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Uptake and Metabolic Fate of [^{14}C]-2,4-Dichlorophenol and [^{14}C]-2,4-Dichloroaniline in Wheat (*Triticum aestivum*) and Soybean (*Glycine max*)

SOPHIE PASCAL-LORBER,* ESTELLE RATHAHAO, JEAN-PIERRE CRAVEDI, AND FRANCOIS LAURENT

INRA, UMR Xénobiotiques, 180 ch. de Tournefeuille, BP3, 31931 Toulouse Cedex 9 France

The uptake and metabolism of [^{14}C]-2,4-dichlorophenol (DCP) and [^{14}C]-2,4-dichloroaniline (DCA) were investigated in wheat and soybean. Seeds were exposed to a nutrient solution containing 50 μM of one of two radiolabeled compounds, and plant organs were harvested separately after 18 days of growth. In wheat, uptake of [^{14}C]-2,4-DCP was 16.67 $\pm$ 2.65 and 15.50 $\pm$ 2.60% of [^{14}C]-2,4-DCA. In soybean, uptake of [^{14}C]-2,4-DCP was significantly higher than [^{14}C]-2,4-DCA uptake, 38.39 $\pm$ 2.56 and 18.98 $\pm$ 1.64%, respectively. In the case of [^{14}C]-2,4-DCP, the radioactivity absorbed by both species was found mainly associated with roots, whereas [^{14}C]-2,4-DCA and related metabolites were associated with aerial parts, especially in soybean. In wheat, nonextractable residues represented 7.8 and 8.7% of the applied radioactivity in the case of [^{14}C]-2,4-DCP and [^{14}C]-2,4-DCA, respectively. In soybean, nonextractable residues amounted to 11.8 and 5.8% of the total radioactivity for [^{14}C]-2,4-DCP and [^{14}C]-2,4-DCA, respectively. In wheat, nonextractable residues were nearly equivalent to extractable residues for [^{14}C]-2,4-DCP, whereas they were greater for [^{14}C]-2,4-DCA. In soybean, the amount of extractable residues was significantly greater for both chemicals. However, in both species, nonextractable residues were mainly associated with roots. Isolation of soluble residues was next undertaken using excised shoots (wheat) or excised fully expanded leaves including petioles (soybean). Identification of metabolite structures was made by comparison with authentic standards, by enzymatic hydrolyses, and by electrospray ionization−mass spectrometric analyses. Both plant species shared a common metabolism for [^{14}C]-2,4-DCP and [^{14}C]-2,4-DCA since the malonylated glucoside conjugates were found as the final major metabolites.

KEYWORDS: ***Triticum aestivum*; *Glycine max*; 2,4-dichlorophenol; 2,4-dichloroaniline; absorption; residues; metabolism; malonylated glycosides**

INTRODUCTION

Chlorophenols and chloroanilines are widespread environmental persistent organic pollutants generated in many industrial processes. Chlorophenolic compounds mainly arise from the manufacture of plastics, dyes, drugs, and the paper pulp industry and are also formed as degradation products of pesticides (*1*). 2,4-Dichlorophenol (2,4-DCP) is used as an intermediate in the manufacture of the 2,4-dichlorophenoxyacetic acid herbicide. Chloroanilines mainly result from herbicide degradation and also from the textile, dye, and leather manufacturing industry (*2*). 2,4-Dichloroaniline (2,4-DCA) is widely used as an intermediate in industrial syntheses. Soil concentrations of chlorophenols range from 0.001 to 0.018 mg kg^{-1} in uncontaminated areas to 10 000 mg kg^{-1} and even higher near some sawmills (*3*). The concentrations of chloroanilines could range from 36 to 480 ppb for raw wastewater (*4*). Chlorophenols and chloroanilines are reported to be harmful to aquatic and terrestrial ecosystems (*3*). Toxic effects of chloroanilines include methemoglobinaemia, hemolysis, nephrotoxicity, and genotoxicity. Furthermore, chlorophenols are putative precursors of the highly mutagenic and carcinogenic dibenzo-*p*-dioxins and dibenzofurans in different incineration processes (*5*). In addition to several anthropogenic sources, Hoekstra et al. (*6*) reported the natural formation of chlorinated phenols from organic matter and inorganic chloride. Owing to their high chronic toxicity, their ubiquity and their low solubility in water, it is not surprising that all these compounds are prominent on lists of priority pollutants (*7*).

Since toxicity of chlorophenol derivatives increases with the number of chlorine atoms, many metabolic studies in plants have focused on pentachlorophenol (*8, 9*) but also on the herbicides 2,4,5-trichlorophenoxyacetic acid and 2,4-dichlorophenoxyacetic acid (*10*). Little is known about the uptake and metabolism of 2,4-DCP in plants, especially in crop plants. Ensley et al. (*11*) identified the glucoside conjugate of 2,4-DCP as the major

* Corresponding author: Sophie Pascal-lorber, Tel: +33 5 61 28 53 92; fax: +33 5 61 28 52 44; e-mail: spascal@toulouse.inra.fr.

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metabolite of this compound in the aquatic angiosperm *Lemna gibba*. Concerning chloroanilines, metabolic studies have for the most part focused on 3,4-dichloroanilines (*12*) and 4-chloroaniline (*13*), which are residues of pesticides such as phenylcarbamates, phenylureas, or acylanilides. Dichloroanilines and monochloroanilines are often metabolized to glycoside and malonylated glycoside conjugates or directly to malonyl conjugates. However, the metabolic fate of 2,4-DCA has not yet been studied in crop plants.

