A PHYTOREMEDIATION APPROACH TO REMOVE PESTICIDES (ATRAZINE AND LINDANE) FROM CONTAMINATED ENVIRONMENT

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Summary

The present thesis is part of a project exploring new possibilities to remediate soils polluted by insecticide lindane and herbicide atrazine. It is the Indo-Swiss project entitled “Development of Phytoremediation Techniques Using Interactive Potential of Plant and Microbial Activities for Pesticides Hexachlorocyclohexane (HCH) and Atrazine”. The purpose of this project was to address the problems of contamination of sites, agricultural soils, groundwater, surface water, and agricultural food products with recalcitrant pesticides, whose production and use are steadily increasing in India due to rapid economic development, industrialization and enhanced food production over the last 20 years.

Preliminary experiments showed that vetiver was resistant to 20 ppm atrazine for 6 weeks, even with a maximum bioavailability created by the use of a hydroponic system. Atrazine resistance could be explained by plant metabolism, dilution of active ingredient into plant biomass, chloroplastic resistance, and sequestration of atrazine before it reaches its target site in leaves. It was found that vetiver thylacoids were sensitive to atrazine, excluding therefore chloroplastic resistance. Plants known metabolism of atrazine relies on hydroxylation mediated by benzoxazinones, conjugation catalyzed by glutathione-*S*-transferases and dealkylation probably mediated by cytochromes P 450. Therefore, these metabolic pathways were explored in vetiver to understand its resistance to atrazine and to evaluate benefits or risks of phytoremediation.

Plant metabolism took place in vetiver: small amounts of dealkylated products were found in roots and leaves, and conjugated atrazine was detected mainly in leaves, confirming *in vitro* tests. No benzoxazinones were detected in plant extracts, in agreement with the absence of hydroxyatrazine in vetiver organs. Altogether, these metabolic studies suggest that hydroxylation was not an important metabolic pathway in vetiver: the plant behaved more like a related species, sorghum, where conjugation clearly dominates on dealkylation. Under transpiring conditions, conjugation in leaves was important, but under non-transpiring conditions, it is suspected that atrazine and its metabolites could be trapped in roots according to the partition-diffusion law. Over-concentration of atrazine was observed in oil from roots grown in soil, suggesting that during plant ageing, partition may play a non negligible role in

retaining atrazine from agricultural runoff. Atrazine metabolism study was successfully conducted in entire vetiver plants thanks to hydroponic system. However, such a system had limitations for understanding plant effect on atrazine removal from a soil environment.

Limitation of hydroponic system was also observed for the study of γ-HCH (lindane) disappearance from the medium by the species chilli and coriander, but for other reasons: persistence of lindane in soil is not easily transposed to persistence in water. Lindane was stripped by the air sparged in hydroponic solution for roots respiration. Mineral components of hydroponic medium might have catalyzed lindane hydrolysis. Plant effect on lindane disappearance was thought to be mainly adsorption/partition in roots and enhanced hydrolysis thanks to increased pH solution by root secretions. Despite of limitations of hydroponic system, it was concluded that lindane concentration could be lowered in soils because of active change of pH in root rhizosphere.

The use of a hydroponic system is a first step toward comprehensive fate of pesticides in plants, but is also a useful tool for assessment of phytotreatment of industrial wastewater, agricultural runoff, surface and groundwater contaminated with pesticides.

Résumé

La thèse présentée fait partie d'un projet ayant pour but d'explorer de nouvelles possibilités de dépolluer des sols contaminés par des pesticides. Ce projet indo-suisse s'intitule « Développement de techniques de phytoremédiation exploitant le potentiel interactif des plantes et de leurs activités microbiennes en vue de la biotransformation des pesticides hexachlorocyclohexane (HCH) et atrazine ». Ces pesticides récalcitrants se retrouvent dans des sites industriels, des terres agricoles, des nappes souterraines, des eaux de surface et dans les denrées alimentaires. L'utilisation et la production de pesticides en Inde a considérablement augmenté ces vingt dernières années, sans doute en relation avec le rapide développement agricole, industriel et économique de ce pays, aboutissant à une contamination environnementale accrue.

Des expériences préliminaires sur le vetiver ont montré que cette plante était résistante à 20 ppm d'atrazine dissous dans un milieu hydroponique, où la biodisponibilité de l'herbicide était maximale. La résistance à l'atrazine d'une plante peut être l'expression de plusieurs phénomènes comme la capacité métabolique de la plante, une dilution de la matière active de l'herbicide dans la biomasse, une résistance chloroplastique, ou encore une séquestration racinaire empêchant l'herbicide d'atteindre sa cible située dans les feuilles. Les expériences ont montré que des thylacoides de vetiver étaient sensibles à l'atrazine, excluant d'emblée une résistance chloroplastique. De ce fait, nous nous sommes intéressé à la métabolisation de l'atrazine dans la plante; les trois principales voies connues sont l'hydroxylation par les benzoxazinones, la conjugaison par des transférases de glutathion, et la déalkylation vraisemblablement catalysée par des cytochromes P 450. Ces voies métaboliques ont donc été explorées pour comprendre le phénomène de résistance du vetiver à l'atrazine, mais aussi pour évaluer les risques et/ou les bénéfices de la phytoremédiation.

Peu de produits déalkylés ont été trouvés dans le vetiver, en regard de conjugués. La plus forte capacité métabolisante a été observée *in vivo* dans les feuilles de vetiver, et cette observation a été confirmée par des activités *in vitro* de conjugaison d'extraits de cette plante. En revanche, l'hydroxylation de l'atrazine ne semble pas être une voie métabolique importante. Aucune benzoxazinone n'a été détectée, et des extraits de vetiver se sont avérés incapables d'hydroxyler l'atrazine. Il semble que le

métabolisme du vetiver soit similaire au sorgo, un proche parent, où la conjugaison domine et confère à la plante sa tolérance à l'herbicide. Il est cependant particulier que les racines de vetiver produisent une huile essentielle, dans laquelle l'atrazine se partitionnait aisément. Dans des conditions non transpirantes, la partition pourrait être le principal facteur permettant au vetiver d'accumuler l'atrazine dans le milieu environnant. Cette huile est produite seulement dans les racines de vetiver qui se sont développées dans un sol, et non pas en hydroponie. L'étude du métabolisme de l'atrazine dans un système hydroponique a été menée avec succès. En revanche, un tel système ne permet pas de tirer des conclusions concernant la capacité du vetiver à décontaminer un sol pollué par de l'atrazine.

Les limites d'utilisation d'un système hydroponique ont aussi été observées dans le cas du γ-HCH (lindane), mais pour des raisons différentes: le lindane dans la solution hydroponique passait en phase gazeuse à cause du bullage du milieu pour oxygéner les racines. De plus, nous avons soupçonné que les nutriments minéraux des plantes ont contribué à l'hydrolyse du lindane en solution aqueuse. La disparition du lindane du milieu était donc multifactorielle, et les seuls effets de la plante étaient difficilement quantifiables. Une estimation a permis de conclure que les phénomènes d'adsorption/partition sur les racines, ajoutés aux capacités de la coriandre et du piment d'élever le pH de la solution ont certainement contribué à la disparition du lindane de la solution hydroponique.

Cependant, malgré ses limitations, le système hydroponique est un premier pas vers la compréhension du devenir de pesticides dans les plantes, et permet d'évaluer leurs capacités pour le traitement d'eaux industrielles, de drainage agricole, des eaux de surface et de nappes phréatiques.

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Abbreviations

ATR	Atrazine
ATR-GS	Glutathione conjugated to atrazine = glutathioned atrazine
BMP	Best Management Practices
BOA	Benzoxazolin-2(3H)-one
CDNB	1-chloro-2,4 dinitrobenzene
DCMU	Diuron = 3-(3,4-dichlorophenyl)-1,1 dimethylurea
DCPIP	2,6-dichloro-phenolindophenol
DDA	Didealkyl atrazine
DEA	Deethyl atrazine = 2-amino-4-isopropylamino-6-chloro-s-triazine = 6-chloro-N-(1-methylethyl)-1,3,5-triazine-2,4-diamine; deisopropyl propazine
Dealkylates	DEA, DIA, DDA
DIA	Deisopropyl atrazine = 6-chloro-N-ethyl-1,3,5-triazine-2,4-diamine; amino-2-chloro-6-ethylamino-s-triazine = deethyl simazine
DIMBOA	2,4-dihydroxy-7-methoxy-2H-1,4-benzoxazin-3(4H)-one
DIBOA	2,4-dihydroxy-2H-1,4-benzoxazin-3(4H)-one
DTE	Dithioerythritol
DW	Dry Weight
ECD	Electron Capture Detector
EDTA	Ethylenediaminetetracetic acid
EPA	Environmental Protection Agency
FW	Fresh Weight
GC	Gas Chromatography
Glc-DIMBOA	Glucosylated 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one
Glc-DIBOA	glucosylated, 2,4-dihydroxy-2H-1,4-benzoxazin-3(4H)-one
GSH	Glutathione
GST	Glutathione-*S*-Transferase
HDEA	Hydroxydeethylatrazine
HDIA	Hydroxydeisopropylatrazine
HDDA	Hydroxydidealkyl atrazine
HPLC	High Pressure Liquid Chromatography

Hydroxylates	HATR, HDEA, HDEA, HDDA
I_{50}	Herbicide concentration for 50% of inhibition of Hill reaction
LLE	Liquid-Liquid Extraction
MBOA	6-methoxy-benzoxazolin-2(3H)-one
TLC	Thin Layer Chromatography
PVP	Polyvinylpyrrolidone
PS II	Photosystem II

The context of the present work

The present thesis is part of a project exploring new possibilities to remediate soils polluted by insecticide lindane and herbicide atrazine. It is the Indo-Swiss project entitled "Development of Phytoremediation Techniques Using Interactive Potential of Plant and Microbial Activities for Pesticides Hexachlorocyclohexane (HCH) and Atrazine" (ISCB project BR1), supported by the Indo-Swiss Collaboration in Biotechnology (http://www.biotech.biol.ethz.ch/india/). ISCB is currently funding several collaborative research projects (1999-2004), three concerning *in situ* degradation and monitoring of pesticides by exploring phytoremediation (BR1), development of biosensors to monitor pesticides (BR2), and bioremediation (BR3). The phytoremediation project involved two Swiss institutes (ITO, ETHZ, Zurich and ISTE-LBE, EPFL, Lausanne) and two Indian institutes (JRF, Valvada and ARI, Pune). The task of Swiss partners was to explore possible phytoextraction and/or phytotransformation of the target pesticides, whereas Indian partners explored rhizospheric micro-organisms aspects of phytoremediation.

The purpose of the ISCB project BR1was to address the problems of contamination of sites, agricultural soils, groundwater, surface water, and agricultural food products with recalcitrant pesticides, whose production and use are steadily increasing in India due to rapid economic development, industrialization and enhanced food production during the last 20 years. India is an agricultural based country and is dependent on agrochemicals including pesticides. Since 30% of the crop was lost due to different pests, the use of pesticides has become inevitable. Pesticides thus make an essential contribution in increasing agribusiness and food production. In the region of Gujarat and Maharashtra intensive agriculture is being practiced.

However, there is a widespread contamination of agricultural products and food with pesticide residues above tolerance limit. A large number of soils in this region are assumed to be contaminated with pesticide residues. Due to their toxic effects, it becomes essential to remove these pollutants from soil and water.

For example, HCH and DDT were reported to be present in a variety of agricultural products, human adipose tissues and mother's milk, during the last decade. In crop residues of rice, wheat and sorghum, collected from Mumbai market, 4-14 ppm of

HCH were detected as early as 1978. They are now banned in India, but the residues are likely to persist in surface soil, groundwater, food and feed including milk. In fact the Indian export market in European Union is affected due to the presence of organochlorines in sesame seed, fibre, leather, milk etc.

The presence of pesticide residues such as atrazine in the soil is known to inhibit germination and growth of some legumes and oilseeds and thus affecting the yield. Another aspect is the presence of pesticide residues in groundwater which poses a risk for many people who use it as a source of drinking water. There is no systematic study at national level using standard procedural protocol for determination of pesticides residues in soil and groundwater, although different concentration levels have been reported in various parts of the country.

Organochlorine compounds being recalcitrant get accumulated in the environment. Although native flora of soil might degrade some of these compounds, the rate of degradation is slow. To enhance or assist this degradation, the use of plants and microbial consortia for phytoremediation of soils is proposed in this joint project.

