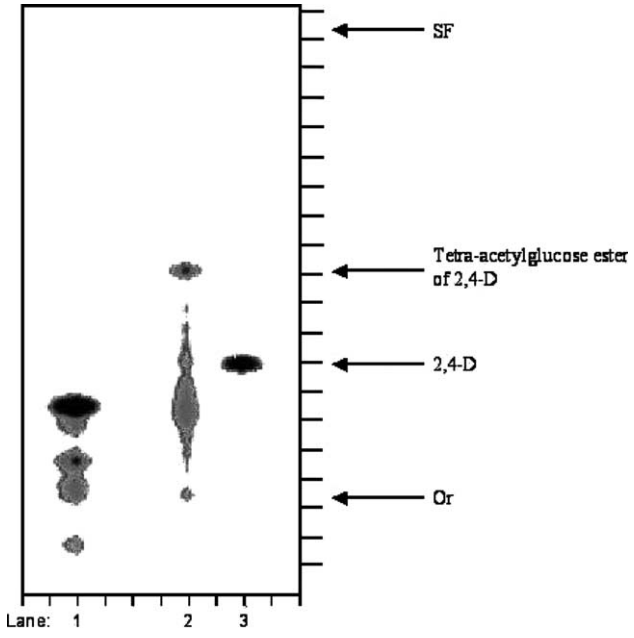

Fig. 3. Radiochromatogram of metabolite I. Metabolite I was isolated and subjected to further TLC as described in Section 4. The plate was developed in toluene:methanol:acetic acid (96:8:4, by volume). The plate was scanned at 0.5 cm intervals as described and the resultant scans assembled into the 2-dimensional representation. Radioactivity is shown on a logarithmic grey-scale with the more active areas being represented by the darker shading. The scale runs between 9 and 1250 counts accumulated over 30 min. Lane 1 shows unmodified metabolite I; lane 2 is metabolite I after acetylation and lane 3 is a radioactive standard for 2,4-D. Tick marks on the borders of the plate are 1 cm apart. The position of the Or and SF are indicated.

with chains of up to 220 amino acids (Nazarova et al., 1980).

2.2.2. Metabolite II

The possibility that metabolite II was an amino acid conjugate, or a mixture of amino acid conjugates, of 2,4-D was investigated by comparing its chromatographic behaviour with that of a number of synthetic 2,4-D-amino acyl conjugates in a variety of solvent systems. In an acidic solvent system of low polarity, diethyl ether:petroleum spirit (60–80 °C):formic acid (70:30:2, by volume), metabolite II did not migrate from the origin nor did the standards for the 2,4-D conjugates with glycine or histidine. The aspartic acid, glutamic acid, tryptophan, alanine, phenyl alanine, valine and leucine conjugates all migrated away from the origin. In a more polar acidic system, of toluene:2-butanone:acetic acid (45:55:3, by volume), only the histidine conjugate stayed on the origin with metabolite II. This evidence suggested that metabolite II is a basic compound. Increasing the polarity further to butanol:acetic acid:water (90:20:10, by volume) (Fig. 4(a)) resulted in both metabolite II and the histidine conjugate migrating from the origin but the two had clearly different R_f values. There was also evidence for at least one other (minor) component of metabolite II. Using a basic solvent system confirmed the basic nature of the main components of metabolite II (Fig. 4(b)). Only a small proportion of metabolite II remained on the origin with the two acidic amino acid conjugates; the major proportion of metabolite II migrated further than all the available standards of 2,4-D amino acid conjugates. Attempted hydrolysis of metabolite II by protease action or by refluxing with ethanolic KOH produced no change in the chromatographic behaviour of the metabolite in several chromatographic systems and no evidence for the release of a radioactive acidic compound.

The chromatographic behaviour of the major component of metabolite II suggests that it is a basic metabolite. The metabolite did not, however, co-chromatograph with any of the synthetic amino acid conjugates of 2,4-D including the conjugate with histidine. Conjugates with lysine or arginine were not available to use as standards but published relative R_f values for amino acid conjugates (Feung et al., 1973) suggest that these also were not probabilities. The resistance of metabolite II to proteolytic digestion rules out the possibility that the conjugate was with a bipeptide (or longer peptide chain) and suggested that the 2,4-D was not conjugated via a peptide (amide) bond. The failure to liberate an acidic metabolite after attempted chemical hydrolysis appears to confirm this view but it is possible that the conditions chosen for hydrolysis, 1 M KOH which is appropriate for lipid hydrolysis, were not sufficiently rigorous for amide bonds, with 7 M alkali being used on occasion (Chkanikov et al., 1976; Nazarova