In this context, the uptake and metabolism of [^{14}C]-2,4-DCP and [^{14}C]-2,4-DCA were investigated in wheat and soybean. The proportion of extractable radioactivity within plant tissues was determined and the biotransformation products were characterized.

MATERIALS AND METHODS

Chemicals and Synthetic Standards. 2,4-DCP (99% pure), [U-*phenyl*-^{14}C]-2,4-DCP (specific activity = 773.3 MBq mmol^{-1}, radiochemical purity > 98% by radio-reverse phase-high performance liquid chromatography, radio-RP-HPLC, analysis), 2,4-DCA (99% pure), malonic acid, dicyclohexylcarbodiimide were obtained from Aldrich (Saint Quentin Fallavier, France). [U-*phenyl*-^{14}C]-2,4-DCA (specific activity = 2886 MBq mmol^{-1}, radiochemical purity > 98% by radio-HPLC analysis) was purchased from Isotopchim (Ganagobie-Peyruis, France). 2,3,4,6-Tetra-*O*-acetyl-α-D-glucopyranosyl bromide, almond β-glucosidase (G-0395), and rabbit liver esterase (E-9636) were purchased from Sigma (Saint Quentin Fallavier, France). Glucose monohydrate was obtained from Prolabo (Fontenay-sous-Bois, France). Solvents used for extractions and HPLC analyses were purchased from Prolabo, Merck (Nogent-sur-Marne, France) or Scharlau Chemie (Barcelona, Spain). 2,4-DCP-Glu was synthesized according to the procedure of Sinnot and Souchard (*14*). 2,4-DCA-Glu was prepared as described by Winkler and Sandermann (*15*), and 2,4-DCA-Mal was prepared as described by Matern et al. (*16*).

Plant Material and Treatments. *Whole Plants (Uptake).* Seeds of wheat cv. Courtot were sown in quartz sand, irrigated with either a [^{14}C]-2,4-DCP (50 μM, 33.5 MBq mmol^{-1}) or a [^{14}C]-2,4-DCA (50 μM, 33.5 MBq mmol^{-1}) nutrient solution (one-fourth strength Hoagland pH 6) and were grown in a climate-controlled cabinet under a 16 h photoperiod (25/30 °C, day/night, 350 μEinstein m^{-2} s^{-1} PAR) during 18 days. The cultivation procedure for soybean (cv. Weber) was the same as described for wheat except that the seeds were individually sown in 250 mL plastic pots to accommodate the larger development of the leaves. In the case of soybean plants, roots, shoots, and cotyledons were rinsed with methanol to remove adsorbed residues, harvested separately, and frozen at −80 °C before further analysis.

Excised Leaves (Metabolism). Excised shoots (wheat) or excised petioles of fully expanded leaves (soybean) of two-week-old plants were placed in small vials containing either 400 μL of a [^{14}C]-2,4-DCP or a [^{14}C]-2,4-DCA solution (16600 Bq, 50 μM). The uptake period, which corresponded to approximately 2 h, was followed by a 48 h water chase under a 16 h photoperiod. Analysis of the remaining radioactivity showed that about 85% of the applied radioactivity was taken up by plants under these conditions. Samples were then stored at −80 °C.

Extraction Procedures. Plant organs were cut into small pieces and ground with a ball grinder (Bioblock, Illkirch, France) for 3 min. An aliquot of the resulting powder was kept to measure the total radioactivity absorbed by plants. Ground tissues were transferred in methanol/dichloromethane/water (2:1:0.8, v/v/v), homogenized for 4 h in a vortex and stored overnight at −20 °C (*17*). The homogenate was then centrifuged at 10000*g* for 10 min and the pellet was washed two times with the solvent mixture. The three supernatants were combined and contained the extractable residues. The residual material, consisting of the nonextractable residues, was allowed to air-dry at room temperature for 48 h to remove organic solvents and was then freeze-dried for 60 h to remove water.

Determination of Radioactivity. The radioactivity of plant organs and nonextractable residues was measured after oxidative combustion of aliquots in a oxidizer (model 306, Packard Instrument Co, Downers Grove, IL), trapping of the resulting $^{14}CO_2$ in an scintillation mixture (Permafluor and Carbo-sorb, Packard) followed by liquid scintillation counting in a Packard Tricarb 2200CA scintillation counter. Aliquots of extractable residues were directly counted with Ultimagold (Packard) as scintillation cocktail. Calculations to obtain the amounts of residues taken up by plants and inside plants were based on the specific activity of the molecules studied.

Isolation of Metabolites. Wheat excised shoots or soybean petioles and leaves were ground with a ball grinder for 2 min, and the resulting powder was extracted with water (2 mL g^{-1}). After centrifugation of the sample at 10000*g* for 10 min, metabolites and the parent compound, if still present, contained in the supernatant were isolated by coupling the HPLC system with a fraction collector (model FC-204, Gilson Medical Electronics, Middleton, WI). The collected fractions, corresponding to HPLC peaks, were evaporated to dryness under vacuum and stored at −20 °C until mass spectrometry (MS) analysis.

Tobacco Cell Cultures [^{14}C]-2,4-DCA Application and Preparation of Cell Extracts. Tobacco (BY2) cell suspension cultures were grown in the dark at 25 °C in 1 L Murashige and Skoog medium (Sigma M-5524) supplemented with KH_2PO_4 (200 mg L^{-1}), sucrose (30 g L^{-1}), myoinositol (100 mg L^{-1}), thiamine (10 mg L^{-1}), and 2 mL of a 5 × 10^{-4} M 2,4-D solution. The medium was adjusted to pH 5.8. A total of 25 mL of cells was transferred every week into fresh sterile medium. Forty micromolar of nonlabeled 2,4-DCA and 410 KBq of [^{14}C]-2,4-DCA (125 μL, dissolved in ethanol) were diluted in the culture medium 2 days after transfer of the inoculum into fresh medium. Incubation was ended 144 h after treatment. The medium was separated from treated cells by centrifugation at 3000*g* for 10 min, and the pellet (cells) was washed two times with water. Cells were then freeze-dried for 60 h to remove water. Thereafter, cells were ground, extracted, and analyzed as described above.

