# 2,4-Dichlorophenoxyacetic Acid Metabolism in Transgenic Tolerant Cotton (*Gossypium hirsutum*)

F. Laurent,* L. Debrauwer, E. Rathahao, and R. Scalla

Laboratoire des Xénobiotiques, INRA, B.P. 3, 180 chemin de Tournefeuille, 31931 Toulouse Cedex 9, France

The metabolic fate of 2,4-dichlorophenoxyacetic acid (2,4-D) was studied in leaves of transgenic 2,4-D-tolerant cotton (*Gossypium hirsutum*), which is obtained by transfer of the *tfdA* gene from the bacterium *Alcaligenes eutrophus*. The *tdfA* gene codes for a dioxygenase catalyzing the degradation of 2,4-D to 2,4-dichlorophenol (2,4-DCP). [*phenyl*-$^{14}$C]-2,4-D was administered by petiolar absorption followed by an 18 h water chase or converted to the isopropyl ester and sprayed onto the leaf surface; the leaves were harvested 48 h later. The herbicide was degraded to 2,4-DCP by the bacterial enzyme expressed in the plants. 2,4-DCP was rapidly converted to more polar metabolites and was never found in detectable amounts. Metabolite structures were deduced from enzymatic hydrolysis studies and mass spectrometric analyses. The first metabolite was the glucoside conjugate of 2,4-DCP (2,4-DCP−*β*-*O*-glucoside). The major terminal metabolites were two more complex glucosides: 2,4-DCP−(6-*O*-malonyl)glucoside and 2,4-DCP−(6-*O*-sulfate)glucoside.

**Keywords:** *Gossypium hirsutum; herbicide metabolism; transgenic plant; 2,4-dichlorophenoxyacetic acid; glucosyl−sulfate conjugate*

## INTRODUCTION

The development of new herbicides meets with increasing costs and more and more stringent toxicological and environmental requirements. In these conditions, an alternative strategy consists of creating herbicide-resistant crop varieties. The introduction of genes for herbicide degradation is an attractive method to reach that goal because it can be applied to herbicides the biochemical target of which is not known or to herbicides with several sites of action, such as auxinic herbicides.

Transgenic cotton or tobacco plants ∼100-fold more tolerant to 2,4-dichlorophenoxyacetic acid (2,4-D) have been obtained by transfer of the *tfdA* gene from the bacterium *Alcaligenes eutrophus* (Streber and Willmitzer, 1989; Bayley et al., 1992; Lyons et al., 1993). That transformation extends the use of 2,4-D to dicotyledonous crops, whereas its use was originally restricted to grasses.

Phenoxyalkanoic acids such as 2,4-D are usually metabolized in plants mainly by the formation of glucose esters and amino acid amides or by ring hydroxylation followed by glucose conjugation (Feung et al., 1978). In transgenic plants, the bacterial *tdfA* gene codes for a dioxygenase catalyzing the degradation of 2,4-D to 2,4-dichlorophenol (2,4-DCP) (Fukumori and Hausinger, 1993), which is much less phytotoxic than the parent compound (Llewellyn and Last, 1996). Degradation of the 2,4-D side chain normally takes place in some plants (Loos, 1976; Aizawa, 1989), but the precise nature of the reaction involved is not fully understood (Owen, 1975). Moreover, that pathway is often of minor importance, and, except for red currant (*Ribes sativum* Syme) (Luckwill and Lloyd-Jones, 1960), it does not seem to play any significant role in the tolerance to 2,4-D.

2,4-DCP is potentially harmful to animal populations and to the environment (Jensen, 1996). It is converted to a glucoside conjugate in the aquatic angiosperm *Lemna gibba* (Ensley et al., 1994). However, contrary to other chlorophenol derivatives such as pentachlorophenol (Schmitt et al., 1985), its metabolism in crop plants has been little studied.

The biochemical fate of 2,4-DCP in resistant, transgenic plants has thus to be clarified. The lack of knowledge in that domain prompted us to examine the metabolism of 2,4-D in transgenic tolerant cotton.