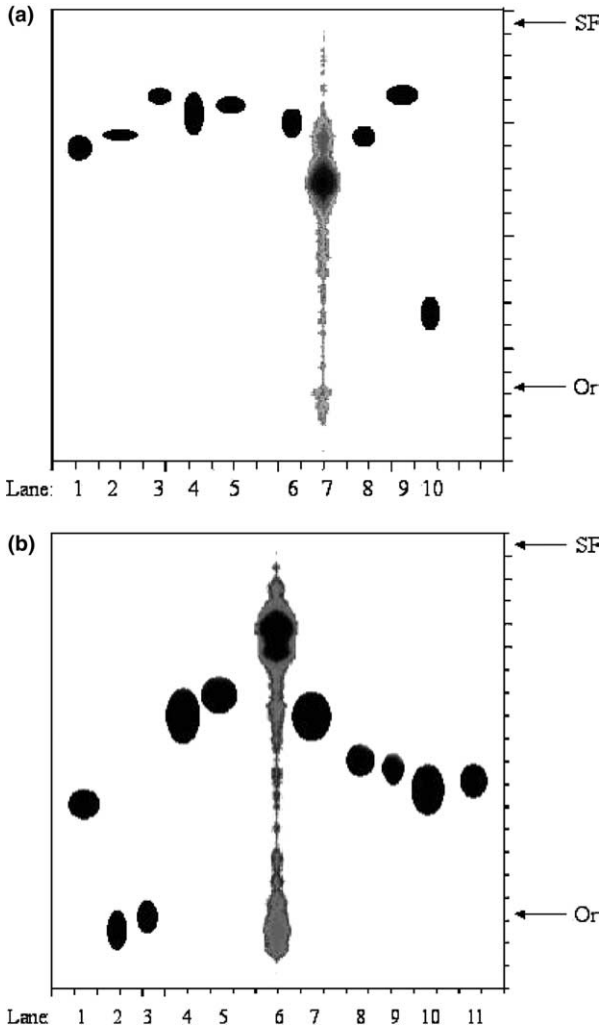

Fig. 4. Radiochromatograms of metabolite II. Metabolite II was isolated and subjected to further TLC as described in Section 4. The plate was developed in (a) butanol:acetic acid:water (90:20:10, by volume) or in (b) chloroform:methanol:concentrated ammonia solution (75:35:2, by volume). The plate was scanned at 0.5 cm intervals as described and the resultant scans assembled into the 2-dimensional representation. Radioactivity is shown on a logarithmic grey-scale with the more active areas being represented by the darker shading. The scale runs (a) between 12 and 250 counts or (b) between 13 and 850 counts accumulated over 30 min. In (a, Lanes 1–6) and 8–10 show the positions of non-radioactive standards visualized by quenching of the fluorescent indicator under UV illumination. The standards are conjugates of 2,4-D with the following amino acids: 1, aspartic acid; 2, glutamic acid; 3, valine; 4, leucine; 5, phenyl alanine; 6, alanine; 9, tryptophan; 10, histidine. Lane 8 shows unmodified 2,4-D and lane 7 is the radioactive scan of metabolite II. In b lanes, 1–5 and 7–11 show the positions of non-radioactive standards. The standards are conjugates of 2,4-D with: 1, glycine; 2, aspartic acid; 3, glutamic acid; 4, valine; 5, leucine; 7, phenyl alanine; 8, tryptophan; 10, histidine.; 11, alanine. Lane 9 shows unmodified 2,4-D and lane 6 is the radioactive scan of metabolite II. Tick marks on the borders of the plates are 1 cm apart. The position of the Or and SF are indicated.

et al., 1980). An amide-linked conjugate of 2,4-D with a non-protein amino acid like ornithine or with a polyamine such as putrescine, spermidine or spermine would yield basic conjugates and remain possibilities despite never having been reported before in plant tissue. The polyamines have been shown to be present in cultured callus of soybean (Du Plessis et al., 1996).

2.2.3. Metabolites III and IV

From the chromatographic behaviour (Fig. 2) it appeared that metabolite IV might be a triacylglycerol and that metabolite III might be elongated forms of 2,4-D as has been reported in alfalfa (Linscott et al., 1968; Linscott and Hagin, 1970). However, neither metabolite migrated exactly with the standards of the elongated acids. This impression was confirmed after TLC in a basic solvent system (results not shown). After alkaline hydrolysis, extracted acids were identified by chromatography on reversed-phase TLC plates (Fig. 5). Under these conditions the elongated versions of 2,4-D from the 2 carbon side-chain (2,4-D itself) to the 16 carbon form (2,4-dichlorophenoxyhexadecanoic acid) can be distinguished. Hydrolysed metabolite IV showed three main products which co-migrated with

