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# 3,4-Dichloroaniline is detoxified and exported via different pathways in *Arabidopsis* and soybean

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**Abstract**

The metabolic fate of [UL-$^{14}$C]-3,4-dichloroaniline (DCA) was investigated in *Arabidopsis* root cultures and soybean plants over a 48 h period following treatment via the root media. DCA was rapidly taken up by both species and metabolised, predominantly to *N*-malonyl-DCA in soybean and *N*-glucosyl-DCA in *Arabidopsis*. Synthesis occurred in the roots and the respective conjugates were largely exported into the culture medium, a smaller proportion being retained within the plant tissue. Once conjugated, the DCA metabolites in the medium were not then readily taken up by roots of either species. The difference in the routes of DCA detoxification in the two plants could be explained partly by the relative activities of the respective conjugating enzymes, soybean containing high DCA-*N*-malonyltransferase activity, while in *Arabidopsis* DCA-*N*-glucosyltransferase activity predominated. A pre-treatment of plants with DCA increased DCA-*N*-malonyltransferase activity in soybean but not in *Arabidopsis*, indicating differential regulation of this enzyme in the two plant species. This study demonstrates that DCA can undergo two distinct detoxification mechanisms which both lead to the export of conjugated metabolites from roots into the surrounding medium in contrast to the vacuolar deposition more commonly associated with the metabolism of xenobiotics in plants.


*Keywords:* Glycine max (Fabaceae) soybean; *Arabidopsis thaliana* (Brassicaceae); Herbicide metabolism; Conjugation; Export; Detoxification; Dichloroaniline; Malonyltransferase; Glucosyltransferase; Roots

## 1. Introduction

The ability of plants to take up and chemically transform xenobiotics has important implications for environmental and food science. Chlorinated anilines such as 3,4-dichloroaniline (DCA) are intermediates in chemical synthesis as well as being metabolites of acylanilide, phenylurea, and carbamate pesticides (Parris, 1980; Van der Trenck et al., 1981). DCA can persist in the environment as insoluble residues in soil and plants and can also photodimerise to form carcinogens (Harvey et al., 2002). Consequently, DCA has been classified as a compound of environmental concern, with substantial interest in its metabolic fate in plants (Bartha et al., 1983; Sandermann et al., 1983; Harvey et al., 2002). DCA metabolism has been investigated in a small number of plants, with differences observed in the proportions and identities of biotransformation products. In soybean, *N*-malonylation was the major route of DCA metabolism in cell cultures (Winkler and Sandermann, 1989; Harms and Langbartels, 1986; Gareis et al., 1992; Schmidt et al., 1995), excised leaves (Gareis et al., 1992) and whole plants (Bockers et al., 1994). In wheat, both plants and suspension-cultured cells metabolised DCA mainly by *N*-glucosylation (Schmitt et al., 1985, 1995; Winkler and Sandermann, 1989, 1995; Bockers et al., 1994), while in carrot cultures, DCA underwent both malonylation and glucosylation (Schmidt et al., 1994). The enzymes responsible for catalysing the conjugation of DCA to glucose and malonic

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acid respectively have been described in plants (Frear, 1968; Sandermann et al., 1991; Matern et al., 1984). In a number of studies, species-dependent differences in DCA metabolism have been attributed to differences in the activity of the respective DCA-*N*-glucosyltransferase and DCA-*N*-malonyltransferase enzymes (Sandermann et al., 1991; Schmidt et al., 1995).

In this study, we have compared DCA metabolism in soybean, a species where the detoxification of this xenobiotic is relatively well characterised, with its metabolism in the model species, *Arabidopsis thaliana*. Ultimately, the intention here is to characterise the enzymes and transporters involved in DCA metabolism in these two species using a combination of biochemical and molecular approaches, with the potential aim of manipulating xenobiotic metabolism in plants.

## 2. Results

### 2.1. Time course of $^{14}C$ DCA uptake

Hydroponically-grown soybean seedlings and *Arabidopsis* root cultures were treated with identical, sub-toxic doses of [UL-$^{14}$C]-3,4-dichloroaniline (45.2 nmol, 37 kBq) for 48 h to investigate the uptake and distribution of radioactivity over time. Plants were extracted with methanol to determine incorporation into soluble metabolites, with any non-extractable radioactivity defined as being a bound residue which was quantified after digestion with alkali. Radioactivity in the medium was quantified directly, with the aqueous medium partitioned with ethyl acetate to recover DCA and its metabolites (average recovery 86% in soybean and 96% in *Arabidopsis*). In each case, the radioactivity present was quantified by liquid scintillation counting (LSC; Fig. 1). In soybean plants, radioactivity in the medium declined rapidly over 0–4 h (Fig. 1A). A major proportion of the loss in radioactivity was due to the uptake of [$^{14}$C]-DCA into the soybean plants, where the radiolabel accumulated as both extractable and bound residues, predominantly in the roots. After 4 h, the distribution of radiolabel between medium and root remained constant. There was a moderate net loss of radioactivity from the system after 48 h, which was attributed to the volatilisation of [$^{14}$C]-DCA from the medium due to aeration. Fluorographs of the [$^{14}$C]-DCA treated soybean plants showed that, at the end of the time course, the majority of the label had accumulated in the roots, with a small proportion in the meristem (Fig. 2).