The use of plants to cleanse water contaminated with organic and inorganic pollutants dates back hundreds of years and has been the basis for the present use of constructed wetlands in treating municipal and industrial waste streams. The concept of using ‘plants in association with micro-organisms’ to remediate soils contaminated with organic pollutants is a more recent development, based on observations that microbial degradation of organic chemicals is accelerated in vegetated soils compared to surrounding non-vegetated bulk soils. To further enhance the rate of biodegradation, microbial inoculates and specific plants which support the growth of these organisms could be used.

Further, it is accepted worldwide that remediation techniques which are economically and ecologically safe should be developed. In this respect microbiologically mediated phytoremediation techniques present a great potential scope.

In addition, higher plants possess a pronounced ability for the metabolism and degradation of many recalcitrant xenobiotic chemicals and can be considered as “green livers”, acting as an important sink for environmental damaging chemicals. It thus appears that wild and cultivated plants could be developed and used for the removal of hazardous persistent organic compounds from contaminated water and

soils. Phytoremediation has been defined as the use of green plants and their associated micro-organisms, soil amendments and agronomic techniques to remove, contain or render harmless environmental contaminants.

Phytoremediation is expected to be complementary to classical bioremediation techniques, based on the use of micro-organisms only. It should be mainly useful for the treatment of recalcitrant organic pollutants, like pesticides. Such innovative bio-treatments should be particularly useful in India, especially in semi-arid areas, since heavily contaminated soils, wastewater, surface and groundwater are widespread in the country.

At present, phytoremediation is still a nascent technology that seeks to exploit the metabolic capabilities and growth characteristics of higher plants: delivering a cheap, soft and safe biological treatment that is applicable to specific contaminated sites, is a relatively recent focus. In such a context, there is still a significant need to pursue both fundamental and applied research to provide low-cost, low-impact, visually benign and environmentally sound decontamination strategies.

The main objectives of this research proposal were thus the development and evaluation of these new, gentle, appropriate and efficient biological processes, based on the use of plants such as herbs , shrubs and wild legumes and agronomic crops either to extract or to degrade or to stabilize two pesticides hexachlorocyclohexane (HCH) and atrazine in sites impacted by these pesticides. This should lead to an optimized economical viable and ecologically site specific phytoremediation technique.

One of the greatest forces driving increased emphasis on research in this area is the potential economic benefit of an agronomy-based technology. Growing plants can be accomplished at a cost ranging significantly lower than the current engineering cost of excavation and reburial.

Objectives of the project BR1 were:

1. To select non-agricultural and agricultural crops for their ability to either degrade or to extract or to stabilize pesticides under investigation in contaminated soils
2. To delineate pathways employed in the uptake and metabolism of hexachlorocyclohexane (HCH) and atrazine by site specific plants and identification of metabolites
3. To isolate readily identified soil microbial consortia present in the rhizosphere of the selected specific plants, which are involved in the metabolism of target pesticides
4. To test one or more selected plants and their rhizosphere co-culture or consortium augmented in soil for transformation and reduction of target pesticides and their metabolites in greenhouse studies
5. To prepare a risk analysis scheme for phytoremediation technique

At the beginning of the present thesis, remediation chances of the target pesticides were assessed. Knowledge of uptake capacities is essential for phytoremediation; agronomists classify pesticides in two categories, namely systemic pesticides entering and moving within the plant to kill it (systemic herbicides such as triazines) or to protect it against insects (systemic insecticides such as imidacloprid), and contact pesticides remaining at the surface of the plant to kill it (contact herbicide such as diquat) or to protect it against insects (contact insecticide such as all organochlorines compounds). It seems that systemicity property of a pesticide is linked to its hydrophobicity. In his review based on numerous studies, the environmentalist Burken (26) showed that phytoextraction is possible for moderate hydrophobic organic chemicals (log K_{ow} between 1 and 3.5). Plant uptake should be limited of hexachlorocyclohexane, a contact insecticide with K_{ow} as high as 3.7, unless plant exudates lower hydrophobicity of the compound before plant uptake. On the other hand, chances of phytoextraction of atrazine (systemic herbicide) is high, since Wilson et al. (189) point out that root uptake of triazines readily occurred with all plants studied regardless of whether they are resistant or susceptible to the herbicides.

The scope of the thesis was to thus identify plants able to extract and possibly transform HCH and atrazine in hydroponic system. Goals of the thesis were formulated as followed:

1. To know if plants are able to accumulate and/or biotransform HCH and its isomers and atrazine and its main soil metabolites
2. To develop hydroponics system in order to identify suitable plants for extraction an/or transformation of tested pesticides
3. To identify main produced metabolites as a start for a risk assessment scheme
4. To understand biochemical mechanisms leading to accumulation and/ transformation of the target pesticides

Part I
phytoremediation of lindane contaminated environment

0 Study of the effect of chilli and coriander on lindane in hydroponic systems

0.1 Introduction

Even at low concentrations, persistent organic pollutants (POPs) are of increasing concern all over the world. Known for their toxicity, lipophilic properties and behaviour in the environment, numerous organochlorine compounds, including many pesticides, are considered as POPs. Due to their impact on the environment and human health, the use of many of them has been reduced or even banned in developed countries, but they are still widely used in developing nations. Among them, India is one of the major source of diffuse and point organochlorine contamination, producing and utilizing the largest chlorinated pesticide quantity throughout southern Asia (131, 132).

The 1,2,3,4,5,6 hexachlorocyclohexane (HCH) (**Figure 0.1**) is an efficient insecticide, available in two formulations: technical HCH (a mixture of different isomers including α, β, δ, and γ-HCH) and lindane (almost pure γ-HCH), also known as benzene hexachloride (BHC) (95). It is toxic but for its low costs production and its effective pesticide properties, it is ubiquitously used in tropical countries to reduce vector-transmitted diseases, to protect livestock and to increase agricultural yields. Being a POP, HCH is nowadays found in air, water and soil samples all over the world. As such, many scientists are now involved in the development of remediation technologies including bioremediation and phytoremediation.

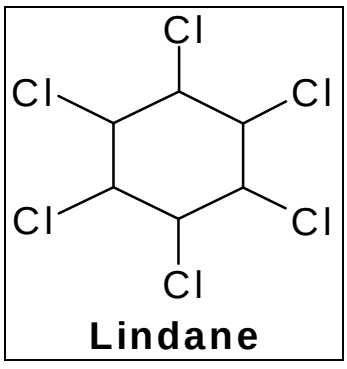


Figure 0.1 Lindane molecule

Penetration and translocation in plants of organic compounds with a log K_{ow} higher than 3 is thought to be very unlikely (26) (**Table 0.1, see Log K_{ow}**). As reflect of a high K_{ow} and a high K_{oc} values, lindane is classified as a non–systemic insecticide, and is known to bind strongly to organic matter on the surface of soil particles (**Table 0.1, see Log K_{oc}**). Bound residues represent up to 75% of the HCH soil contamination (4). However, even if mobility in soil is weak, lindane adsorbed on soil particles can be eroded and therefore found far away from the original application place (188).

Table 0.1 Physical-chemical properties of γ-HCH

	Solubility	**Log K_{ow}**	**Log K_{oc}**	**Vapour pressure**	**Henry law constant**
	7.3	3.72	3-3.57	0.72	2.16 X 10^{-5}
Units	[mg/l]	-	-	[Pa]	[atm m^3/mol]
Source	(137, 166)	(137, 166)	(137, 166)	(180)	(137, 166)

With regards to HCH isomers, in theory, phytoextraction should also not be possible. HCH isomers are hydrophobic chemicals (alpha log Kow 3.8, beta 3.9. gamma 3.72, delta 4.1) and theoretically bound strongly to soil and also to the roots of plants, preventing plant uptake (145).

Persistence of all isomers was found to be higher in uncropped plots compared with cropped plots of maize (*Zea mays*), wheat (*Triticum spp*) or pigeon pea (*Cajananus cajan*) (159) therefore sustaining the idea of remediation of polluted sites with plants. Screening of literature concerning contamination of the aerial part of plants was done, as a first step to select the appropriate plant species for phytoextraction of lindane from environment. Alpha, beta and gamma isomers in plants have been detected in many species, including *Lactuca sativa* (lettuce) (89), *Sesamum indicum* (sesame) (18), *Hydrilla verticillata* (hydra) (178), *Lagernia siceraira* (bottle gourd), *Memordica charantia* (bitter gourd), *Luffa cylindrica* (sponge gourd), *Citrullus varifistulosus* (tinda punjab) and *Spinacia oleracea* (spinach) (72), *Brassica campestris* (rape) (181). These species were not selected to be tested in hydroponics, since the detected residues on these plants were due to direct contact with lindane, and

not as a result of translocation from roots to shoots. However, chilli (*Capsicum annuum*) and coriander (*Coriander sativum*) cultivated in lindane contaminated soil were reported to contain γ-HCH residues in their aerial parts (84, 85). This fact was considered as a clue for the selection of plants able to extract lindane from a hydroponic system.

Wide range of lindane half-life in water is found in literature: it is 47.9 hours at pH 9 and 100.7 hours at pH 7, and no hydrolysis is observed at pH 5 according to Herbst (78). Tomlin (166) reported half-life of lindane of 11 hours at pH 9 and 191 days at pH 7, whereas in sea water, at pH 8, half-life is 75 days at 30°C and 42 years at 5°C (113). These data show clearly that hydrolysis of lindane depends on pH, temperature, and the presence of catalytic components such as minerals in water. Therefore, hydroponic system should be handled with care.

0.2 Material and methods

Plant preparation

Seeds of chilli (*Capsicum annuum*, Samen Mauser, Switzerland) and coriander (*Coriander sativum*, Samen Mauser, Switzerland) were sown in quartz sand and after germination, plants were transferred in hydroponic reactors for 1 month before uptake experiments. Plants were maintained under a cycle of 18 hours light and 6 hours dark, with artificial light (Glow ®, Switzerland), 25°C during day and 22°C during the night. Humidity was not constant, with an average of 50% relative humidity.

Reactors

Vetiver reactors were Erlenmeyers of 100 [mL] wrapped in aluminium foils to prevent any possible photolysis of tested compounds and growth of algae. Vetiver plants in hydroponics were supplemented with commercially available ready Hoagland Basal Salt full strength (Sigma). The level of water was checked through a mobile aluminium window. Plants were watered with nutrient solution every 2-3 days through refill pipe with a syringe of 5 [mL] to 100 [mL], the original volume.

Aeration

Aeration and refill pipes were made with a Teflon capillary of 0.5 [mm] internal diameter (Maagtechnic). Aeration of plants was done by air sparging. A hydrophilic filter of 0.2 [µm] (Sarstedt) was installed between the aquarium pump (ACO-9530,

Jun®, Switzerland) and the reactor to avoid microbial contamination from ambient air. It could be observed with the help of an O_2 electrode (Oxy 96, WTW), that plants at 25 °C consumed 70% of dissolved oxygen within 2 hours. Therefore, intermittent sparging was controlled with a timer (20 min every 2 hours).

pH of hydroponic solution

As hydrolysis of lindane is pH dependent, the study of the effect of plant on lindane in hydroponic solution was performed at pH 5. This pH was automatically obtained after addition of 1.6 [g/L] Hoagland salts in water. No buffer was used to avoid unknown interactions between the buffer and the plant.

Study of the effect of plant on lindane in hydroponic solution

Autoclaved Hoagland solution was spiked with 1000 ppm lindane dissolved in methanol filtered with a hydrophobic filter of 0.2 [µm] (Sarstedt) to a final concentration of 2 ppm. Every day for 9 days, the effect of plant on lindane concentration in solution was followed and compared to its corresponding control without plant.

Adsorption/partition on roots

Roots of chilli and coriander freshly cut (about 20 g) were dried on paper and introduced in bottles. The bottles were filled to avoid any gas phase with sterile Hoagland solution spiked with 1000 ppm to a final concentration of 2 ppm. Bottles were airtightly closed with a butyl septum, to avoid any gas leakage. Bottles were agitated on a horizontal shaker at 200 rpm for 9 days at room temperature. Sampling was done with a microlance syringe through the septum at time 0, 1, 17, 77 hours and 9 days. Adsorption and partition values were deduced from the value of disappeared lindane in solution compared to control bottles without roots.