Enzymatic and Chemical Hydrolysis Procedures. All the samples, which contained about 85 Bq, were evaporated to dryness under vacuum or a nitrogen stream before treatment by enzymes or hydrochloric acid (HCl) hydrolyses.

β-D-Glucoside Glucohydrolase or β-D-Glucosidase Hydrolysis. Samples were incubated with one unit of almond β-glucosidase in 200 μL 0.1 M pH 5.0 sodium acetate buffer at 30 °C for 2 h.

Esterase Hydrolysis. Samples were treated with two units of rabbit liver esterase in 200 μL 0.05 M pH 7.5 potassium phosphate buffer at 30 °C for 10 min.

Malonyl Esterase Hydrolysis. Samples were incubated with 250 μL of a crude enzyme preparation made from parsley (*Petroselinum hortense*) stems (*18*) in 0.1 M pH 7.5 potassium phosphate buffer at 30 °C for 2 h.

Acid Hydrolysis. Samples were dissolved in 200 μL of 2 N HCl and heated at 100 °C for 2 h. After acid hydrolysis, distilled water was added to the samples and the radioactivity was extracted from the aqueous phase using chloroform (1:1, v/v). The chloroform fraction was evaporated and the dry residue was reconstituted in the appropriate HPLC mobile phase.

Reverse-Phase HPLC Analyses. RP-HPLC was performed on an Alltech adsorbosphere C18 reverse-phase column (5.0 μm, 4.6 × 250 mm) using a Spectra-Physics P4000 system (Les Ulis, France) equipped with a P1000 Spectra Physics UV detector. Radioactivity was monitored with an on-line Packard Flow-One A250 β scintillation detector (cell volume, 0.5 mL; scintillation cocktail ratio, 2 mL scintillation liquid/1 mL HPLC effluent), using Flow-scint 2 as scintillation counting cocktail (Packard). 2,4-DCP and related metabolites were separated at ambient temperature by a 30 min linear gradient beginning with 100% solvent A (water/acetonitrile/acetic acid, 88:10:2, v/v/v) and ending with 100% solvent B (water/acetonitrile/acetic acid, 58:40:2, v/v/v). The elution profile was also recorded at 285 nm. Separation of 2,4-DCA and related metabolites was performed at ambient temperature using mobile phases composed of solvent A (ammonium acetate buffer 20 mM pH 5.0) and solvent B (ammonium acetate buffer 20 mM pH 5.0/acetonitrile, 10:90, v/v). The column was equilibrated with a mixture of 85% solvent A and 15% solvent B. Elution conditions were as follows: a 20 min linear increase of solvent B from 15 to 30%, 30% solvent B for 15 min, and then a 10 min linear increase of solvent B from 30 to 100%. The elution profile was also recorded at 250 nm. The flow rate was 1

mL min^{-1} in both cases. Acetonitrile was added to samples before analyses, 10% for 2,4-DCP and 15% for of 2,4-DCA; samples were thereafter centrifuged at 10000*g* for 10 min and the supernatant injected into HPLC.

Retention times of standards and metabolites were as follows: 2,4-DCP, 40 min; 2,4-DCP-Glu-Mal, 24 min; 2.4-DCP-Glu, 20 min; 2,4-DCA, 46 min; 2,4-DCA-Glu, 22 min; 2,4-DCA-Mal, 20 min; 2,4-DCA-Glu-Mal, 18 min.

Mass Spectrometric Analyses. All experiments were carried out using an electrospray/quadrupole ion trap mass spectrometer (Finnigan LCQ, Thermo Finnigan, Les Ulis, France). The positive ion mode was used except for 2,4-DCA-Mal, which was analyzed in the negative ion mode. The following conditions were used for the positive ion mode: needle voltage (5 kV); heated capillary temperature (220 °C); capillary voltage (4 V); tube lens offset (−17 V). The conditions used for recording the negative mass spectrum of DCA-malonyl were as follows: needle voltage (−4 kV); heated capillary temperature (200 °C); capillary voltage (−16 V); tube lens offset (17 V). Nitrogen was used as sheath gas at a flow rate of 80% (arbitrary units).

For standard compounds and 2,4-DCP metabolites, the analyte solution in methanol/water (50:50, v/v) mixture was directly introduced into the ESI source at a flow rate of 3 μL min^{-1}. To enhance positive ionization efficiency, metal chloride was also added to the analyte solution (1 mM lithium chloride).

Analyses of purified 2,4-DCA metabolites required a separation before analysis by mass spectrometry. Liquid chromatography (LC) was used to introduce the samples into the electrospray ionization (ESI) source at a 0.2 mL min^{-1} flow rate. This was carried out using a Thermo Separation P4000 pump (Thermo Quest, Les Ulis, France) fitted with a Rheodyne injector. The LC column used was a Kromasil 5.0 μm C18 reverse-phase column (250 × 2 mm). The following elution gradient was used: 15% B to 30% B from 0 to 20 min, then 30% B from 20 to 35 min, 30% B to 100% B from 35 to 40 min, and finally 100% B from 40 to 50 min, with A, 0.1% formic acid and B, 0.1% formic acid in water/acetonitrile (10:90, v/v).