In *Arabidopsis*, the rate of net uptake of radioactivity into cultured roots was also rapid, with soluble residues in the roots comprising 65% of total label after 24 h of treatment (Fig. 1B). Unlike soybean, only 3–4% of the administered label was incorporated into bound residues in *Arabidopsis* roots. The percentage of the dosed radiolabel in the *Arabidopsis* growth medium continued to decline during the course of the experiment, to a final value of 20% after 48 h treatment.

### 2.2. Metabolic fate of DCA in soybean and Arabidopsis

The radioactive metabolites present in the plant tissues and respective media were analysed by thin layer chromatography (TLC) and quantified, following Phosphorimaging. For reference, the profiles of metabolites seen in both soybean and *Arabidopsis* after a 48 h incubation with [$^{14}$C]-DCA are shown in Fig. 3. The identification of DCA metabolites was based on their co-chromatography in two TLC solvent systems and by HPLC (data not shown) with prepared standards of DCA-*N*-malonate (M-DCA) and [$^{14}$C]-DCA-*N*-β-D-glucoside (G-DCA), which are both well characterised metabolites of DCA in plants (Winkler and Sandermann, 1989, 1992; Gareis et al., 1992).

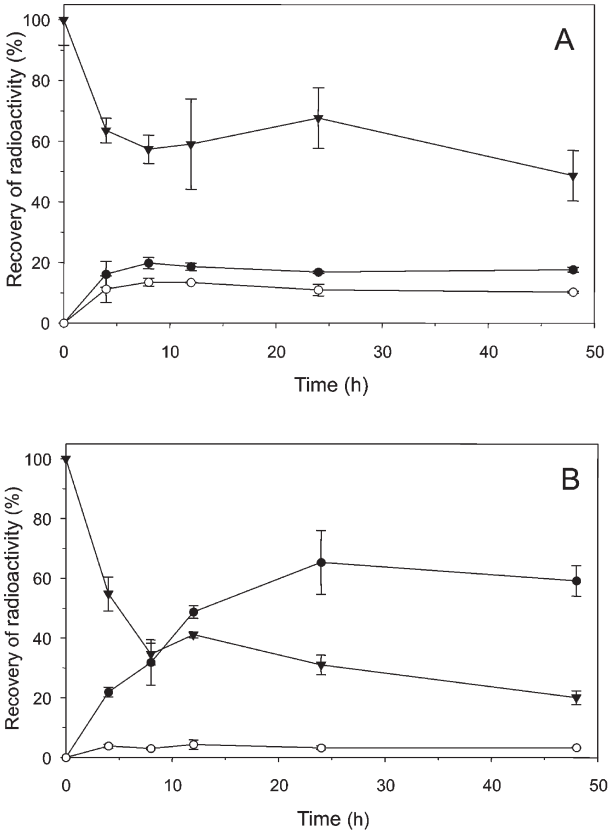


Fig. 1. Distribution of radioactivity following addition of [$^{14}$C]-DCA to the root medium of soybean and *Arabidopsis*. Hydroponically-grown soybean seedlings (A), and *Arabidopsis* root cultures (B) were treated with 45.2 nmol (37 kBq) [UL-$^{14}$C]-3,4-dichloroaniline for 48 h. At various time points, the radioactive metabolites were extracted from the plants and medium and quantified by LSC as: soluble residues in the plant (closed circles), bound residues in the plant (open circles), radioactivity in the growth medium (closed triangles). Data points represent the means of 2–4 replicates, with error bars showing standard deviations. Representative of four experiments.

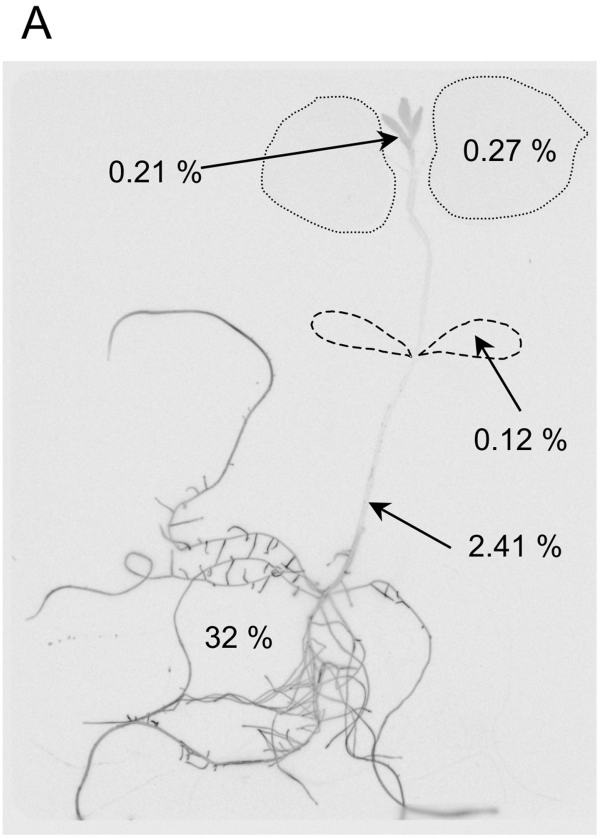


Fig. 2. Distribution of radiolabel in soybean plant fed [$^{14}$C]-DCA through the root medium. Hydroponically-grown, 10 day-old soybean seedlings were treated with 45.2 nmol (37 kBq) [UL-$^{14}$C]-3,4-dichloroaniline. After 3.5 days, plants were analysed by phosphor-imaging (A). Radioactivity in the dissected plant parts was quantified by LSC and the results expressed as % of the original dose (A) and nmol of [$^{14}$C]-DCA equivalents $g^{-1}$ FW (B), with results showing the means of duplicated experiments ± S.E.

