Pestic. Sci. 1997, **49**, 157–163

Kinetics of Uptake and Metabolism of Atrazine in Model Plant Systems

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(Received 15 July 1996; accepted 8 October 1996)

Abstract: The present work concerns atrazine absorption and metabolism by corn (*Zea mays*.) seedlings immersed in an aqueous medium in comparison with *Acer pseudoplatanus* cell cultures. At the point of equilibrium, the apparent concentration inside the *A. pseudoplatanus* cells (with a moderate lipid content: 0·17% of dry weight) was about twice that of the medium. This equilibrium was probably due to a simple partition process; part of the atrazine was dissolved in the cell water and reached the same concentration as in the external medium while the rest was concentrated inside the cellular lipids. The theoretical calculation of the lipid/water partition, taking into account the value of log P measured not with the lipids but with octanol (log $P = 2{\cdot}5$), gave a value of 1·5 for concentration inside the plant material. Such an equilibrium, resulting from a partition process between water and lipids, was also obtained in non-living corn seedlings. In living seedlings, an over-concentration of radioactivity due to [^{14}C]atrazine derivatives was rapidly obtained inside roots and shoots giving concentrations respectively 7- and 12-fold higher than that of atrazine in the external medium. This was due to very rapid chemical transformation of atrazine into its hydroxy derivatives, especially hydroxyatrazine. This hydrolysis of atrazine in corn was due to the presence of high levels of benzoxazinone derivatives in corn seedling cells. The hydroxylated metabolites were able to concentrate in the cells very rapidly and were unable to diffuse freely into the external medium. As a consequence, this process facilitated the penetration of large quantities of atrazine which became rapidly hydroxylated, allowing therefore the passive penetration of atrazine to be further improved, since the concentration C_1 in the receiver compartment was always close to zero. The passive transfer of atrazine, following Fick's law: $dq/dt = -Pa\,(C_0 - C_1)$, was therefore optimized.

Key words: atrazine, hydroxyatrazine, corn seedlings, *Acer pseudoplatanus* cells, lipid/water partition

1 INTRODUCTION

When studying the fate of herbicides inside plants, there are several key factors which determine the physiological effects of the compounds. These factors are: (1), the ability of the herbicides to reach, inside plant cells, what is called the 'critical concentration',[1] i.e. the minimal concentration giving lethal symptoms, (2) their ability to move and distribute throughout the plant, specifying either a contact effect or, a systemic effect, (3) their ability to be chemically stable over a long enough period of time to bring about cell death, (4) their ability to partition between lipids and water. This partitioning might result, for lipophilic compounds, in a critical concentration inside biological membranes which may contain the biochemical target of these herbicides. This

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Pestic. Sci. 0031-613X/97/$09.00

is the case, for example, for photosystem II inhibitors acting inside the thylakoid membrane and which must be lipophilic in order to be effective.[2] In order to understand all these factors, detailed studies are required on different types of plant material and with contrasting types of active ingredient.

The objective of this paper is to characterize the uptake of atrazine into plants and to determine the influence of degradation and partition upon this. For this purpose, this work compares the atrazine distribution inside two types of plant material: *Acer pseudoplatanus* L. cells and corn (*Zea mays* L.) seedlings, under three fundamental conditions: (a) atrazine was absorbed either by *A. pseudoplatanus* cells or corn seedlings immersed in an aqueous medium in which the concentration was maintained throughout the experiment, (b) none of the plant material under study contained the biochemical target (i.e. the D1 protein), (c), *A. pseudoplatanus* cells were unable to metabolize atrazine and the corn seedlings were known to metabolize it through different pathways.[3–5] The scientific results which were the objective of this study are necessary, at a first step, to develop predictive models of (1) herbicide uptake by corn cultures and (2) the accumulation of the active ingredient and its metabolites inside the different parts of the plant material.

2 EXPERIMENTAL METHODS

2.1 Chemicals

Atrazine, hydroxyatrazine, deethylatrazine, deisopropylatrazine and didealkylatrazine (purity >99%) were purchased from Cluzeau (Interchim, Sainte Foy La Grande, France). [*Ring-U*-^{14}C]atrazine (radiochemical purity >98%, specific activity 0·92 GBq nmol^{-1}) was purchased from Sigma. Atrazine, deethylatrazine, deisopropylatrazine and didealkylatrazine stock solutions were prepared with ethanol as solvent, whereas hydroxylated derivatives were dissolved in water + acetic acid (19 + 1 by volume).