MS^n experiments were performed on the mass selected ion in the ion trap mass spectrometer using helium as collision gas. Ion isolation and collision energies around 20% (100% collision energy corresponds to 5 V peak to peak) were optimized separately to get maximal structural information for each experiment. All spectra were acquired using AGC (Automatic Gain Control).

2,4-DCP-Glu (Standard). PI-ESI-MS^2 385/383 $[M+Li]^+$ (% relative abundance: 100): *m/z* 169 $[M+Li\text{-}2,4\text{-}DCP]^+$ (85).

2,4-DCP-Glu-Mal. PI-ESI-MS^2 419/417 $[M + Li]^+$ (% relative abundance: 100): *m/z* 375/373 $[M+Li-CO_2]^+$ (78), *m/z* 255 $[M+Li\text{-}2,4\text{-}DCP]^+$ (35), *m/z* 211 $[M+Li\text{-}2,4\text{-}DCP-CO_2]^+$ (21).

2,4-DCA-Glu. PI-ESI-MS^2 326/324 $[M+H]^+$ (% relative abundance: 100): *m/z* 308/306 $[MH-H_2O]^+$ (38), *m/z* 164/162 $[MH\text{-}Glu]^+$ (42).

2,4-DCA-Glu-Mal. PI-ESI-MS^2 412/410 $[M+H]^+$ (% relative abundance: 40): *m/z* 394/392 $[MH-H_2O]^+$ (100), *m/z* 254/252 $[MH-C_6H_6O_5]^+$ (5), *m/z* 249 $[MH\text{-}2,4\text{-}DCA]^+$ (3), *m/z* 231 $[MH\text{-}2,4\text{-}DCA\text{-}H_2O]^+$ (3).

2,4-DCA-Mal (Standard). NI-ESI-MS^2 248/246 $[M-H]^-$ (% relative abundance: 100): *m/z* 204/202 $[M-H-CO_2]^-$ (55).

NI-ESI-MS^3 204/202 (% relative abundance: 100): *m/z* 162/160 $[2,4\text{-}DCA\text{-}H]^-$ (48).

2,4-DCA-Glu-pentose (Metabolite from Nicotiana tabacum Cell Suspension Cultures). PI-ESI-MS^2 458/456 $[M + H]^+$ (% relative abundance: 100): *m/z* 440/438 $[MH-H_2O]^+$ (75), *m/z* 326/324 $[MH\text{-}pentose]^+$ (55), *m/z* 164/162 $[DCA+H]^+$ (3).

Data Analysis. Results, expressed as μg of equiv parent compound g^{-1}, are always relative to fresh weight (FW). They are the average of at least three measurements for each experiment and were analyzed for statistical significance by two-tailed Student's *t* test ($p < 0.05$).

RESULTS AND DISCUSSION

Uptake of [^{14}C]-2,4-DCP and [^{14}C]-2,4-DCA by Wheat and Soybean Whole Plants. The results on the uptake of [^{14}C]-2,4-DCP and [^{14}C]-2,4-DCA by wheat and soybean whole plants are shown in Table 1. The total uptake was calculated on the basis of the counts obtained from the individual plant organs. The amount of radioactivity absorbed by plants and the radioactivity still present in the nutrient solution accounted for the total applied radioactivity. Eighteen days after growth on sand containing either a [^{14}C]-2,4-DCP or a [^{14}C]-2,4-DCA water solution, wheat and soybean had taken up both molecules. The octanol−water partition coefficient (K_{ow}), in particular, log K_{ow}, is often used as a key parameter in the estimation of plant uptake of xenobiotic compounds and their mobility within plant after root absorption. Uptake of organic chemicals is also reported to be important for compounds with log K_{ow} values between 1 and 3.5 (*19*). Since log K_{ow} values of 2,4-DCP and 2,4-DCA are 3.06 (*20*) and 2.79 (*21*), respectively, efficient uptake of these molecules was thus expected. However, differences in uptake between the two species were highlighted. In wheat, the uptake was 16.67 ± 2.65% of the applied radioactivity for [^{14}C]-2,4-DCP and 15.50 ± 2.60% for [^{14}C]-2,4-DCA (Table 1), indicating that the uptake of these compounds was equivalent. In contrast, uptake of [^{14}C]-2,4-DCP in soybean was significantly greater ($p < 0.05$) than that of [^{14}C]-2,4-DCA (Table 1). Since pK_a values of 2,4-DCP and 2,4-DCA are 8.51 (*3*) and 2.05 (Handbook of Physics and Chemistry), respectively, 2,4-DCA is expected to be neutral around pH 5 (pH of the apoplast) or around pH 7 (pH inside cells) and 2,4-DCP to be very slightly ionized. Therefore, both molecules were expected to enter plant roots in almost equal quantities. It was the case for wheat, but it was not true for soybean. Since excretion of malonylated 3,4-DCA into the medium of hydroponic soybean plants was reported by Bockers et al. (*12*), it is not unrealistic to surmise that excretion of malonylated 2,4-DCA could occur into the nutrient solution and could explain the apparent lower uptake of 2,4-DCA as compared to 2,4-DCP in soybean. Furthermore, apparent differences in the absorption and translocation of [^{14}C]-herbicides between a dicotyledonous (soybean) and a monocotyledonous (oat) plant have already been reported (*22*).