In all chromatograms, the parent [$^{14}$C]-DCA ran as two radioactive entities, with the proportion of radioactivity in each band determined by the solvent system. In the chloroform:methanol:water system, 92% of the radioactivity in the [$^{14}$C]-DCA dosing solution co-chromatographed with authentic unlabelled DCA, while the

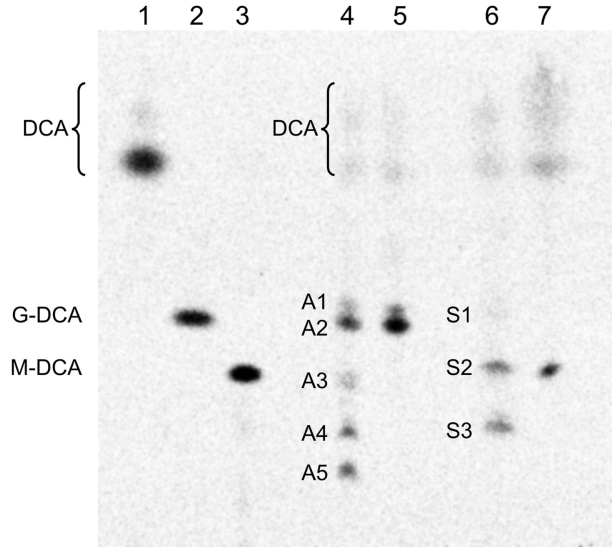


Fig. 3. Identification of DCA metabolites by TLC. Radioactive DCA metabolites were extracted from plants and culture medium and analysed by TLC using chloroform/methanol/water (60:35:8; v/v/v). Lanes: (1) [$^{14}$C]-3,4-dichloroaniline, (2) *N*-$^{14}$C-D-glucopyranosyl)-3,4-dichloroaniline, (3) *N*-$^{14}$C-malonyl-3,4-dichloroaniline, (4) *Arabidopsis* plant soluble extract, (5) *Arabidopsis* growth medium, (6) soybean plant soluble extract, (7) soybean growth medium.

other 8% ran as a diffuse spot near the solvent front. When the "authentic" [$^{14}$C]-DCA was scraped off and re-run in the same chromatography system, the radioactivity again partitioned into the two moieties in a similar ratio (9:1). It was concluded that the minor, more hydrophobic radioactive entity was an artefact of chromatography, rather than an impurity and in all subsequent analyses, these two radioactive bands were collectively quantified as parent [$^{14}$C]-DCA. When analysed by HPLC, [$^{14}$C]-DCA ran as a single radioactive entity (data not shown).

Previous studies have demonstrated that DCA can spontaneously form the respective *N*-β-D-glucopyranosyl conjugates when incubated with D-glucose (Winkler and Sandermann, 1992). It was important, therefore, to demonstrate that DCA metabolism in the root cultures was due to reactions mediated in planta, rather than due to non-enzymic reactions. When [$^{14}$C]-DCA was incubated with *Arabidopsis* growth medium containing 1% (w/v) D-glucose in place of sucrose, G-DCA was seen to accumulate at a rate of 0.15 nmol $h^{-1}$ under the dosing conditions used in the plant feeding studies. When sucrose, the sugar used routinely in the plant studies was tested, [$^{14}$C]-DCA was not converted to polar conjugates. Similarly, the rate of DCA conjugate synthesis in conditioned media taken from the *Arabidopsis* root cultures or soybean plants was also below the limit of detection. These experiments confirmed that any metabolism of DCA observed in the feeding studies was due to metabolism in planta.

After a 48 h incubation with [$^{14}$C]-DCA, distinct profiles of radioactive metabolites were seen in soybean and *Arabidopsis* (Fig. 3). Extracts from soybean plants contained three major metabolites of DCA which were designated S1, S2 and S3, respectively. Based on co-chromatography, S1 was identified as G-DCA and S2 as M-DCA. Compound S3 did not co-chromatograph with any available standards. In the soybean root medium, only S2 (M-DCA) was observed in addition to [$^{14}$C]-DCA. Extracts from *Arabidopsis* plants contained five major metabolites of DCA, designated A1–A5. Compound A1 was not identified but migrated very closely to A2, which in turn co-chromatographed with G-DCA. Metabolite A3 appeared to be M-DCA while A4 was identical to the unknown soybean DCA metabolite S3. The polar compound A5 was also not characterised. Although metabolites A1, A4 and A5 were not identified, their lability to acid hydrolysis and associated release of DCA suggested that they were all *N*-conjugates of the parent compound. In the *Arabidopsis* root medium, only A1 and G-DCA were observed as DCA metabolites.