2.2 Plant material

Corn seeds (*Zea mays* L. cv. Furio) were soaked for 2 h in water and allowed to germinate in Petri dishes at 30°C in the dark for four days. Approximately 50 etiolated seedlings were harvested and incubated for three days in darkness at room temperature in a 2-litre Erlenmeyer flask containing 1 litre of distilled water. The seedlings were fully immersed inside the medium which was vigorously bubbled with filtered and hydrated air at 4·5 litres min^{-1}, as previously described for wheat seedlings.[6] This unusual system used in growing the corn seedlings totally submerged was an absolute condition to reach an optimal and constant atrazine penetration during all the experimental period.

Acer pseudoplatanus L. cells were cultured as previously described.[7]

2.3 Treatment and radioactivity measurements

A. pseudoplatanus cells were treated for a week with solutions of radiolabelled atrazine (final concentrations: 4 or 50 μM). The specific activities of [^{14}C]atrazine solutions were 1·85 and 0·15 kBq nmol^{-1}, respectively. Atrazine, dissolved in ethanol, was added just prior to initiation of the culture, which was brought about by the aseptic transfer to 90 ml nutrient medium of a 10-ml aliquot of cell suspension in the exponential phase of growth. Different parameters were measured; cell fresh weight and the number of cells per millilitre suspension, dry weight per cell and the corresponding protein content. Cell structure and size were also observed by light microscopy. Cell fresh weight was determined from a 5-ml aliquot, filtered on Millipore filters (SWP 02500) under vacuum, and repeatedly rinsed with distilled water to remove residual medium. Dry weight was measured after desiccation at 75°C overnight. Cell counting was performed with a haematocytometer (Malassez cell) on an aliquot which was suspended in chromic acid (60 g litre^{-1}) for 10 h, and was thereafter vigorously shaken before counting. Experiments were done in triplicate. Protein estimation was performed following the procedure of Bradford[8] using bovine serum albumin as a reference.

For seedling treatments, an ethanolic solution of [^{14}C]atrazine (final concentration 4 μM, specific activity 1·85 kBq nmol^{-1}) was added in the reaction medium (distilled water) at the beginning of the experiment in Erlenmeyer flasks. At the end of the experiments, the plant material was washed in water for 5 s. After this washing, all organs from the seedlings were separately weighed, crushed with a mortar and extracted with ethanol + water (1 + 1 by volume).

The radioactivity of the different fractions obtained from plant material (supernatants and pellets) was counted for 1 min, using an Intertechnique liquid scintillation spectrophotometer (model SL 30). Supernatants (1 ml) and weighed pellets were suspended in 4 ml distilled water + 10 ml a liquid scintillation cocktail for aqueous samples (Ready Safe™, Beckman).

When necessary, live material was killed by either nitrogen freezing or by boiling for 5 min at 100°C.

2.4 Lipid contents

Five grams of fresh material were extracted with acetone + water (4 + 1 by volume; 3 × 50 ml) and the brei filtered. The filtrate was extracted with light-petroleum distillate (3 × 10 ml). The lipophilic phase

was concentrated to dryness under vacuum and weighed.

2.5 Efflux experiments

After 72 h treatment as previously described, corn seedlings (50–60 g fresh weight) were rapidly washed in water (5 s) and reincubated for 24 h in a 2-litre Erlenmeyer flask containing 1 litre of distilled water. During this period, aliquots of water (5 ml) and plant material (five seedlings) were harvested and counted for the establishment of a kinetic efflux curve.

2.6 Chromatographic analysis

Thin layer chromatography (TLC) was carried out on silica-gel plates (Merck, 60F 254). Lipophilic compounds were separated with light petroleum distillate (35–60°) + ethyl acetate (1 + 1 by volume) and more polar products (hydroxyderivatives) were analysed with ethyl acetate + acetic acid + formic acid + water (20 + 1 + 1 + 2 by volume). Autoradiography of TLC plates was performed with Kodak films (DEF 5).

