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Comparative uptake and metabolism of 2-[^{14}C]-2,4-dichlorophenoxyacetic acid in callus cultures of monocot (*Dioscorea* spp.) and dicot (*Nicotiana tabacum* L.) plants

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ABSTRACT - (Comparative uptake and metabolism of 2-[^{14}C]-2,4-dichlorophenoxyacetic acid in callus cultures of monocot (*Dioscorea* spp.) and dicot (*Nicotiana tabacum* L.) plants). The uptake and metabolism of 2-[^{14}C]-2,4-dichlorophenoxyacetic acid (2,4-D) were investigated in leaf calluses of *Nicotiana tabacum*, tuber calluses of *Dioscorea opposita* and calluses derived from zygotic embryos, leaves and petioles of *Dioscorea composita*. Striking similarities were evident in the patterns of 2,4-D metabolites and their chemical characteristics in the three callus types of *D. composita* compared, but significant differences were detected among the patterns of rnetabolites in the three species studied. Preliminary investigations on the stability of various metabolites (separated using TLC) by hydrolysis showed that sugar esters appeared to be the major metabolites in tobacco whilst in yams (*D. opposita*) glycosides were shown to be the main ones, which indicated a similarity between plants of Gramineae and Dioscoreaceae in terms of 2,4-D metabolism. Release of 2,4-D from tobacco callus cells upon their transfer to 2,4-D-free medium was detected and the implications of this are discussed in relation to the cultural conditions necessary to induce morphogenesis *in vitro*.

RESUMO - (Absorção e metabolismo do ácido 2-[^{14}C]-2,4-diclorofenoxiacético (2,4-D) em calos de *Dioscorea* spp. e de *Nicotiana tabacum* L.). A absorção e o metabolismo do ácido 2-[^{14}C]-2,4-diclorofenoxiacético (2,4-D) foram investigados em calos de folhas de *Nicotiana tabacum*, em calos de tubérculos de *Dioscorea opposita* e em calos derivados de embriões zigóticos, folhas e pecíolos de *Dioscorea composita*. Fortes semelhanças ficaram evidentes com relação aos padrões de metabólitos e suas características químicas nos três tipos de calo de *D. composita* comparados, mas diferenças foram detectadas entre os padrões de metabólitos nas três espécies estudadas. Investigações preliminares sobre a estabilidade dos metabólitos à hidrólise mostraram que os ésteres de açúcares parecem ser os mais importantes metabólitos em tabaco enquanto em *D. opposita* são os glicosídeos, o que indica uma semelhança entre plantas das famílias Gramineac e Dioscoreaceae em termos de metabolismo do 2,4-D. A liberação de 2,4-D das células de calos de tabaco após sua transferência para meio livre de 2,4-D foi detectada e as implicações deste fato são discutidas em relação às condições de cultura necessárias para induzir morfogênese *in vitro*.

Key words - *Dioscorea* spp., *Nicotiana tabacum*, plant tissue culture, 2,4-dichlorophenoxyacetic acid (2,4-D)-uptake, auxin metabolism, radioactively labelled 2,4-D

Introduction

Auxins such as 2,4-dichlorophenoxyacetic acid (2,4-D) and its related compounds, naphthaleneacetic acid (NAA) and indoleacetic acid (IAA), are used widely in plant tissue culture media formulations for the induction of calluses, adventitious rooting and somatic embryogenesis from excised plant tissue explants and microcuttings. Of the above auxins, 2,4-D is the most extensively used for the induction of somatic embryos in both monocotyledonous and dicotyledonous plant species (Ammirato 1978). In the case of yam (*Dioscorea* spp.), 2,4-D has been used successfully for callus induction and plant regeneration in *D. floribunda* and some other species of the genus (Ammirato 1983) and in the case of *D. composita* and *D. cayenensis* we have demonstrated that this auxin is effective at 18 μM for induction of embryogenic calluses from mature zygotic embryo explants (Viana & Mantell 1989). Despite the widespread use of auxins in tissue culture media, little information is available on the speed with which auxin is effectively removed from media and how it is metabolised to be made available to various cells and tissues in the excised explant. Such information may be critical in determining whether or not a pulse treatment is more effective in induction of calluses and morphogenesis in plant tissue explants than the continual presence of an auxin for a complete subculture generation. For example, auxin exposure of tissue explants for short periods (i.e. a few hours or days) are known to be more effective than continual exposure of explants to a sustained level of auxin in instances of induction of adventitious rooting of woody plant microcuttings,

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somatic embryogenesis (Alizadeh & Mantell 1991) and organogenesis (Christiansen & Warnick 1984).

Auxins may not necessarily produce the appropriate conditions for induction of somatic embryogenesis. For example, tobacco leaf tissues have been reported to be induced by cytokinins rather than by auxins (Gill & Saxena 1993). In addition, Ebert & Taylor (1990) found that by using radiolabelling techniques they could detect that 2,4-D is heavily bound to components of tissue culture media such as activated charcoal and that this could influence significantly the induction of calluses and uptake of 2,4-D and other growth regulators such as benzylaminopurine (Ebert et al. 1993).

The use of radiolabelled auxin in the tissue culture medium facilitares the study of the initial metabolism of auxin into conjugated forms with primary sugars and amino-acids as described in the scheme shown in figure 1. Such conjugates can be detected by relatively simple methods of thin-layer chromatography.

