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3,4-DICHLOROANILINE *N*-GLUCOSYL- AND *N*-MALONYLTRANSFERASE ACTIVITIES IN CELL CULTURES AND PLANTS OF SOYBEAN AND WHEAT

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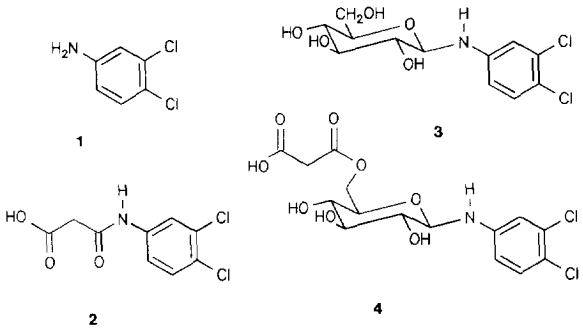
Key Word Index—*Glycine max*; Fabaceae; soybean; *Triticum aestivum*; Poaceae; wheat; suspension culture; 3,4-dichloroaniline; *N*-glucosyltransferase; *N*-malonyltransferase.

Abstract—The activities of UDP-glucose: 3,4-dichloroaniline *N*-glucosyltransferase (*N*-GT; EC 2.4.1.71) and malonyl-CoA: 3,4-dichloroaniline, *N*-malonyltransferase (*N*-MT) were determined in soybean and wheat cell suspension cultures, and in individual organs of the respective plants. In the soybean suspensions, *N*-GT and *N*-MT activities were quite constant over the subculture interval, and ranged from 0.48 to 1.65, and 34.3 to 80.1 pkat · mg^{-1} protein, respectively. No *N*-MT activity was detected in wheat cultures, while the specific *N*-GT activities were between 1.3 and 4.5 pkat·mg^{-1} protein. In soybean plants, *N*-MT was most active in the roots (9.6 pkat·mg^{-1} protein) and *N*-GT activity was maximal in the cotyledons (1.7 pkat·mg^{-1} protein). No *N*-MT activity was detected in the wheat plants, whereas *N*-GT activities of the wheat roots and cotyledons amounted to 1.4 and 1.0 pkat·mg^{-1} protein, respectively.

INTRODUCTION

The metabolism of xenobiotic compounds in a plant is subdivided into three phases. Phase I: simple biotransformations; phases II and III: conjugation of the parent molecule or its metabolites with endogeneous plant substances, resulting in soluble conjugates containing carbohydrates (e.g. glucose), organic acids (e.g. malonate), etc. on the one hand, and insoluble, macromolecular structures (e.g. copolymerisation with lignin), respectively, on the other [1]. The latter are usually referred to as bound, non-extractable residues. In most cases, biotransformations of xenobiotics are considered as detoxification reactions. Due to this fact, and certain similarities to natural plant and soil constituents [2–4], interest especially in phase II reactions of foreign compounds is growing.

The metabolic fate of the pesticide metabolite and priority pollutant 3,4-dichloroaniline (**1**) has been investigated in plant systems *in vivo* and *in vitro*. The plant systems studied include soybean and wheat cell suspension cultures, excised leaves, and intact plants [5–11]. The studies demonstrated phase II transformations of the chemical to its *N*-malonyl (**2**), *N*-β-D-glucosyl (**3**), and the proposed 6′-*O*-malonyl-*N*-β-D-glucosyl (**4**) conjugate (Scheme 1) in addition to bound residues (phase III). Among the systems, considerable differences with regard to the proportions of the biotransformation products



Scheme 1.

were observed. In principle, metabolic differences can be attributed to different uptake rates and metabolic channelling of the respective compounds due to the activities of enzymes involved, or a combination of both. For example, it was pointed out that compared to plants, cell suspension cultures show a greater uptake capacity for organic chemicals [12]. However, the results of the metabolism studies with 3,4-dichloroaniline pointed to species-specific biotransformations. Thus *N*-malonylation of **1** prevailed in soybean whereas wheat preponderantly showed *N*-glucosylation and the formation of bound residues. It was supposed that the different metabolite patterns resulted mainly from activity differences of the crucial enzymes, namely the *N*-malonyl-and *N*-glucosyl-

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transferase. The UDP-glucose: 3,4-dichloroaniline *N*-glucosyltransferase (*N*-GT; EC 2.4.1.71) of soybean seedlings and cell suspension cultures has been studied [13, 14], and was found to show a marked substrate specifity for aniline **1**. In addition, malonyl-CoA: 3,4-dichloroaniline *N*-malonyltransferase (*N*-MT) has been investigated in soybean cell suspensions [14] and peanut seedlings [15]. These investigations concentrated on the isolation and properties of the respective enzymes. The reasons for the present investigation were to: (1) determine whether or not the apparent lack of agreement between the metabolism studies discussed above can be traced back to different *N*-GT and *N*-MT activities, and (2) examine the comparability of different *in vivo* and *in vitro* plant systems of the same species. It was hoped the cell cultures would prove to be a convenient model for further studies of the different mechanisms employed by soybean and wheat to detoxify **1**. We report here the first comparison of the 3,4-dichloroaniline *N*-GT and *N*-MT activities in cell suspension cultures and plants of the two species.