2.7 Benzoxazinone activity

DIMBOA (2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one) was extracted from four-day-old plantlets (50 g fresh weight) with acetone (100 ml). After centrifugation (4000*g*, 10 min), the supernatant was concentrated to 30 ml under vacuum and extracted with hexane (3 × 10 ml). The aqueous acetone phase was separated, concentrated and analysed by TLC (silica-gel plate Merck, 60F 254, solvent: ethyl acetate + acetic acid + formic acid + water, (20 + 1 + 1 + 2 by volume). DIMBOA ($R_f = 0{\cdot}88$) was compared to an authentic sample and also identified by spectrophotometry. The kinetics of hydroxylation of atrazine (6 μM, specific activity 2·5 kBq nmol^{-1}) in the presence of DIMBOA (100 μM to 10 mM) were analysed, at room temperature, in aqueous media at different pH values (between 5 and 9).

3 RESULTS

3.1 Passive transfer of [^{14}C]atrazine from the nutrient medium to *Acer pseudoplatanus* cells: kinetics and equilibrium state

No difference in the number of cells, the weight of fresh and dry organic material, and the amount of soluble proteins was found between an untreated *A. pseudoplatanus* culture and cultures treated with atrazine at 4 or 50 μM. At the end of the experiment, the absorbed [^{14}C]atrazine was shown to be present without chemical change (TLC studies, not shown). Figure 1 shows the kinetics of atrazine penetration into the cells. In the ratio C/C_0, C represented the apparent concentration of atrazine in the cells and C_0 the concentration in the medium. The C/C_0 ratio reached a constant value close to 1·8–1·9 rapidly (8 h), and remained stable for a week (end of the experiment). This experiment showed that the apparent concentration inside the cells reached almost twice that of the medium. Almost the same results were obtained with cells killed through liquid nitrogen freezing (results not shown). The value of C/C_0 obtained was probably the result of a partition process, one part of the atrazine reaching, in the cell sap, the same concentration as in the external medium, another part being concentrated through a partition mechanism inside the cellular lipids. Such a situation, controlled by a passive partition process, was previously described for pentachlorophenol partitioned in *A. pseudoplatanus* cell cultures.[7] The amount of extractable lipids from *A. pseudoplatanus* cells was previously evaluated as being 0·165% of the fresh weight of the pellets. The amount of atrazine associated with *A. pseudoplatanus* lipids can be estimated theoretically through the use of the partition

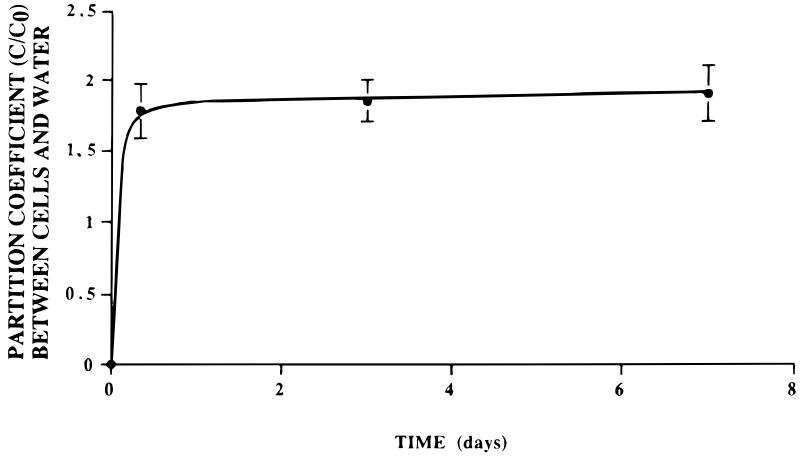

Fig. 1. Distribution of [^{14}C]atrazine (4 μM) between *Acer pseudoplatanus* cells and the aqueous nutrient medium, as a function of time. Error bars: $\pm\sigma$ (mean value of three experiments), C_0 : concentration in the aqueous medium, C: concentration in the cells.

value of atrazine between lipids and water, supposed to be identical to the value of the octanol/water partition (log $P = 2{\cdot}5$). This amount was shown to reach 6 nmoles g^{-1} fresh weight with an external concentration at 4 μM (for calculation explanations see Ref. 7).

