Uptake, Translocation, and Metabolism of 3,4-Dichloroaniline in Soybean and Wheat Plants

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Glycine max, Triticum aestivum, 3,4-Dichloroaniline, Metabolism, Translocation

Introduction

3,4-Dichloroaniline is the aromatic moiety of certain pesticides, and liberated during metabolism of these compounds (Ashton and Crafts, 1981). The metabolic intermediate proved to be considerably stable, for instance as soil and plant non-extractable residues (Bartha et al., 1983; Sandermann et al., 1983), resulting in a contamination of the environment. Thus, 3,4-dichloroaniline is currently considered as a priority pollutant. The metabolic fate of the xenobiotic was studied in different in vitro plant systems including soybean and wheat cell cultures and soybean excised leaves (Harms and Langebartels, 1986; Winkler and Sandermann, 1989; Gareis et al., 1992). Large differences were observed between both soybean and wheat cell cultures, and soybean cell cultures and excised leaves. These include the percentages of non-extractable residues and metabolites formed, and the partitioning of the xenobiotic metabolites between culture medium and cell extract. Wheat plants produced considerable 3,4-dichloroaniline-derived bound residues (Arjmand and Sandermann, 1985; Sandermann et al., 1992) in addition to soluble metabolites. The results were similar to those from the corresponding cell cultures (Winkler and Sandermann, 1992); unfortunately, no details were published concerning uptake into the plants and subsequent translocation.

Excised leaves and plant cell cultures (mainly chlorophyll-deficient) are often used in plant metabolism studies (Shimabukuro and Walsh, 1979; Mumma and Hamilton, 1979). However, the in vitro methods are artificial systems and represent different degrees of simplification. Data thus obtained may qualitatively and quantitatively differ from each other and from intact plants.

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Consequently, it is reasonable to validate the respective in vitro data with regard to plants. As completion of the investigations cited above, the present paper deals with the fate of 3,4-dichloroaniline in soybean and wheat plants, including uptake and translocation of the xenobiotic and its metabolites. Due to the mode of application of the parent pesticides (Ashton and Crafts, 1981; Hassall, 1982) and soil contamination resulting from plant and microbial metabolism (Marco and Novak, 1991), roots rather than the foliage are exposed to the xenobiotic; therefore, the roots were chosen as site of application. To exclude interfering soil processes the plants were incubated in nutrient solution. The plant metabolites of 3,4-dichloroaniline previously identified or proposed are shown in Fig. 1.

Materials and Methods

Chemicals and analytical methods

\[[\text{Ring-U-}^{14}\text{C}\text{-3,4-dichloroaniline was purchased from Sigma Chemical Company (477.3 MBq/mmol; radiochemical purity } > 98\%\text{); non-labeled 3,4-dichloroaniline was supplied by Riedel-de Haën (Pestanal Quality). } N-(\beta-\text{D-glucopyranosyl})-3,4-dichloroaniline (\text{Glc-}\beta-3,4-dichloroaniline)\text{, and } N\text{-malonyl-3,4-dichloroaniline (Mal-3,4-dichloroaniline) were prepared as outlined (Gareis et al., 1992). Thin-layer chromatography (TLC) and radioanalysis were essentially performed according to Gareis et al. (1992). TLC solvent systems used were A: CH}_2\text{Cl}_2\text{ and B: CHCl}_3/\text{MeOH}/H_2O 65/25/4 (v/v/v). Multiple development was used for enhanced separation of radioactive zones.}\]