To see the effect of “pre-saturated lindane roots” on lindane concentration in medium, an other pool of chilli were pre-saturated for 9 days in 2 ppm lindane solution, then roots were washed briefly in distilled water, dried on paper and put back in a new solution containing 2 ppm lindane in Hoagland solution. Lindane concentration in solution was followed at time 0, 1, 24 hours.

Liquid-liquid extraction of lindane (LLE)

Two [mL] of hexane were added to an aliquot of 200 [µL] of hydroponic solution. One [mL] of sulphuric acid was then added to the biphase to purify the sample to be analyzed by GC-ECD. The mix was shaken with a vortex for 2 min. and centrifuged for 5 min at 2000 g to obtain a biphase, from which hexanic supernatant was transferred into GC vials after a concentration step under a nitrogen flux. Lindane extraction by LLE was 90%.

GC-ECD

Analysis of lindane disappearance in water was performed by injection of 5 [µL] of sample in a Gas Chromatograph (GC Varian Star 3400*cx*) equipped with an electron captor detector (ECD) and a capillary column (CP-Sil 8CB). Operating temperatures were constant through the analysis and were 200°C for the column, 280°C for the injector and 300°C for the detector. Nitrogen flux on column was 2.6 [mL/min], with a split mode of 50 [mL/min]. The linearity of the GC-ECD was tested from 0.2 [mg/L] to 5 [mg/L]. The calibration curves had a coefficient of determination r^2 = 0.991 for all isomers.

0.3 Results

Controls without plants

Controls without plants showed 90% disappearance of γ-HCH within 24 days showing that the chosen hydroponic system was irrelevant without modifications for the study of lindane behaviour in hydroponic solution with a plant. Amongst different hypothesis, i.e. stripping, evaporation at the step of volume reduction of samples by nitrogen flux, metabolization by micro-organisms and hydrolysis, stripping and hydrolysis were the most important factors explaining γ-HCH disappearance. γ-HCH disappearance was 45% within 15 days under sterile conditions at pH 5 in Hoagland solution and constant sparging. Respective contribution of stripping and hydrolysis that might be catalyzed by some nutritive component of the medium was not determined.

The loss was reduced to 17% within 9 days by using minimum air sparging (see material and methods).

Adsorption and partition of lindane on roots

Adsorption/partition of lindane on chilli roots was taken place within few hours as shown in (**Figure 0.2**)**.** Equilibrium was reached after 17 hours. Pre-satured chilli roots showed a decreased capacity for adsorption/partition compared to non satured roots (**Figure 0.3**)**.**

Effect of plants on lindane concentration in hydroponic solution

Within 9 days, lindane concentration in medium decreased by 70% with chilli plants and 86% with coriander. 29% and 40% disappearance of γ-HCH was due to adsorption on the roots of chilli and coriander respectively, as demonstrated by adsorption experiments (**Figures 0.4 and 0.5**)**.**

The 23% and 30% remaining loss were called “plant effects”. The pH of reactors with plants increased to 6.8 after 9 days incubation in hydroponic system, showing that plant activity changed the pH of the solution. Plant effects included the increasing of pH from 5 to 6.8, leading to increased hydrolysis, and a possible uptake with transpiration flux of γ-HCH in unknown quantities.

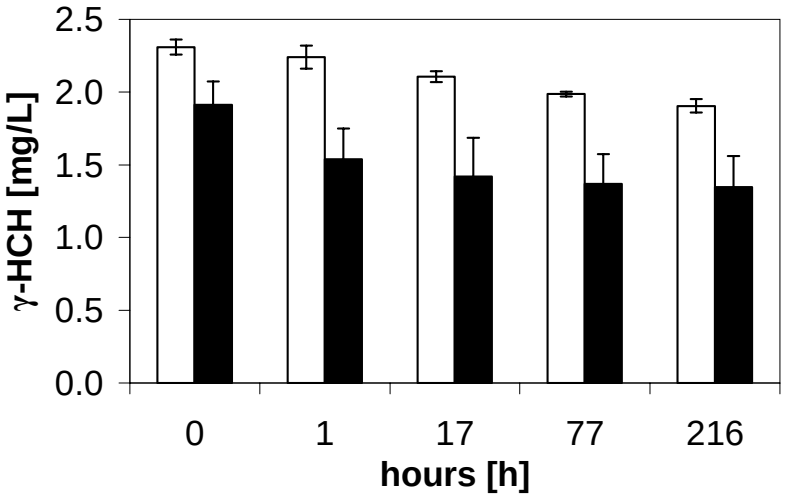


Figure 0.2 Effect of chilli roots on lindane concentration in the media

Control without roots (white), chilli roots (black). Data points represent the mean of 5 replicates determinations. Bar = SD

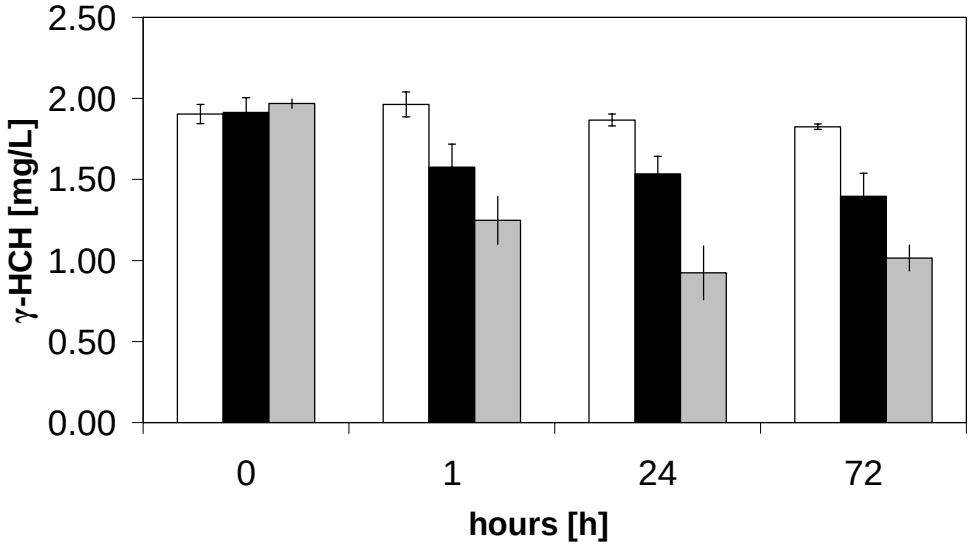


Figure 0.3 Effect of pre-saturated and non saturated chilli roots on lindane concentration in the media

Control without roots (white), pre-saturated chilli roots (black), non saturated chilli roots (grey). Data points represent the mean of 5 replicates determinations. Bar = SD

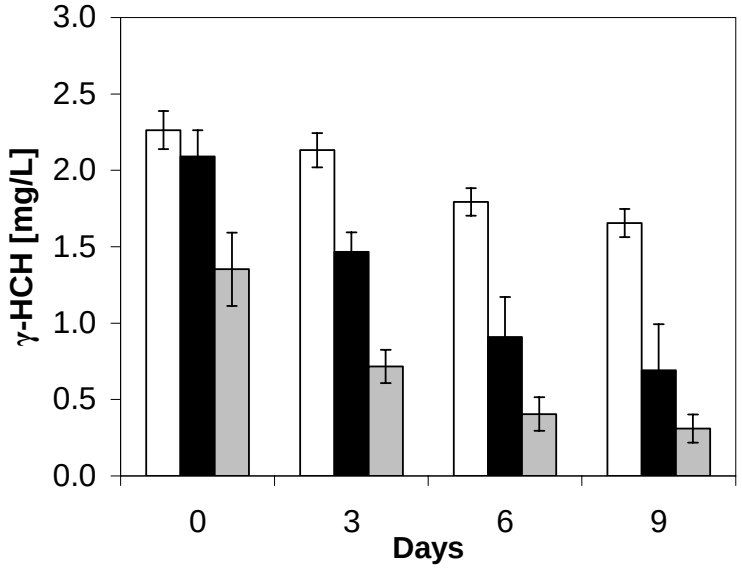


Figure 0.4 Time course experiment of γ-HCH disappearance in solution with chilli and coriander

Controls (white columns), chilli (black columns), coriander (grey column). Data points represent the means of 5 replicates determinations. Bar = SD

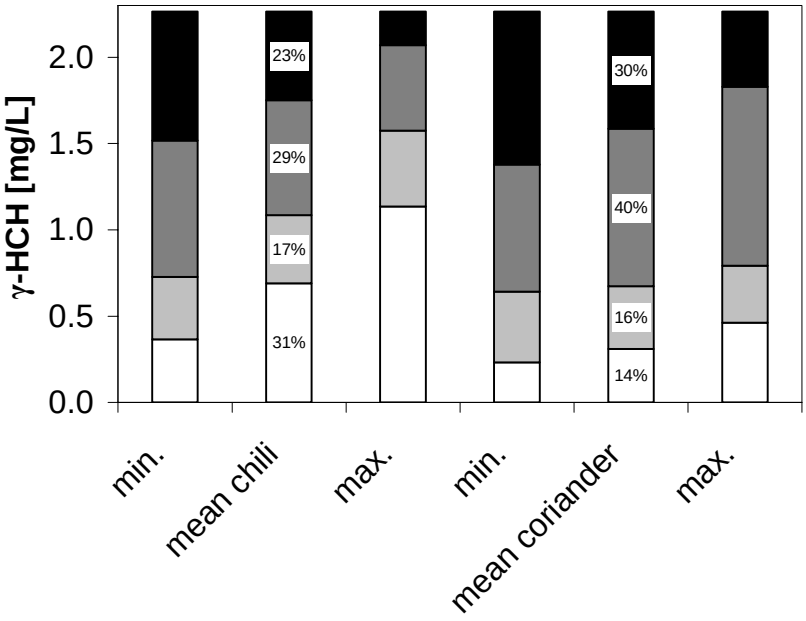


Figure 0.5 Minimum, maximum, and mean effects of chilli and coriander on γ-HCH disappearance in solution

Final remaining concentration of lindane (white), loss of the system (pale grey), adsorption/partition in roots (dark grey), and plant effect (black). Data points represent the means of 5 replicates determinations. Bar = SD

0.4 Discussion

Adsorption/absorption on roots values have to be taken with care. Although volatilization was minimized in bottles by reduction of air compartment, enhanced hydrolysis by increased pH could also contribute to lindane disappearance from the medium. Lindane disappearance linked to plant effect was defined as a combination of increased pH leading to increased hydrolysis and uptake with transpiration of the plant. Without radioactivity, it seemed that the respective contribution of these two phenomena to lindane disappearance in the medium was not possible.

The deduced plant effects of coriander and chilli were in the same range. As aerial plant analysis was not done, the contribution of transpiration could not be evaluated. However, it is believed that this latter would be small compared to the effect of increasing pH and substances excreted in the medium that could hydrolyze lindane.

Important stripping of lindane from hydroponic solution by direct air sparging in the medium was confirmed by a project partner, Dr Raghu[1], with the use of ^{14}C-lindane allowing mass balance between medium, air and content in the plant. Stripping of organics is possible for compounds with slight water solubility and polarity, despite of a low vapour pressure (7). Persistence of γ-HCH in soils seems to be best explained by a partition on the solid phase of the soil preventing hydrolysis and leading to poor bioavailability to micro-organisms and plants; a parallel of persistence in soil and in a liquid system (hydroponic) should not be done. Moreover, temperature and pH are also parameters strongly influencing lindane concentration in aqueous solution, as well as hydroponic medium containing essential mineral nutriments for the plant, probably acting as catalysts of hydrolysis. As a comparison, half-life of the pesticide carbosulfan at pH 7 is estimated to be 1 year in aqueous solution, while the half-life in montmorillonite suspensions is only 5 days (182). Therefore, study of plant effect on lindane concentration in hydroponic medium is possible, but delicate.

There is an interesting parallel to be done with Harms and Zehnder publication (73) about the relevance of bacterial activities on hydrophobic organics in liquid medium compared to soil environment: it is difficult to extrapolate the bacterial activities observed in liquid cultures in the laboratory to field conditions. This notice is also

[1] Dr K. Raghu, Jai Research Foundation (JRF), Valvad, Gujarat, India

relevant for the study of lindane in hydroponics, a high hydrophobic compound with low bioavailability in soils for organisms.