## MATERIALS AND METHODS

**Chemicals.** [U-*phenyl*-$^{14}$C]-2,4-dichlorophenoxyacetic acid (specific activity = 18.2 *μ*Ci/mmol; radiochemical purity > 97% as determined by HPLC), nonlabeled 2,4-D, and 2,4-DCP were purchased from Sigma (St. Quentin Fallavier, France). The isopropyl ester of 2,4-D was prepared as described by Sànchez-Brunete et al. (1991). 2,4-DCP−*β*-D-glucoside was synthesized according to the procedure of Koenigs-Knorr (Conchie and Levvy, 1963). 2,4-DCP−(6-*O*-malonyl)-*β*-D-glucopyranoside was prepared following the one-step method developed by Roscher et al. (1996). 2,4-DCP−(6-*O*-sulfate)-*β*-D-glucopyranoside was synthesized by mole to mole reaction of synthetic 2,4-DCP−*β*-D-glucoside with pyridine−sulfur trioxide in anhydrous dimethylformamide (Whistler et al., 1963). Glucosides were purified by HPLC as described below (see Chromatography).

Without other specifications, all other chemicals were of analytical grade.

**Plant Material and 2,4-D Applications.** Transgenic 2,4-D-tolerant cotton (*Gossypium hirsutum* cv. Cooker 315) was kindly provided by Dr. D. J. Llewellyn (CSIRO). Seeds were individually sown in 300 mL plastic pots containing a compost/vermiculite mixture (2:1) and grown for 5 weeks in a climate-controlled cabinet set at 30/25 °C (day/night) with a 16 h photoperiod.

Two methods of herbicide administration were used. First, leaves of intact cotton plants were sprayed with [$^{14}$C]-2,4-D-isopropyl ester (300 mg/L, 2.24 *μ*Ci/mmol), dissolved in a 0.1% Tween 80/50% acetone solution, until dripping wet. That herbicide concentration was half the lowest concentration inducing damage to the transgenic cotton (Llewellyn, 1996).

* Author to whom correspondence should be addressed [fax (33) 05 61 28 52 44; e-mail flaurent@toulouse.inra.fr].

10.1021/jf990672c CCC: $19.00 © 2000 American Chemical Society
Published on Web 10/13/2000

Treated leaves were harvested 48 h after the spray application. In other experiments, fully expanded leaves were excised and their petioles inserted in vials containing 1 mL of a solution of [$^{14}$C]-2,4-D (18 $\mu$Ci/mmol, 0.5 $\mu$Ci, without unlabeled 2,4-D). The labeled 2,4-D solution was absorbed in ~2 h and was replaced by distilled water. The treatment was continued for 18 h prior to metabolite extraction.

**Preparation of Plant Extracts.** When 2,4-D was absorbed from the upper surface of leaves of intact plants, the leaves were rinsed with acetone to remove surface residues of treatments. In all experiments, the tissues were frozen at −80 °C and then ground with a ball grinder for 5 min. The resulting powders were extracted with water (5 mL/g of fresh weight) and then with a methanol/dichloromethane mixture (2:1, v/v). The surface washings and the aqueous and organic extracts were concentrated and then directly analyzed by radio-HPLC or further purified.

In some experiments, metabolites were purified by solid phase extraction (SPE). Water extracts were acidified (pH 3), loaded on C18 SPE cartridges (Supelco, France), and eluted with increasing methanol concentrations. Eluates from 0 to 50% methanol were dried and solubilized with 10 mM $NaHCO_3$ and then transferred to anionic exchange cartridges (Chromabond SB, Macherey-Nagel). Metabolites were eluted with a formic acid/methanol mixture (1:9, v/v) and then with 0.3 M $H_3PO_4$.

**Chemical or Enzymatic Metabolite Hydrolyses.** Hydrolyses were carried out in 200 $\mu$L of reaction media, according to the method of Schmitt et al. (1985). Samples containing at least 4000 dpm of the compound under investigation were evaporated to dryness.

*Acid Hydrolysis.* Samples were dissolved in 2 N HCl and heated at 100 °C for 2 h.

*Alkaline Hydrolysis.* Samples were incubated with 0.1 N NaOH for 1 h at 50 °C.

*$\beta$-Glucosidase Hydrolysis.* Samples were incubated during 2 h at 30 °C with almond $\beta$-glucosidase (2 units) (G-0395, Sigma) in 0.1 M, pH 5.0, sodium acetate buffer.