The metabolism of [$^{14}$C]-DCA in soybean plants and *Arabidopsis* root cultures was studied over a 48 h feeding period and the major recovered metabolites (Fig. 3) quantified in both media and plant tissue (Table 1). With soybean, parent [$^{14}$C]-DCA steadily declined from the medium, to be replaced with M-DCA. In contrast, the M-DCA content in the soybean plant remained virtually constant throughout this period. By comparison with the proportion of DCA metabolised by malonylation, the amounts of G-DCA and metabolite S3 formed were modest, with G-DCA accumulating transiently at 2 h and then declining.

In *Arabidopsis*, G-DCA was the dominant metabolite at all time points in both the roots and the media. In contrast, M-DCA remained a minor metabolite, confirming that glucosylation was the major route of DCA conjugation in *Arabidopsis*. In the roots, the accumulation of G-DCA was particularly marked over the first 4 h, after which time its relative abundance steadily declined. During this loss of G-DCA, the amounts of DCA and M-DCA remained constant, A4 and A5 slowly accumulated and A1 slowly declined. The selective disappearance of G-DCA from the roots was associated with an increase in the glucoside in the medium, suggesting that the conjugate was being exported. Similarly, the slower decline in A1 in the roots was mirrored by its steady accumulation in the medium.

Table 1
Quantification of major DCA metabolites formed over 48 h in soybean plants and *Arabidopsis* root cultures dosed with 45.2 nmol [$^{14}$C]-DCA[a]

| Time (h) | Extracted metabolites (nmol) | | | | | |
|---|---|---|---|---|---|---|
| | DCA | A1 | G-DCA | M-DCA | A4/S3 | A5 |
| *Soybean root extract* | | | | | | |
| 0.5 | 1.5 | | ND | 0.6 | 0.1 | |
| 2 | 4.4 | | 0.4 | 2.2 | 0.4 | |
| 4 | 4.2 | | 0.3 | 1.5 | 0.4 | |
| 8 | 5.5 | | ND | 0.6 | 0.5 | |
| 12 | 5.0 | | ND | 0.6 | 0.2 | |
| 24 | 4.2 | | ND | 0.9 | 0.2 | |
| 48 | 3.8 | | ND | 1.3 | 0.2 | |
| *Soybean medium* | | | | | | |
| 0.5 | 29.5 | | | 2.0 | | |
| 2 | 15.8 | | | 4.7 | | |
| 4 | 7.8 | | | 7.0 | | |
| 8 | 4.1 | | | 11.3 | | |
| 12 | 4.4 | | | 11.7 | | |
| 24 | 3.3 | | | 15.1 | | |
| 48 | 3.2 | | | 12.0 | | |
| *Arabidopsis root extract* | | | | | | |
| 4 | 2.7 | 3.1 | 18.2 | 0.5 | 0.9 | 0.5 |
| 8 | 2.2 | 2.5 | 9.7 | 0.3 | 0.9 | 0.5 |
| 12 | 3.4 | 2.5 | 10.9 | 0.5 | 1.4 | 0.6 |
| 24 | 2.2 | 2.0 | 6.5 | 0.6 | 1.7 | 1.4 |
| 48 | 1.3 | 0.9 | 3.0 | 0.8 | 2.5 | 2.0 |
| *Arabidopsis medium* | | | | | | |
| 4 | 2.7 | 0.3 | 6.1 | ND | | |
| 8 | 3.0 | 2.2 | 9.5 | ND | | |
| 12 | 5.6 | 3.0 | 12.8 | 1.1 | | |
| 24 | 2.2 | 5.8 | 22.0 | 0.5 | | |
| 48 | 2.8 | 6.7 | 17.5 | 0.6 | | |

[a] Results represent the means of duplicated studies with the variation in the plants being $<10\%$ of the mean. ND = not detected.

## 2.3. Transferase activities

In order to account for the differences in DCA conjugation in soybean and *Arabidopsis*, the activities of 3,4-dichloroaniline-*N*-malonyltransferase (DCA-*N*-MT) and 3,4-dichloroaniline-*N*-glucosyltransferase (DCA-*N*-GT) were determined in crude root extracts from the two species. In both cases, product formation was monitored by quantifying the amount of radiolabelled M-DCA or G-DCA formed which partitioned into ethyl acetate (Brazier et al., 2002). With both assays, the activities reported significantly underestimate the true catalytic efficiencies of the respective enzymes, as in both cases they were incubated with low concentrations of the radiolabelled co-substrate, either UDP-[$^{14}$C-glucose] or [$^{14}$C-malonyl]-CoA. This was done to enhance the sensitivity of the assays by keeping the radioactive specific activities of the substrates high. However, with both DCA-*N*-MT and DCA-*N*-GT activities, it was demonstrated that product formation was strictly dependent on protein content for all comparative assays performed. In soybean roots, DCA-*N*-MT activity was high (12 nkat $g^{-1}$ protein), whereas DCA-*N*-GT was barely detectable (0.5 pkat $g^{-1}$ protein). In *Arabidopsis* root cultures, DCA-*N*-GT and DCA-*N*-MT activities were readily detectable although DCA-*N*-GT activity was two orders of magnitude greater than DCA-*N*-MT (6.3 nkat $g^{-1}$ protein and 52 pkat $g^{-1}$ protein, respectively). No DCA-*N*-MT or

DCA-*N*-GT activity could be determined in the media immersing either the soybean or *Arabidopsis* roots, confirming that the synthesis of the respective conjugates occurred within the plant tissues.