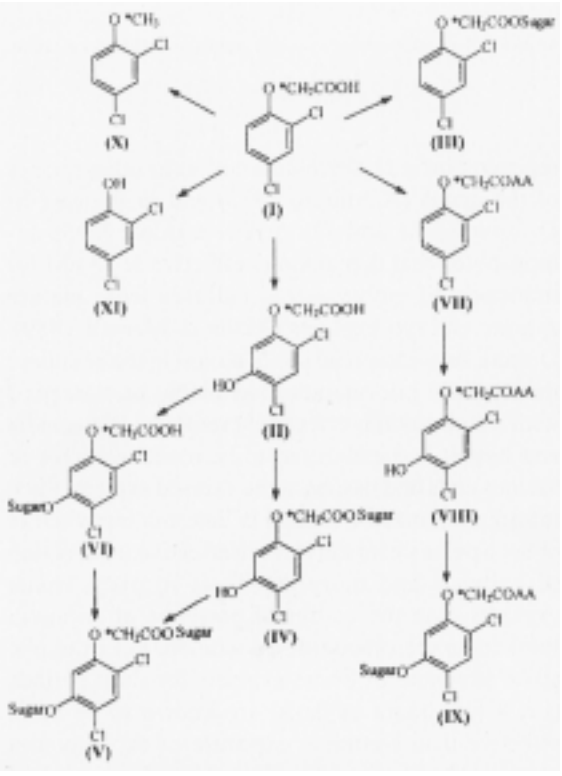

Figure 1. General scheme of 2,4-D structure and its main metabolites in plants; AA = amino-acid.

The aim of the work reported here was to compare the uptake of radioactively labelled 2,4-D from culture medium into calluses of a dicotyledonous species (*N. tabacum*) and a monocotyledonous plant (*Dioscorea* spp.) and to assess the early patterns of conjugation of labelled auxin in these tissues. In so doing we wished to develop practical procedures for researching the uptake and metabolism of 2,4-D into calluses of *Dioscorea* and *Nicotiana* to determine whether or not differing auxin conjugation metabolism patterns in the contrasting callus systems might possibly be responsible for and could explain the contrasting growth behaviours that occur in these cultures when grown under identical media and environmental conditions.

Materials and methods

Origins of callus materials - Tobacco (*N. tabacum*) cv. NC2512 calluses were initiated on 8 mm diam. leaf discs using standard tissue culture techniques. Young leaves of *in vitro* shoot cultures maintained on basal MS salts medium (Murashige & Skoog 1962), as supplied by Flow Laboratories, UK, and supplemented with 2% sucrose and 0.8% (w/v) Technical Grade III agar (Oxoid Ltd., UK) were used to generate a supply of sterile leaf discs which were then placed for callus induction on MS medium supplemented with 3% sucrose, 8.9 mM NAA and 0.88 μM 6-benzylaminopurine contained in 2.5 cm diam. x 10 cm long glass tubes capped with sterilised polypropylene covers held in place with rubber bands. Inoculated tubes were incubated under dark conditions at 25ºC for 28 days, by which time calluses had been induced on the wounded surfaces of the leaf disc explants. Small pieces of callus were then subcultured onto fresh samples of the same medium for a further two 28-day subcultures before being used for the experiments described below. *D. opposita* calluses were induced on sliced sterile microtubers induced on *in vitro* shoot cultures maintained and subcultured as described previously by Mantell & Hugo (1989). Microtuber slices of 1 mm thickness were placed on MS media supplemented with 3% (w/v) sucrose, 18 μM 2,4-D and 0.8% (w/v) Technical Grade III agar contained in identical culture vessels and incubated under the same conditions as the tobacco callus cultures. Microtuber calluses were induced after 40-60 days and were subcultured at least once for a further 40-day period away from the original microtuber tissue explants before being used for the labelling experiments. *D. composita* calluses were induced from mature zygotic embryo explants on MS medium supplemented with the same 2,4-D, sucrose and agar levels described above for *D. opposita* microtuber calluses using the method described by Viana & Mantell (1989). Calluses produced after 60 days of culture of the original explants were used for experiments.

Radioactive labelling experiments - For the purpose of following the uptake and subsequent metabolism of small amounts of 2,4-D by callus tissue, it was necessary to use radiolabelled chemical. For the purpose, 2-[^{14}C]-2,4-dichlorophenoxyacetic acid with a specific activity of 36 μCi/μM was used. In preliminary experiments, the activity was reduced

by the use of some unlabelled "cold" 2,4-D, but for later experiments no dilution was carried out. Radioactivity was assessed by liquid scintillation counter (LKB Rac B). Metabolites were analysed by thin layer chromatography on silica gel plates (Merck GF 254) which were scanned for radioactivity on a Berthold 2722-2 scanner. Agar media containing 18 μM 2,4-D were prepared as described in Ebert & Taylor (1990). Aliquots (2 g) of the labelled ("hot") medium were dispensed accurately into glass tubes (75 x 25 mm) and autoclaved. Pieces of callus (50 to 150 mg fresh weight) were transferred aseptically to the agar, the tubes sealed and incubated at 28ºC in darkness. Three replicates were used per sampling time at which point pieces of callus were removed, reweighed and frozen in liquid nitrogen. The frozen tissue was ground and acetone added. Maceration was completed by holding the tube in an ultrasonic bath for 2-3 minutes and the extractions carried out overnight at – 15ºC. The total uptake of [^{14}C] was determined by counting 50 μl aliquots of the extract using Fluoran UV scintillant. In some cases the residual agar was also counted. Aliquots (50-100 μl) of extracts containing a suitable level of radioactivity were applied to the origin of a silica gel thin layer chromatography plate as 30 mm bands and developed in one of following solvent systems:

I - benzene: acetic acid (75:12) (v/v) for the separation of free 2,4-D from its conjugates; II - ethylacetate : methyl ethyl ketone : formic acid : water (50:30:10:10) (v/v) for the separation of conjugates which remained on the origin in solvent I; III - ether : benzene (70:30) (v/v), a solvent which has been used previously, and for which some R_f values of possible metabolites were available (Dr. H.F. Taylor, personal communication).