*Esterase Hydrolysis.* Samples were incubated for 10 min at 30 °C with rabbit liver esterase (2.5 units) (E-9636, Sigma) in 0.05 M, pH 7.5, potassium phosphate buffer or with 60 $\mu$L of crude esterase preparation from parsley stems (Matern, 1983) in 0.1 M, pH 5.0, sodium succinate buffer. After hydrolysis, alkaline samples were acidified to pH 3 with HCl. Then acid and acidified alkaline hydrolysates were extracted three times with ethyl ether and analyzed by HPLC. Enzymatic hydrolysates were acidified with HCl to pH 3 and directly analyzed by HPLC.

**Chromatography.** HPLC separations were performed on a Spectra-Physics P4000 liquid chromatograph equipped with a P1000 Spectra-Physics UV detector set at 216 nm. Radioactivity was monitored with an on-line Canberra-Packard Flow-One\beta scintillation detector, operated with Flow-Scint II scintillation counting cocktail (Canberra-Packard). The binary mobile phase was A = $H_2O$ plus 2% acetic acid and B = acetonitrile plus 2% acetic acid. Separations were carried out on a C18, 6 $\mu$m Bischoff column (4.6 mm × 250 mm) at ambient temperature with a flow rate of 1 mL min$^{-1}$. Two-step elution was as follows: step 1, 25% B, 12 min; step 2, 40% B, 18 min.

2,4-D and its metabolites were compared to synthesized standards on the basis of their retention times: 2,4-D, 20.8 min; 2,4-DCP, 22.6 min; 2,4-DCP glucoside, 6.8 min; 2,4-DCP (malonyl) glucoside, 11.9 min; 2,4-DCP (sulfate) glucoside, 3.8 min.

**Mass Analysis.** Mass spectra were obtained with a Finnigan LCQ quadrupole ion trap mass spectrometer (Thermo Quest, Les Ulis, France) equipped with an electrospray or an APCI ionization source and operating under negative ionization conditions. For ESI/MS experiments, a typical needle voltage of 5 kV and a heated capillary temperature of 220 °C were used. Sample solutions (5−10 ng/$\mu$L in methanol/water, 50:50) were infused at a flow rate of 3 $\mu$L/min into the ESI source. Sheath gas flow rate was adjusted in each case to obtain a stable spray and a maximum signal level. No auxiliary gas was used. In the case of APCI-MS experiments, the vaporizer and heated capillary temperatures were set to 450 and 150 °C, respectively. The source voltage and current were 1.90 kV and 5 $\mu$A, respectively. The gas flow rate was adjusted for good spraying conditions. Metabolites were analyzed by direct flow injection (typically 20−50 ng per injection) using methanol/water (50:50) at a flow rate of 1 mL/min. All analyses were performed in normal scan mode (unit resolution) under automatic gain control conditions. Helium was used as the collision gas for MS-MS experiments. Ion isolation and collision conditions were optimized separately for each metabolite to gain maximal structural information.