Table 1. Residues of [^{14}C]-2,4-DCP and [^{14}C]-2,4-DCA in Wheat and Soybean[a]

	[^{14}C]-2,4-DCP		[^{14}C]-2,4-DCA	
	wheat	soybean	wheat	soybean
roots	19.14 ± 3.81 (14.03 ± 1.65)	48.49 ± 6.97 (27.13 ± 2.01)	11.99 ± 1.42 (11.73 ± 2.29)	22.23 ± 5.52 (7.30 ± 1.35)
leaves	1.32 ± 0.18 (2.63 ± 1.18)	7.59 ± 0.77 (4.48 ± 0.53)	1.92 ± 0.26 (3.77 ± 0.40)	16.44 ± 3.87 (7.13 ± 0.95)
cotyledons		56.67 ± 14.06 (6.78 ± 1.54)		36.13 ± 6.80 (4.55 ± 0.66)
whole plant	8.54 ± 2.62 (16.67 ± 2.65)	35.77 ± 7.61 (38.39 ± 2.56)	6.09 ± 0.69 (15.50 ± 2.60)	19.97 ± 3.52 (18.98 ± 1.64)

[a] Results are expressed as micrograms of equivalent parent compound per gram of fresh weight or as percentage of the applied radioactivity (parentheses). Values are means ± SD from three samples.

In the case of [^{14}C]-2,4-DCP, 85% of the radioactivity absorbed by wheat was found associated with roots, giving a residue concentration of 19.14 ± 3.81 μg equiv 2,4-DCP g^{-1}, while only 15% of the radioactivity was present in shoots, corresponding to 1.32 ± 0.18 μg equiv 2,4-DCP g^{-1} (Table 1). In the case of [^{14}C]-2,4-DCA, 75% of the radioactivity was found in roots. The distribution of the radiolabeled material was slightly different in soybean: while 75% of the radioactivity was associated with roots in the case of [^{14}C]-2,4-DCP uptake, only 36% remained associated with this compartment for [^{14}C]-2,4-DCA uptake (Table 1). Leaves and cotyledons of soybean

Table 2. Extractable (ER) and Nonextractable Residues (NER) of [^{14}C]-2,4-DCP and [^{14}C]-2,4-DCA in Wheat[a]

	[^{14}C]–2,4-DCP		[^{14}C]–2,4-DCA	
	ER	NER	ER	NER
roots	7.13 ± 1.36	10.09 ± 2.74	2.60 ± 0.35[b]	7.67 ± 0.72[b]
leaves	1.07 ± 0.55	0.33 ± 0.11	1.14 ± 0.92	0.86 ± 0.30
whole plant	3.32 ± 0.90	3.99 ± 1.36	1.70 ± 0.76[b]	3.40 ± 0.27[b]

[a] Results are expressed as micrograms of equivalent parent compound per grams of fresh weight. Values are means ± SD from three samples. [b] Statistically significant, ER as compared to NER ($p < 0.05$).

Table 3. Extractable (ER) and Nonextractable Residues (NER) of [^{14}C]-2,4-DCP and [^{14}C]-2,4-DCA in Soybean[a]

	[^{14}C]–2,4-DCP		[^{14}C]–2,4-DCA	
	ER	NER	ER	NER
roots	36.09 ± 13.70	24.08 ± 5.56	7.84 ± 2.11	11.02 ± 2.16
leaves	6.13 ± 1.14[b]	1.63 ± 0.09[b]	9.66 ± 2.31[b]	3.32 ± 0.75[b]
cotyledons	39.77 ± 1.90[b]	7.78 ± 5.40[b]	22.07 ± 3.11[b]	1.43 ± 0.11[b]
whole plant	20.40 ± 3.68[b]	10.55 ± 0.55[b]	10.69 ± 2.22[b]	5.85 ± 1.10[b]

[a] Results are expressed as micrograms of equivalent parent compound per gram of fresh weight. Values are means ± SD from three samples. [b] Statistically significant, ER as compared to NER ($p < 0.05$).

could easily be separately harvested then analyzed. These results showed that the uptake by cotyledons of both chemicals was statistically equivalent ($p < 0.05$). The difference in the amount of radioactivity found in the aerial parts between [^{14}C]-2,4-DCA-treated and [^{14}C]-2,4-DCP-treated soybean was thus only due to a significantly higher amount of radioactivity in leaves (Table 1). Due to its pK_a value, 2,4-DCA is supposed to better move across membranes, which is also needed for long-range translocation of pesticides. pK_a values of parent molecules may thus partly explain the different distribution of 2,4-DCA and 2,4-DCP inside the plants. Nevertheless, compounds seemed to be rapidly metabolized and could be transported already as transformed products. Indeed, radio-HPLC profiles obtained from whole plant extracts only showed the presence of 2,4-DCA and 2,4-DCP derived metabolites in roots. Differences in 2,4-DCP and 2,4-DCA distribution could be then due to different rates of metabolism and also instability of N-glucosides, which could return to the 2,4-DCA parent molecule that could be more easily translocated.

Finally, it is worth noting that the concentration of residues, expressed as μg equiv parent compound g^{-1}, was considerably higher in cotyledons than in leaves of soybean. It is reasonable to assume that this higher level of radioactivity may be partly due to closer contact between cotyledons and the radioactive solution in early plant growth. Physiological processes could in addition conveniently account for the higher concentration of residues in cotyledons. Since they are main energy providing organs of the seedlings and metabolically active in early plant growth, nutrients and other compounds including xenobiotics could accumulate in these organs, carried by the transpiration stream.