Plates were scanned at the settings indicated on each chromatogram presented in the Results section. Accurate values for the conjugated and "free" 2,4-D levels in extracts were obtained by removing the corresponding area of silica gel from chromatograms developed in solvent system 1. Acetonitrile was added to the silica gel and this was then dispersed in acidified Triton X/ toluene scintillant. While it was not possible to establish the identity of metabolites giving the peaks of radioactivity on the scans they could nevertheless be characterised by their solubilities in ether and stabilities to certain hydrolytic procedures as follows: procedure I - relative partition between ether and water at pH 3.0; procedure II - relative stability to mild alkaline hydrolysis at room temperature; procedure III - relative stability to β-glucosidase (pH 5.0).

Samples of extracts (150 μl each) were subjected to these treatments and then chromatographed in either solvent systems II or III and with a sample of the original extract for direct comparison purposes.

The pulse/chase procedure was adopted to differentiate between early- and late-formed metabolites. For these treatments, tobacco callus tissues were allowed to remain in a 2-[^{14}C]-2,4-D source for about three days, by which time little detectable free 2,4-D was present. Some calluses were extracted in the normal way whilst others were transferred aseptically to fresh tubes containing medium with 18 μM "cold" 2,4-D. After further incubation at 28ºC in darkness both tissue and agar were extracted with acetone. Aliquots of these extracts were counted and also subjected to chromatographic analysis in the manner previously described.

Results and Discussion

The first experiments were conducted on tobacco calluses that had been previously induced on NAA containing media from excised leaf pieces, because tobacco calluses were more homogenous than *Dioscorea* ones due to the nature of the explants used. The objective was to set up the conditions of radiolabelling of culture media that would ensure recovery of sufficient levels of label to allow accurate determinations to be made in subsequent experiments.

2,4-D uptake by *N. tabacum* calluses - In a preliminary experiment, small pieces of tobacco callus (100 mg fresh weight) were transferred aseptically into culture tubes containing 4 ml agar solidified medium supplemented with "hot" 2,4-D at 9 μM in combination with "cold" 2,4-D at 9 μM. Samples were taken, weighed, and extracted as previously described.

Table 1 presents the levels of radioactivity in three replicate extracts of tobacco calluses after

Table 1. Radioactivity in leaf callus of *N. tabacum* incubated for 90, 190 and 330 h in media containing 2-[^{14}C]- 2,4-D.

Time (hours)	Total radioactivity (Kdpm)	Fresh weight (mg)	Concentration (Kdpm/mg)	Mean ± SD
90	129.39	232	0.557	
	57.88	121	0.478	
	131.22	257	0.510	0.515 ± 0.390
190	275.67	275	1.002	
	308.66	241	1.280	
	302.10	248	1.218	1.166 ± 0.168
330	601.39	436	1.379	
	372.20	354	1.051	
	571.06	570	1.001	1.143 ± 0.205

incubation for either 90, 190 or 330 h. Values for the corresponding amounts of free and conjugated 2,4-D are shown in figure 2. In this experiment the reduction in the amount of radioactivity in the tissue observed between the 190 and the 330 h sampling times suggested that 2,4-D uptake was being accompanied by losses of radioactive substances from the callus into the agar. Evidence for this was obtained by transferring tissue after 90 h to fresh medium (containing "cold" 2,4-D). After a further 240 h, agar in these cultures was extracted and the radioactivity assessed. The effects of this manipulation are shown in figure 2 and indicated that about 50% of the count had left the tobacco callus tissues. Scans of typical chromatograms used to separate 2,4-D from its conjugates (using solvent system I) are shown in figure 3A and those used to separate the conjugates produced in calluses after 90 h (using solvent system II) are shown in figure 3B. Using solvent system II, it was possible to distinguish three conjugates in the tobacco calluses. A portion of the calluses remaining were transferred to "cold" media (i.e. without 2,4-D) at 90 h for a further 240 h. This was done in order to determine the fate of the remaining 2,4-D left within the calluses. From the scans presented in figure 4, it can be seen that the radioactivity which remained in calluses was mainly as conjugates (figure 4B) while the radioactivity which was exported from calluses was mainly in the form of free 2,4-D (figure 4A). The free nature of the exported 2,4-D was confirmed by the cochromatography of the standard radioactive 2,4-D with au extract of "cold" agar medium following contact with radioactive ("hot") callus for 240 h (figure 5). Analyses of extracts of tobacco tissue following incubation on "hot" medium for either 190 or 330 h showed progressive conjugation and these effects are reported in full in the next experiment which was carried out to test the reproducibility of the experimental setup.

Tobacco leaf callus was again used to follow the pattern of conjugation of 2-[^{14}C]-2,4-D after 72, 168, 264 and 408 h. Levels of radioactivity are shown in table 2 and this data presented also in figure 6 to indicate the patterns of radiolabelling in calluses and culture media at the four sampling times. The total label levels at each sampling time are also given.

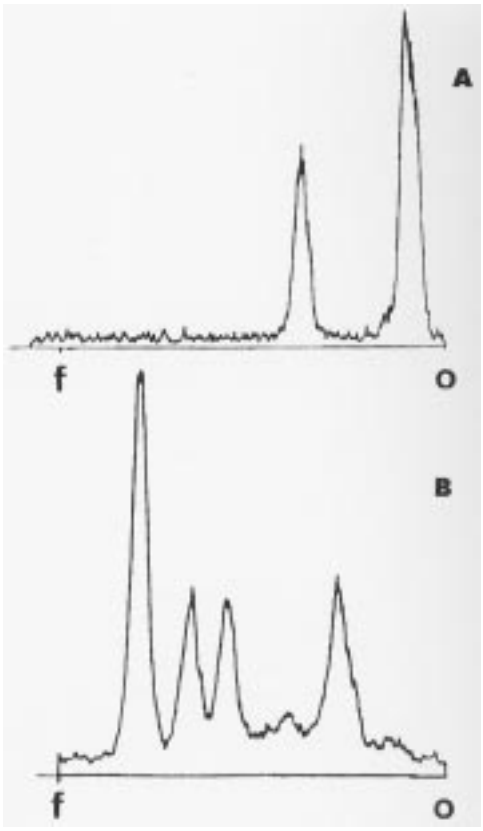

Figure 3. Scans of callus extracts of *N. tabacum* chromatographed (TLC) in solvent systems I (A) and II (B), after 90 h in 2-[^{14}C]-2,4-D; o = origin, f = front.