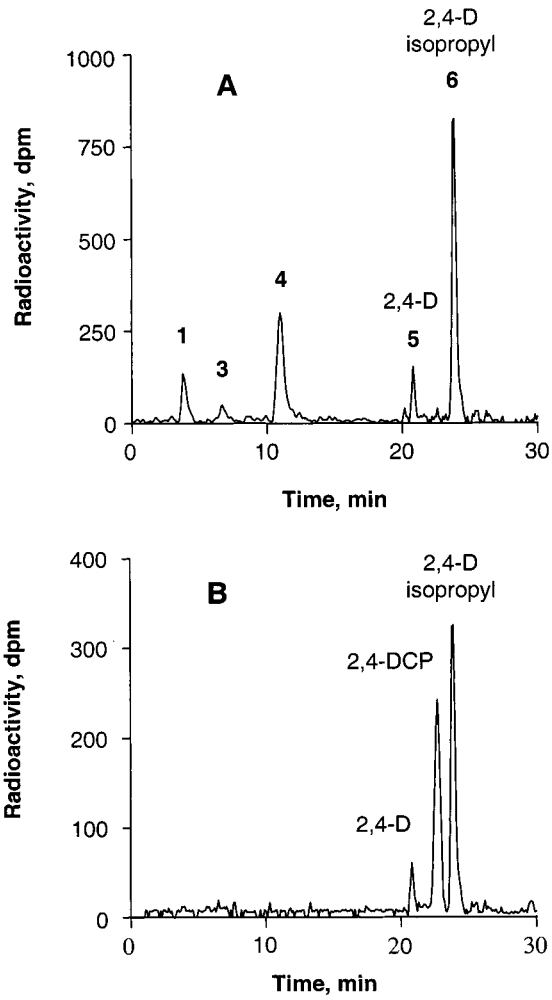


**Figure 1.** HPLC radiochromatograms of extracts from 2,4-D-tolerant cotton leaves sprayed with [$^{14}$C]-2,4-D isopropyl ester: (A) aqueous extract; (B) aqueous extract after acid hydrolysis [half level of radioactivity of (A)].

## RESULTS AND DISCUSSION

After transgenic cotton plants had been sprayed with [$^{14}$C]-2,4-D isopropyl ester, five radioactive peaks were observed in HPLC chromatograms of aqueous extracts (Figure 1A). Peaks 5 and 6 were identified as 2,4-D and 2,4-D isopropyl ester, respectively. The three other peaks (1, 3, and 4) were more polar. In organic extracts, only the parent compound and its de-esterified product, 2,4-D, were observed. 2,4-DCP was absent from both extracts, even though they accounted for 97% of the total radioactivity.

After acid hydrolysis of water extracts, all of the radioactivity of polar compounds was found under a 2,4-DCP peak (Figure 1B). No increase of the 2,4-D radioactivity was detected. Therefore, as expected, 2,4-DCP