**Plant materials and treatments**

Soybean plants

Seeds (\textit{Glycine max} \textit{L.} var. Harosoy 63) were germinated and grown in soil. After 10 d the seedlings were removed, cleaned of adhering soil particles with tap water and rinsed with double distilled water. The seedlings were placed into 80 ml of modified Hoagland nutrient solution (Table I; Hoagland and Arnon, 1950) and 20 ml tap water (1 plant each) in 5.0 cm×8.5 cm (diameter × height) cultivation flasks wrapped in aluminum foil. The top of each flask was covered with a disk of silicone equipped with incisions to hold the seedling; only the roots were immersed in the nutrient. The volume of the nutrient solution was restored to 100 ml each day by the addition of tap water. After 3 d at 20 °C with a 16 h photoperiod (1500 lx; Osram-L 65 W/25 Universal White), 100 μg 3,4-dichloroaniline (2.5 ×10^6 dpm; 0.62 μmol) in 20 μl MeOH was added to the medium; the cultivation flask was shaken carefully.

Table I. Composition and preparation of the modified Hoagland nutrient solution. All stock solutions were prepared separately (excepting FeSO_4·7H_2O and Na_2EDTA·2H_2O). Appropriate volumes of these were mixed, adjusted to final volume with double distilled H_2O as indicated, and were autoclaved.

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Molarity of nutrient solution [mmol/l]</th>
<th>Concentration [mg/l]</th>
</tr>
</thead>
<tbody>
<tr>
<td>KH_2PO_4</td>
<td>1.00</td>
<td>136.1</td>
</tr>
<tr>
<td>KNO_3</td>
<td>5.00</td>
<td>505.5</td>
</tr>
<tr>
<td>Ca(NO_3)_2·4H_2O</td>
<td>25.00</td>
<td>5904.0</td>
</tr>
<tr>
<td>MgSO_4·7H_2O</td>
<td>4.00</td>
<td>985.9</td>
</tr>
<tr>
<td>Na_2B_4O_7·10H_2O</td>
<td>0.022</td>
<td>8.4</td>
</tr>
<tr>
<td>MnCl_2·4H_2O</td>
<td>0.009</td>
<td>1.8</td>
</tr>
<tr>
<td>ZnSO_4·7H_2O</td>
<td>0.0007</td>
<td>0.2</td>
</tr>
<tr>
<td>CuSO_4·5H_2O</td>
<td>0.0008</td>
<td>0.2</td>
</tr>
<tr>
<td>Na_2MoO_4·2H_2O</td>
<td>0.00012</td>
<td>0.03</td>
</tr>
<tr>
<td>FeSO_4·7H_2O</td>
<td>0.05</td>
<td>13.9</td>
</tr>
<tr>
<td>Na_2EDTA·2H_2O</td>
<td>0.05</td>
<td>18.6</td>
</tr>
</tbody>
</table>
Two plants were incubated for 2 d and two for 5 d. The plants were extracted and analyzed individually. During incubation, 1 ml aliquots of the media (2 samples each) were analyzed for $^{14}$C at 6, 24, 48, 72 and 96 h (results shown in Fig. 2).

In control experiments, 3 soybean plants were grown, transferred to the nutrient solution, and were cultivated as described. The plants were removed from the cultivation flasks, and 10 μg 3,4-dichloroaniline (250,000 dpm) in 5 μl MeOH was added to each medium. The control flasks were closed with aluminum foil and incubated for 48 h.

**Wheat plants**

Seeds (*Triticum aestivum* L. var. Ares) were germinated on wet filter-paper for 48 h at 25 °C in the dark. Six of the resulting seedlings were transferred onto a steel wire grating (1 mm mesh width). The grating was placed upon the surface of 100 ml nutrient solution by means of a handle, so only the roots passing through the wire grating were immersed in the solution. The cultivation flask was covered with a disk of silicone perforated to hold in place the wheat seedlings. The plants were cultivated as described for the soybeans for 5 d. Subsequently, 40 μg of 3,4-dichloroaniline (1.0×10^6 dpm; 0.25 μmol) in 20 μl MeOH was added to the medium, and the seedlings were incubated for 3 d. Duplicate aliquots (1 ml) of the medium were analyzed for $^{14}$C every 24 h (results shown in Fig. 2). Plants cultivated in the same flask (3 replicates) were worked up together.