As lindane is classified as non-systemic insecticide, even if not proved experimentally, phytoextraction followed by translocation in leaves should be very low or nil in coriander and chilli; it seems that residues of DDT in the foliage of plants were shown to result from vapour uptake of pesticides (112). Similarly, the levels of DDT, HCB and lindane detected in foliage of *Phaseolus vulgaris* (dwarf bean) are not dependent on pesticide concentration in soil. The xylem translocation of the pesticide in the bean plants is negligible, and the main route of uptake by the foliage appeared to be through vapour absorption (10). Limitation of phytoremediation to remove hydrophobic pesticides from soils has been recently summarized in the review of Chaudhry et al. (37).

In conclusion, the use of a hydroponic system to understand the role of phytoextraction and/or phytotransformation of lindane from liquid medium is not really relevant without the use of radioactivity and in regard to lindane low bioavailability in soils. Translocation chances of lindane from roots to shoots are low, due to lindane hydrophobicity, unless special molecules exuded by plants able of increasing the apparent aqueous solubility of hydrophobic pollutants, as shown by Campanella and Paul for dioxin (31).

Part II phytoremediation of atrazine contaminated environment

1 Triazines in soils and agriculture run-off

1.1 Use of atrazine

Biziuk et al. (20) pointed out that the number of known organic compounds is estimated to be about 16 millions, from which 2 millions are produced by chemical synthesis. Every year, approximately 250'000 new compounds are synthesized, from which 1000 are produced at an industrial scale. In 1996, 70'000 organic compounds were commercially available with an annual global production of 100-200 million tons. Approximately one-third of all organic compounds end up in the environment. Annually, the worldwide production of pesticides is several hundred thousand tons.

Annually, in the U.S.A., atrazine use has been estimated to be 40'000 tons (129), and 5'000 tones in China (83). Before the ban of atrazine in France in 2003, Ravanel et al. (133) mentioned that 10'000 tons of atrazine were spread on cultivated areas in this country. As been told by Mr R. Kumar[2], pesticides in India have contributed significantly to the development of agriculture during the "Green Revolution". According to a study carried out by scientists of Indian Agriculture Research Institute, the use of pesticides on Indian soil has been increasing at a rate of 20% per annum since 1989. Atrazine constitutes the most widely used herbicide in India, in corn and sugarcane crops, of which India is one a leading producer. Producers of atrazine in India are: Novartis Ltd, Rallis India Ltd, Sanachem Ltd, Drexel Chemical Co, and Markfed Agro Chemicals Co. The present total consumption estimation done by one of the leading manufacturers in India is approximately 1'000 tons per annum. The major atrazine consuming states are Punjab, Haryana, Uttar Pradesh, Bihar, and Southern states.

Atrazine has been the most important herbicide used over the last 30 years for non selective weed control on industrial and non cropped land (129). But it is also used as pre-emergence herbicide for selective control of weeds mainly in maize, sorghum and sugarcane cultures, as well as in aspargus, vines, coffee, oil palms, roses, grassland, forestry, fruit orchard such as citrus, bananas, pineapples, guavas, and macadamia (166), and in irrigation channels for cotton production (170). It is phytotoxic for most

[2] Dr Raj Kumar, Chief Chemist of Markef Agrochemic, India

species including agricultural crops, such as most vegetables, potatoes, soybeans, and peanuts (166).

1.2 Persistence of atrazine in soils

Atrazine is classified as a moderately persistent chemical, with half-life ranging from several weeks to months in soil (99, 129), with an average of 40 days (193). But atrazine is extremely persistent in clay and sandy loam soils at 15°C, with half-life of 105 and 166 weeks respectively (170), whereas half-life of atrazine and its derivatives has been shown to exceed 170 days in aquifer sediments (129).

Yanze Kontchou and Gschwind (193) cited studies where it was observed that mineralization in soils proceeds slowly: Wolf and Martin (190) recovered only 20 % of ^{14}C-atrazine applied on soil as $^{14}CO_2$ after 2 years of incubation, and Mc Mahon et al. (105) recovered only 0.1% $^{14}CO_2$ from ^{14}C-atrazine after 23 days. In contrast, after 38 weeks, biotransformation of atrazine in anaerobic wetland sediments ended with 20% of non–triazine compounds (40). In surface soils, several studies indicate that atrazine is degraded by micro-organisms mainly into dealkylated metabolites (2, 22, 107, 144, 160) (see also chapter 2 and chapter 10) or it is adsorbed tightly to the soil matrix (96), whereas in vadose zone and aquifers, persistence seems to be linked to low temperature and to the lack of degrading organisms.

Coastal plains are believed to be particularly sensitive to groundwater contamination, because of their low organic matter content (88). Tasli et al. (163, 164) showed that due to the importance of heavy rainfall in a fluvio-glacial soil type, leaching of atrazine occurs after the first month following treatment of 1 [kg/ha]. Businelli et al. (30) assessed the potential danger of groundwater pollution by atrazine in the Po Valley in Italy, and concluded that atrazine should be avoided in sandy soil, and could not be used in irrigated crops. Piccolo et al. (124) and Loiseau (96) explained that atrazine mobility in the soil profile and its potential contamination of groundwater are linked to the humic substances content of the soil: atrazine adsorption on humic substances is mostly controlled by the hydrophobic content of humic material. Dramatic increase of atrazine adsorption is observed when aliphatic character increases. This was confirmed later by Loiseau et al. (96) who observed that the release of non-extractable (bound) residues of atrazine from fulvic organic matter is 89% atrazine and the rest is hydroxyatrazine. Meiwirth (106) showed that

contamination of alluvial aquifer is high in the Rhone Valley plain of Switzerland, due to poor content in organic matter, lack of biological activity, and rapid transport of the applied pesticide on sloppy fields.

1.3 Atrazine contamination of water

The European Union edicted the maximum concentration limit (MCL) of a single pesticide in drinking water to 0.1 [µg/L] and the total concentration of all pesticides to 0.5 [µg/L] (49). U.S. Environmental Protection Agency (EPA) fixed the maximum level of atrazine in water at 3 [µg/L] (129), whereas no total concentration of pesticides is defined in soil. Switzerland is considering to follow the European Union regulations related to drinking water: the maximum allowed level of atrazine in fresh water is also 1 ppb (110). The use of atrazine in Switzerland has been restricted to pre-emergence treatment in maize fields ten years ago and was forbidden as non selective herbicide on industrial and non cropped land (30, 65). Atrazine was banned in 1991 in Germany (49), and more recently in Italy and France (30). Although several countries gave up the use of atrazine, it is still one of the most popular herbicide in the world (83), and many countries did not fix any MCL in water, such as India (http://www.pmfai.org).

In 1998, in the U.S.A, a national water quality assessment study showed that atrazine was the most frequently detected compound, representing 38% of water samples contamination from 1034 sites, from which several sites exceeded MCL (Kolpin et al., cited by Businelli et al. (30)). Miller et al. (108) explained that the Midwest States Nebraska, Minnesota, Iowa, Illinois, Indiana, and Ohio commonly known as the cornbelt are a major source of atrazine to the Great Lakes because of intensive use. In China, atrazine was detected in large areas of the Yang River and Guanting Reservoir and as a results from the discharge of pesticide plant (point source pollution), with level of atrazine ranging from 0.22 to 26.1 [µg/L] (83). Atrazine is also the most widely and frequently detected herbicide in Northern rivers of Australia, up to 15.9 [µg/L] (170). In Slovenia, the most frequently detected herbicides in rivers and groundwater are atrazine and alachlor, up to 1 to 6 [µg/L] (194). In Switzerland, in 1999 and 2000, despite of restricted use of atrazine, concentration of the herbicide exceeded 0.1 [µg/L], in Lake Greifensee, during the main application period from mid-May to end of June (65). Average concentration of 0.6 [µg/L] atrazine

concentration was measured in groundwater in corn growing regions of Germany at the beginning of the 90's (49).

Several authors showed that atrazine occurrence in water is not constant, but peaks of contamination coincide with first rain event following pre-emergence application on nude soils (65, 106, 163, 164). Early in the season after application of herbicides, the concentration of atrazine is high, but later in the season after degradation, at least 50% of the load are dealkylated metabolites, also able to enter surface water and groundwater (165).

In summary, the level of water contamination by atrazine and other pesticides in general depends on physical-chemical and biological degradation of the parent compound, soil type, frequency of use, and rainfalls following application.

But physical-chemical properties of pesticides can also explain water contamination, with no exception for atrazine (**Table 1.1**): when applied to fields, it has little tendency to volatilize because of its low Henry constant and low vapour pressure. A moderate log K_{ow} together with a nil charge at almost all encountered soil pH (low pKa) can explain horizontal and vertical move of atrazine in soils, and uptake by plants: Ravanel et al. (133) showed that on mineral or organic soil thin-layer chromatography, migration of lipophilic pesticides with moderate log K_{ow} in water solvent development system is the highest, like diuron (log K_{ow} 2.7) and phenmedipham (log K_{ow} 3.75); the plant uptake and translocation of neutral organic compounds from soil through the plant root occurs for chemicals not too hydrophobic, with optimal transfer range between log K_{ow} 1.5 and 3 (25, 38, 141, 146).

Table 1.1 Physical-chemical properties of atrazine

	pKa	**Solubility**	**Log K_{ow}**	**Vapor pressure**	**Henry law constant**
	1.68	33	2.5	0.039	2.48 X 10^{-4}
Units	-	[mg/l]	-	[mPa]	[Pa m^{-3} mol^{-1}]
Source	(137, 166)	(137, 166)	(137, 166)	(137)	(32)

1.4 Health and environmental hazard of atrazine

Until recently, it was widely believed that because plants differ from animals in their morphology and physiology, herbicides would be of relatively low risk to animals and humans. But recently, some data indicated that this assumption is not valid.

Low concentration of atrazine in water causes health problems: Van Zwiten (170) cited different studies, where it was demonstrated that the formation of N-nitroatrazine by ingestion of atrazine in conjunction with nitrite at low pH causes up to 10'000 times the normal chromosome breakage in human lymphocytes, and that atrazine in groundwater supplies increases incidences of non-Hodgin's lymphoma. Leeuwen et al. (169) found that atrazine and nitrate contamination levels in drinking water are positively associated with stomach cancer incidence in Ontario (Canada) for the period 1987-1991. Recently (2002), the U.S. Environmental Protection Agency (EPA), Office of Pesticide Programs (1), issued a report where triazines are grouped on the basis of a common mechanism of toxicity: it was observed that atrazine, simazine, propazine and their common dealkylated derivatives are toxic because of disruption of the hypothalamic pituitary gonadal (HPG) axis, leading to attenuation of the luteinizing hormone (LH). Cumulative low doses of triazines and their derivatives dealkylates lead to delayed puberty in rats, loss of pregnancy, disruption of the oestrous cycle (anovulation), and increased risk of mammary gland tumors in rats. Aromatase activity responsible for oestrogen synthesis of human cells is inhibited by atrazine and its major metabolites, explaining apparent endocrine disrupting effects of atrazine in rats (116).

Low concentration of atrazine in soils also causes environmental problems: the choice of crops that can be grown on the land the year after herbicide application can be reduced if it persists the following season, as shown by Delmonte et al. (54) who observed reduced oat growth caused by residual phytotoxicty of atrazine treated soil.

Aquatic fauna is especially exposed to atrazine pollution, since it lives permanently in contact with polluted water. In the aquatic system, Koncan (194) observed considerable adverse effects of atrazine on net biomass production of algae in rivers. Low exposure concentrations (0.01 to 500 ppb) of atrazine increases male production of *Daphnia pulicaria* (57). It was observed by Bisson et al. (19) that atrazine applied to cells of rainbow trout disrupts adrenal steroidogenesis. In Atlantic salmon, the

reproductive endocrine function is altered: steroid synthesis of the testes is inhibited at atrazine concentration of 0.04 [μg/L] (109).

Atrazine is also deleterious to amphibians: it depresses plasma corticosterone in larval tiger salamander, decreases intracellular dopamine and norepinephrine of neuronal cells (19). In the U.S.A., atrazine is mainly applied as a pre-emergence treatment in corn fields during spring time, which coincides with heaviest contamination of water and breeding activity of many amphibians; Hayes et al. (77) observed that male larvae of *Rana pipiens* exposed to 0.1 to 25 ppb atrazine develop testicular oocytes and that gonadal development is retarded. Allran and Karasov (5) observed deformed larvae of *Rana pipiens*, *Rana sylvatica* and *Buffo americanus* with atrazine concentration of 0.2-20 [mg/L], and concluded that these atrazine concentrations deleterious to amphibian are considerably higher than actual concentration found in surface water in America, and that direct toxicity of atrazine is probably not significant factor in recent amphibian declines.