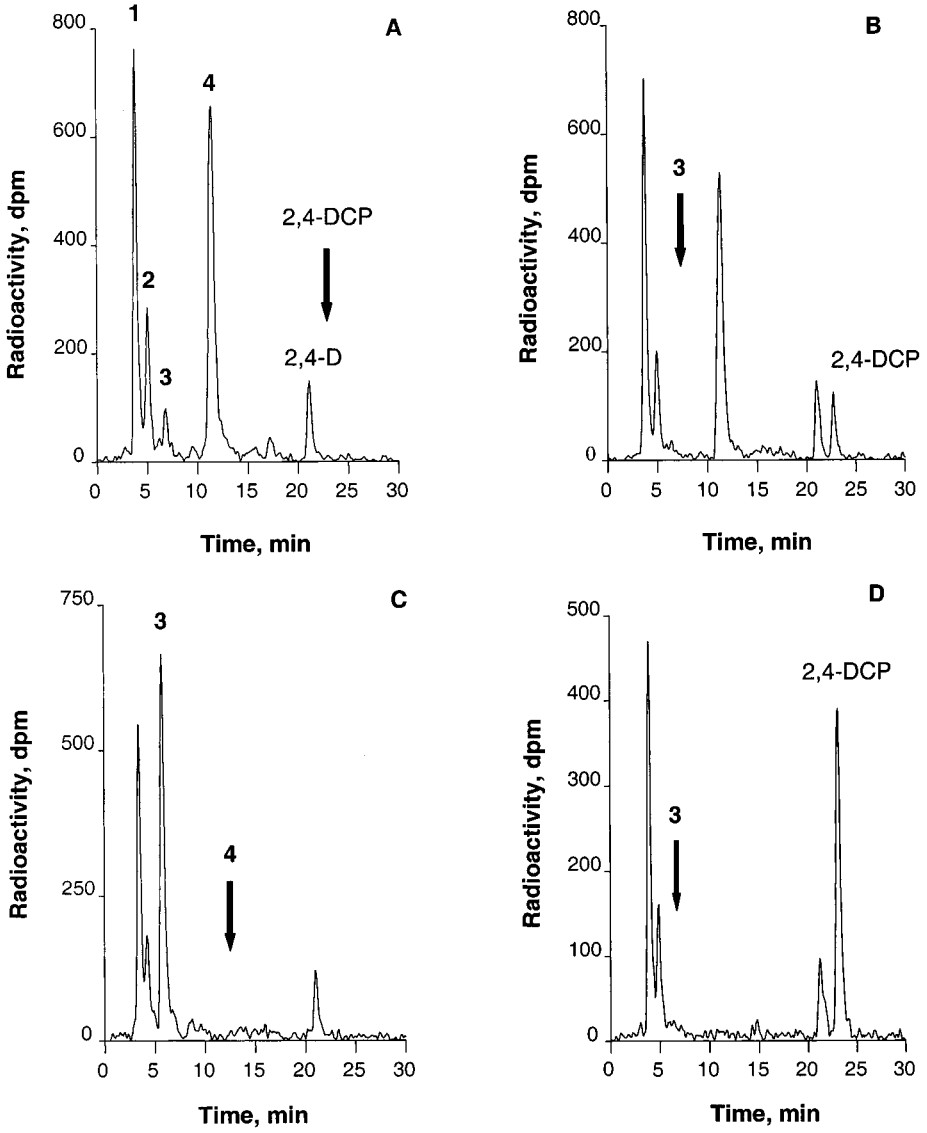


**Figure 2.** HPLC radiochromatogram of water extracts from excised, 2,4-D-tolerant cotton leaves after petiolar absorption of [$^{14}$C]-2,4-D: (A) water extract; (B) water extract after treatment with $\beta$-glucosidase; (C) water extact after treatment with a crude esterase preparation from parsley stems; (D) same extract as in (C), after a further treatment with $\beta$-glucosidase.

was formed in transgenic cotton but as an apparently transient intermediate that is rapidly metabolized to more polar compounds.

When loaded on SPE columns, 2,4-D and the components of peaks 1 and 4 were retained by the anion exchanger, contrary to peak 3. Peak 4 and 2,4-D were eluted in weakly acidic conditions (formic acid/methanol, 1:9), whereas elution of peak 1 required a strong acid (0.3 M $H_3PO_4$). These results indicated that peaks 1 and 4 were acidic compounds, with different acidic functions.

The structures of the three metabolites were next examined by enzymatic hydrolysis studies and mass spectrometric analyses. To increase the amounts of metabolites, excised leaves were allowed to metabolize 2,4-D for 18 h, after an initial absorption period of 2 h. In these conditions, the same polar metabolites as above were detected, with in addition a new peak representing <10% of the total polar metabolites (Figure 2A; peak 2). The identity of that compound was not determined, but it was identified as a 2,4-DCP metabolite by acid hydrolysis and mass spectrometry (negative mode APCI-MS).

Digestion of water extracts by $\beta$-glucosidase converted metabolite **3** to 2,4-DCP (Figure 2B). In addition, metabolite **3** coeluted in HPLC with authentic 2,4-DCP−$\beta$-*O*-glucoside and yielded by ESI/MS-MS the same mass spectrum as the authentic 2,4-DCP−$\beta$-*O*-glucoside with a base peak quasi-molecular ion at *m*/*z* 323, a characteristic dichlorine isotopic pattern, and a diagnostic *m*/*z* 161 fragment ion corresponding to dichlorophenol moiety of the molecule.

The other metabolites were not degraded by $\beta$-glucosidase or other glycosidases (results not shown). Metabolite **4** (Figure 2A) was hydrolyzed by 0.1 N NaOH to yield a product coeluting with the $\beta$-glucoside, suggesting the presence of an ester linkage in a complex glucoside conjugate. As this metabolite was acidic, it was tentatively identified as a 2,4-DCP−(6-*O*-malonyl)glucoside. Upon treatment with a parsley esterase preparation, specific to the hemiester of malonic acid (Matern, 1983), metabolite **4** was hydrolyzed to yield the glucoside, which could be further hydrolyzed with $\beta$-glucosidase to give 2,4-DCP (Figure 2C,D).

As shown by its retention characteristics on an anion exchanger, metabolite **1** was a strongly acidic compound.