**Extraction and identification of metabolites**

**Extraction of nutrient solution**

The nutrient media from the soybean plants were adjusted to pH 3 and extracted with EtOAc (2×50 ml). The nutrient media from the wheat seedlings were extracted without pH adjustment. Recoveries were >90% in all experiments. The extracts were analyzed by TLC.

**Soybean plants**

The plants were separated into roots, hypocotyl, cotyledons, epicotyl, primary leaves, and secondary leaf (including preceding internode). The individual parts were cut into pieces, introduced into 50 ml of CHCl₃/MeOH 1/2 (v/v; Bligh and Dyer, 1959), kept overnight at −20 °C, and extracted by means of an Ultraturrax mixer (Jahnke & Kunkel). Insoluble plant material was separated from the extract by suction filtration and washed with CHCl₃/MeOH/H₂O 1/2/0.8 (v/v/v). The Bligh–Dyer extracts were analyzed for $^{14}$C (used for calculations shown in Table II), while the insoluble residues were air-dried and subjected to $^{14}$C combustion analysis. The organic fractions from the Bligh–Dyer extracts were removed *in vacuo*. The remaining aqueous phases were diluted to 300 ml and extracted 2× with 100 ml portions of n-BuOH (recovered radioactivity: 75% (48 h) and 68% (120 h) of original Bligh–Dyer extracts). Then, the combined n-BuOH phases were evaporated to dryness. In the case of the roots, the residue was dissolved in MeOH and analyzed by TLC. To remove green pigments from extracts of the hypocotyls, cotyledons, epicotyls, and leaves, the corresponding residues were dissolved in 100 ml MeOH/H₂O 9/1 (v/v). The resulting solution was extracted 3× with 50 ml of cyclohexane; the cyclohexane phase was discarded. The aqueous methanol phase was evaporated, and the concentrate was chromatographed on Sephadex LH-20-100 with CH₂Cl₂/MeOH 2/1 (v/v). Fractions containing radioactivity (4 to 7, 10 ml each) were collected (recovered radioactivity: 91%), concentrated and were analyzed by TLC (results shown in Fig. 3).

**Wheat plants**

The plant material was divided into root, cotyledon and primary leaf fractions, and treated as described above. n-BuOH phases (recovered radioactivity: 96%) of the root extracts were concentrated, the residues were dissolved in MeOH and directly analyzed by TLC. The green pigments from the cotyledon and leaf extracts were removed as described for soybeans. Subsequently, the remaining methanolic phases (81% of the recovered radioactivity) were concentrated and analyzed by TLC (results shown in Fig. 4).

**Results and Discussion**

**Soybean plants**

Partitioning and distribution of metabolites

In the soybean experiments, a moderate decrease in radioactivity found in the nutrient solu-
Fig. 2. Root uptake of 3,4-dichloroaniline-derived radioactivity into soybean and wheat plants (average values of two and three replicates are shown in the case of soybean and wheat, respectively).

Table II. Distribution of 3,4-dichloroaniline-derived radioactivity in soybean and wheat plants in % of total applied radioactivity (percent distribution of $^{14}C$ in the seedlings); average values of two and three replicates are shown in the case of soybean and wheat, respectively.