The apparent concentration of atrazine inside the cells would therefore be 6 μM, giving a calculated C/C_0 value of 1·5. This result is slightly lower than the experimental value ($C/C_0 = 1{\cdot}9 \pm 0{\cdot}2$, Fig. 1). The difference between the measured and the calculated values could be due to the presence of a non-extractible lipophilic phase inside *Acer* cells or to a possible non-negligible difference between the partition coefficient for octanol/water and lipids/water.

3.2 Transfer of [^{14}C]atrazine from the medium to immersed growing corn seedlings

The use of cell suspension cultures growing in a nutrient medium allows a study of the distribution equilibrium of a xenobiotic compound between external water and cells. This cannot be done for plants growing under field or greenhouse conditions. For a comparison between *A. pseudoplatanus* cells and corn, the use of a suspension culture of this latter species would have been possible. However, the use of whole seedlings was chosen as it allowed a concurrent study of the different cell types which are normally present in a plant, and are characteristic of roots, coleoptiles and leaves, the metabolic capacity of which might be quite different. It was possible to obtain a normal growth of the corn seedlings (and also of their isolated organs) in an aerated water medium for several days, using a method previously described.[6] Under immersed conditions, corn seedlings were shown to be able to grow very rapidly. In the dark, they remained etiolated and were therefore unable to elaborate functional chloroplasts which contain the D1 protein, the biochemical target of atrazine.

Figure 2 shows the penetration curve of atrazine into the growing parts of the seedlings, the roots and shoots being studied separately. The amount penetrating inside the seed itself remained very low.

In contrast with *A. pseudoplatanus* cells, the values of C/C_0 found for roots and for shoots were very high, being 4 and 12 respectively. These results could not be explained by a simple partition process between lipids and water (which explained the C/C_0 value in the case of *A. pseudoplatanus* cells) even if an especially high content of lipids inside the seedlings was found. In fact, the extractable lipids, which remained quite stable during the period of experiment, were found to reach 1·2% of the fresh weight in roots but only 0·5% in shoots.

Therefore, the very high concentrations obtained in the shoots and roots were clearly not explained by their lipid content alone. Furthermore, these high values of C/C_0 were severely decreased when boiled material was used (Fig. 2). Thus, the high concentrations obtained with living material appeared to be the result of a physiological process. In killed material, C/C_0 seemed to correspond to a simple passive partition process concerning atrazine itself (C/C_0 value between 1·5 and 2) and was close to the value found for living and killed *A. pseudoplatanus* cells. The hypothesis of an active penetration process of atrazine in corn was unlikely for a compound with so high a lipophilicity. This point was reinforced by the fact that a thiol inhibitor, *p*-chloromercuribenzenesulfonic acid, (*p*-CMBS; 10 μM), was unable to alter the penetration rate of atrazine during the first 5 h of experiment. The hypothesis of a rapid metabolism followed by the segregation of metabolites was therefore considered.

3.3 Atrazine metabolism inside corn seedlings

The TLC chromatography and autoradiography of extracts of seedlings treated with [^{14}C]atrazine showed

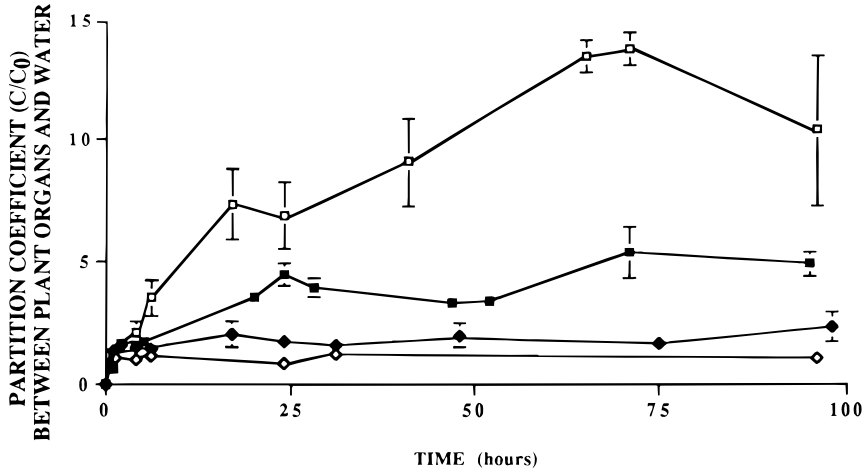

Fig. 2. Kinetics of atrazine distribution between water (1 litre) and immersed corn seedlings (50–60 g fresh weight). C/C_0 : ratio between the apparent concentration in the plant material (C) and the concentration in the aqueous medium (C_0). (■) living roots, (□) living shoots, (◆) killed roots, (◇) killed shoots. Error bars: $\pm\sigma$ (mean value of three experiments).