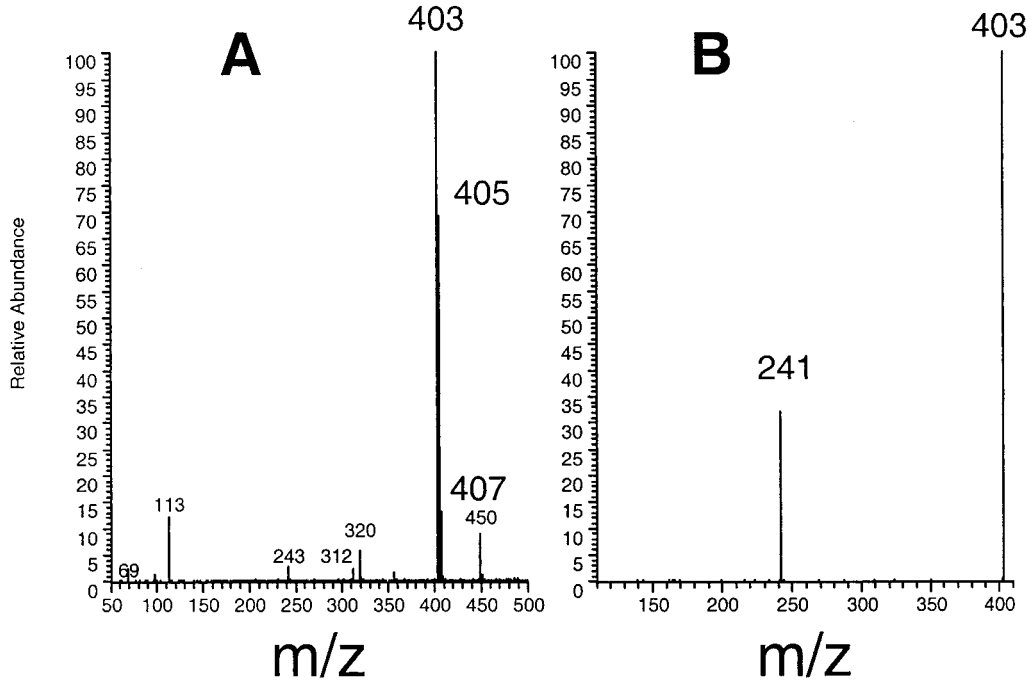


**Figure 3.** Negative ESI/MS spectra of metabolite **1** isolated from an aqueous extract of 2,4-D-treated tolerant cotton plant: (A) full scan mass spectrum; (B) CAD mass spectrum of $(M - H)^-$ ions (*m/z* 403).

Its ESI mass spectrum showed a quasi-molecular ion cluster at *m/z* 403/405/407 (Figure 3A), characteristic of a molecule with two chlorine atoms. The MS-MS decomposition of the *m/z* 403 parent ion gave a fragment ion at *m/z* 241 corresponding to a loss of 2,4-DCP (Figure 3B). The same *m/z* 241 daugther ion was observed from the selected *m/z* 405 quasi-molecular ion, showing that the *m/z* 241 ion did not contain any chlorine atom. Thus, this ion was tentatively identified as a complex glucoside with a hydroxyl subtituted by an acidic function. The mass increase of 80 th compared to the unchanged glucoside was compatible with a sulfate derivative. A synthesized 2,4-DCP−(6-*O*-sulfate)glucoside yielded the same CAD mass spectrum and coeluted with metabolite **1** in HPLC. Sulfate ester conjugates are generally bound to the aglycon moiety of secondary metabolites—mainly on aryl compounds (Paulson, 1976; Fenwick et al., 1983). Direct carbohydrate sulfating is less frequent. Some sulfate ester glucosides of natural metabolites, such as brucinium, have been described (Whistler et al., 1963), but such derivatives are relatively rare among pesticide metabolites. In a recent study (Capps et al., 1996), a 6-*O*-sulfate glucoside conjugate was described as a unique conjugate for a dihalogenated molecule in cotton, too. The other reported case concerns the metabolism of phemedipham in sugar beet, in which 2-*O*-sulfate glucosides are formed (Lamoureux, 1989).

In conclusion, a metabolic pathway of 2,4-D in transgenic 2,4-D-tolerant cotton is proposed (Figure 4). The main metabolites in transgenic cotton were complex derivates of 2,4-DCP−glucoside, malonyl, and sulfate, conjugates. These conjugates were not described in wild plants treated with 2,4-D, even in plants in which 2,4-D dealkylation is the major degradative pathway, such as black currant (Luckwill and Lloyd-Jones, 1960), because the 2,4-D used is labeled on the alkyl chain.