RESULTS AND DISCUSSION

N-*GT and* N-*MT activities in cell suspension cultures*

More than 90% of the transferase activities (cell cultures and plants) was found in the soluble cytosol fraction. Upon freezing the plant material for 48 hr, minor losses of the *N*-GT and *N*-MT activities were observed (below 7%). However, those of the roots were reduced to 19% of the initially assayed activity. Consequently, enzyme activities were determined immediately following harvest of the plant material. The parameters of the assays for both *N*-GT and *N*-MT were in accordance with published data [13–15]. With regard to time and protein concentration, linearity of the transferase assays existed within the range of the incubation intervals used. The assays relied on direct measurement of the production of malonyl conjugate **2** and *N*-glucoside **3**, respectively.

In the soybean suspension cultures, the activities of *N*-GT and *N*-MT were more or less constant over the subculture interval (within experimental error and biological variation) and ranged from 0.48 to 1.65 pkat, and 34.3 to 80.1 pkat·mg^{-1} protein, respectively. Protein concentrations of the cell were between 2.21 and 3.21 mg·g^{-1} fr. wt. The data demonstrated that both transferase activities were constitutive. During the entire subculture interval, the *N*-MT activity was 30 to 100 times greater than that of *N*-GT. Generally, both transferases were studied over several growth cycles. Though differing to some extent, the enzyme activities were of the same order of magnitude, and more important, the same ratio of *N*-GT activity to *N*-MT activity was observed.

The growth kinetics of the wheat cultures in relation to *N*-GT activity are shown in Table 1. In the wheat cell suspensions, no *N*-MT activity could be determined within the parameters of the experimental design. On the other hand, the *N*-GT activity was constitutive, ranging from 1.3 to 4.5 pkat·mg^{-1} protein in the course of the subculture interval (1.29–2.81 mg·g^{-1} fr. wt protein content). In wheat cells, a maximum of *N*-GT activity was observed from 2 to 5 days following inoculation and coincided with the start of linear growth.

Table 1. Activities of *N*-glucosyltransferase during one cell culture cycle of the wheat suspension culture

Time* (day)	Cells (g fr. wt)	Activity† (pkat·mg^{-1} protein)
0	0.95	1.3
1	1.05	3.4
2	0.85	4.5
5	1.43	2.7
7	1.59	2.6
9	2.41	2.1
12	2.47	2.5
14	2.63	3.1

*Days after subculture.
†Mean values of 4 enzyme assays.

The *N*-GT and *N*-MT activities of the cell suspension cultures account for the results of the metabolism studies cited above. In soybean cultures, the aniline was predominantly metabolised to malonyl conjugate **2** [5, 6]. Malonylation (20 μg **1** per suspension) occured rapidly within 15 min [10]. This corresponds with the high *N*-MT activity level in the soybean suspensions. Neglecting uptake and diffusion, 1–5 μg min^{-1} of aniline **1** should be malonylated by about 3.5 g of the soybean cells. Metabolism studies with wheat cell suspension cultures demonstrated that aniline **1** was primarily biotransformed to the glucosides **3** and **4**, in addition to a large quantity of non-extractable residues [5, 7]. This agrees with the fact that in the present investigation no *N*-MT activity, and a larger *N*-GT activity was determined. The lower total conjugating potential of the wheat cells seems to make possible the formation of a large portion of non-extractable residues.

Regarding the *N*-MT of the soybean cell suspension cultures, the specific activities found in the present study agree with published data [14] which amounted to 32 pkat·mg^{-1} protein. The presence of the soybean *N*-GT activity was also reported, but no specific activities were given [14]. Investigations on an *N*-glucosyltransferase for metribuzin in tomato suspension cultures demonstrated specific activities comparable to those found in the soybean cultures for aniline **1** [16]. However, the metribuzin glycosyltransferase of tomato cultures was maximal at 3 days after subculturing, then it declined sharply.