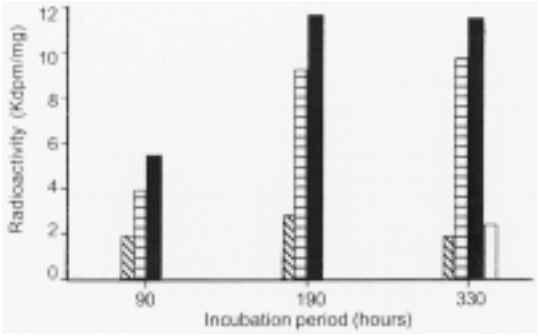

Figure 2. Uptake of 2-[^{14}C]- 2,4-D by *N. tabacum* calluses after incubation for 90, 190 and 330 h (■) total radioactivity, (▤) radioactivity of conjugates, (▧) radioactivity of free 2,4-D, (□) total radioactivity of callus tissue after further transferring to "cold" agar.

Table 2. Radioactivity in leaf callus of *N. tabacum* after incubation for 72, 168, 264 and 408 h on media containing 2-[^{14}Cl- 2,4-D.

Period (hours)	Total radioactivity (Kdpm)	Fresh weight (mg)	Concentration (Kdpm/mg)	Mean ± SD
72	925.77	310	2.98	
	784.69	239	3.28	3.05 ± 0.204
	846.96	293	2.89	
168	1002.82	186	5.39	
	1037.69	225	4.61	4.87 ± 0.447
	1349.3 5	292	4.62	
264	1288.91	227	5.68	
	1891.24	260	7.27	6.80 ± 0.977
	1455.05	195	7.46	
408	1381.57	31 8	4.34	4.34

The patterns of accumulations of "hot" 2,4-D were similar to those obtained in the previous experiment. Figure 7 shows scans of three replicated extracts made at 72 h compared to a "cold" culture control thus confirming the retiability of the radiolabelling and chromatography methodologies being employed. At subsequent sample times, only a single scan has been presented of two chromatograms: one produced using solvent system I and the other using solvent system II. The scans shown in figure 8 are of extracts of tobacco calluses which were cultured on "hot" media for 168, 264 and 408 h. There were no apparent differences in the peak patterns obtained using solvent system I but there were some differences detectable using solvent system II since the conjugate patterns changed with time particularly with respect to the numbers of peaks present. Significantly, levels of free labelled 2,4-D accumulated in calluses.

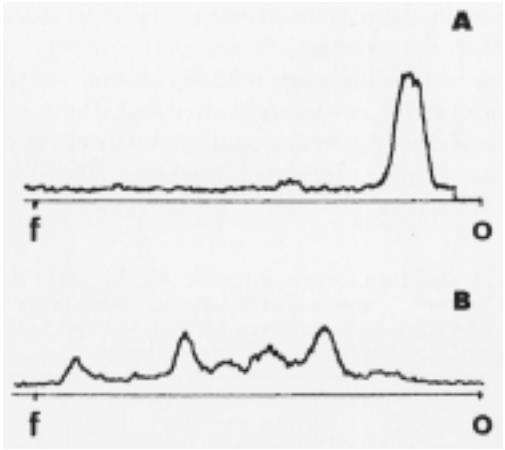

Figure 4. Scans of TLC - separated extracts of agar (A) and callus (B) of N. *tabacum* after 90 h in 2-[^{14}C]-2,4-D and further transferring to "cold" agar for 240 h, in solvent system I; o = origin, f = front.

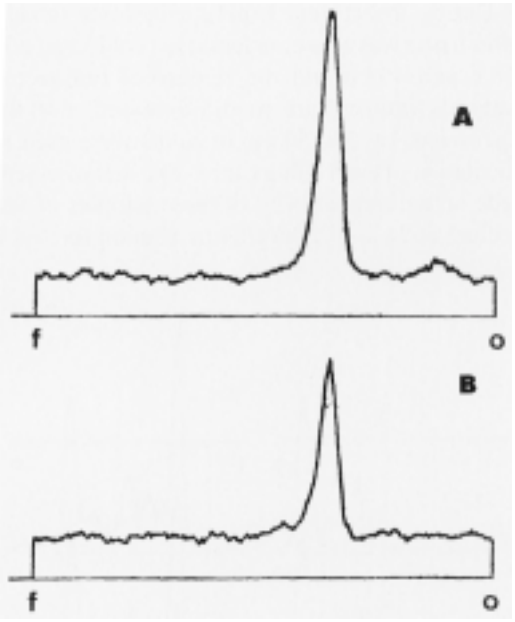

Figure 5. Scans of a standard 2-[^{14}C]- 2,4-D solution (A) and of "cold" agar after contact with radioactive callus of *N. tabacum* (B); o = origin, f = front.

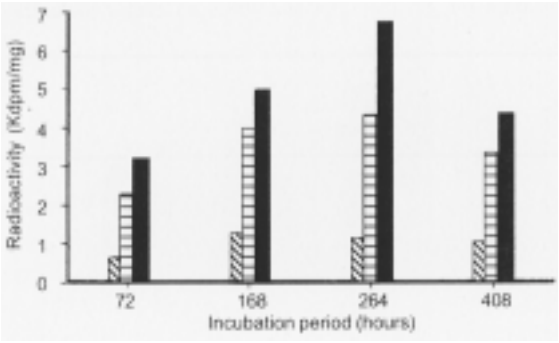

Figure 6. Uptake of 2-[^{14}C]-2,4-D by *N. tabacum* calluses after 72, 168, 264 and 408 h: (■) total radioactivity; (▭) radioactivity of conjugates; and (▨) radioactivity of free 2,4-D.