The metabolism of 2,4-D in wild cotton has been scarcely described. Only one study shows that alkyl-labeled 2,4-D undergoes some side-chain degradation,

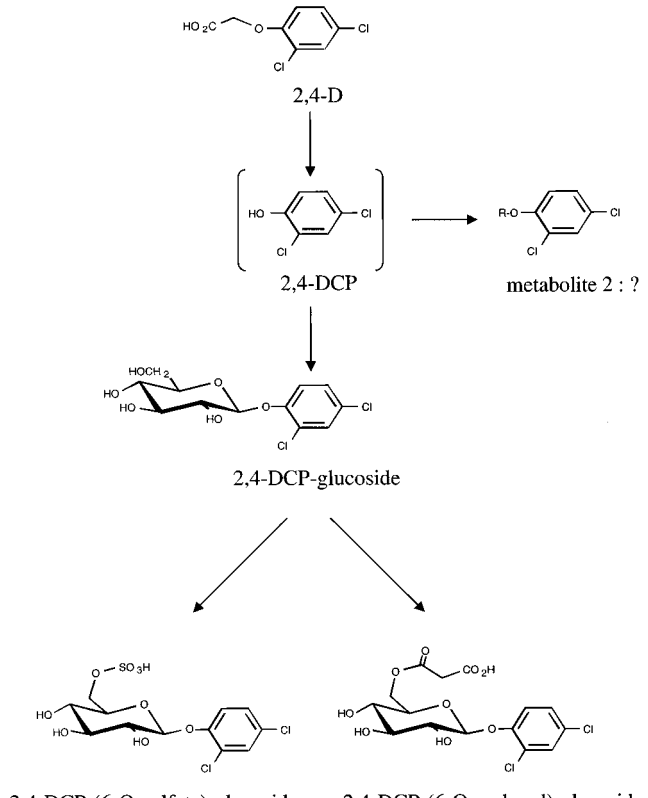


**Figure 4.** Proposed metabolic pathway of 2,4-D degradation in transgenic cotton.

but the degradation is slow, and the metabolites have not been identified (Morgan and Wayne, 1963). We have examined that point by supplying wild cotton with [$^{14}$C]-2,4-DCP, to avoid the phytotoxic effect of 2,4-D. In these conditions, wild cotton synthesizes the same three glucosylated metabolites as transgenic cotton (results not shown). Hence, transgenesis does not modify the native metabolism of 2,4-DCP in cotton.

The effect of 2,4-DCP on health depended on the subconjugate items. Malonyl conjugate and glucoside, like the natural glucosides such as isoflavone conjugates, could be hydrolyzed in the gastrointestinal tract and liberate free 2,4-DCP. The latter, like the other polychlorophenols, is directly toxic for humans (Jensen, 1996), and its toxicity should be increased by its transformation to dioxin-like compounds in the organism (Wittsiepe et al., 2000). On the other hand, greater chemical or enzymatic stability of the sulfate glucoside should decrease the health hazard.

Generally, chlorophenols were degraded by soil microorganisms. However, it seems that 2,4-DCP was less rapidly mineralized than the parent compound, 2,4-D. For this reason, the use of transgenic plants, tolerant for 2,4-D, could increase the environmental contamination by chlorophenols due to the use of phenoxy acid herbicides. Nevertheless, the 2,4-D-tolerant cotton has been developed to protect sensitive cottons from the 2,4-D spray drift from applications to wild tolerant crops (Lyon et al., 1993) and not to widen the use of the 2,4-D to the sensitive crops.

## ABBREVIATIONS USED

2,4-D, 2,4-dichlorophenoxyacetic acid; 2,4-DCP, 2,4-dichlorophenol; SPE, solid phase extraction; HPLC, high-performance liquid chromatography; ESI/MS, electrospray ionization/mass spectrometry; MS-MS, mass spectrometry−mass spectrometry; APCI, atmospheric pressure chemical ionization.

## ACKNOWLEDGMENT

We thank Dr. D. Llewellyn from CSIRO, Division of Plant Industry (Canberra City, Australia), for providing the transgenic cotton seeds.

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Received for review June 21, 1999. Revised manuscript received July 18, 2000. Accepted July 28, 2000.

JF990672C