Since many xenobiotic-metabolising enzymes have been shown to be inducible (Marrs, 1996; Tommasini et al., 1997; Robineau et al., 1998), the effects of DCA pre-treatment on DCA-*N*-MT and DCA-*N*-GT activity (assayed at saturating DCA) were determined. Pre-treatment of *Arabidopsis* root cultures with 100 μM DCA for up to 48 h had no effect on DCA-*N*-MT or DCA-*N*-GT activity (data not shown). In soybean roots however, DCA-*N*-MT activity was increased by 2- to 6-fold depending on the length of treatment with 100 μM DCA (Fig. 4A). Increasing the DCA concentration to 200 μM gave no further enhancement in activity compared to 100 μM pre-treatment. Kinetic analysis of the effect of DCA pre-treatment indicated that although $V_{\mathrm{maxDCA}}$ was somewhat variable between experiments (control: 248.83±272.40 nkat $g^{-1}$; DCA-treated: 1376±474 nkat $g^{-1}$), this parameter was always increased by DCA pre-treatment (fig. 4B). In contrast, $K_{\mathrm{mDCA}}$ did not change substantially (control: 23.09±3.17 μM; DCA-treated: 30.75±5.15 μM).

### 2.4. Cross-feeding study

Since metabolic fate studies indicated that DCA was exported from roots in a species-specific manner, predominantly as M-DCA in soybean and G-DCA in *Arabidopsis*, a cross-feeding experiment was conducted to determine whether or not these metabolites could be reabsorbed by either plant. Soybean seedlings and *Arabidopsis* root cultures were grown in the presence of 37 kBq [UL-$^{14}$C]-DCA (45.2 nmol). After 48 h, the metabolites released into the medium by each plant species were extracted with ethyl acetate, concentrated and quantified by LSC. Untreated plants from both species were then incubated for 24 h in 20 ml fresh medium containing 6.67 kBq $^{14}$C-labelled metabolites from each species and plants and media analysed by LSC to determine the partitioning of $^{14}$C between the growth medium and plant material.

Soybean seedlings took up approximately equal amounts of $^{14}$C-labelled compounds whether they were derived from soybean or *Arabidopsis* growth medium. In each case, 12% of the supplied radioactivity was recovered in the extractable fraction from the plants while 5% became incorporated into bound residues, regardless of source of the metabolites. *Arabidopsis* root cultures showed a slight preference for uptake of radiolabelled compounds derived from *Arabidopsis* culture medium: the soluble fraction recovered from plants corresponded to 13% of the dosed radioactivity when *Arabidopsis* metabolites were supplied, compared with only 5% for soybean. Almost no bound residues were formed in *Arabidopsis*, in agreement with the DCA feeding studies (Fig. 1). After 48 h of DCA treatment, about 5–10% of the $^{14}$C in the medium is unchanged DCA (see Table 1) and it is possible that the small amount of uptake and incorporation in the cross-feeding study was due to the presence of this parent compound. In both soybean and *Arabidopsis*, the majority of the radioactivity (76–93%) remained in the medium during the cross-feeding experiment, irrespective of the source of the labelled metabolites, suggesting that the export of M-DCA or G-DCA constitutes an effective mechanism of exclusion which extends across the species.