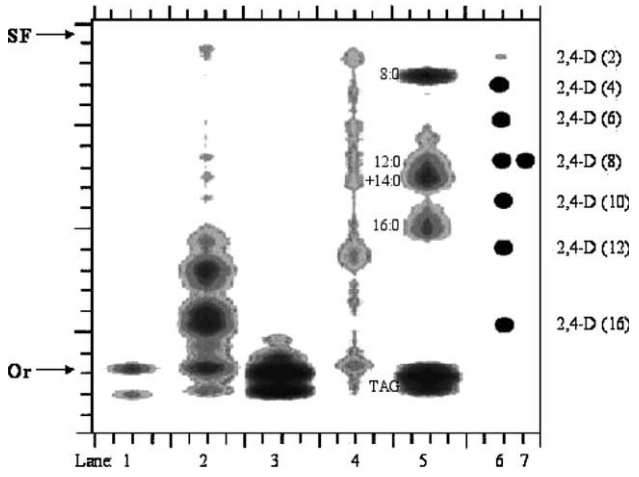

Fig. 5. Radiochromatogram of metabolites III and IV. Metabolites III and IV were isolated and subjected to further TLC as described in Section 4. The reversed phase plate was developed in acetonitrile:methanol:water (80:10:30, by volume). The plate was scanned at 0.5 cm intervals as described and the resultant scans assembled into the 2-dimensional representation. Radioactivity is shown on a logarithmic grey-scale with the more active areas being represented by the darker shading. The scale runs between 9 and 1600 counts accumulated over 30 min. Lanes 6 and 7 show the positions of non-radioactive standards visualized by quenching of the fluorescent indicator under UV illumination or by staining as described in Section 4. Lanes 1 and 2 are metabolite IV before and after hydrolytic treatment respectively; lanes 3 and 4 are metabolite III before and after hydrolytic treatment respectively; lane 5 is a mixture of radioactive lipid standards (TAG, triacylglycerol; 16:0, palmitic acid; 14:0 myristic acid; 12:0, lauric acid; 8:0, octanoic acid); lane 6 is a mixture of 2,4-D homologues with the number in parentheses indicating the length of the carbon side-chain such that 2,4-D (2) is 2,4-D itself; lane 7 is the methyl ester of 2,4-D. Tick marks on the borders of the plate are 1 cm apart. The position of the Or and SF are indicated.

the 16 carbon and the 12 carbon elongated 2,4-D standards, the third migrating between these standards where the 14 carbon elongated form would be expected (standard not available). A proportion (17%) remained on the origin with natural TAG standards and very small amounts migrated with the shorter forms, perhaps as a result of incomplete separation of metabolites III and IV on the original TLC plate. Metabolite III, after hydrolysis, yielded a number of radioactive bands which migrated with the shorter forms of elongated 2,4-D from 12 carbon to 4 carbon and 2,4-D itself. Again there is evidence of unhydrolysed TAG on the origin and incomplete separation as judged by the appearance of some minor bands characteristic of metabolite IV. In this reversed phase TLC system, 94.3% of non-hydrolysed metabolite III and 100% of non-hydrolysed metabolite IV remained on the origin. While the standard for methyl-2,4-D also appears on the plate, results on normal-phase TLC show that it was not associated with any radioactivity.

Absolute certainty in the identification of the components of these metabolites could only come via mass spectrographic (MS) analysis. This proved not to be feasible because the techniques used for isolation of the component acids, while very successful at separating the radioactive (i.e. 2,4-D-derived) acids, were not capable of separating them from the non-radioactive natural fatty acids, which were present in massive excess compared to the 2,4-D metabolites. This makes analysis of the resulting spectra almost impossible as had been found on a previous occasion (Dodds et al., 1995).

The evidence presented is consistent with both metabolites III and IV being triacylglycerol (TAG) analogues, with one of the three fatty acids being replaced with a chain-elongated 2,4-D. At this stage we have no evidence to enable us to distinguish the three possible sites of esterification. The acids were liberated from the TAG by hydrolysis and identified by a novel application of reversed-phase TLC. Similar but shorter acids have been reported before in alfalfa (Linscott and Hagin, 1970) fed 2,4-D and, in alfalfa (Linscott et al., 1968), soybean and cocklebur (Wathana and Corbin, 1972) fed 2,4-dichlorophenoxybutanoic acid; elongated forms, with side chains in the range 4–10 carbons, have been identified and further (longer) acids were also observed but not identified or quantified.