Distribution of Radioactivity in the Soluble and Nonextractable Fractions. The partitioning of the soluble and nonextractable radioactivity in wheat and soybean is shown in Tables 2 and 3, respectively. In wheat, nonextractable residues represented 7.8 and 8.7% of the applied radioactivity for [^{14}C]-2,4-DCP and [^{14}C]-2,4-DCA, respectively. The balance between soluble and nonextractable residues in whole plants was equivalent for [^{14}C]-2,4-DCP, whereas the extraction procedure resulted in a concentration of residues in the pellet 2-fold higher than in the total extract for [^{14}C]-2,4-DCA (Table 2). Since the distribution of extractable and nonextractable residues was quite similar in leaves, this difference was mainly due to the presence of high levels of nonextractable residues in roots (Table 2). In soybean whole plants, nonextractable residues amounted to 11.8 and 5.8% of total radioactivity for [^{14}C]-2,4-DCP and [^{14}C]-2,4-DCA, respectively (Table 3). In both cases, no statistical difference ($p < 0.05$) was found between soluble and nonextractable residues in roots, whereas nonextractable residues were considerably lower in leaves and especially in cotyledons (Table 3).

The higher proportion of nonextractable residues associated with roots as compared to aerial parts was in good agreement with published data concerning the distribution of [^{14}C]-3,4-dichloroaniline and derived metabolites in wheat and soybean. In addition, a lower percentage of absorbed radioactivity in nonextractable residues in soybean as compared to wheat has been reported (*12*). In support of this finding, the majority of chloroaniline-derived metabolites are found in the soluble fraction in soybean cells, whereas in wheat cells, nonextractable residues predominate (*15*). Even though soybean is expected to form high levels of aniline-derived nonextractable residues (*23*), differences in cell wall structures could partly explain lower levels of bound residues in dicotyledonous as compared with monocotyledonous plants. However, no difference could be seen between the partitioning of radioactivity into the soluble and the nonextractable fractions, relative to absorbed radioactivity, in soybean treated with either [^{14}C]-2,4-DCP or [^{14}C]-2,4-DCA. This result was somewhat surprising since chloroaniline compounds are known to form bound residues as final metabolization products (*24*).

Extractable Metabolites of [^{14}C]-2,4-DCP and [^{14}C]-2,4-DCA in Wheat and Soybean. To investigate [^{14}C]-2,4-DCP and [^{14}C]-2,4-DCA metabolism and to obtain large amounts of soluble residues, uptake by the petiole of soybean excised leaves or by wheat excised shoots was used as a relevant model for whole plant uptake. Indeed, radio-HPLC profiles from whole plant extracts and excised leaves qualitatively closely resembled each other. HPLC analyses revealed rapid metabolism by excised leaves since within 48 h, almost no trace of [^{14}C]-2,4-DCP or [^{14}C]-2,4-DCA was observed (**Figures 1** and **3**). The structures of the metabolites were identified by comparison with retention times in HPLC of authentic standards, by enzymatic hydrolyses, and by mass spectrometric analyses. There are no HPLC analytical data on the media.

Extractable Metabolites of [^{14}C]-2,4-DCP in Wheat and Soybean. Most of the applied radioactivity was metabolized to one main metabolite both in wheat and in soybean (**Figure 1A,B**).

This peak was resistant to almond β-D-glucosidase and carboxyl esterase hydrolysis but was hydrolyzed by parsley esterases (*18*), which are known to hydrolyze diacid hemiesters of glucosides, to yield a product coeluting in HPLC with authentic 2,4-DCP-Glu. After malonyl esterase treatment, the hydrolyzed metabolite could then be converted by almond β-D-glucosidase to the parent compound. The structure of the intact main metabolite was further characterized using ESI-MS. The mass spectrum is shown in **Figure 2**.

Under positive ionization conditions with the presence of metal chloride (lithium chloride), the compound was ionized, yielding a quasi-molecular [M+Li]$^+$ ion cluster at *m*/*z* 419/417, characteristic of a dichlorine isotopic pattern. MS/MS experiments carried out on these ions yielded (i) a major fragment

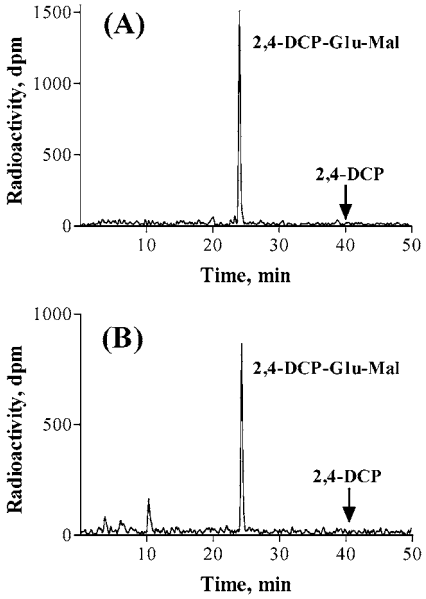

Figure 1. Typical radio-HPLC profiles of [^{14}C]-2,4-DCP soluble residues obtained from aqueous extracts of (A) wheat excised shoots and (B) soybean excised leaves.