In conclusion, atrazine is generally moderately persistent in soil, but its intensive use associated with a relative high mobility in soil contributes significantly to water contamination, causing adverse effects on human health and environment. Studying plant uptake and metabolism of atrazine is indeed relevant to prevent contamination of water, and to remove atrazine from contaminated soil or water.

2 Resistance and tolerance of plants towards atrazine

Several authors have studied plant metabolism of agrochemicals to understand its role in herbicide selectivity (41, 42) and to predict effective control of weeds (82). Such fundamental understanding is useful to discover new selective herbicides, as well as to obtain genetically modified plants resistant to herbicides. Plant metabolism of pesticides is well documented for primarily annual agricultural crops and grasses (82), but it is scarce in woody perennials plant species, except for poplar trees (27, 28, 110).

In the present study, vetiver plant metabolism was explored for phytoremediation of soil contaminated with atrazine, and for risk assessment of this remediation technique.

It is important to highlight that atrazine is mainly used as a pre-emergence herbicide, and therefore, concerning plant resistance and/or tolerance, germinating and small plantlets have to be considered. Van Asche (168) described that death of weed is only obtained for a total inhibition of photosynthesis for more than one week. With increasing plant leaf biomass, saturation becomes impossible due to the increasing number of target sites and atrazine dilution in biomass. Therefore, when high biomass is considered, any plant species is “resistant” to atrazine.

Many plants are not sensitive to atrazine. Some of them acquired with time a resistance and other tolerates atrazine thanks to degradation of the active moiety by chemical pathway (hydroxylation) and enzymatic pathway (N-dealkylation and conjugation). Selectivity of the herbicide atrazine is based on its tolerance by plants. For example, maize and sorghum tolerate atrazine by chemical and enzymatic transformation of the herbicide.

2.1 Chloroplastic resistance

The appearance of herbicide resistant weeds has become an important agricultural concern all over the world. Triazines are the herbicide family with more resistant weed biotypes reported and characterized than any other group. In fact, most of the resistant biotypes are found in fields that have been in continuous monoculture and where the same herbicide has been repeatedly applied for several years (67). In general, herbicide developed resistance in plants can be described in 3 categories: (i)

limited uptake and translocation; (ii) enhanced metabolism and detoxification; (iii) modified target site. Due to the selective pressure, it was found that with time, some plant species became resistant to atrazine. For the period 70's to the 90's, it was commonly admitted, that the modified target site was the widest spread mechanism in plants treated with photosystem II inhibitors. A point mutation of the gene was identified as responsible for D1 protein modification, causing a chemical or conformational change of the binding site of atrazine. Because of this modification, D1 protein is no longer able to fix atrazine (79).

Chloroplastic resistance was described in biotypes of *Senecio vulgaris* (140), *Chenopodium album* (161), *Brassica campestris*, *Solanum nigrum*, *Poa annua*, *Setaria viridis*, and *Phalaris paradoxa* (143). In 1982, Le Baron and Gressel (94) indexed 76 biotypes resistant to atrazine.

At the beginning of 90', some authors also showed that enhanced metabolism of plant species could explain plant acquired resistance to atrazine (51, 52, 67, 68, 179).

2.2 Tolerance via metabolic pathways

2.2.1 N-dealkylation

The N–dealkylation is an enzymatic reaction leading to deethyl atrazine (DEA), deisopropyl atrazine (DIA) and didealkyl atrazine (DDA) (**Figure 2.1**). This metabolization pathway is dominant in *Asperillus fumigatus* (87), pea (*Pisum sativum)* (152), *Spartina alternifora* (125), and potato (*Solanum tuberosum*) (61). But dealkylation was also found in maize, as a minor metabolic process (39). A study on isolated chloroplast from oat showed that the phytotoxic activitiy was reduced 23 times for monoalkylates, as compared to atrazine (153, 156).

Studied species undergo dealkylation to different extent. Only in pea, dealkylation is fairly important, and confers intermediate resistance to atrazine (152). The other species with slight dealkylation are sensitive to atrazine such as *Asperillus fumigatus* (87), *Spartina alternifora* (125), potato (*Solanum tuberosum*) (61), soybean (*Glycin max*), and wheat (*Triticum sp.*) (155) (**see also Figure 2.1**).

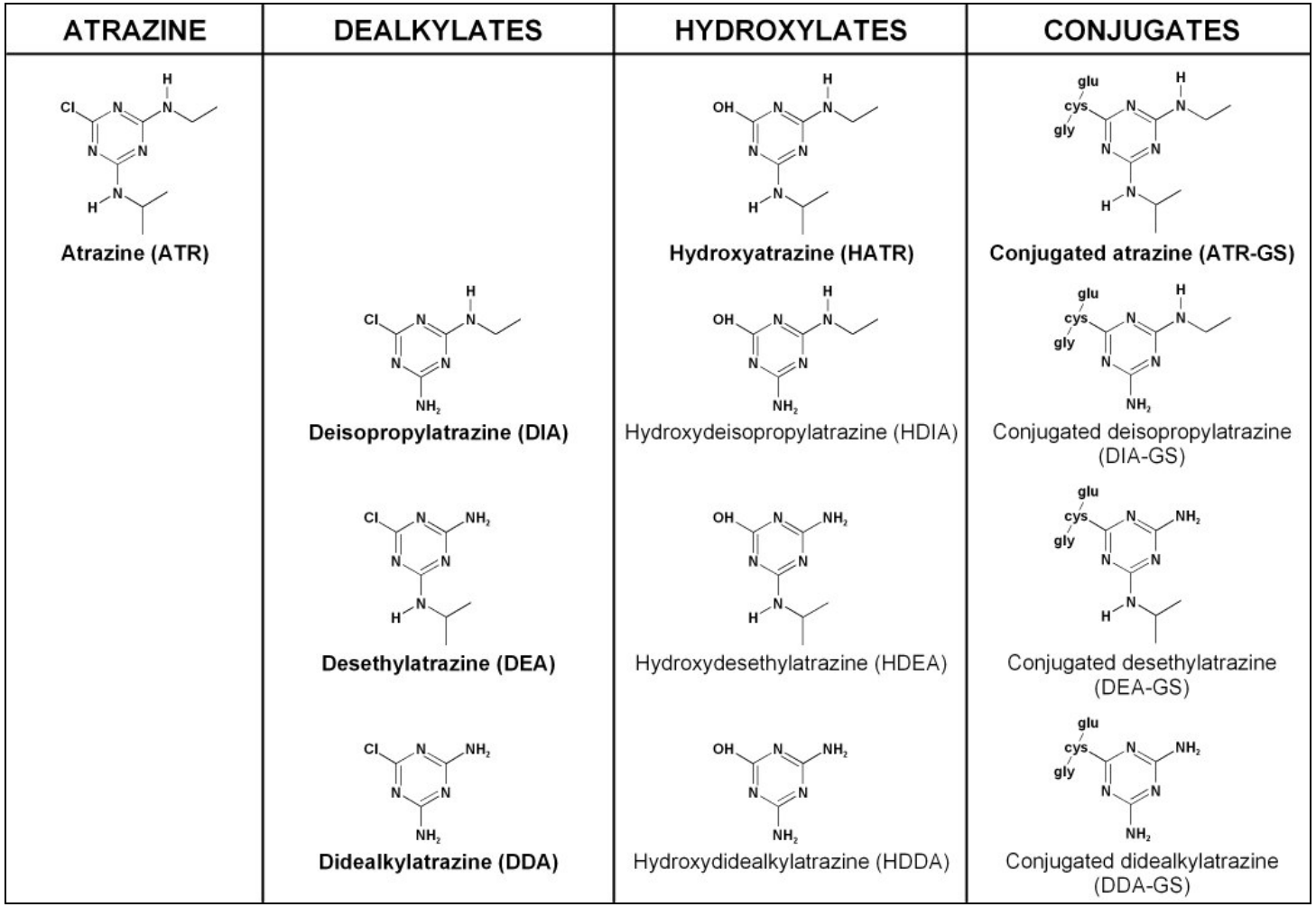


Figure 2.1 Main known metabolites of atrazine

Detectable molecules in the present thesis are represented with bold legends

Monuron, chlorotoluron, prosulfuron, metolachlor, alachlor herbicides are dealkylated by cytochrome P 450 enzymes (P 450). Plant P 450s are membrane-bound to the endoplasmic reticulum (43). They are powerful oxidizing catalysts, which activate molecular oxygen and insert typically one oxygen atom (as a hydroxyl group) into lipophilic substrates. But oxidation of heteroatoms like N was also found to give dealkylated products (70). P 450 involvement of *in vitro* plant microsomal preparations could be shown by its CO inhibition (21, 186). The cytochrome P 450 inhibitor 1-aminobenzotriazole (ABT), used in combination with simazine in *Lolium rigidum,* causes a greater reduction in dry mass of resistant plants than simazine applied alone (29). This suggests involvement of oxidative enzymes in the mechanism of dealkylation of simazine. Triazines are analogues and share exactly the same dealkylates, therefore conclusion on simazine could be extended to atrazine (**Figures 2.1 and 2.2**).

Although atrazine dealkylation occurs in many plant species, no literature mentions which are the enzymes involved in this metabolic process. Two reasons could explain this lack of information concerning dealkylation of atrazine: (i) N-dealkylated metabolites of chlorotriazines herbicides do not result in complete detoxification and are generally associated with species which are sensitive to these herbicides (61), unlike chlortoluron for example where two successive dealkylations resulted into non phytotoxic metabolites (187). (ii) Studying P 450 in plants is rather difficult. P 450 content is generally low (5-50 pmoles/mg microsomal proteins) and estimations are often unreliable, because of plant pigments such as flavonoids, xanthophylls, carotenes and especially chlorophylls, which interfere strongly with P 450 determination by the standard CO difference spectra (59).

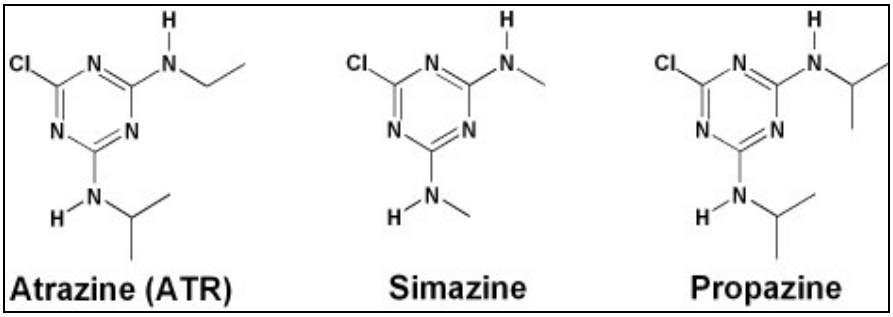


Figure 2.2 Triazines analogues of atrazine

2.2.2 Hydroxylation

Chemical transformation of atrazine into hydroxyatrazine has been well studied in maize plant (34, 134) (**Figure 2.1**). Hydroxylation was described not only on atrazine, but also on dealkylates DEA, DIA and DDA (150). The obtained metabolites are namely hydroxyatrazine (HATR), hydroxydeethyl atrazine (HDEA), hydroxydeisopropyl atrazine (HDIA), hydroxydidealkyl atrazine (HDDA) (**Figure 2.1**). The replacement of the chlorine atom by a hydroxy group results in non phytotoxic metabolites (34), explaining mainly maize tolerance to atrazine. Chemical pathways leading to the formation of the inactive hydroxyatrazine is the pre-eminent form of metabolization inside the roots and, during the first week, inside the leaves of maize (39). The formation of a glutathione-atrazine conjugate, due to the activity of a glutathione–*S*-transferase (GST), although existing, is small, and beside all, is only fully effective after 1 week culture for maize plantlets. Under *in vitro* experimental conditions, a benzoxazinones mixture extracted from corn plantlets is able to transform 90% of atrazine into hydroxyatrazine within 24 hours (135). This *in vitro* experiment was correlated with pre-eminent hydroxyl atrazine metabolites present *in vivo* in maize plantlets (136).