One novel aspect of the findings is that two distinct populations of elongated 2,4-D acids were identified. Presumably, they arose by the action of fatty acid synthase enzymes and the presence of two thioesterase activities with different chain length specificities is suggested. If this were the case, it would be consistent with the normal functioning of the enzyme in coconut, which produces both medium chain and long chain fatty acids. TAGs may be subjected to lipolysis at a later stage of development and it is not known whether the released elongated forms of 2,4-D will exert any physiological effects in their own right or be β-oxidised back to active 2,4-D.

The findings here are of significance compared to the previous reports and differ from those reports in three respects: (i) the 2,4-D chains were elongated by as many as 16 carbons, which is more than has been reported before; (ii) the elongated acids have been identified as conjugates analogous to natural TAG and (iii) they are identified in a commercially exploited food-oil crop. The formation of the TAG may suggest a quasi-physiological role where the triacylglycerol acts as a sink for 2,4-D, when it is supplied in excessive (toxic or mutagenic) quantities, and as a source of 2,4-D, which could be supplied by the action of lipolytic and β-oxidation enzymes at times of lipid mobilisation when 2,4-D concentrations may be sub-optimal. The finding that TAG can act as an end-product of xenobiotic metabolism suggests that other xenobiotic compounds may also be metabolised in this way and persist in the oil after the water-soluble metabolites have been removed.

2.3. Effects of culture conditions on the proportions of 2,4-D metabolites

Major differences between tissues in the metabolism of 2,4-D were observed. Cultured inflorescence tissue of either developmental stage produced metabolites of only one of the four classes, the polar metabolite I, whereas the plumular tissue produced measurable quantities of all four metabolite classes. All tissues contained some unchanged non-esterified 2,4-D. The results with inflorescence tissue at stage −4 and −5, incubated under standard conditions of 0.116 M sucrose and 0.15 mM 2,4-D, showed that the proportion of metabolite I decreased from 85.3 ± 5.4% (mean ± SEM from five separate explants) to 75.6 ± 6.8% (stage −4) or from 91.6 ± 1.8% to 80.9 ± 2.6% (stage −5), the remainder being unchanged 2,4-D, as the total 2,4-D taken up increased between days 32 and 64 of incubation. In plumular tissue (Fig. 6), cultured over a longer period under standard conditions of 0.116 M sucrose and 0.4 mM 2,4-D, a steady increase in the total 2,4-D radioactivity present in the tissue was observed. A progressive decrease in the percentage (but not the total amount) of metabolite I present (from about 55% of all radioactivity at 30 days to about 37% at 120 days) was observed as the total tissue uptake of 2,4-D increased. Metabolites II–IV were each present at between 8% and 13% up to 90 days of incubation but fell as a proportion thereafter. Only the proportion of unchanged 2,4-D increased throughout the incubation period. No attempt was made to measure 2,4-D metabolites that may have been released back into the medium. Since release was likely to have been a low proportion of the approximately 3% of the total 2,4-D taken up from the medium,

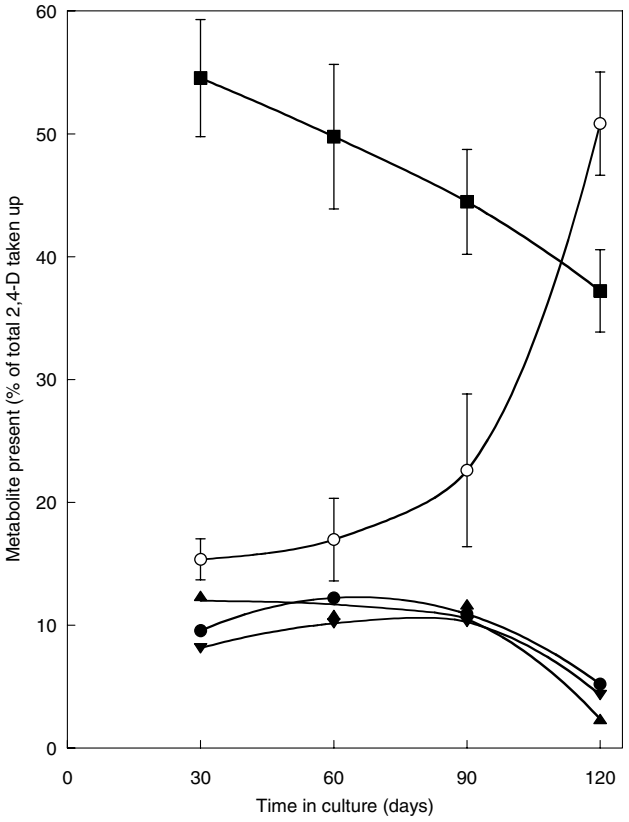

Fig. 6. Effect of time in culture on the composition of metabolites of 2,4-D in coconut plumular tissue. Plumular tissue was obtained and cultured in the presence of radioactive 2,4-D as described in Section 4. Tissue was extracted in methanol and analysed by TLC also as described in the text. The percentage of methanol-extracted radioactivity appearing as 2,4-D (○), as metabolite I (■), metabolite II (●), metabolite III (▲) or metabolite IV (▼) is shown. The data represent radioactivity accumulated continuously from day 0 until the day of analysis. The error bars represent the means ± standard error of the mean from five separate explants. The standard errors for metabolites II–IV cannot be shown because of the crowding of the points on the graph but were all in the range 0.31–2.85%.

it would represent a very low proportion of radioactivity outside the explant.