During the current experiment, some tobacco callus tissue was also transferred to "cold" agar after 168 h and 408 h and the release of radioactive materials into culture media assessed. For this experiment, ca. 50-150 mg of callus were used for inoculation of each culture tube. The measurements made on the radioactivity of three samples of agar medium at 24 and 72 h (after incubation for 168 h) were 299.76 and 519.44 Kdpm, respectively, and at 168 h (after a 408 h incubation period) 793.92 Kdpm which indicated that ca. 50% of labelled 2,4-D moved into the culture medium within the first week, confirming the findings of the preliminary experiment. The movement of 2,4-D back into the media after transferring callus tissue to a new medium may be of particular significance in terms of providing the appropriate growth regulator balance in the tissue, which would trigger morphogenetic pathways. Similar results were achieved by Montague et al. (1981) for cultured soybean cells and embryogenic carrot cells in which the higher loss of free 2,4-D by the embryogenic cultures contrasted to the lower releasing characteristics of comparable non-embryogenic ones.

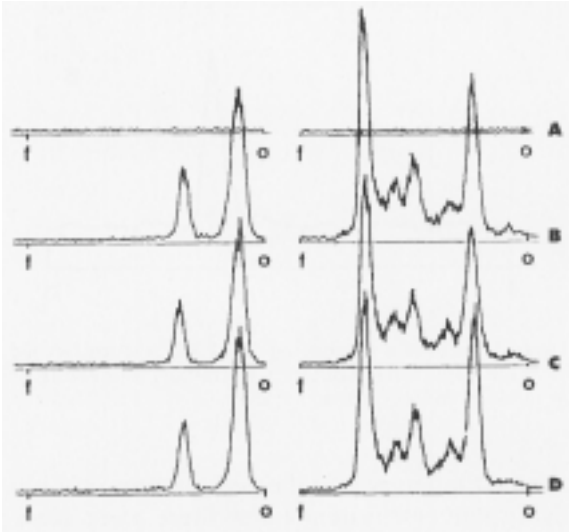

Figure 7. Scans of three replicate extracts of *N. tabacum* in solvent systems I (left) and II (right) after incubation for 72h in 2-[^{14}C]-2,4-D (B,C,D). Scan A is of a TLC-separated extract of a tobacco callus grown for the same period ou a medium containing "cold" 2,4-D instead of "hot" 2,4-D; o = origin, f = front.

Uptake of 2,4-D by *D. composita* and *D. opposita* - In this experiment, callus derived from *D. composita* embryos and microtubers of *D. opposita* were investigated along with tobacco callus for the purposes of comparison. Tissues were extracted after 72, 168 and 408 h and extracted as previously described. The comparative uptake of radiolabelled 2,4-D into the three sets of calluses are presented in figure 9. No significant differences in the patterns of uptake by these could be detected. The scans of the extracts obtained from these materials are presented in figure 10. The different numbers, positions and sizes of peaks indicated that for each species the levels of 2,4-D and its various metabolites changed with time of culture. The pattern of metabolites present in tissues were different in the three species studied. For example, *D. opposita* had only two major metabolites after 168 h incubation whereas tobacco and *D. composita* showed four. The overall level of conjugation achieved by the tissues at the various time are indicated in table 3. These data

Table 3. Percentage of conjugated metabolites by callus tissues of *N. tabacum*, *D. opposita* and *D. composita*, incubated for 72, 168 and 408 h in media containing 2-[^{14}C]- 2,4-D. Each value is a mean of three replicates ± standard deviation.

Species	Conjugated metabolites		
	72 h	168 h	408 h
N. tabacum	77.0 ± 10	87.2 ± 10	90,2 ± 10
D. opposita	73.9 ± 21	84.8 ± 9	90.2 ± 10
D. composita	57.7 ± 8	79.7 ± 10	89.2 ± 4

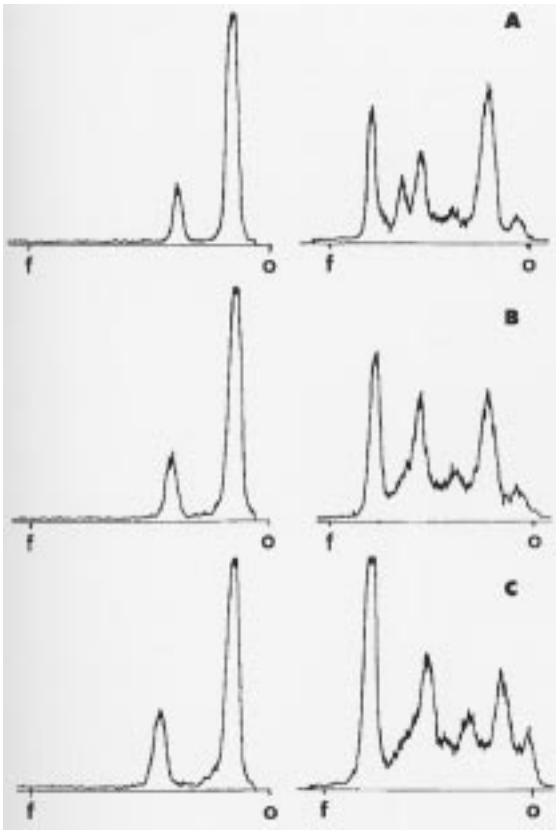

Figure 8. Scans of TLC-separated callus extracts of *N. tabacum* incubated for 168 (A), 264 (B) and 408 h (C) in 2-[^{14}C]- 2,4-D and chromatographed in solvent systems I (left) and II (right); o = origin, f = front.

show that there were no significant differences between the patterns of 2,4-D accumulation and conjugation in the respective dicotyledonous and monocotyledonous cell systems. However, the caliuses of *D. composita* appeared to show a slower accumulation compared to those of *D. opposita* and tobaceo. The percentage of conjugated metabolites was also smaller for *D. composita* at 72 h but this difference had disappeared by 408 h.