TABLE 1
Atrazine and Derivatives[a] found in Corn Roots and Shoots after a 72-Hour Period of Treatment

	Quantity found (% of original atrazine)								
	A	*DEA*	*DIA*	*DDA*	*OHA*	*OH–DEA*	*OH–DIA*	R_f 0	R_f 0·34
Live roots	3·5	0·8	0·8	0·4	51·5	15	9	18	1
Live shoots	1·5	1	0·5	0·8	73	10	4	7·5	1·7
Killed roots	83	0	0	0	17	0	0	0	0
Killed shoots	79	0	0	0	21	0	0	0	0

[a] A: atrazine; DEA: deethylatrazine; DIA: deisopropylatrazine; DDA: didealkylatrazine; OHA: hydroxyatrazine; OH-DEA: hydroxydeethylatrazine; OH-DIA: hydroxydeisopropylatrazine; R_f 0: unidentified products at the TLC start; R_f 0·34: unidentified products having a R_f value at 0·34 in the TLC system used (SiO_2 with ethyl acetate + acetic acid + formic acid + water, (20 + 1 + 1 + 2 by volume) as solvent).

that, at the end of the treatment period (72 h, see Table 1), less than 5% of the ^{14}C was present as atrazine. Most of it was represented by OH-atrazine (as shown by co-chromatography with a reference sample) and by the two other hydroxylated derivatives having lost one of their alkyl chains (OH-deethylatrazine and OH-deisopropylatrazine). A small amount of the radioactivity probably corresponded to more polar products, which may be OH-didealkylatrazine and/or glutathione conjugates. The conclusion of this experiment was that the transformation of atrazine to OH-derivatives was responsible for the high concentration of these products inside the living corn cells, which a simple partition process failed to explain.

Concurrently, either treated living shoots or roots accumulating the OH-derivatives were frozen and then

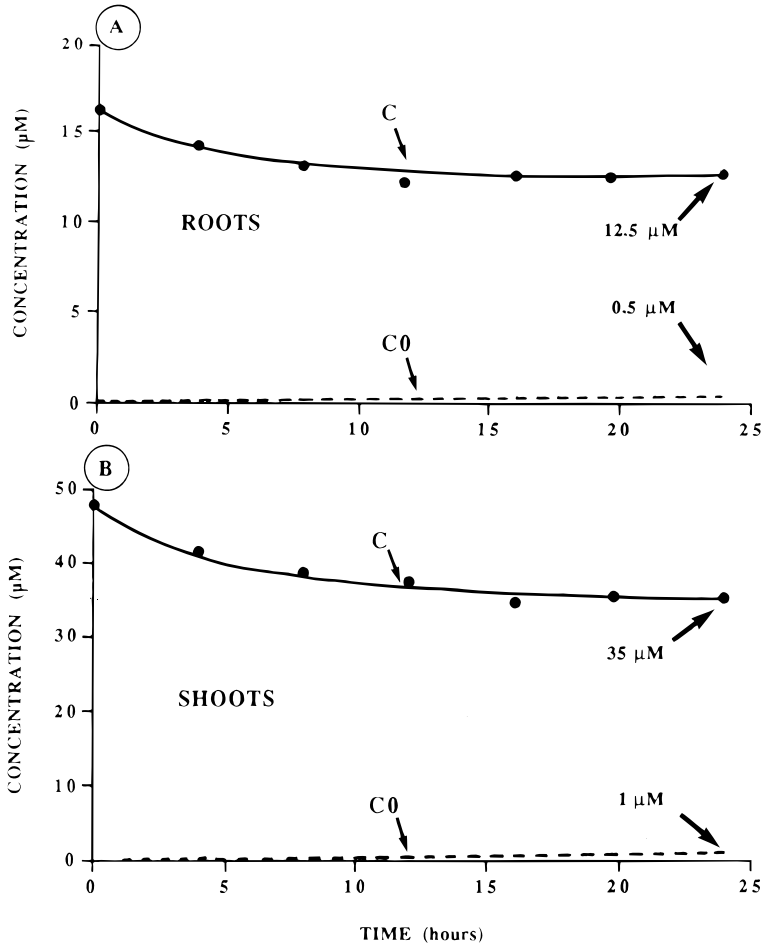

Fig. 3. Kinetics showing the efflux of atrazine and derivatives between treated corn seedlings (4 µM atrazine during three days) into a fresh aqueous medium. (A) roots, (B) shoots. *C*: concentration in the plant material, C_0 : concentration in the medium.