Further experiments with plumular tissue, cultured in the presence of increasing concentrations of sucrose (0–0.233 M) or of 2,4-D (0.6 μM–0.6 mM) also showed that metabolite I was the major metabolite present in cases of low incorporation but decreased at higher sucrose concentrations as metabolites II–IV and unchanged 2,4-D all increased and represented between 9% and 13% of total metabolites at 0.232 M sucrose. Similar findings were obtained when the concentration of 2,4-D was increased: the proportion of metabolite I fell as the other metabolites increased.

The total absence of metabolites II–IV, in cases of low uptake, suggests that the enzymes that catalyse their synthesis were also being induced as the rate of uptake increased. These may be enzymes that play a role in the development of the tissue in culture.

Our results are consistent with those published and reviewed elsewhere inasmuch as the major metabolite group in all our experiments were polar, probably sugar, conjugates (Bärenwald et al., 1994; Edwards et al., 1982; Schneider et al., 1984).

2.4. Relationships between metabolism and differentiation of the tissue

In histological terms, these observations corresponded to no observable de-differentiation of the plumular tissue at 0.6 μM 2,4-D and to a high degree of de-differentiation, as evidenced by a formation of meristematic centres and by proliferation of parenchymal cells in the callus, at 0.4 and 0.6 mM 2,4-D. The response at 0.2 mM 2,4-D was intermediate between the two extremes.

The capacity to metabolise 2,4-D by coconut explants was associated with the de-differentiation of the tissue. Higher absolute quantities and proportions of the novel non-polar metabolites (TAG containing elongated forms of 2,4-D) were contained in highly de-differentiated tissue derived from plumules. Furthermore, inflorescence explants failed both to produce callus and to synthesise the TAG analogues. It is difficult to ascertain whether or not this association represents a mechanistic link between 2,4-D metabolism and its action on callus proliferation; the possibility that the above metabolites may just be products of callus metabolism, with no further significance, cannot be excluded.

3. Concluding remarks

The findings presented herein demonstrate that 2,4-D can be metabolised to novel chain-elongated forms which can potentially be stored as TAG. The formation of such conjugates responds to manipulations of the tissue culture conditions and are associated with improved performance of the cultures. The finding may provide clues to the understanding of the variable efficacy of methods used for embryogenesis. The precise physiological and toxicological significance of these findings will require further investigation.

4. Experimental

4.1. Materials

Activated charcoal (acid washed), amino acids, β-glucosidase from almond, protease type XXI from *Streptomyces griseus*, sucrose (from sugar cane, 99% pure), Phytagel and lipid standards were obtained from Sigma (Sigma-Aldrich Company Ltd., Poole, UK); 6-bromohexanoic, 8-bromo-octanoic, 12-bromododecanoic and

16-bromohexadecanoic acids, 2,4-dichlorophenol and thionyl chloride were from Aldrich (Sigma–Aldrich Company Ltd., Poole, UK); 10-bromodecanoic acid was from Pfaltz and Bauer (Waterbury, USA); 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranoyl bromide were obtained from Acros (Geel, Belgium); 2-(2,4-dichlorophenoxy)-2-^{14}C-acetic acid ([^{14}C]2,4-D) was obtained from ARC inc, St Louis, MO, USA; Ecolite scintillation cocktail was from ICN (Costa Mesa, USA); solvents and other chemicals were obtained from the normal chemical suppliers.

Plates for TLC were obtained from the following suppliers: silica gel 60 F_{254} 250 μm thick 20 cm × 20 cm or 7.5 cm × 2.5 cm (''microscope-slide'' sized) plates, from Merck (Darmstadt, Germany); silica gel 60 A, 250 μm thick, 20 cm × 20 cm ruled into 19 channels, with a pre-adsorbant layer, from Whatman International Ltd. (Maidstone, UK); reversed phase C-18 bonded silica gel 60–250 μm thick, 20 cm × 20 cm were also from Whatman.