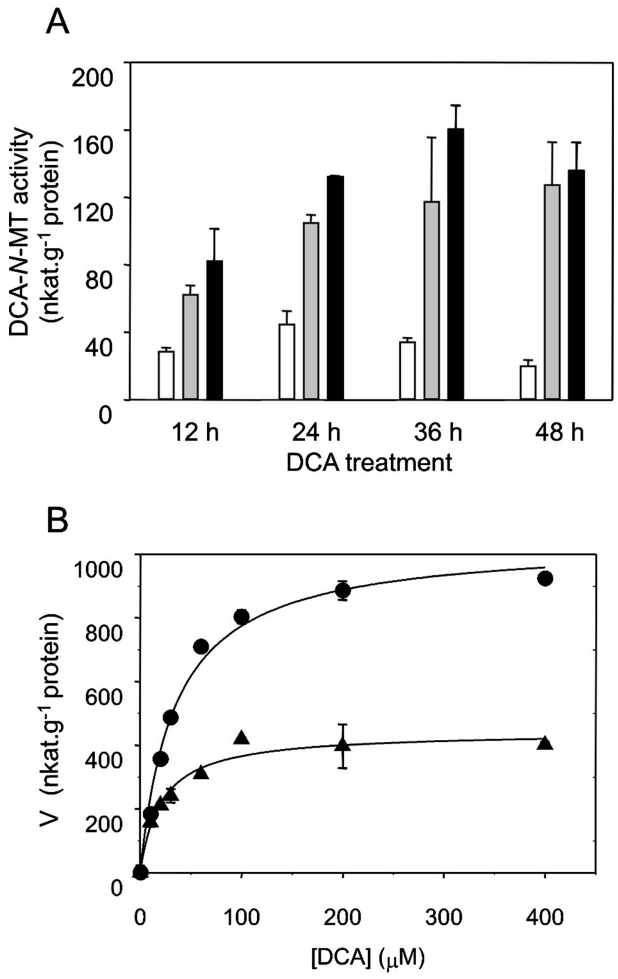


Fig. 4. Effect of DCA treatment on DCA-*N*-MT activity. (A) Hydroponically-grown soybean plants were treated with 100 μM DCA (light grey bars) or 200 μM DCA (black bars) for 12–48 h. Roots were harvested at 10 days and DCA-*N*-MT assayed. Open bars represent controls (methanol treatment). (B). Soybean roots were treated with 100 μM DCA (circles) or an equal vol. of methanol (triangles) for 24 h, harvested at 10 days and DCA-*N*-MT activities were then determined for a range of concentrations of DCA with each replicated four times (means±S.E.). $K_{\mathrm{mDCA}}$ and $V_{\mathrm{maxDCA}}$ were determined by calculating the least-squares fit to a Michaelis–Menten function. In this experiment, $K_{\mathrm{mDCA}}$ values were 20.84±4.04 μM and 34.39±3.91 μM in control and DCA-treated plants, respectively. $V_{\mathrm{maxDCA}}$ values were 441.45±21.73 nkat $g^{-1}$ and 1040.75±35.1 nkat $g^{-1}$ in control and DCA-treated plants, respectively.

## 3. Conclusions

Our results demonstrate that *N*-malonylation of DCA is the major route of detoxification in soybean, while in *Arabidopsis* *N*-glucosylation predominates. In both cases, the respective conjugates were rapidly exported from their site of synthesis in the roots into the surrounding medium. The *N*-malonylation of DCA in soybean and carrot has been reported previously (Winkler and Sandermann, 1989; Gareis et al., 1992; Bockers et al., 1994; Schmidt et al., 1994, 1995). In contrast, *N*-glucosylation is a minor route of metabolism of DCA in soybean, but a major route in monocots such as wheat and rice (Still, 1968; Schmitt et al., 1985; Winkler and Sandermann, 1989; Schmidt et al., 1995; Bockers et al., 1994). Interestingly the *N*-malonylation of DCA in soybean was linked with insoluble residue formation (Gareis et al., 1992; Bockers et al., 1994). The *N*-malonylation of DCA in soybean can be readily explained by the presence of a highly active DCA-*N*-MT in the roots with the small proportion of G-DCA formed reflecting the very low activities of DCA-*N*-GT. In earlier studies, the 6″-*O*-malonate of G-DCA was reported to be a significant metabolite of DCA in soybean (Gareis et al., 1992; Bockers et al., 1994). Attempts to demonstrate the presence of this metabolite proved inconclusive, although its chromatographic properties resemble those of metabolite S3 (= A4).

In *Arabidopsis*, the major metabolite was identified as G-DCA. In addition to G-DCA and M-DCA, three other polar, radiolabelled metabolites could be detected, all of which appear to be *N*-conjugates. In terms of abundance, compound A1 was the second most significant metabolite, accumulating along with G-DCA in the medium over time. The presence of a small amount of M-DCA in *Arabidopsis* was presumably due to the low, but measurable DCA-*N*-MT activity present, which was 100-fold less active than the DCA-*N*-GT.

Induction experiments revealed that DCA-*N*-MT is differentially regulated in *Arabidopsis* and soybean. *Arabidopsis* root cultures exhibited a low, constitutive activity which was not elevated by DCA pre-treatment, whereas DCA-*N*-MT was increased in soybean roots following pre-incubation of roots in 100 μM DCA. The DCA pre-treatment of soybean roots resulted in an increase in $V_{\mathrm{maxDCA}}$ for the DCA-*N*-MT, but only a modest change in $K_{\mathrm{mDCA}}$, suggesting that pre-treatment increases the abundance of the enzyme present either by increasing synthesis or preventing protein turnover. This could provide a potential route to the molecular identification of DCA-*N*-MT.

In both soybean and *Arabidopsis*, a major sink of DCA metabolism involved export of the M-DCA and G-DCA conjugates into the respective media. In soybean, the export of M-DCA into the media has previously been reported to be a major route of DCA metabolism in both suspension cultured cells (Winkler and Sandermann, 1989) and the roots of whole plants (Bockers et al., 1994). Significantly, we have also demonstrated that once exported, the M-DCA was not readily re-imported into the roots of soybean or *Arabidopsis*. Since M-DCA is chemically stable, its export would therefore represent an effective route and long-term mechanism for detoxification (Winkler and Sandermann, 1989, 1992). Extracellular export of M-DCA may result from the inability of soybean to deacylate this metabolite (Matern et al., 1984). The insecticide, phoxim [*O*,*O*-diethyl α-cyanobenzylideneamino-oxyphosphononthioate; 2-(diethoxyphosphinothioloxyimino)-2-phenylacetonitrile] is also exported from soybean suspension culture cells as its *N*-malonyl conjugate (Höhl and Barz, 1995), suggesting that plasma membrane transport of *N*-malonylated xenobiotics is a general detoxification mechanism for synthetic amines. This is in contrast to the *N*-malonates of natural products such as the *N*-malonyl-conjugate of the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC), which is selectively transported into the vacuole (Yang and Hoffman, 1984). In *Arabidopsis*, the small amount of M-DCA formed was also exported, though this was negligible compared to the efflux of G-DCA (Table 1). This was in contrast to wheat, with neither G-DCA nor other related glycosides being exported from the root (Harms and Langbartels, 1986; Bockers et al., 1994). Neither soybean nor *Arabidopsis* roots supported an appreciable net uptake of the *N*-glucosyl conjugate when it was supplied to hydroponic growth medium. However, unlike the *N*-malonate, G-DCA is labile to both non-enzymic and microbial hydrolysis (Winkler and Sandermann, 1992), suggesting that under field conditions the DCA would be released in the rhizosphere and become available for uptake by plants.