<table>
<thead>
<tr>
<th></th>
<th>Soybean 48 h</th>
<th>Soybean 120 h</th>
<th>Wheat 72 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nutrient solution</td>
<td>58.8</td>
<td>54.6</td>
<td>18.4</td>
</tr>
<tr>
<td>Seedlings</td>
<td>37.3</td>
<td>24.1</td>
<td>78.3</td>
</tr>
<tr>
<td>Bligh–Dyer extracts</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Roots</td>
<td>(49.1)</td>
<td>(25.7)</td>
<td>(47.6)</td>
</tr>
<tr>
<td>Hypocotyls</td>
<td>(13.1)</td>
<td>(9.5)</td>
<td>(—)</td>
</tr>
<tr>
<td>Cotyledons</td>
<td>(1.1)</td>
<td>(1.7)</td>
<td>(5.4)</td>
</tr>
<tr>
<td>Epicotyls</td>
<td>(2.7)</td>
<td>(2.5)</td>
<td>(—)</td>
</tr>
<tr>
<td>Primary leaves</td>
<td>(8.3)</td>
<td>(13.3)</td>
<td>(1.4)</td>
</tr>
<tr>
<td>Secondary leaves</td>
<td>(2.2)</td>
<td>(12.1)</td>
<td>(—)</td>
</tr>
<tr>
<td>Non-extractable residues</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Roots</td>
<td>(14.7)</td>
<td>(21.6)</td>
<td>(42.7)</td>
</tr>
<tr>
<td>Hypocotyls</td>
<td>(7.5)</td>
<td>(9.2)</td>
<td>(—)</td>
</tr>
<tr>
<td>Cotyledons</td>
<td>(0.3)</td>
<td>(1.2)</td>
<td>(2.3)</td>
</tr>
<tr>
<td>Epicotyls</td>
<td>(0.5)</td>
<td>(1.2)</td>
<td>(—)</td>
</tr>
<tr>
<td>Primary leaves</td>
<td>(0.5)</td>
<td>(0.8)</td>
<td>(0.6)</td>
</tr>
<tr>
<td>Secondary leaves</td>
<td>(0.0)</td>
<td>(1.2)</td>
<td>(—)</td>
</tr>
<tr>
<td>Total</td>
<td>96.1</td>
<td>78.7</td>
<td>96.7</td>
</tr>
</tbody>
</table>

Fig. 3. Partitioning of 3,4-dichloroaniline-derived metabolites in soybean plants (incubation intervals of 48 and 120 h; mean of two replicates shown).
0.05; Mal-3,4-dichloroaniline, 0.15; Glc-β-3,4-dichloroaniline, 0.32; 3,4-dichloroaniline, 0.83.

The majority of radioactivity detected inside the soybean plants was associated with the roots (63.8 and 47.7% of absorbed 14C after 48 and 120 h, respectively). A considerable decrease of the soluble radioactivity in the roots was observed between 48 and 120 h (49.1 to 25.7%), whereas the bound residues increased. The soluble 14C in the roots consisted of 3,4-dichloroaniline, the N-glucosides and the N-malonyl conjugate. While the portions of both Mal-O(6')Glc-β-3,4-dichloroaniline (10.5/12.4%) and Mal-3,4-dichloroaniline (7.5/5.8% after 48/120 h) nearly remained constant, the amounts of Glc-β-3,4-dichloroaniline decreased from 27.3 to 6.2%. The data suggest that primarily this disappearance of the N-glucoside was responsible for the decrease of total soluble 14C in the roots.

After 48 and 120 h, respectively, 36.2 and 52.7% of the absorbed radioactivity were found translocated up the soybean plants. Totals of 20.6 and 18.7% were found in the hypocotyl; the major portion of this was soluble and predominantly consisted of Mal-3,4-dichloroaniline. Analogous results were obtained for the epicotyl. In both plant parts the percentage of the N-malonyl conjugate slightly decreased in the course of the experiment. Negligible amounts of 14C were found in the cotyledons, mainly consisting of Mal-3,4-dichloroaniline. The primary and secondary leaves accumulated somewhat larger amounts of radioactivity, while the soluble 14C in the roots, hypocotyls and epicotyls decreased. The radioactivity in the leaves consisted primarily of the N-malonyl conjugate and increased between 48 and 120 h. This increase was due to the accumulation of the N-malonyl derivative (7.5% to 10.4% and 2.1% to 10.4% in the primary and secondary leaves, respectively).