4.2. Chemical synthesis of 2,4-D metabolites and standards for chromatography

Amino acid conjugates of 2,4-D were prepared by reaction of the amino acids with 2,4-dichlorophenoxyacetyl chloride (2,4-D chloride) in a ''Schotten-Bauman''-type reaction (Wood and Fontaine, 1952). 2,4-D chloride, made from 2,4-D and thionyl chloride (Freed, 1946), was reacted with L-(−)-alanine, aspartic acid, glutamic acid, glycine, histidine, leucine, phenylalanine, tryptophan or valine at room temperature for 3 h. After extracting unreacted acyl chloride and acidifying with 2 M HCl to pH 2.0, the amino acid conjugate precipitated as a white solid which was collected, washed three times with diethyl ether, dried, recrystallised twice from 50% (v/v) ethanol and dried again giving 55–67% of the theoretical yield. The structure was confirmed by (proton) nuclear magnetic resonance spectroscopy using a Varian T-60 NMR spectrometer (60 MHz). The NMR analyses were performed by Merlin Synthesis, Kent, UK.

Elongated forms of 2,4-D were prepared by reacting the potassium salt of 2,4-dichlorophenol with an ω-bromo-1-alkanoic acid by a method modified from that of Linscott et al. (1968). The reactants, 10 mol of 2,4-dichlorophenol, 10 mmol of 6-bromohexanoic acid, 8-bromooctanoic acid, 10-bromodecanoic acid, 12-bromododecanoic acid or 16-bromohexadecanoic acid, and 25 mmol of K_2CO_3, were dissolved in 50 mL of anhydrous dimethylformamide. The mixture was refluxed for 4 h then cooled and extracted with 100 mL of diethyl ether to remove unreacted precursors. The remaining phase was adjusted to pH 2.0 with 2 M HCl and the product extracted into 100 mL of diethyl ether. The extract was further washed and dried and recrystallised from petroleum spirit (40–60 °C boiling fraction). A solution of the product in petroleum spirit was applied to a 70 cm by 2.5 cm (diameter) column of silica gel, which had been prepared in petroleum spirit, and eluted with dichloromethane:petroleum spirit (40–60° fraction):diethyl ether (90:10:5, by volume). Fractions containing product were identified by TLC on ''microscope-slide'' silica gel plates developed in dichloromethane:petroleum spirit (40–60° fraction):diethyl ether (90:10:5, by volume) and pooled. Structures were confirmed by NMR spectroscopy.

Attempts to synthesise a glucose ester of 2,4-D proved problematic. Both tetra-acetylated and tetrabenzoylated derivatives were synthesised but various attempts to remove the blocking groups, for instance by hydrogenolysis as described for indol-3-acetic acid (Jakas et al., 1993), also resulted in the hydrolysis of the glucose-2,4-D ester bond. In the end, a different approach was adopted in which the possible glucose ester metabolite was acetylated, and compared with a synthetic tetra-acetyl-glucose ester of 2,4-D.

The tetra-acetyl-glucose ester of 2,4-D was prepared according to the method of Stock (1979). 2,4-D, dissolved in acetone containing 4.7% (v/v) triethylamine, was reacted with 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranoyl bromide at room temperature for 20 h, when a precipitate of triethylammonium bromide had formed. The addition of ethyl acetate promoted more precipitation of the bromide which was removed by filtration. Unreacted 2,4-D acid was extracted from the filtrate with sodium hydrogen carbonate solution and the ethyl acetate phase was washed, dried and filtered. The tetra-acetyl-glucose ester of 2,4-D was further purified by recrystallisation from 50% (v/v) ethanol. The identity of the product was confirmed by NMR spectroscopy as above.

The methyl ester of 2,4-D was synthesised by the method of Appleby et al. (1974).

4.3. Plant material

Immature inflorescences of coconut palm (*C. nucifera* L.) were obtained from Coconut Industry Board (CIB), Jamaica. Cores of endosperm containing mature zygotic embryos were obtained from The Coconut Plantation for Hybrid Production, Colegio de Postgraduados en Ciencias Agrícolas de México, Tabasco, Mexico. The ecotype used exclusively throughout the studies was Malayan Yellow Dwarf (MYD).

4.4. Culture media

Full strength Murashige and Skoog medium (Murashige and Skoog, 1962) was supplemented with 3 g of Phytagel and 2.5 g of activated charcoal per litre. When required, the medium was adjusted to contain up to