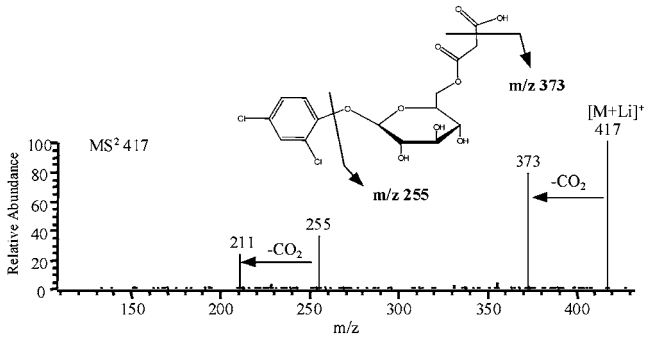

Figure 2. Mass spectrum, obtained by positive ESI-MS, of the 2,4-DCP main metabolite isolated from aqueous extracts of wheat excised shoots and soybean excised leaves.

ion at *m*/*z* 375/373 by loss of carbon dioxide, consistent with the presence of a carboxylic acid function, and (ii) a fragment ion at *m*/*z* 255 resulting from loss of 2,4-DCP, which underwent consecutive decomposition leading to the formation of *m*/*z* 211 ion by loss of carbon dioxide. The chemical structure of the main metabolite was therefore attributed to the malonyl-*O*-glucoside conjugate of 2,4-DCP. A minor peak (retention time 20 min) was tentatively assigned to 2,4-DCP-Glu, because it cochromatographed with authentic 2,4-DCP-Glu in HPLC and was hydrolyzed by almond β-D-glucosidase to a product coeluting with parent 2,4-DCP. Due to its presence in very low quantities, it could not be formally identified by ESI-MS.

The observed results were in good agreement with published data. Direct conjugation to glucose has been frequently described in the transformation of many xenobiotic compounds in plants (*25*) and more recently in 2,4-DCP metabolism in *Lemna gibba* (*11*). However, subsequent malonyl ester formation has not been detected in these organisms, probably due to the weak structure of malonate hemiesters of glucosides (*18*). Further acylation of glycoside conjugates by malonic acid is a common process in the plant kingdom and may represent a significant final step in the metabolism of xenobiotic compounds (*26*).

Extractable Metabolites of [^{14}C]-2,4-DCA in Wheat and Soybean. As reported above for [^{14}C]-2,4-DCP metabolism, analysis by HPLC of aqueous extracts from [^{14}C]-2,4-DCA-treated wheat and soybean gave similar radio-HPLC profiles (**Figure 3A,B**).

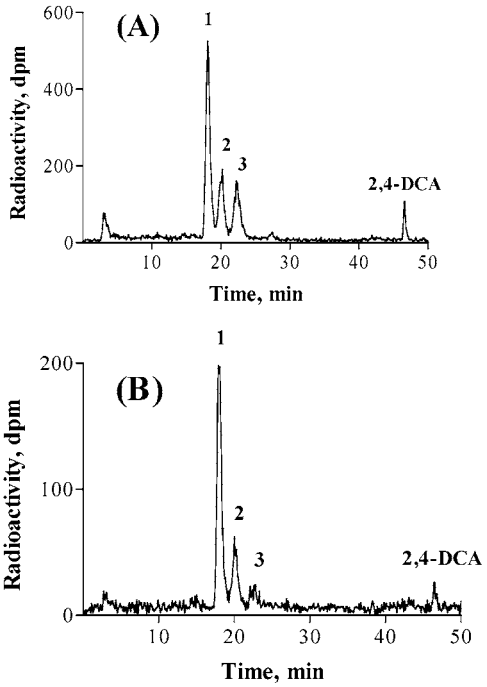

Figure 3. Typical radio-HPLC profiles of [^{14}C]-2,4-DCA soluble residues obtained from aqueous extracts of (A) wheat excised shoots and (B) soybean excised leaves. Peaks are numbered according to their elution order. Peak 1: 2,4-DCA-Glu-Mal; peak 2: unknown; peak 3: 2,4-DCA-Glu.

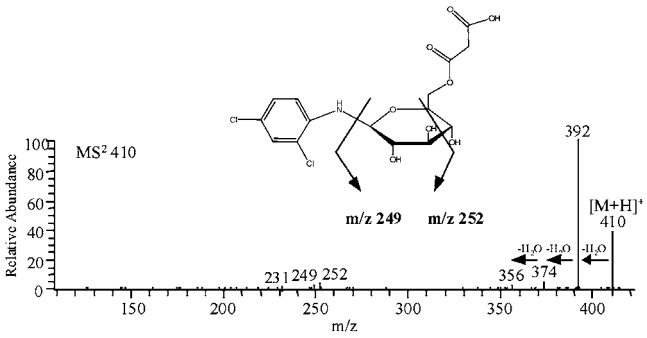

Figure 4. Mass spectrum, obtained by LC-ESI-MS (positive ionization), of the malonyl glucoside metabolite of 2,4-DCA isolated from aqueous extracts of wheat excised shoots and soybean excised leaves.

One major and two minor peaks were detected and were numbered according to their elution order. The main metabolite (peak 1) accounted for almost 85% of the total soluble metabolites and was hydrolyzed by parsley malonyl esterases to a product coeluting with peak 3. The structure of the first metabolite was further characterized by ESI-MS analysis. In the case of 2,4-DCA metabolites structure characterization, the ionization source was highly susceptible to matrix signal suppression effects. Two-dimensional (LC-LC) separation was therefore an effective way to address signal suppression for the LC-ESI-MS analysis of the complex samples (*27*). **Figure 4** shows the typical mass spectrum obtained for the major metabolite.

Its positive ESI mass spectrum displayed a dichlorine isotopic pattern at *m*/*z* 412/410. In the MS/MS experiments, these ions mainly decomposed by loss of water generating the *m*/*z* 394/392 ions. The presence of a low abundant fragment ion at *m*/*z* 249 was symptomatic of the elimination of the 2,4-DCA moiety.