Uptake of 2,4-D by callus originating from different explants of *D. composita* and *D. opposita* – Evidence that the friable calluses of *D. opposita* (derived from microtubers) and the calluses of *D. composita* (derived from embryos and containing embryogenic globular structures) had given different metabolic products when cultured on 2,4-D (figure 10) prompted a further experiment in which the 2,4-D uptake by calluses derived from different explants of *D. composita* were also compared. Explants included friable calluses derived from leaves, petioles and mature zygotic embryos. All calluses were extracted after 192 h. Aliquots (50 µl each) of all the samples were assessed for radioactivity and the total label which had been taken up over the treatment period was calculated for each callus. Table 4 represents the levels of label in replicate extracts of calluses from different explants. Data presented in figure 11 show striking similarities between the scans of extracts of the different explants of *D. composita* while there were major diferences between the peaks present in *D. opposita* extracts. It was concluded therefore that the different tissue origin of calluses of *D. composita* did not appear to affect the patterns of 2,4-D uptake and metabolism. More significant effects were associated with the different species of yam rather than with explant types.

Investigations on the stabilities of 2,4-D metabolites to hydrolysis - The scheme presented in figure 1 shows the groups of compounds which might be expected when 2,4-D is metabolised by plants and it gives an indication of the likely biochemical routes in which this occurs and the possible biochemical mechanisms involved. Structures III to IX represent types of compounds with differing sugar and/or amino-acid residues. The most commonly occurring sugar in plants is glucose while the aspartate and glutamate are generally the most frequently occurring amino-acid conjugates. Reactions next to the -COOH group and ring hydroxylation are

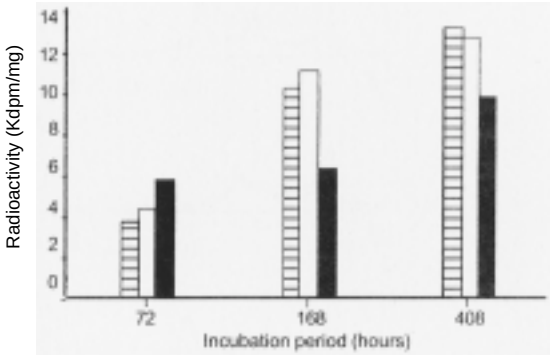

Figure 9. Uptake of 2-[^{14}C]-2,4-D by cailus tissue of *N. tabacum* (▤), *D. opposita* (□) and *D. composita* (■) after incubation for 72, 168 and 408 h.

Table 4. Radioactivity in calluses derived from different explants of *D. opposita* and *D. composita* after incubation for 192 h on media containing 2-[^{14}C]-2,4-D.

Species	Initial fresh weigth (mg)	Final fresh weight (mg)	Growth (mg)	Total radioactivity (Kdpm)	Concentration (Kdpm/mg)	Mean ± SD
D. opposita	47	84	37	736	8.76	
(tuber)	64	76	12	727	9.56	
	73	95	22	800	8.42	8.91 ± 0.585
D. composita	56	96	40	582	6.06	
(zygotic embryo)	81	205	124	779	3.80	
	58	81	23	530	6.54	5.47 ± 1.463
D. composita	64	120	56	848	7.06	
(leaf and petiole)	65	103	38	750	7.28	
	61	150	89	699	4.66	6.33 ± 1.453
D. composita	65	76	11	541	7.12	
(petiole)	70	105	35	563	5.36	
	71	83	12	538	6.48	6.32 ± 0.891

generally not considered to occur separately. They are more likely ou available biochemical evidence to occur simultaneously and may even interact (Loos 1975). In addition to the hydroxylation of the ring in the 5 position there is now increased evidence of changes in the position 4 resulting in the formation of the respective 5-chloro-4-hydroxy and 3-chloro-4-hydroxy compounds. The formation of sugar esters may begin with the synthesis of the glucose ester by a reversible reaction but the subsequent formation of more polar esters could indicate that disaccharides and trisaccharides may be involved. No information on the reversibility of such conjugates is available. This is of importance as the retention of physiological auxin activity may depend upon the hydrolysis to the parent compound (i.e. 2,4-D) (H.F. Taylor, personal communication).

Amino-acid conjugates are metabolites frequently associated with tissues of many monocots and dicots but seem to be a major metabolite in leguminous plants (Feung et al. 1973, 1975). Conjugates containing many amino-acids have been

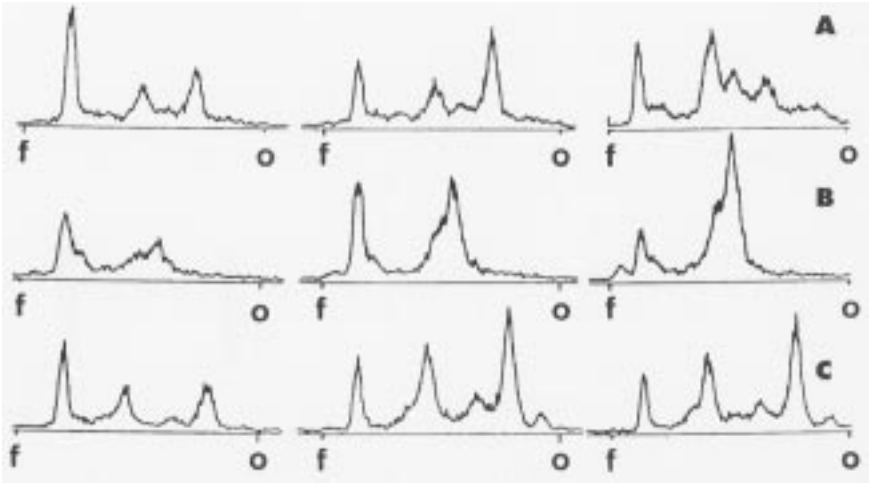

Figure 10. Scans of TLC-separated callus extracts of *D. composita* (A), *D. opposita* (B) and *N. tabacum* (C) after incubation for 72 h (left), 168 h (middle) and 408 h (right) in 2-[^{14}C]-2,4-D; o = origin, f = front.