Malonylation and glucosylation of natural products are well known to influence the transport and storage of natural products. Thus *O*-malonylated glucosides of natural products such as anthocyanins and the isoflavone phytoalexin medicarpin, as well as *O*-malonates of chlorophyll degradation products, are compartmentalised within the vacuole (Harborne and Self, 1987; Mackenbrock et al., 1993; Barz and Mackenbrock, 1994; Hinder et al., 1996; Tommasini et al., 1998; Lu et al., 1998). It has been proposed that the malonyl group not only protects the β-glucosyl group from attack by β-glucosidases but also acts as a signal for vacuolar sequestration (Matern et al., 1983; Köster et al., 1984; Schmitt et al., 1985; Sandermann, 1994). In all these examples, *O*-malonylation can be considered to be a freely reversible modification through the action of malonylesterases located in the cytoplasm or the tonoplast (Matern, 1983; Mackenbrock et al., 1992). In contrast, *N*-malonates of xenobiotics are recalcitrant to

hydrolysis and appear to be more effectively detoxified by extracellular deposition. It also appears that *N*-glycosides of DCA are readily exported from the roots of *Arabidopsis* plants, but not from other species such as wheat. These observations raise some interesting questions about the specificity and regulation of *N*-conjugate transporters in plants and understanding these processes could be an important tool in manipulating xenobiotic metabolism in plants in the future. To that end, future work will concentrate on identifying the genes encoding the proteins responsible for the conjugation and transport of xenobiotics in *Arabidopsis* and other plants.

## 4. Experimental

### 4.1. Chemicals

3,4-Dichloroaniline (DCA) was obtained from Riedel-de-Haën (Germany) and unlabelled malonic acid from Fluka. [UL-$^{14}$C]-3,4-Dichloroaniline (817.7 MBq/mmol) was purchased from Sigma-Aldrich (UK) and [2-$^{14}$C-Malonyl]-Coenzyme A (2.035 GBq/mmol) and Uridine diphospho-D-[UL-$^{14}$C]-glucose (11.84 GBq/mmol) from Amersham Pharmacia Biotech (UK). *N*-malonyl-3,4-dichloroaniline (M-DCA) was prepared by chemical synthesis (Matern et al., 1984).

### 4.2. Feeding studies with [$^{14}$C]-DCA

*A. thaliana*, ecotype Col 0 seeds (Lehle Seeds, TX USA) were sterilised in 70% (v/v) ethanol for 1 min followed by sodium hypochlorite (20% v/v) for 10 min. After rinsing with sterile distilled water, 10 seeds were transferred to 50 ml Gamborg B5 liquid medium (Sigma-Aldrich, UK), pH 5.8, supplemented with 1% (w/v) sucrose. After growing for 17 days in the dark with constant shaking, 37 kBq (45.2 nmol) [$^{14}$C]-DCA was added in 50 μl methanol to each flask. At timed intervals, the root cultures and medium were separated by vacuum filtration and frozen in liquid nitrogen prior to storage at −80 °C.

Soybean seeds (*Glycine max* L. var. Chapman) were sown in vermiculite and grown at 24 °C, with 110 μE $m^{-1}$ $s^{-1}$ light intensity (16 h photoperiod), for 10 days. For DCA metabolism studies, the seedlings were transferred to Erlenmeyer flasks, each containing 50 ml Hoagland's No. 2 basal salt mixture (Sigma-Aldrich, UK), pH 6.0 and incubated for 24 h with constant aeration. [UL-$^{14}$C]-3,4-dichloroaniline (37 kBq; 45.2 nmol) was added to the media (50 ml), and the seedlings incubated with constant aeration and illumination. Duplicate batches, each of three seedlings, were harvested at timed intervals, the roots were rinsed with distilled water, blotted dry and plants separated into roots, cotyledons, primary leaves, secondary leaves, and stem. Samples were weighed, frozen in liquid nitrogen and stored at −80 °C.

### 4.3. Extraction of radioactive metabolites

Frozen plant tissue was ground in a pestle and mortar with acid-washed sand in the presence of ice-cold methanol (10 v/w). After centrifugation (3000 *g*, 5 min), the supernatant was decanted and the pellet frozen at −20 °C. The volume of the supernatant was noted and a sample assayed by liquid scintillation counting (LSC). The extract was then concentrated under reduced pressure at 50 °C and the residue resuspended in methanol (1 ml) and re-assayed by LSC. Non-extractable radioactivity in the plant material was determined after extensively washing the pellet from the initial extraction with methanol. The pellet was then incubated in 2 ml 2 M NaOH, at 37 °C for 20 h prior to the addition of 320 μl concentrated HCl. Samples (100 μl) were then analysed by LSC. Growth medium was made up to a known volume with water and 200 μl analysed by μLSC. The medium was then partitioned two times against one v/v ethyl acetate and the organic phases combined and concentrated to 1 ml under vacuum, at 40 °C. The residue was redissolved in 1 ml methanol and re-assayed by LSC.