This ion could undergo consecutive decomposition yielding a fragment ion at *m*/*z* 231 by water loss. On the basis of these data, the major metabolite was thus identified as the malonyl-*N*-glucoside conjugate of 2,4-DCA. The structure of the metabolite corresponding to peak 2 could not be determined by ESI-MS probably due to its presence in low quantities. Upon HCl hydrolysis, metabolite 2 was converted to the parent compound, suggesting the presence of a conjugated product. Peak 2 coeluted in HPLC with authentic 2,4-DCA-Mal. However, this metabolite also showed identical retention time to a diglycoside conjugate that was isolated from tobacco cell cultures and identified by ESI-MS analysis (mass spectrum described above in materials and methods). Since aniline diglycoside conjugates are easily cleaved by HCl treatment in contrast to malonyl aniline conjugate, metabolite 2 could thus be a diglycoside conjugate of 2,4-DCA. Peak 3 coeluted in HPLC with authentic standard 2,4-DCA-Glu and was further identified by ESI-MS. Mass spectrometric analysis showed a quasi-molecular ion cluster at *m*/*z* 326/324, characterizing a molecule containing two chlorine atoms. This was consistent with the *m*/*z* values of the $[M+H]^+$ protonated ions of 2,4-DCA-Glu. This was confirmed by MS/MS spectra, which displayed decomposition by loss of the glucoside moiety leading to the formation of fragment ions at *m*/*z* 164/162, corresponding to the 2,4-DCA moiety. The loss of water leading to fragment ions at *m*/*z* 308/306 was also observed but did not provide any structural information. In accordance with MS data, the glucoside conjugate of 2,4-DCA is proposed here as the possible structure for the third metabolite.

In whole plants, 2,4-DCA-Glu-Mal and to a lesser extent 2,4-DCA-Glu, were predominantly associated with plant aerial parts, whereas the glucoside and more polar derivatives probably higher saccharide conjugates for example, were detected in roots (not shown). The accumulation of glucosides in soybean roots can also be observed during the metabolization of 3,4-dichloroaniline. However, equal amounts of the *N*-malonyl and the *N*-glucoside conjugates of 3,4-dichloroaniline have been found in wheat roots (*12*). Several data on the metabolism of 3,4-dichloroaniline and 4-chloroaniline have shown the predominant formation of the direct *N*-malonyl conjugate in particular from carrot and soybean cell suspension cultures (*28*, *29*), the *N*-glucoside and the (malonyl)-*N*-glucoside being minor pathways. These results are slightly different from those obtained with 2,4-DCA metabolization as the (malonyl)-*N*-glucoside conjugate was generally found to be predominant. Nevertheless, Winkler and Sandermann (*15*) mainly detected the *N*-glucoside of 3,4-dichloroaniline in intact wheat plants and wheat cell cultures by thin-layer chromatography. As previously discussed, the bond between glucose and malonic acid is very labile and consequently many simple glucoside conjugates may actually be malonylated (*18*). Oxidation of the aromatic amine to the corresponding hydroxylamine is perhaps to be expected as it occurs in vertebrates such as rat (*30*) and rainbow trout (*31*), but under our conditions neither *N*-hydroxy-2,4-DCA or its glycoside conjugate could be detected. *NO*-glycoside formation is indeed rarely found in plants (*32*−*34*) and has never been described in the case of chlorinated anilines.

In conclusion, the results presented in this study showed that metabolism of 2,4-DCP and 2,4-DCA was similar in wheat and soybean, as the malonyl glucoside conjugates were the major metabolites. Formation of *O*- and *N*-glucosyl conjugates of 2,4-DCP and 2,4-DCA, respectively, was expected since distinct conjugation enzymes acting on xenobiotic substrates such as chlorinated phenols and chlorinated anilines have already been isolated (*35*). *O*- and *N*-glucosyltransferases are widespread in the plant kingdom and have been highly purified and characterized (*36*).

The important question concerning these metabolites is, once they are ingested by animals or humans, can the conjugate be hydrolyzed by the gastric juice and/or intestine enzymes, and the parent compound be released and cause toxicity (*3*, *37*, *38*)? This question is all the more important because 2,4-DCP and 2,4-DCA are known as carcinogenic chemicals for mammals and may be responsible for a direct attack upon the gastrointestinal tract with a concomitant increase in the risk of tumor development at this site. Concurrently, the safety or toxicological significance of bound residues has to be evaluated before attempting any risk assessment approach. Of particular relevance to the bound residue issue is information on its bioavailability for humans, which is usually obtained from rat model studies (*39*). Although numerous data exist on plant-bound residues (*40*), this issue remains to be investigated in the case of 2,4-DCP and 2,4-DCA. Nevertheless, since results on studies of plant-bound residues bioavaibility in animals are difficult to interpret in most cases, depending on digestibility of the food component, variety of plant species, and type of plant parts, further studies are still needed to assess toxicological importance of these particular residues.

ABBREVIATIONS USED

2,4-DCP: 2,4-dichlorophenol; 2,4-DCP-Glu: *O*-β-D-glucopyranosyl-2,4-dichlorophenol; 2,4-DCP-Glu-Mal: (6-*O*-malonyl)-O-β-D-glucopyranosyl-2,4-dichlorophenol; 2,4-DCA: 2,4-dichloroaniline; 2,4-DCA-Glu: *N*-β-D-glucopyranosyl-2,4-dichloroaniline; 2,4-DCA-Mal: *N*-malonyl-2,4-dichloroaniline; 2,4-DCA-Glu-Mal: (6-*O*-malonyl)-*N*-β-D-glucopyranosyl-2,4-dichloroaniline; ESI-MS: electrospray ionization-mass spectrometry; eq: equivalent; FW: fresh weight; RP-HPLC: reverse phase-high performance liquid chromatography; LC-MS: liquid chromatography−mass spectrometry; Kow: octanol−water partition coefficient

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