Natural benzoxazinones were discovered 40 years ago, when resistance against pathogenic fungi was investigated. They play a major role in the defense of cereals against insects (114, 115), fungi and bacteria (114), chelation of Fe^{3+}(120, 121), in allelopathic effects (12, 111) and in the detoxification of herbicides (71, 114, 135). The family of benzoxazinones is divided in several classes, namely the cyclic hydroxamic acids, lactams, methyl derivatives, and benzoxazolinones. Benzoxazinones are predominantly found in the family *Poaceae* including genera *Aegilops*, *Arundo*, *Chusquea*, *Coix*, *Elymus*, *Secale* (rye*)*, *Tripsacum*, *Triticale*, *Triticum* (wheat), and *Zea mays* (maize). They are not present in *Avena* (oat), *Hordeum* (barley*)*, or *Oriza* (rice) (114). But they were also identified in *Acanthaceae*, *Ranunculaceae*, and *Scrophulariaceae*. Interestingly, sorghum (*Poaceae*) is subject to contradictory information: Hamilton and Shimabukuro (71, 149) found no benzoxazinone in sorghum, whereas the review of Niemeyer (114) mentioned that other authors detected benzoxazinones in this plant.

It seems that plants lacking benzoxazinones fail in producing hydroxylates. On the other hand, wheat contains benzoxazinones and soybean not, whereas both plants are sensitive to atrazine (71).

Within cereals, DIMBOA is the main hydroxamic acid derivative occurring in wheat and maize, whereas DIBOA is pre-eminent in rye (114). Acronyms are used in the literature that are derived from the chemical designation, e.g. DIMBOA, the best-studied benzoxazinone, is derived from 2,4-**di**hydroxy-7-**m**ethoxy-2*H*-1,4-**b**enz**o**x**a**zin-3(4H)-one. Since DIMBOA and DIBOA are highly toxic, they are glucosylated and stored in the vacuole. These glucosides are readily hydrolysed when the structural integrity of the tissue is destroyed and the toxic aglucone is set free. DIBOA in rye and DIMBOA in maize seedlings are present at relatively high concentrations, up to 1 mg/g fresh weight (158), explaining why hydroxylation of atrazine and simazine are so pre-eminent.

2.2.3 Conjugation

The reaction of conjugation means that the chlorine atom of the triazinic cycle of atrazine is replaced by a substance produced by plant, the tripeptide glutathione (**Figure 2.3**). Glutathione-*S*-transferases (GSTs) are enzymes which act on hydrophobic, electrophilic, and cytotoxic substrates. GSTs have been found in virtually all living organisms. Cytotoxic substrates include xenobiotics, and they have been well studied with regard to herbicide detoxification. Natural role of GSTs include involvement in stress response: oxidative stress, heavy metal toxicity, response to auxins during plant secondary metabolism of anthocyanins and cinnamic acids, and targeting molecules for transmembrane transport (101), thanks to a vacuolar ATP-dependent glutathione-conjugate transporter (102). In sorghum, conjugated atrazine is not an end product, but is subjected to later transformation. Four other successive conjugates have been identified, namely γ-glutamylcysteine, L-cysteine, *N*-acetyl-L-cysteine and lanthionine conjugates (91, 93). Conjugation of atrazine was reported to occur in many species, in cultivated plants such as maize (56, 150, 151, 155) and sorghum (91, 93), but also in weeds, such as *Digitaria sanguinalis* (76), *Echinochloa crus-galli* (76), *Panicum miliaceum* (52, 76), *Setaria sp* (51, 67, 76, 179), *Abutilon theophrasti* (68, 127). Very interestingly, if grouped taxonomically, atrazine tolerant species are members of the subfamily *Panicoideae*, whereas sensitive

species, such as quarkgrass, oats and barley are in the *Festucoideae,* as shown by Jensen et al. (82). To sustain this observation, more than 40 grass species belonging to *Festucoideae* and *Panicoideae* subfamily. They found that high yield of conjugation was observed in tolerating *Panicoideae* grasses such as *Setaria sp,* and *Sorghum sp.* compared to low or nil conjugation in sensitive *Festucoideae* such as *Avena sp, Bromus sp*. and *Hordeum sp.* As vetiver is belonging to *Panicoideae* subfamily, it is interesting to know if vetiver confirms or invalidate Jensen et al. observation.

Recently, Pflugmacher et al. (123) showed that conjugation of atrazine could be detected in many plants, including marine macroalgae. It was concluded that the evolutionary "green liver" concept derived for xenobiotic metabolism in higher plants is also valid for lower plant species macroalgae, distributed worldwide with enormous biomass.

Some GSTs have been reported to be inducible by safeners, elicitors and ozone (123). To increase tolerance of the treated plant, "safeners" are commonly used to enhance GSTs activities (62, 90), but these compounds are used above all for chloroacetanilide herbicides.

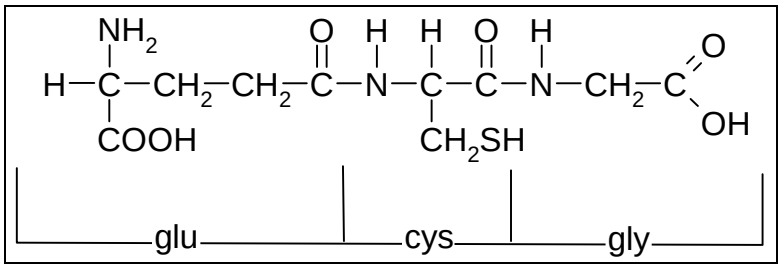


Figure 2.3 Glutathione chemical structure

glu = glutamate; cys = cysteine; gly = glycine

Conjugation can be explained as follow: electrophilic reagents react preferentially with nucleophilic sites. This selectivity can be best explained by the concept of hard and soft electrophiles/nucleophiles. The softest cellular nucleophilic site is the nonbonded pair of electrons in the sulphur atoms of the thiol group of cysteine residue in glutathione, allowing conjugation with electrophiles. This reaction is either spontaneous, or can also be enhanced by GSTs (43).

The percentage of non enzymatic conjugation of atrazine is reported as being as high as 79% in some maize varieties (80), but only 6% for other varieties (76). In sorghum, non enzymatic conjugation is reported to be 10-20% by Lamoureux et al. (93).

Maize tolerance tightly relies on GSTs action when leaf treatment is applied, but when atrazine is applied to roots, hydroxylation seems to confer protection (39, 136, 151, 155). In sorghum, tolerance was found to be related to high capacity of conjugation (91, 93, 149, 150).

2.2.4 Plant atrazine metabolism

Maize undergoes the 3 pathways of atrazine metabolization: hydroxylation, dealkylation and conjugation (155), and sorghum performs dealkylation and conjugation (91) (**Figure 2.4**). Tolerance of these 2 crops plants as well as the weeds *Setaria adherens* and *S. verticillata* is native thanks to high metabolization (67). Tolerance of crop plants to atrazine can be best explained by a high intensity of one metabolic pathway, like sorghum, or by the addition of several metabolic pathways, like in maize (**Table 2.1**). In contrast, the absence of metabolic pathway explains the sensitivity of species like wheat and soybean, whereas dealkylation alone confers intermediate tolerance to atrazine in pea.

Acquired resistance due to selective pressure is multiple: (i) weeds *Setaria glauca* (67), *Polygunum lapathifolium* (52), *Amaranthus rudis* and *A. tuberculatus* (119) are resistant because of a single mutation affecting D1 protein, the target site of atrazine; (ii) some have enhanced dealkylation (*Lolium rigidum* (29)), or enhanced conjugation (*Abutilon theophrasti* (68, 127), *Panicum dichotomiflorum* (52)); and finally (iii) some weeds seem resistant to atrazine thanks to mutation and enhanced conjugation (*Setaria faberi* and *S. viridis* (51)). Plant acquired resistance to atrazine is complex, but is well studied because of increasing worries for agriculture.

Some evidence leads to the idea that vetiver is resistant and/or tolerant to atrazine (chapter 5). Therefore, vetiver plant metabolism is relevant to be studied and explored for a possible use in phytoremediation.

Table 2.1 Metabolization pathways of atrazine in selected plant species

Plant		Dealkylation	Hydroxylation	Conjugation	Response to atrazine	Source
Corn [1]	shoot	+	++++	+	tolerant	Raveton et al, 1997 (135) Cherifi et al 2001 (39)
	roots	+	++++	+		
Corn [2]	shoot	+	+++	++	tolerant	
	roots	+	+++	+		
Sorghum	shoot	++	-	++++	tolerant	Shimabukuro et al, 1970 (153)
	roots	++	-	+		
Pea	shoot	++++	-	+	intermediate	
	root	++++	-	+		
Soybean		++	-	+	sensitive	
Wheat		+	+	+	sensitive	

[1] from germination until 1 week old plant (39)

[2] 1 month old plant (39)

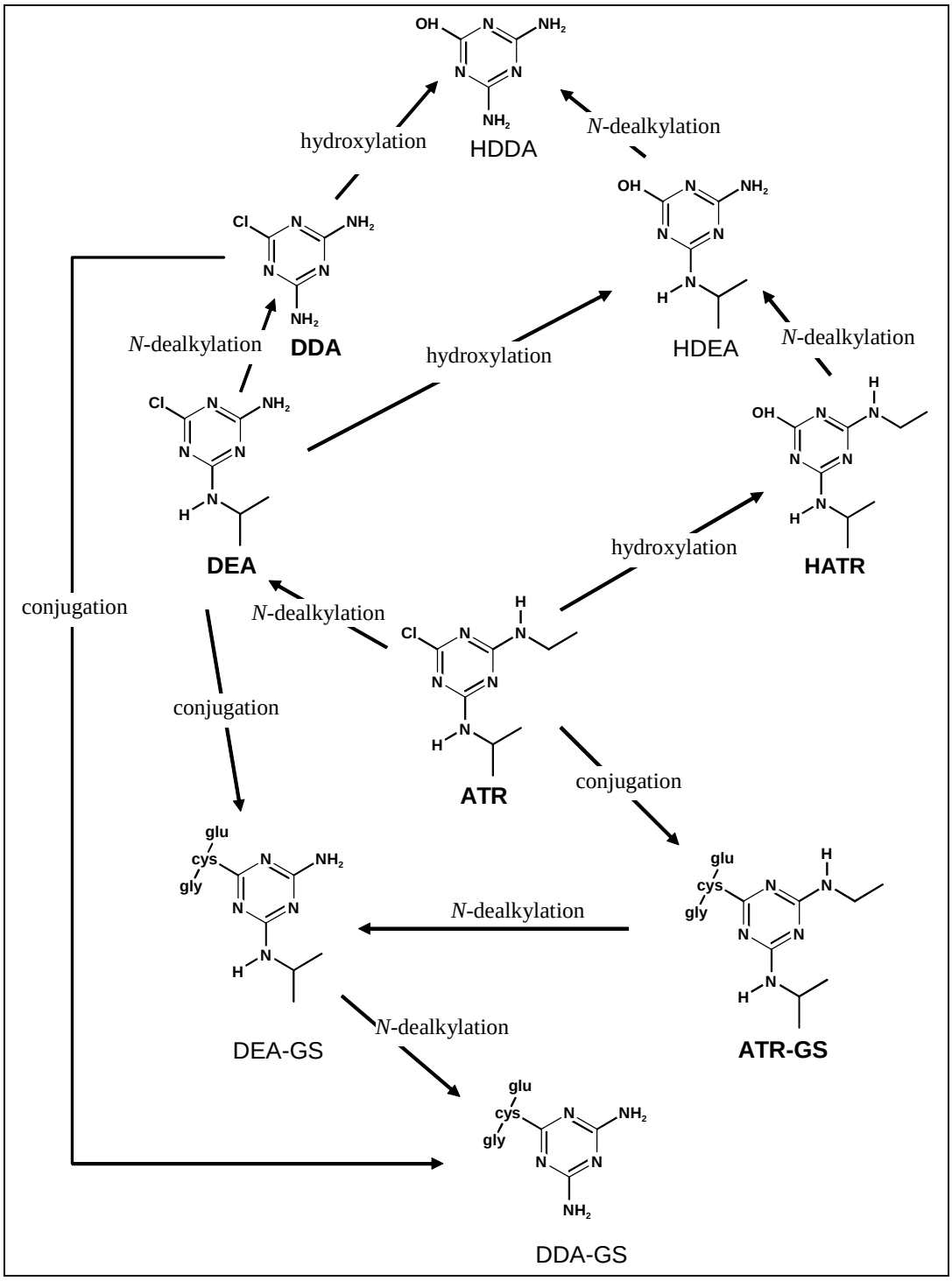


Figure 2.4 Enzymatic pathways (dealkylation and conjugation) and chemical transformation (hydroxylation) of atrazine in maize and sorghum.