### 4.4. Cross feeding study

Four soybean seedlings and four *A. thaliana* root cultures were transferred into 50 ml Hoagland's medium and Gamborg B5 basal salt medium respectively, each containing 37 kBq [UL-$^{14}$C]-DCA. After 48 h, plants were removed and the radiolabelled metabolites present in the growth media were assayed by LSC and then extracted by partitioning with 1 v/v ethyl acetate. The organic phase was evaporated under vacuum and the residue redissolved in 1 ml of methanol and assayed by LSC. After concentrating the methanolic extract to 50 μl under a stream of $N_2$, the equivalent of 6.67 kBq of $^{14}$C-labelled metabolites was added to 20 ml of fresh media in the following combinations: (1) fresh soybean plants treated with DCA metabolites from soybean (2) fresh *Arabidopsis* root cultures treated with DCA metabolites from soybean (3) fresh soybean plants treated with DCA metabolites from *Arabidopsis* (4) fresh *Arabidopsis* root cultures treated with DCA metabolites from *Arabidopsis*. After 24 h, the plant material and growth media were radioassayed as described above.

### 4.5. Identification of metabolites

Samples were applied onto silica gel TLC plates precoated with fluorescent indicator ($UV_{254}$, Sigma-Aldrich, UK). The compounds were separated in chloroform/methanol/water (60:35:8; v/v/v) or ethyl

acetate/propan-2-ol/water (63:23:11; v/v/v). UV-absorbing spots were detected under UV light at 254 nm and 366 nm, respectively. Radioactive metabolites were detected by autoradiography using BioMax MR Film in combination with BioMax TranScreen LE intensifying screens (Kodak Scientific Imaging System, Cambridge) and individual metabolites quantified using a Bio-Rad GS-525 Phosphor-imager which had been calibrated with known amounts of $^{14}C$ metabolites. The identity of metabolites was confirmed by their co-chromatography in both TLC systems with authentic unlabelled or $^{14}C$-labelled standards. In addition, radioactive metabolites were co-chromatographed with authentic standards using a reverse-phase HPLC column (Symmetry C18, 3.5 μm, 4.6×30 mm). The initial solvent system of acetonitrile/1% (v/v) phosphoric acid (5:95; v/v) was changed after 2 ml to give a linear gradient to 100% acetonitrile at 7.6 ml. The flow rate was 0.8 ml.min$^{-1}$.

### 4.6. Enzyme extraction and assay

Frozen soybean tissue was ground to a fine powder using a pestle and mortar and suspended in 1.5 vol. homogenising buffer [200 mM Tris–HCl, pH 7.5, 2 mM $MgCl_2$, 5% (w/v) PVPP, 5 mM DTT, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 1 mM PMSF]. The slurry was strained through four layers of muslin and the filtrate centrifuged (20,000*g*, 10 min at 4 °C). The resulting supernatant was employed for enzyme assays.

For *Arabidopsis*, the frozen tissue was ground under liquid nitrogen using a pestle and mortar and the resulting powder was resuspended in 3 vol. extraction buffer [0.2 M Tris–HCl pH 8, 5% (w/v) PVPP, 1 mM DTT]. The preparation was filtered and centrifuged as above and proteins in the supernatant precipitated after adjusting to 70% (w/v) with respect to ammonium sulphate and collected by centrifugation (10,000*g*, 40 min). Protein pellets were resuspended in extraction buffer and desalted on a G25 column. In all cases, the protein concentration was determined using Coomassie dye Reagent (BioRad) and γ-globulin as reference protein.

### 4.7. Enzyme assays

For DCA-*N*-MT assays, the mixture consisted of 100 mM Bis-Tris Propane–HCl, pH 6.5, 300 μM DCA, 9 μM [2-$^{14}C$]-malonyl-CoA (3.7 kBq), 22 μM cold malonyl-CoA, 0.1% (w/v) BSA and 20 μg crude protein, in a final vol. of 100 μl. After incubation at 35 °C for 5 min, the reaction was stopped with of 3 μl glacial acetic acid and the malonylated conjugates partitioned into 200 μl ethyl acetate and 100 μl of the organic phase quantified by LSC.

The DCA-*N*-GT assay mixture consisted of 200 mM Tris–HCl, pH 8.0, 5 μl of 1 mM DCA, 10 μl of [UL-$^{14}C$]-UDP-glucose (962 Bq) and 100 μg protein, in a final vol. of 75 μl. After incubation at 30 °C for 20 min, 125 μl Tris–HCl, pH 8.0 was added and the glucosylated conjugates were partitioned into 200 μl ethyl acetate and 100 μl quantified by LSC. Controls consisted of incubating the protein extract under identical conditions except that DCA was substituted with methanol.

To prepare radioactive reference metabolites of M-DCA and G-DCA, the respective organic phases from the reactions were pooled and then concentrated under a stream of $N_2$ prior to analysis by TLC.

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