rapidly thawed and centrifugated (15 000*g*). A large proportion (79·5% for roots and 75·1% for shoots) of the radioactivity present in the plant material was found, after centrifugation, as OH-derivatives in the water supernatant and the radioactivity remaining in the pellet (20–25%) corresponded, for a large part (close to 60%) to unchanged atrazine. These results demonstrate that OH-derivatives were not substantially bound to cell membranes, in contrast with atrazine itself, which was mostly associated with membrane lipids, as could be surmised from its log *P* value.

3.4 Hydroxyatrazine segregation inside corn seedling cells

The analysis of the external medium in which corn seedlings were grown and treated showed only traces of OH-derivatives (less than 10%), and atrazine represented 90% of the remaining labelled compounds of the medium by the end of the experiment.

Figure 3 shows efflux experiments in which living seedlings (50–60 g fresh weight) previously treated during 72 h were immersed for one day in one litre of a fresh aqueous medium. The values of C/C_0 were very high at the end of this experiment, reaching 25 for roots and 35 for shoots. Furthermore, the greatest part (close to 90%) of the label released by the plants was atrazine. As a whole, it appears that OH-derivatives were highly accumulated in corn seedlings but were almost unable to diffuse into the fresh medium.

3.5 *In-vitro* hydroxyatrazine formation in the presence of extracted DIMBOA

The rates of atrazine hydroxylation were measured in water, at 25°C and at pH values between 5 and 9, in the presence of DIMBOA previously extracted from corn seedlings. This rate reached a maximum value of 60 nmoles h^{-1} which occurred at pH 5–5·5 in the presence of 1 mM DIMBOA. At pH 7 this rate was very low and zero at pH 9. In the absence of DIMBOA no hydroxylation of atrazine occurred *in vitro*.

4 DISCUSSION

The experiments carried out demonstrated that the OH-derivatives of atrazine represent the greater part of the metabolites developed by corn seedlings. Similar results had been suggested previously.[9]

These hydroxylated metabolites, mainly OH-atrazine, were able to concentrate in the cells, and were unable to diffuse freely into the medium. As a consequence, this rapid metabolism allowed the passive penetration of atrazine to increase continually, as the concentration C_1 of free atrazine in the receiver compartment was constantly close to zero. The passive transfer of atrazine following Fick's law: $dq/dt = -Pa(C_0-C_1)$, was therefore optimized.

It seems likely that the accumulation of OH-atrazine occurred mainly inside the vacuole in the presence of DIMBOA and other hydrophilic representatives of the benzoxazinone family. These compounds are present at high concentrations in the cell sap, reaching 10 mM in corn seedling material and they have been shown elsewhere to stimulate atrazine hydroxylation.[10–12] A vacuolar localization of OH-atrazine seemed likely because we could demonstrate that hydroxylation of atrazine in the presence of corn benzoxazinones was very low at pH 7 (which is a pH value close to that of cytosol, stroma and matrix) but highly active at slightly acidic pH (which is the case of vacuolar pH).

In view of the rate of atrazine hydroxylation *in vitro* and *in vivo* it can be suggested that this transformation occurs very readily inside the seedling cells. This means that the internal distribution of free atrazine from cell to cell inside the seedlings can only be very limited. As a consequence, it appears that only the very first step of atrazine penetration into the plant involves the parent compound. This step would be limited to plasmalemma penetration and, perhaps, diffusion inside cytosol of the superficial cell layer of the seedlings. The segregation of OH-atrazine inside the cells needs to be discussed, as its log *P*, close to 1·5,[13] seems high enough not to prevent permeation through plant cell membranes.[14] A first hypothesis is that ionization of the hydroxyl group of OH-atrazine (pKa = 5·15)[15] might induce a repulsion of the product by the electronegatively charged membranes. This would be more effective inside the cytoplasm than inside the vacuole. A second hypothesis is that OH-atrazine was able to bind tightly to cellular protein as it binds to soil components (Tasli, pers. commun.).