Detectable molecules in the present thesis are represented with bold legend. Monodealkylate DIA undergoes the same pathways as DEA (not represented)

For phytoremediation, plants must be resistant to the target compound to be removed. The candidate for phytoremediation has first to be screened for its resistance to herbicides. Metabolism of atrazine by vetiver plant for phytoremediation is of interest, and a resistance explained by chloroplastic resistance alone would not be of great value. For this reason, assessment of vetiver chloroplastic resistance was explored.

No mineralization of atrazine in vetiver is expected, as literature never mentioned total mineralization of atrazine by plants, but the observed tolerance of vetiver to atrazine was assumed to be best explained by classical detoxification pathways, such as N-dealkylation, hydroxylation and conjugation, and/or chloroplastic resistance.

In addition to endogenous substrates, plants have systems of xenobiotic detoxification that rely heavily on cytochromes P 450. This is true particularly in the monocotyledon species, including rice, wheat and maize (59). For this reason, as vetiver is a monocotyledon, the presence of possible dealkylates of atrazine in this plant is expected.

Hydroxylation exploration is relevant, since many *Poaceae* plants contain benzoxazinones secondary compounds able to replace the chlorine atom by a hydroxy group. As vetiver belongs to this family, it is interesting to try to detect benzoxazinones and to identify putative hydroxylated compound of atrazine.

Conjugation of atrazine is also possible, as vetiver is phylogenetically close to sorghum (17), a plant species reported to tolerate atrazine because of GSTs replacing the chlorine atom by a peptide, glutathione. Moreover vetiver belongs to the *Panicoideae* subfamily, from which 11 species were reported to tolerate atrazine because of high conjugation ability (82).

2.3 Environmental and health hazard of atrazine metabolites

Plants act globally as detoxifiers, thanks to active P 450s, benzoxazinones and GSTs, and participate in xenobiotic decrease in the environment. In contrast to plants, many micro-organisms degrade and mineralize organic pollutants and use them as a source of energy and carbon skeletons for cell protein synthesis (74). Unlike animals, where most transformation products of xenobiotics are excreted, plant tissues store them in conjugated soluble form, or as insoluble bound residues. First studies of xenobiotic metabolism were performed using whole animals. Animal studies established that

xenobiotic transformation and conjugation reactions (phase I and phase II, respectively) are largely localized in the liver, where the main liver enzymes of phase I are P 450 monooxygenases. Those of phase II include glucuronyl transferases, GSTs, and amino conjugation systems. In phase III (excretion), the glutathione pump recognizes GSH conjugates for transfer across membranes, allowing in animals their excretion from the body via urine and feces. In 1972, the dechlorination of atrazine was already considered as a clear case of detoxification, since lethal dose (LD_{50}) of glutathione conjugates is greater than 1000 [mg/kg] in the rat compared with [294 mg/kg] for the herbicide (46). Similarly, plants undergo the same steps, namely phase I (transformation), followed by phase II (conjugation) and phase III compartmentation into vacuole or apoplastic space (142). This led to the concept of "green liver": plants possess active enzymes which can "detoxify" xenobiotics. Although mineralization (complete degradation of pesticides) is the desired endpoint in remediation, usually a few transformation reactions are sufficient to drastically change their biological activity (38).

Pesticides that are particularly hazardous to all organisms are those that contain electrophilic sites, i.e. compounds that have centers of low electron density and can accept an electron pair to form a covalent bond. These chemicals can exert toxic effects by covalent binding to nucleophilic sites on cellular molecules. Electrophilic xenobiotics are particularly harmful, because they can be cytotoxic or genotoxic (43). Atrazine possess the atom chlorine which is an electrophile. The removal of the chlorine atom could therefore be already considered as a beneficial transformation, as do hydroxylation and conjugation.

Plant metabolites can differ from animal metabolites, but the toxicological assessment of pesticides and other xenobiotics is usually based only on animal feeding studies with the parent compound. A systematic consideration of plant metabolites with regard to bioavailability and toxicology is strongly recommended, since most linkages of conjugation are cleaved in the animal digestive tract, releasing parent compounds or reactive intermediates (142). But these kinds of data are not very numerous concerning atrazine metabolites. Belfroid et al. [61] and Cole and Edwards (41) pointed out that there is a global lack of information about transformation products of pesticides. In registration procedures, the environmental fate and toxic effect of major

transformation products are generally taken into account. However, most of this information has never been published in the open scientific literature.

Belfroid et al. (14) mentioned a reference about DDA where it is described to be slightly toxic and chronically non-toxic for crustaceans. But other ecotoxicological data are not found for the other atrazine metabolites, with the exception of a study carried out on algae. It appears that DEA, DIA, DDA and HATR are less toxic for 8 algae species than the parent compound. Cole and Edwards (41) pointed out that conjugates arising from the plant metabolism of agrochemicals have been reviewed as a metabolic "dead-end", but since several years, it is now clear that conjugation is only one step in the processing of xenobiotics into final end-products. The lack of knowledge about further processing of conjugates is mainly due to technical difficulties (41). The same authors did not find any study about the processing of atrazine conjugates into bound residues and the fate of plant atrazine conjugates in the mammalian gut with toxicological consequences.

Recently (2002), the U.S. Environmental Protection Agency (EPA), issued a report where atrazine, simazine, propazine and their common metabolites DEA, DIA and DDA could be grouped by a common mechanism of toxicity for disruption of the hypothalamic – pituary-gonadal (HPG) axis in rats (1). The exact mechanism of this endocrine disruptor action is currently actively studied, and it seems that atrazine and DDA act on aromatase activity responsible for estrogen synthesis (116).

Based on the present knowledge of toxicity of atrazine metabolites, transformations of atrazine can be subjectively ranked in decreasing order of interest: total mineralization > conjugation = hydroxylation > dealkylation. As previously mentioned, mineralization of atrazine in vetiver is unlikely to occur, as total mineralization of pesticides in plants was never described in literature. As dealkylates were found to exhibit endocrinal effects, this transformation is not of high benefit for health. Nevertheless, phytotoxicity is reduced and this is already positive for the environment. Conjugation and hydroxylation are the most interesting transformations, as these metabolites were not described to be acutely toxic, neither exhibiting endocrinal effects.

3 Vegetation against agricultural runoff of atrazine

3.1 Interception of pesticides in runoff water

Heavy environmental contamination by herbicides may arise from industrial point sources such as accidental spillage during production, wastewater from pesticide production, leakage of old stockpiles, storage and transport, or as leachates from former dumping sites and municipal waste (20).

In contrast, sources of pollution arising from agricultural uses of herbicides are considered to be diffuse as the compounds are distributed over large areas. Herbicides that are applied to the soil (pre-emergence herbicides), as well as some of their degradation products such as atrazine, reach surface water and groundwater through leaching and runoff (44). Most of the transport of atrazine in runoff occurs during the first rain or irrigation events after application; most of the annual load in streams occurs over a relatively short period (32). Once atrazine reaches the surface water system, it is transported to the ocean without substantial loss (32), or end in long-term storage in lakes, reservoirs and alluvial aquifers, where atrazine shows minimal loss by volatilization, sorption or transformation.

Capel et al. (32) defined runoff as following: there is a continuum in the movement of water, solids, and solutes like atrazine from a terrestrial environment, such as an agricultural field, through a surface water system and eventually to the marine environment. This continuum is divided in 2 parts, soil and stream. The process that connects these 2 parts of the continuum is termed “field runoff”. The surface water system begins in the field in the form of interflow and concentrates to stream flow. The water moves through some combination of drainage ditches, streams, small rivers, and large integrating rivers, ultimately ending in the marine environment.

The occurrence of soil particles and agricultural chemicals in field runoff depends on the kind of soil, weather, pesticide properties, and agricultural management practices, such as erosion control practices, residue management, irrigation and vegetative filter strips.

Improving quality of surface water has led to emphasis on best management practices (BMP) for controlling agricultural non point source pollution (11). One practice, which has received widespread interest, is the use of wetland vegetation or of natural

or artificial vegetative filter strips to remove chemicals from the flow prior to their entry into stream, lake or sea.

3.2 Relevant places of phytoremediation for treatment of runoff water

Interesting cases where phytoremediation of pesticides could be useful includes:

- **Decontamination of wastewater from ornamental plant nurseries**

According to Fernandez et al. (63), herbicide contamination of nursery runoff water is becoming a major concern for the ornamental plants production industry. The problem is caused by nurseries themselves, applying granular pre-emergence herbicides to prevent weeds from becoming established. Residual amount of herbicides can be beneficial as traces by controlling sensitive weeds, but phytotoxicity problems can result when sensitive nursery crops are irrigated with recycled water containing herbicide residues. Therefore, water treatment by plants before water re-use could be of high benefit.

- **Decontamination of pesticide wastewater from golf course**

Wilson et al. (189) worked with the Yellow King Humbert canna (*Canna hybrida*), a tropical and warm temperate herbaceous perennial plant, which is able to take up simazine without adverse effects, and can suit to decontamination of water from golf course polluted with simazine.

- **Decontamination of soils in mixing and loading**

Areas often contain pesticides at concentrations that are above the field application rates and usually contain mixtures of chemicals. Anhalt et al. (6) tested the germination and plant survival on atrazine, metolachlor, and pendimethalin highly contaminated soil. Tested plants were giant foxtail (*Setaria faberi*), birdsfoot trefoil (*Lotus corniculatus*), kochia (*Kochia scoparia*) and cannola (*Brassica napus*). It was noted that with time, atrazine concentration is decreasing, but the respective contribution of plant uptake, and/or micro-organisms and/or increasing adsorption (decrease of bioavailability) is not known.

- **Decontamination of wastewater from agricultural source ending in wetlands**

Natural and constructed wetlands have been shown primary to be effective in reducing the amount of agricultural runoff, such as nitrogen (nitrate, ammonium) and phosphorus. Past research found that vetiver (*Chrysopogon zizanioides*) can remove 74% of total N and 99% of dissolved P (17). The use of constructed wetlands for the treatment of agricultural runoff is gaining in popularity as relatively inexpensive alternative to traditional treatments. Currently, more than 300 constructed wetlands are used in the treatment of agricultural, municipal, industrial, and storm water in the U.S.A. (139).

Many BMPs involve the loss of agricultural surface for the farmer for reducing the amount of pesticides, sediments and nutrients that run off from fields and enter rivers and lakes. So one low alternative cost is to use agricultural drainage ditches as a new BMP for reducing effects of agricultural runoff.

Wetlands receiving agricultural runoff containing herbicides could also help in decreasing their concentration before they reach water system ; although constructed wetlands are able to successfully treat many types of water, little evaluation of their action on pesticides have been done (139). The activity of micro-organisms degrading atrazine in wetland sediments was slight, leading to the hypothesis that observed disappearance of atrazine in water was due to plant uptake. In contrast, Mc Kinlay and Kasperek (104) observed decontamination of 6 ppm atrazine polluted water by marsh plant commun club-rush *(Schoeplectus lacustris),* Bulrush *(Typha latifolia),* yellow iris (*Iris pseudacorus),* common reed *(Phragmites australis)* and concluded that disappearance of atrazine from water is due to the action of rhizosphere micro-organisms. The action of plants themselves was not explored, but was not excluded. Fernandez et al (63) also evaluated semiaquatic herbaceous perennial plants for their use in herbicide phytoremediation, such as canna *(Canna generalis),* pickerel *(Pontaderia cordata*), and iris *(Iris X Charjoys Jan),* and concluded that these taxa were not optimal for phytoremedation, since the plants exposed to herbicides showed significantly reduced biomass. It seems on the contrary that vetiver can tolerate 2 ppm atrazine without adverse effect (47).

- **Interception of pesticides runoff along slope of cultivated area**

  Vegetative filter strips (VFS) are defined as areas of vegetation located along streams, water bodies, field borders, and terraces, to entrap sediments and improve water quality from an up-slope pollutant source (Chaubey et al, cited by Fernandez et al. (63)). Primary effects of vegetative filter strips are: (1) reducing flow velocity, resulting in loss of transport capacity which leads in turn to deposition of sediments and adsorbed chemicals; (2) adsorbing chemicals onto the litter, vegetation, and surface layer of soil, all of which reduce the outflow concentration; (3) storing chemicals in the surface layer allowing time for plant uptake and subsequent biological or chemical transformation; (4) providing wildlife habitat. Effectiveness of VFS is depending on flow depth and velocity of run-off water, vegetation density, incoming sediment and pollutant loads, size and slope of the VFS.