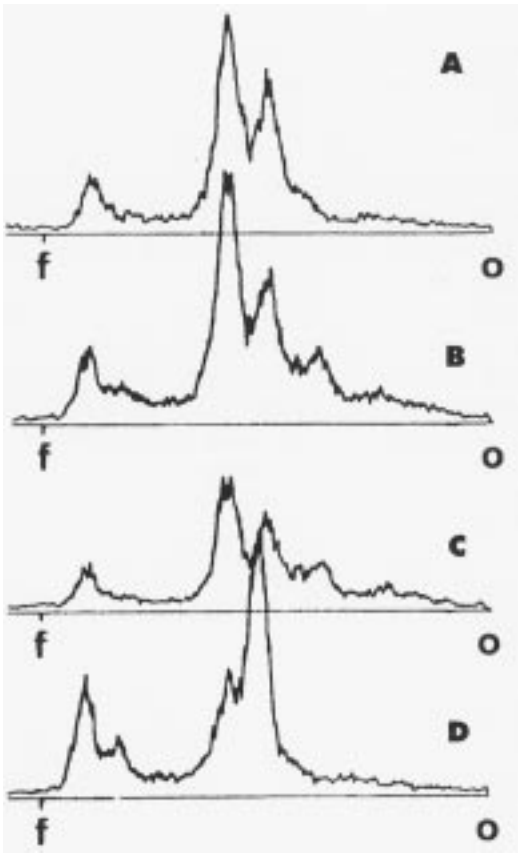

Figure 11. Scans of callus extracts of *D. composita* of petiole (A), leaf and petiole (B), zygotic embryo (C), and tuber (D) of *D. opposita*, incubated for 192 h in 2-[^{14}C]-2,4-D; o = origin, f = front.

synthesised in the laboratory and evidence that they can be formed in plant tissues has been obtained (Feung et al. 1974). On the other hand, ring-hydroxylated metabolites appear to predominate in monocotyledonous plants (H.F. Taylor, personal communication).

During the present work, the unavailability of reference compounds made the identification of 2,4-D metabolites revealed on chromatograms impossible, but much information could be obtained on the chemical natures of the metabolites by assessing their relative solubilities in solvents and their relative stabilities to hydrolysis. Treatment of extracts with alkali at room temperature would be predicted to hydrolyse esters of 2,4-D to the parent acid (Cohen & Badurski 1982). Such reactions include III to I, IV to II and V to VI but would leave the glycosides including VI unchanged (viz. Figure 1). On the other hand, glycosides would not be stable to hydrolysis by β-glucosidase when incubated with the enzyme at pH 5.0. Hydrolysis of compounds V to IV or II, VI to II, IX to VIII, however, would be expected always to yield an aglycone, i.e. a hydroxyphenoxyacetic acid (Davidonis et al. 1978, Feung et al. 1972). The hydroxyl group could not be assumed to always be in position 5 as shown in figure 1 as rearrangements involving the para chlorine are known to occur in some plant tissues (H.F. Taylor, personal communication). Ether solubility of compounds in aqueous solution at pH 3.0 or less might be expected with free acids and possibly amino-acid conjugates. Thus the unchanged 2,4-D (I), the aglycones II, VIII and the amino-acid conjugates VII shown in figure 1 would be expected to be present in ether extracts. The acetone extract obtained from tobacco callus which had been incubated with 2-[^{14}C]-2,4-D for 408 h hydrolysed with alkali and reacidified was chromatographed in solvent system II (figure 12 A, B, C, D) and solvent system III (figure 12 E, F, G, H). The sample peaks in Scan B (figure 12) indicated that the four metabolites present in the original extract (Scan A) had all been hydrolysed to compounds with the same R_f as a 2,4-D reference spot (figure 13, Scans E and J). lt was therefore concluded that the four peaks were most likely all esters of 2,4-D. Scan F in figure 12 showed a similar pattern except that there was a small peak at the origin. This could have been a trace amount of a polar amino-acid conjugate, the aspartate or glutamate conjugate, but its non-appearance in Scan B (figure 12) does not support this assumpion. Incubation of extracts overnight at 29ºC with β-glucosidase at pH 5.0 and separation using the solvent system II again hydrolysed the conjugation products as shown by Scan C (figure 12). However, when chromatography of this extract was carried out using solvent system III, in addition to the supposed amino-acid conjugate at the origin there was also evidence of a compound with a slightly lower R_f than 2,4-D (Scan G, figure 12). This was almost certainly an aglycone (II in figure 1) released by the β-glucosidase and this probably indicated the presence of small amounts of compounds IV or more