Throughout the plant, only a small amount of the applied 14C was found as non-extractable residues (8.5% to 8.8%) and most of this was in roots and hypocotyls. Relative to absorbed radioactivity, this fraction amounted to 23.5 (48 h) and 35.1% (120 h).

Metabolism in soybean plants

Presumably, 3,4-dichloroaniline was passively absorbed into the soybean roots, a small percentage by-passing the Casparian strip via the symplast. In the roots, the xenobiotic was transformed rapidly to the soluble conjugates and non-extractable residues. The major portion of the applied 14C was metabolized to Mal-3,4-dichloroaniline (total amounts of 67.9/63.9% after 48/120 h). The conjugates were channeled differently. While the glucosides remained inside the roots, Mal-3,4-dichloroaniline seemed to be extensively sequestered into the root-free space (apoplast). Subsequently, the most of the N-malonyl conjugate diffused into the nutrient solution; a smaller amount was translocated via xylem to hypocotyl, epicotyl and the leaves of the soybeans. Acidic compounds diffuse across plasma membranes only in the unionized form (Pillmoor and Gaunt, 1981). Due to its pKa value (pKₐ of malonanilic acid 3.7; Ostwald, 1889), Mal-3,4-dichloroaniline should be ionized and ion-trapped inside the root cells of the soybean plants. Thus, the transfer into the root apoplast is likely to occur via a remarkably effective transporter. So, during the first 24 h the uptake of 3,4-dichloroaniline was accompanied by an efflux of its malonyl conjugate.

After 48 h the upward movement and accumulation of Mal-3,4-dichloroaniline in the leaves had increased. Presumably, a portion of the glucoside initially found in the soybean roots was transformed to Mal-3,4-dichloroaniline, the major part of which arrived at the nutrient solution and leaves. Data on the chemical hydrolysis of Glc-β-3,4-dichloroaniline and conversion into the N-malonyl conjugate were published (Winkler and Sandermann, 1992; Schmidt et al., 1994).

Regarding the soybean leaves, the present results obviously differ from those obtained with excised leaves (Gareis et al., 1992). Whereas the excised leaves were exposed to the parent xenobiotic, it was rather Mal-3,4-dichloroaniline reaching the leaves of the intact soybeans. The processes observed in the soybean roots resembled those obtained with soybean cell cultures (Harms and Langebartels, 1986; Winkler and Sandermann, 1989; Gareis et al., 1992). 3,4-Dichloroaniline was metabolized by the soybean suspensions primarily to the N-malonyl conjugate mainly found in the medium. Metabolism studies with 4-chloroaniline and phoxim (Winkler and Sandermann, 1989; Barz et al., 1990; Höhl, 1988) in soybean cells also demonstrated the formation of N-malonyl conjugates,
Metabolism of 3,4-Dichloroaniline in Soybean and Wheat

It was speculated (Winkler and Sandermann, 1989; Barz et al., 1990) the secretion of the N-malonyl metabolites may be related to translocation phenomena in plants. The present results support this assumption. So far, root excretion processes have scarcely been investigated. A simple leakage out of the root free space was observed with some pesticides (Graham and Buchholtz, 1968; Shimabukuro et al., 1976; Domir, 1980; Marquis et al., 1979). The excretion of the moderate acidic Mal-3,4-dichloroaniline by the soybean roots closely resembles phenomena observed after foliar application of certain herbicides, such as benzoic and phenoxyalkanoic acids, or picloram (Chang and van den Born, 1968; Basler et al., 1970; Coble et al., 1979; Sanad and Müller, 1973; Lym and Moxness, 1989). It may be speculated that the same mechanisms underlie root exudation of both these acidic pesticides and Mal-3,4-dichloroaniline. The excretion mechanisms are unknown, and warrant further research (Pillmoor and Gaunt, 1981).