N-*GT and* N-*MT activities in plants*

In the soybean plants, the *N*-GT and *N*-MT activities were constitutive, and were present in all of the tissue examined (Table 2). While *N*-MT was most active in the

Table 2. Activities of *N*-glucosyltransferase (*N*-GT) and *N*-malonyltransferase (*N*-MT) in soybean and wheat plants

Plant part	Protein (mg·g^{-1} fr. wt)	Activity* (pkat·mg^{-1} protein)	
		N-GT	*N*-MT
Soybean:			
Root	3.68	0.74	9.6
Cotyledon	13.87	1.7	0.60
Primary leaf	18.18	0.037	0.20
Wheat:			
Root	0.93	1.4	n.d.†
Cotyledon	4.78	1.0	n.d.

*Mean values of 4 enzymes assays (soybean root and cotyledon), or 2 enzyme assays (soybean primary leaf, wheat root and cotyledon).

†Not detectable.

roots (9.6 pkat·mg^{-1} protein), *N*-GT activity was maximal in the cotyledons (1.7 pkat·mg^{-1} protein). The activities detected in the primary leaves were comparably low, amounting to 0.037 (*N*-GT)'and 0.20 pkat mg^{-1} protein (*N*-MT). In the wheat plants, no *N*-MT activity could be detected. The *N*-GT activities in the wheat roots and cotyledons were 1.4 and 1.0 pkat·mg^{-1} protein, respectively (Table 2).

The *N*-GT and *N*-MT activities in intact soybean and wheat plants can explain the results obtained in previous metabolism experiments. In soybean plants grown on hydroponic solution [10, 11], the root applied xenobiotic was transformed to the glucosides **3** and **4**, and the main product, conjugate **2**. Comparable percentages of conjugates **2**–**4**, and a considerable fraction of bound residues were detected in excised soybean leaves [6]. On the whole, these data correspond with the pattern of *N*-MT and *N*-GT activities observed in the soybean plants. The remarkably high *N*-MT activity in the soybean roots providing the explanation as to why aniline **1** is primarily conjugated with malonic acid. The main metabolites detected in the roots of wheat plants were the glucosides **3** and **4**, and bound residues, in addition to the *N*-malonyl conjugate **2** [5, 8–11]. In the present study, no *N*-MT activity was detected in the wheat plants. Thus, the difference between soybean and wheat regarding the metabolism of **1** becomes evident.

A previous investigation using 6-day-old soybean seedlings [13] also demonstrated that *N*-GT activity was present in all of the tissues examined. Activity was found to be maximal in seeds and cotyledons (0.76 and 0.71 pkat·mg^{-1} protein, respectively); 0.1 pkat·mg^{-1} protein was observed in both the roots and leaves. With regard to cotyledons and roots, the activities determined in the present study were higher. However, the relative distributions of *N*-GT activities in the individual plant parts were similar to the levels published. In 2- to 64-day-old peanut plants, *N*-MT activity was found to be maximal in the hypocotyls and leaves of especially young seedlings and was absent from older plants (19 days and older) [15]. An *N*-glucosyltransferase catalysing the conjugation of metribuzin was investigated in tomato leaves [17], and demonstrated a specific activity smaller than that of the *N*-GT obtained in the present study.

The results of the present study demonstrated that the species and organ specific biotransformations of the model xenobiotic 3,4-dichloroaniline published previously can be traced back to the activities of the crucial *N*-malonyl- and *N*-glucosyltransferases in the different plant systems. In detail, the cell suspensions of soybean and wheat seemed to mimic closely the roots of the respective intact plants, rather than the leaves.

EXPERIMENTAL

Chemicals. 3,4-Dichloro[*Ring*-U-^{14}C]-aniline (sp. act 477.3 MBq·mmol^{-1}) purchased form Sigma Chemical Company was diluted with unlabelled 3,4-dichloroaniline, (*p. A.* quality) (**1**). *N*-(β-D-glucopyranosyl)-3,4-dichloroaniline (**3**) and *N*-malonyl-3,4-dichloroaniline (**2**) were prepd by the methods outlined in [6].

Analytical methods. TLC: silica gel developed in solvents 1: $CHCl_3$–MeOH (4:1); 2: $CHCl_3$–MeOH–H_2O (65:25:4). ^{14}C TLC zones were located with a radiochromatogram scanner; quantitative ^{14}C measurements were performed by liquid scintillation counting (LSC).