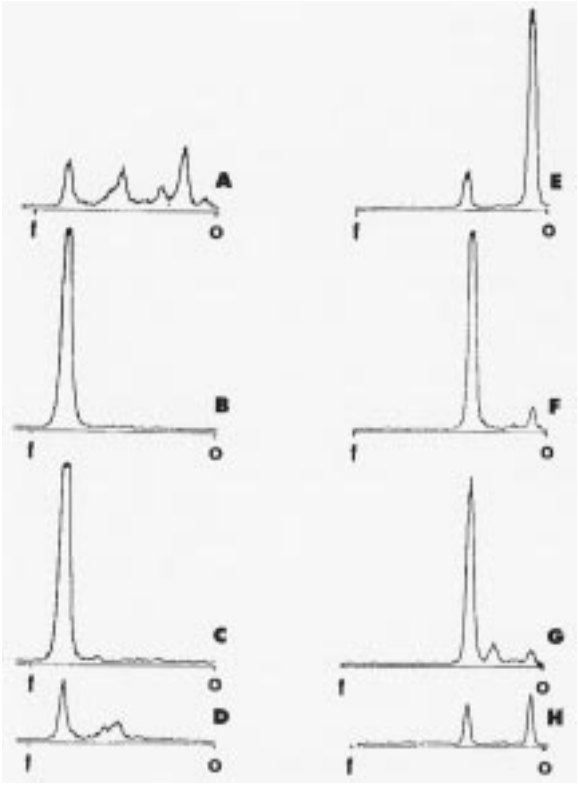

Figure 12. Scans of TLC-separations of callus extracts of *N. tabacum* chromatographed in solvent systems II (A, B, C, D) and III (E, F, G, H). A, E - original extract; B, F – after alkaline hydrolysis; C, G - after incubation with β-glucosidase; D, H - partition in ether; o = origin, f = front.

probably V (indicated in figure 1) in the original extract. Ether soluble compounds present in the extract included 2,4-D and some small peaks shown in Scan D (figure 12) further suggested that there were probably traces of amino-acid conjugares of the types VII and VIII (viz. figure 1) present in the extracts.

In figure 13, the products of hydrolysis (Scans B, G, C, H, D, and I) were compared with the compounds present in the original extract for yam tuber tissue (Scans A, F). Treatment with alkali did not hydrolyse all of the conjugates and large apparently unchanged peaks remained in both solvent systems (i.e. Scans B and G, in figure 13). This observation indicated that sugar esters (compound III, figure 1) are probably not the major metabolites present in yams. However, it did not exclude the possibility that there were metabolites present in the extracts which were only partially hydrolysed, i.e. sugar esters of glycosides (compounds such as V in figure 1). The products of alkaline hydrolysis would then be expected to be glycosides such as those with a structure like VI as shown in figure 1.

Treatment with β-glucosidase hydrolysed the majority of the conjugates with the appearance of a major component as seen in Scan H in figure 13. This was assumed to be the aglycone derived by the hydrolysis of glycosides, and this would be in agreement with the interpretation of the alkaline hydrolysis results given above. It was apparent that both 2,4-D and the aglycones were chromatographed together in solvent system II (Scan C in figure 13).

Separations of ether-soluble fractions were not conclusive but did show the presence of 2,4-D and the apparent partial solubility of a conjugate (Scan I

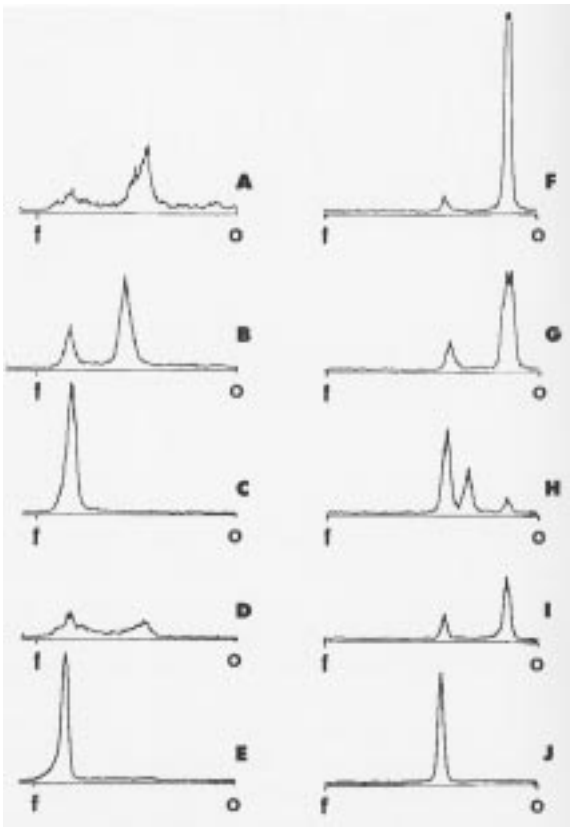

Figure 13. Scans of TLC-separations of callus extracts. of *D. opposita* chromatographed in solvent systems II (A, B, C, D, E) and III (F, G, H, I, J). A, F - original extracts; B, G – after alkaline hydrolysis; C, H - after incubation with β-glucosidase; D, I - partition in ether; E, J - standard 2-[^{14}C]- 2,4-D; o = origin, f = front.

and D, figure 13). It was difficult to interpret these results as both amino-acid conjugates were glycosides (compound IX, figure 1) and as such may have been able to partition between ether and water (pH 3.0) in an unpredictable way.

From the above chromatograms of the extracts of the two yam callus tissues it was apparent that large differences occured not only in the patterns of metabolites (Scan A in figure 12, Scan A in figure 13) but also in the chemical characteristics of the metabolites between tobacco (scans in figure 12) and *D. opposita* (scans in figure 13). lt was therefore concluded that in tobacco the sugar esters appeared to be major products of 2,4-D metabolism and that the stability of these compounds may always make it possible for hydrolysis to occur in the callus with the regeneration of the physiologically active 2,4-D. This however was probably not the case in the *D. opposita* calluses which were shown to produce glycosides which, even when they were hydrolysed naturally in the tissue, would be expected to release only aglycones. The physiological significance of these compounds is not known, but it is likely that they did not have the same physiological activity as the parent 2,4-D and probably constituted irreversible degradation of the auxin. Such aromatic ring hydroxylation has been reported in plants of the Gramineae (Feung et al.1975) and therefore the possibility that such a behaviour could be operating in Dioscoreaceae, another monocotyledonous plant, is of substantial interest.

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