0.232 M sucrose and up to 0.6 mM 2,4-D. For incubations containing radioactive 2,4-D, the final mixture contained 1000 Bq of [^{14}C]2,4-D per mL for inflorescences or 2650 Bq mL^{-1} for plumular tissue. The resultant specific radioactivities were 6.64 MBq mmol^{-1} for inflorescence tissue and a range from 2035 to 1.75 MBq mmol^{-1} corresponding to non-radioactive 2,4-D concentrations from 0 to 0.6 mM respectively for plumular tissue. The media were autoclaved at 103.5 kPa and 120 °C for 20 min and used the following day. It has previously been shown that autoclaving under these conditions does not affect the integrity of 2,4-D (Ebert and Taylor, 1990). The culture medium of cultures of plumular tissue was removed after every 30 days and replaced with medium having a composition and a radioactive content identical to the original medium. Half of the cultures of inflorescence tissue were established in the presence of radioactive 2,4-D and half in its absence. After 32 days, the radioactive cultures were taken for analysis and the remainder moved to a medium having identical composition but now supplemented with radioactive 2,4-D and maintained for a further 30 days. Consequently, total uptake of [^{14}C]2,4-D at time 62 days reflects only the uptake between days 33 and 62 in inflorescence tissue. In plumular tissue, in contrast, the results reflect continuous accumulation from day 0 to the day of sampling.

4.5. Tissue culture

Spathes sheathing immature inflorescences at both stage −4 and stage −5 (Hornung, 1995b) were wiped with 70% (v/v) ethanol before excision of the inflorescence materials. The inflorescences were cut into segments 0.5 cm in length prior to incubation for 72 h in liquid medium (López-Villalobos et al., 2001). Subsequently, inflorescence segments of 0.5–1.0 mm length were cut and transferred to gelled medium. Throughout the incubation period, tissues were maintained in the dark at 29 ± 1 °C.

Mature zygotic embryos were excised from endosperm cores and incubated as described by López-Villalobos et al. (2001). After 10 days in culture, plumules were excised following the procedure of Hornung (1995a). The plumular tissue was transferred to gelled medium and cultured as described for immature inflorescences.

4.6. Extraction and analysis

The extraction of 2,4-D and its metabolites was carried out using a method similar to that described by Schneider et al. (1984). At the end of the culture period, plant tissues were removed from the culture vessels and weighed after carefully removing the gel medium adhering to their surface. The tissue was washed three times with distilled water, to eliminate traces of 2,4-D, and dried with tissue paper. They were transferred into 5 mL plastic cryogenic vials in an ice-bath and cut into smaller pieces. Immediately 2 mL of ice-cold methanol was added to each vial, which was then stored at −20 °C for at least 48 h. After this period, the frozen tissues were crushed with a glass rod and a clear methanol supernatant was obtained for further analysis after sedimentation of the tissue at 3000 rpm in a bench centrifuge. The residues, after a further three washes in methanol, were transferred to counting vials and the radioactivity associated with the tissue debris determined by liquid scintillation counting. The total volume of methanol extract (including rinsings) was adjusted to 3.0 mL. Aliquots (200 μL) were taken for liquid scintillation counting to determine the content of 2,4-D in tissue.

Aliquots (500 μL) of the total methanol extract were loaded onto individual lanes of 20 cm × 20 cm 19-channel TLC plates. The aliquots were applied to alternate lanes with standards in the intervening lanes. The plates were developed in toluene:2-butanone:acetic acid (45:55:3, by volume). Appropriate authentic 2,4-D metabolites and natural lipids were used as qualitative standards. Plates were visualized by fluorescent quenching under UV light (254 nm) and scanned for radioactivity. Alternatively, the plates were stained to visualise free acids by spraying sequentially with 0.1% (w/v) 2′,7′-dichlorofluoroscein in 95% (v/v) methanol, 1%(w/v) $AlCl_3$ in ethanol then 1% (w/v) aqueous $FeCl_3$, warming at 45 °C between sprayings (Fried, 1989).

Individual radioactive areas, identified only as metabolites I–IV were scraped from the plates and pooled from different samples for further analysis. Polar metabolites (I and II) were extracted from the silica gel in ethanol and non-polar metabolites (III and IV) were extracted in diethyl ether.

4.6.1. Acetylation

It proved impossible to prepare a glucose ester of 2,4-D from its blocked chemical precursor, the 2,3,4,6-tetra-*O*-acetyl-D-glucopyranose ester of 2,4-D. It was therefore decided to acetylate metabolite I, to see whether any of the products shared properties with the authentic tetra-acetyl-glucose ester. Pooled metabolite I extracted from 10 samples was dissolved in 1 mL of dry pyridine and reacted with 1 mL of acetic anhydride at room temperature for 12 h. Then 2 mL of water at 4 °C was added to stop the reaction and acetylated products were extracted with 3.5 mL of ethyl acetate. The extract was washed, dried and concentrated (Morillo et al., 2001). Concentrated samples were applied to silica gel TLC plates and developed in toluene:methanol:acetic acid (96:8:4, by volume). Radioactive products and authentic standards were located as described above.