Wheat plants

Partitioning and distribution of metabolites

The uptake of 3,4-dichloroaniline was much more rapid in wheat than in soybean (Fig. 2). In wheat, after 72 h, a residual portion of 18.4% of the applied radioactivity was found in the nutrient solution. Due to TLC analysis (systems A and B) of the EtOAc extracts, the radioactivity consisted primarily of 3,4-dichloroaniline (38%); Mal-3,4-dichloroaniline was not detected. The unidentified 14C portion probably reflects artefacts (Gareis et al., 1992; Marco and Novak, 1991). A fraction of 78.3% of the applied 14C was absorbed by the wheat plants. Thus, total recovered radioactivity amounted to 96.7%.

The partitioning of radioactivity in the wheat plants (Table II) shows that the major portion (90.3%) of the absorbed 14C was detected in the roots. A considerable percentage of this (42.7%) was in the insoluble plant fraction, while 47.6% was soluble. The distribution of the extractable radioactivity in the wheat roots into the parent and the various metabolites (Fig. 4) was comparable to that observed in soybean, and was as follows: A small percentage of 3,4-dichloroaniline was detected. The major portion consisted of the glucosides, Glc-β-3,4-dichloroaniline (19.0%) and Mal-O(6')Glc-β-3,4-dichloroaniline (3.3%), while 18.5% of the root associated 14C were Mal-3,4-dichloroaniline.

Only limited translocation of 3,4-dichloroaniline or its metabolites was observed in wheat plants. Portions of 7.7 and 2.0% were translocated from the roots to the cotyledons and primary leaves, respectively. In contrast to soybean, in wheat, the applied radioactivity showed no marked trend to be translocated nor to accumulate in specific epigeous plant parts. The results suggest that 3,4-dichloroaniline rather than Mal-3,4-dichloroaniline was translocated. The main metabolite detected in the cotyledons was Glc-β-3,4-dichloroaniline (2.6%), whereas similar amounts of the glucosides and Mal-3,4-dichloroaniline were detected in the primary leaves.

Metabolism in wheat plants

Unlike soybean, the absorbed 14C remained inside the wheat plants, primarily in the root. The results suggest that 3,4-dichloroaniline was rapidly taken up, and then partitioned equally between apoplastic and symplastic root parts. A rapid initial root uptake has been observed with various...
herbicides (Ashton and Crafts, 1981). Then, in the wheat roots, 3,4-dichloroaniline was metabolized to Glc-β-3,4-dichloroaniline, Mal-O(6')Glc-β-3,4-dichloroaniline, and Mal-3,4-dichloroaniline. A considerable fraction of the 3,4-dichloroaniline presumably accumulating in the non-living, apoplastic root parts, was accessible to an extensive co-polymerization into cell wall macromolecules. This latter finding is one of the main differences between wheat and soybean. In contrast to the soybean roots, all soluble conjugates remained inside the wheat root cells. Data on the metabolism of 3,4-dichloroaniline in wheat cell cultures and plants (Sandermann et al., 1983; Harms and Langebartels, 1986; Winkler and Sandermann, 1989; Harms, 1992) and rice plants (Still et al., 1981) have been published; analogous findings were obtained with 4-chloroaniline in wheat (Winkler and Sandermann, 1989; Harms, 1992; Langebartels and Harms, 1986). On the whole, the present data agree with these results.

The main purpose of the study with soybean and wheat plants was the validation of results obtained with two in vitro systems, the respective cell suspension cultures and excised leaves. According to the present data, the processes observed in the roots of the intact plants largely resembled those of the respective cell cultures. It is questionable, whether this analogy can be extrapolated to other xenobiotics and plant species. It would be desirable to draw this additional information from cell culture experiments.

**Acknowledgements**

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Langebartels C. and Harms H. (1986), Plant cell suspension cultures as test systems for an ecotoxicologic evaluation of chemicals. Growth inhibition effects and
comparison with the metabolic fate in intact plants. Angew. Bot. 60, 113–123.