It is noteworthy that OH-atrazine is not a substrate for glutathione conjugate formation, this enzymatic transformation being limited to the leaves. The two known pathways of atrazine metabolism in corn are therefore quite independent. In the corn seedlings used (cv. Furio), cultured under submerged conditions as described, chemically induced hydroxylation seems to be the predominant pathway for atrazine transformation. Under field conditions and with other varieties, the rate of OH-atrazine accumulation under the influence of benzoxaquinones might be lower relative to the enzymatic conjugation with glutathione described by Shimabukuro *et al.*[16]

ACKNOWLEDGEMENTS

The authors of this study are grateful to the French Ministry of Agriculture for financial support and especially to our correspondent, Jean Yves Sommier.

REFERENCES

1. Arnaud, L., Nurit, F., Ravanel, P. & Tissut, M., Distribution of glyphosate and of its target enzyme inside wheat plants. *Pestic. Sci.*, **40** (1994) 217–23.
2. Arnaud, L., Taillandier, G., Kaouadji, M., Ravanel, P. & Tissut, M., Photosynthesis inhibition by phenylurea: a QSAR approach. *Ecotox. Environ. Safety*, **28** (1994) 121–33.
3. Shimabukuro, R. H., Atrazine metabolism and herbicidal selectivity. *Plant Physiol.*, **42** (1967) 1269–76.
4. Shimabukuro, R. H., Atrazine metabolism in resistant corn and sorghum. *Plant Physiol.*, **43** (1968) 1925–30.
5. Shimabukuro, R. H., Swanson, H. R. & Walsh, W. C., Glutathione conjugation: atrazine detoxication mechanism in corn. *Plant Physiol.*, **46** (1970) 103–7.
6. Zimmerlin, A. & Durst, F., Xenobiotic metabolism in plants: aryl hydroxylation of diclofop by a cytochrome P-450 enzyme from wheat. *Phytochem.*, **29** (1990) 1729–32.
7. Albertin, H., Chiron, S., Nurit, F., Ravanel, P. & Tissut, M., Penetration, distribution and effects of ^{14}C pentachlorophenol inside cultured *Acer* cells. *Phytochem.*, **30** (1991) 3553–8.
8. Bradford, M. M., A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal. Biochem.*, **72** (1976) 248–54.
9. Darmstadt, G. L., Balke, N. E. & Price, T. P., Triazine absorption by excised corn root tissue and isolated corn root protoplast. *Pestic. Biochem. Physiol.*, **21** (1984) 10–21.
10. Hamilton, H. & Moreland, D. E., Simazine: degradation by corn seedlings. *Science* (*Washington*), **135** (1962) 373–4.
11. Wahlroos, O. & Virtanen, A. I., Free 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one in maize. *J. Pharm. Sci.*, **53** (1964) 844–5.
12. Peng, S. & Chilton, W. S., Biosynthesis of DIMBOA in maize using deuterium oxide as tracer. *Phytochem.*, **37** (1994) 167–71.
13. Schmitt, Ph., Garrison, A. W., Freitag, D. & Kettrup, A., Separation of *s*-triazine herbicides and their metabolites by capillary zone electrophoresis as a function of pH. *J. Chromatogr. A*, **723** (1996) 169–77.
14. Briggs, G. R., Bromilow, R. H. & Evans, A. A., Relationships between lipophilicity and root uptake and translocation of nonionized chemicals by barley. *Pestic. Sci.*, **13** (1982) 495–504.
15. Vermeulen, N. M. J., Apostolides, Z., Potgieter, D. J. J., Nel, P. C. & Smit, S. H., Separation of atrazine and some of its degradation products by high-performance liquid chromatography. *J. Chromatogr.*, **240** (1982) 247–53.
16. Shimabukuro, R. H., Frear, D. S., Swanson, H. R. & Walsh, W. C., Glutathione conjugation: an enzymatic basis for atrazine resistance in corn. *Plant Physiol.*, **47** (1971) 10–14.