4.6.2. Glucosidase

Metabolite I, obtained from 30 samples as above, was divided into three tubes. They were subjected to digestion by β-glucosidase as described by Scheel and Sandermann (1981). A solution of 0.1 mg β-glucosidase mL^{-1} was made up in 100 mM sodium acetate buffer, pH 5.0, solution. To the three tubes, 1 mL of enzyme solution, 1 mL of buffer solution (control for non-enzymic hydrolysis) or 1 mL of distilled water (control for hydrolysis at acid pH) was added. The tubes were incubated at 37 °C. After 60 min, the reaction was stopped by the addition of 20 μL of acetic acid followed by 4 mL of diethyl ether to each tube. The radioactive products from the ether or aqueous phase were applied to a 19-channel silica gel TLC plate, developed in toluene:methanol:acetic acid (96:8:4, by volume) then visualised by radioscanning.

4.6.3. Protease

Metabolites I or II was obtained from TLC plates as described for the glucosidase incubations and incubated with a protease of low specificity in an attempt to hydrolyse peptide-like bonds between 2,4-D and the amino group of amino acids. Pooled metabolites from 30 samples were again used for three incubations per metabolite. The enzyme was dissolved to give 20 μg of enzyme mL^{-1} in 10 mM sodium acetate, 5 mM calcium acetate solution adjusted to pH 7.5 with acetic acid. The recovered metabolite was mixed with 2 mL of this enzyme solution or of buffer alone or of distilled water and incubated at 37 °C for 60 min. The tubes were extracted as described for glucosidase incubations and subjected to TLC followed by radioscanning.

4.6.4. Chemical hydrolysis

Alkaline hydrolysis of metabolite II (obtained as above) and of metabolites III and IV was carried out so that the form of the radioactive acid could be identified. Metabolites III and IV were scraped from 14 lanes of TLC plates and extracted with diethyl ether; metabolite II was extracted with ethanol from silica scrapings from 10 lanes. The solvent was removed in a stream of dry nitrogen gas. Hydrolysis was carried out, as described by Christie (1989) by adding 2 mL of 1 M KOH in 95% (w/v) ethanol and refluxing for 60 min. After cooling, 5 mL of water and 5 mL of hexane:diethyl ether (1:1, v/v) were added, the tube contents mixed and the phases separated in a bench centrifuge for 7 min at 3500 rpm. Liberated acidic compounds were extracted as their potassium salts in the aqueous phase, which was combined with two further 2.5 mL aqueous washes of the organic phase. The resulting combined aqueous phase was acidified with 0.4 mL of 6 M HCl and the, now protonated, acids extracted three times with 4 mL of hexane:diethyl ether (1:1, v/v). The combined extract was concentrated in a stream of dry nitrogen to approximately 1 mL which was applied to a reversed-phase TLC plate, developed in acetonitrile:methanol:water (80:10:30, by volume) and subjected to radioscanning. The synthetic elongated 2,4-D standards and a variety of natural lipid standards were also applied to the plate.

4.7. Determination of radioactivity

The radioactive content of samples was determined by liquid scintillation counting in 22 mL plastic scintillation vials (Meridian, Surrey, UK) containing 7 mL of EcoLite scintillation fluid. Each vial could contain up to 1 mL of methanolic or aqueous sample and was counted for 10 min. Homogeneous samples were counted using an external standard ratio method to correct for quench and non-homogeneous samples used a samples channel ratio method. Colourless samples were corrected for chemical quench by reference to quench-curves prepared using quenched standards (Amersham Life Science, Bucks, UK); coloured samples were corrected by reference to quench-curves of coloured standards prepared in-house. The scintillation counter was a LKB-Wallac Rackbeta 1211 (Perkin–Elmer Life Sciences, Cambridge, UK) and counts-per-minute measurements were corrected for quench using the software provided.

TLC plates were scanned for radioactivity using a Bioscan System 200 Imaging Scanner (Washington, D.C., USA). The percentage of total radioactivity detected associated with individual bands was determined using the software Win-Scan, version 1.6, (Lab Logic, Sheffield, UK) provided. The data from a whole plate were viewed as a single (''2-dimensional'') representation using Win-Scan 2D, version.1.6, (Lab Logic, Sheffield, UK).

Acknowledgements

We acknowledge Mr. T. Gilkerson of Krown Synthesis, Imperial College, London, Wye campus, for help with the chemical syntheses and Dr. R. Nash of Merlin Synthesis, Imperial College, London, Wye campus, for help with the NMR spectroscopy. Dr. J.L. Garraway helped with the IR spectroscopy and Mr. C. Ladley provided technical assistance with radiochemical methods. Financial support for A.L.-V. was received from CONACYT and Colegio de Postgraduados en Ciencias Agrícolas de México.

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