Plant materials. Soybean cell suspension cultures (*Glycine max* L.) were grown in 20 ml Gamborg B5 medium (pH 5.5) supplemented with 4.5 μmol l^{-1} of 2,4-D on a rotary shaker at 110 rpm and 27° in the dark [6]. Every 7th day, 1 g of the cells was introduced into fresh medium by passing them through a spoon-shaped sieve (0.8 mm mesh width). To minimize strain selection effects, an appropriate number of parent cultures were pooled before inoculation. The cells exhibited a lag-phase for 4 days and then rapid growth for another 2 days, when the cells entered the stationary phase. *N*-GT nd *N*-MT activities were followed by harvesting 2 replicates every day of the subculture (suction filtration). Average fr. wt yields of the cells were 0.70 (0 days after subculture), 0.93 (1), 1.40 (2), 1.92 (3), 3.14 (4), 3.35 (5), 4.94 (6), and 4.81 g (7).

Wheat suspension cultures (*Triticum aestivum* L). were grown in B5 medium (9.0 μmol l^{-1} 2,4-D; pH 5.2) as described above. For subculture (every 14th day), cells were withdrawn from the preceding suspension by use of a spoon-shaped sieve (1.00 mm mesh width), and 1 g was introduced into fresh medium using a spatula [18]. The wheat suspensions demonstrated a 2 day lag-phase, linear growth for 10 days followed by a stationary phase. Two replicates were harvested every 2 or 3 days.

Soybean seeds were germinated and grown in soil for 10 days. The seedlings were placed into 100 ml of 80% Hoagland [19] nutrient soln (1 plant each; pH 5.0), and were cultivated at 23° with a 16 hr photoperiod (3750 lx) for another 3 days, essentially as described in [11]. Wheat seeds were germinated on filter paper for 72 hr at 25° in the dark. Then, the seedlings were transferred into 100 ml of modified Hoagland soln (6 plants each), and were grown for another 5 days as described in [11].

Buffer systems and solutions. A: 50 mM Tris–HCl (pH 7.4); B: buffer A with 2 mM $MgCl_2 \cdot 6H_2O$, 2 mM dithiothreitol and 1 mM phenylmethylsulphonyl fluoride added; C: buffer A with 2 mM UDP–glc added; D: buffer A with 0.4 mM malonyl–CoA added.

Enzyme extraction. Generally, the *N*-GT and *N*-MT activities were determined simultaneously. The data are taken from representative experiments. Plant material (1.0 g fr. wt) was ground under liquid N_2; the dry powder was suspended in 3.5 ml buffer B and centrifuged for 20 min at 3300 g. The supernatant (2.5 ml) was placed on a PD–10™ Sephadex G-25M column (Pharmacia) equilibrated with 25 ml buffer A. Proteins were eluted with 3.5 ml of buffer B and the resulting eluant assayed for *N*-GT and *N*-MT activity. The same volumes were used in the cases when the available plant material was less than 1 g. All transferase assays were measured at 2 incubation intervals. Experiments using cell suspensions were performed with 2 replicates. Sepd parts of 10 soybean plants were pooled; fr. wt primary leaf 0.10, cotyledon 0.67, and root 0.55 g per plant. One replicate of the primary leaf, and 2 replicates each of the root and cotyledon extract were subjected to Sephadex purification. Parts of 11 wheat plants were combined (root 0.23, and cotyledon 0.10 g fr. wt per plant). One replicate each of the root and cotyledon extract was assayed for transferase activities.

The protein content of the Sephadex G-25M eluants was determined according to ref. [20] with BSA as standard protein (2 replicates of each eluant).

N-*Glucosyltransferase assay.* Sephadex G-25 eluant (0.6 ml) was added to 0.5 ml soln C. The reaction was started by addition of 5 μg **1** (125,000 dpm) in 5 μl MeOH. After incubation at 28° for 30 or 60 min, respectively, the reaction was terminated by addition of 0.5 ml MeOH. Subsequently, 80 μl of the resulting mixt. (*ca* 6,000 dpm) was subjected to TLC (solvent 1) and analysed. Glucoside **3** was identified by cochromatography with an authenic sample. The respective ^{14}C zones were scraped off, suspended in cocktail, and were analysed after deposition of the silica gel by LSC.

N-*Malonyltransferase assay.* In the case of soybean cells, 0.2 ml of the Sephadex G-25 eluant were added to 0.4 ml of buffer B and 0.5 ml soln D. Incubation (10 and 20 min) and TLC analysis (solvent 2) were performed as described for *N*-GT activity. *N*-MT activities of the individual soybean plant parts were assayed with 0.6 ml of the protein extract (no buffer B added); incubation intervals were adjusted appropriately (10 to 40 min). In the case of wheat suspensions and plants, 0.6 ml of the protein extract was incubated for 30, 60, or 120 min.

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