Publications about plant metabolism in species used for phytoremediation are scarce compared to plant metabolism publications for agronomic purposes: Barfield et al. (11) studied efficiency of a natural filter of bluegrass (*Poa annua*) and fescue (*Festuca sp.*) strips located immediately down slope from standard erosion plot of 9% slope. Trapping efficiency of atrazine of a 4.57 m wide strip was 93%, in the same magnitude as dissolved phosphorus, nitrate, ammonium and sediments. This study emphasises the relevance of grass filers as buffer strips, but the mechanism underlying atrazine disappearance was not studied. However, Jensen et al. publication (82) gives a clue on one possible explanation of atrazine disappearance in buffer strips: *Poa annua* and *Festuca sp.* species belongs to the subfamily *Festucoideae*, which is reported by the authors to take up atrazine but without subsequent transformation.

Hybrid-poplar buffer strips were first initiated planted in row along portion of stream at the end of the 80's (110). Plant buffer zones with deep-rooted trees installed next to streams have the potential to reduce nitrate concentration up to 90%, and trichloroethylene (TCE) can be accumulated into poplar leaves, followed by volatilization. Later, it was documented that poplar could take up atrazine with transpiration stream, showing that poplar tree buffer strips are also effective in removing atrazine from agricultural percolation and runoff water. The only extensive study of plant metabolism of atrazine for a phytoremediation purpose is in fact in

poplar tree. Burken and Schnoor (27, 28) showed that poplar (*Populus deltoides X nigra)* can take up atrazine and metabolize it mainly into dealkylates and to a lesser extend, into polar HDDA (ammeline).

Although atrazine has been applied in the field for over 30 days, no enhanced degradation to complete mineralization by bacteria leading to adaptive soils has been reported on large scale yet, thus indicating the difficulty of rapid microbial breakdown in the field (185) and showing the usefulness of plants as vegetative strips or in constructed wetland.

In conclusion, as Coleman et al. (44) suggested, the most interesting possible use of phytoremediation of atrazine contaminated soil and water (1) to prevent run-off to the rivers when atrazine is applied as a pre-emergence treatment just after sowing or planting when field is not yet vegetated (2) to prevent surface run-off to the rivers either by maintaining buffer zones planted with species capable of metabolizing atrazine and (3) to detoxify ditches, storm basins or wetlands.

4 Vetiver as a candidate against atrazine runoff

4.1 Taxonomy

Vetiver belongs to the family *Poacea*, subfamily *Panicoideae*, tribe *Andropogonae* and subtribe *Sorghinae.* and the genus includes ten species. The genus is related to the genera *Sorghum* and *Chrysopogon*. Adams et al. (3) by using DNA finger printing revealed that *Vetiveria* and *Chrysopogon* cannot be distinguished and lead to merge both genera. Previously, vetiver was classified as *Vetiveria zizanioides*. However, it has now been reclassified, and it should now be known botanically as *Chrysopogon zizanioides,* as recommended today by the 2003 Catalogue of New World Grasses (CNWG) (195).

Vetiver is a perennial tropical grass, also known as khus-khus. The generic name Vetiveria comes from the Tamil word “vetiver” meaning “root that is dug up”. Vetiver is native to India, but the exact location of origin is not precisely known; some say that is native to northern India, others say that it is native of the region near Mumbai. Vetiver is by nature a hydrophyte, but often thrives under xerophytic conditions: vetiver grows particularly well on river-banks and in rich marshy soil. It can withstand periods of flood, as well as extreme drought, survives at temperatures of between -9°C and 45°C, is fire resistant, and is able to grow in any type of soil regardless fertility, salinity, or pH. Vetiver grows large, densely tufted clumps form a stout, compact rhizome (crown) with erect clums up to 3 meters high (97). The distribution of vetiver is pantropical, but was introduced recently in Mediterranean regions, such as Italy, Portugal and Spain (http://www.vetiver.org/).

Vetiver is normally established vegetatively by slips because growing it from seeds is extremely difficult and slow (48). Moreover, sterile cultivars are recommended to reduce the vetiver potential to spread as a weed. Multiplication of vetiver is done as following: clumps of vetiver are dug out from soil, and roots are cut about 20 cm below the surface. The leaves are cut about 30 cm above the roots, and the clump is cut into pieces, or slips, of about five tillers. Tillers are defined as being shoots of the plant springing from the bottom of the clump.

4.2 Vetiver grass as a tool for soil conservation applications

Vetiver is a tall, fast growing, and perennial huge grass, with dense packed stiff and though stems which form a dense hedge when planted closely in rows. Some boundary strips in vetiver's native region of India are thought to be 200 years old, but plant age is believed to be 60 years old (45). Vetiver has deeply penetrating root system that works as anchor. In general the fibrous roots of grasses spread out from the underground part of the culm and hold the soil in horizontal pattern, but in vetiver they do not expand horizontally but penetrate mainly vertically deep into the soil acquiring 3 m in good conditions (17). The roots have a weak tendency to branch (http://www.vetiver.org/): 90% of the roots are found within a radius of 20 cm from the vetiver plant, and therefore, vetiver does not interfere with plants cultivated nearby and can be used in natural hedges beside crops.

Because of these properties, non-seeding vetiver plants are used in many countries for soil erosion control and many other applications (48, 97): vetiver grass was first used for soil conservation and land stabilization in Fiji in the early 1950s (48). Recognizing the potential in combating land degradation, the World Bank has promoted in the mid 1980s the vetiver grass system and now vetiver grass is used worldwide as a low-cost, low-technology and effective means of soil and water conservation and land stabilization in developing countries. The U.S. Board of Science and Technology for International Development (45) mentioned successful vetiver applications for stabilization of slopes, terraces and channel banks in numerous tropical and subtropical countries: Australia, Bolivia, Brazil, China, Costa Rica, Ecuador, El Salvador, Guatemala, Honduras, India, Indonesia, Madagascar, Malawi, Malaysia, Mexico, Nepal, Nicaragua, Nigeria, Philippines, Sri Lanka, South Africa, Thailand, Zambia, and Zimbabwe. Vetiver plantation for soil erosion control is mainly performed linearly, along fields, terraces, canals, streams, or rivers, where the erosive force of water is at its greatest, lakeshores, artificial embankment, and little canals for irrigation or water drainage. It even can be planted across the river itself to slow down the flow of water.

More recently, it has been observed that vetiver is tolerant to wide ranges of pH, salinity, acidity, and heavy metals such as As, Cd, Cr, Ni, Pb, Zn, Hg, Se, and Cu (157, 167, 191, 192), showing that vetiver has a wide potential in the restoration of mine wastes and heavy metal contaminated soils. However, as not been reported yet

in literature, the mechanism of metal tolerance of this species is not known and would be of great value to assess the potential risk of metal accumulation and transfer through the food chain.

Beside use of vetiver for soil protection, roots produce essential oil used by perfumery industry. The aroma of this essential oil is heavy and extremely persistent, and therefore roots are put in sachets among clothes to keep insects away, or when distilled, oil is used in perfumes, deodorants, and soap (50). Vetiver oil is of interest to the cosmetic and perfumery industry, not only due to its scent, but also due to its ability as fixative, preventing other volatile oils to evaporate (97). In medicine, a stimulant drink is made from fresh roots in India, and in Madya Pradesh (India), the vetiver plant is used as an anthelmintic (50, 97). Young cut leaves of vetiver can be used as fodder for cattle and goats, whereas dried leaves are used for making brooms or for thatching of huts (50, 97). Handicraft products are made with dried vetiver leaves, such as hats, bags, baskets and other useful items (17). Interestingly, after cutting of the leaves, new ones start to grow from the base of the plant very quickly, renewing leaves for a next use.

Some evidence of vetiver resistance to atrazine was found before starting the present work: vetiver plants in pots are resistant to 2 ppm atrazine (47), and Pareek et al. (118) suggested that improvement of vetiver cultivation for obtaining high root and essential oil yield include the use of 0.5 [kg a.i/ha] atrazine as a pre-emergence treatment.

Interestingly, Cull et al. (47) ended their paper by this statement: “ further research is needed to determine the mechanism underlying vetiver’s tolerance to atrazine and diuron, and indicate the extent to which residues are absorbed by the roots and translocated to the shoots”.

This call was heard, and the scope of the present work addresses the question of the fate of atrazine in vetiver species. Vetiver relevance for control of atrazine runoff is believed to be high, since vetiver was observed to resist atrazine (first requirement of phytoremediation); it is a non invasive plant (vegetative multiplication); it is a low competitor to adjacent cultivated plants (low root horizontal spread); it is tolerant to different ecological conditions (wide distribution, large pH tolerance, tolerance to drought and flood). The deep and dense roots allow a slow runoff of water, to catch

soil and sediments on which atrazine is adsorbed, allowing plant uptake and micro-organisms to lower atrazine in water by biotransformation. As vetiver is a huge grass, it is expected that this plant could remove atrazine as do smaller grasses festuca and poa in temperate climates (11). Vetiver is not a crop plant, but is easily available to each developing country thanks to the Vetiver Network (http://www.vetiver.org/). To our knowledge, most of the plants used for phytoremediation of atrazine are most of the time adapted to temperate climate of developed countries, but as the present thesis was a part of an Indo-Swiss project, it was a matter of fact that selected plants should be adaptable to India.

Although vetiver has already a wide range of applications, understanding the fate of atrazine in vetiver could open a new application window.

5 Evaluation of chloroplastic resistance

5.1 Introduction

Plants and different micro-organisms with chlorophyll pigments have the ability to synthesize carbohydrates from atmospheric CO_2. Carbon assimilation by plants can be summarized in the global chemical reaction occurring in the presence of light as follow (global equation of photosynthesis):

$$nCO_2 + nH_2O = (CH_2O)_n + nO_2$$

This reaction is endothermic and is possible only thanks to light giving the necessary energy to the chlorophyllian system. This reaction is in fact resulting from many different reactions that can be classified in two series of processes. (1) Water photolysis and associated phosphorylation. These reactions occur in the presence of light and are characterized by a release of O_2 from water photolysis and by the formation of a reductor able later to reduce CO_2. (2) Reduction of CO_2 and carbohydrate production (obscure reactions namely Benson, Bassham and Calvin cycles) (162).

The light reaction activities can be studied by measuring O_2 release from isolated chloroplasts or from intact leaves. It is also possible to detect proton release from isolated chloroplasts by photometry in the presence of an artificial electron acceptor like 2,6 dichloro-phenolindophenol (DCPIP) (**Figure 5.1**). This latter compound changes reversibly from oxidized state (blue by eyes, absorbing in a red band) to reduced state (transparent by eyes, with disappearance of absorption in the red band); it is then possible to measure the photosynthetic transfer of electrons from water to DCPIP (Hill reaction). Finally, the light reactions can also be studied by following variations of chlorophyll fluorescence. The fluorescence rise follows biphasic kinetics, which has been explained by a photochemical reduction of two successive electron acceptors of photosystem II (PS II), the primary acceptor Q_A (photochemical phase, 0→ I) and the plastoquinone pool A or PQ (thermal phase I→P). Thus the kinetics of fluorescence induction, reflecting the Q_A^-/Q_A ratio, can be used for probing the oxido-reduction state of PS II centers. Many herbicides cause the interruption of photosynthetic flux of electrons, by acting on photosystem I (like paraquat), whereas others act on quinone acceptor complex of the transport chain between photosystems

II and I: inhibitors like DCMU and triazines prevent the electron transfer from Q_A^- to a secondary acceptor Q_B, without affecting the reduction of Q_A (**Figure 5.2**). It seems that when chloroplasts are treated with atrazine, the phase 0→ I reaches a maximum fluorescence higher than a control without atrazine, showing that the extra energy received from chlorophyll is dissipated mainly by fluorescence when the electronic transfer is blocked. Fluorescence rise parameters directly reflect the PS II inhibition by herbicides, and reference to curves of effect/concentration in chloroplasts is possible (58, 134).

In summary, photosynthetic inhibition of photosystem II can be highlighted by measuring decreased release of O_2 with an oxygen electrode (68), by measuring decreased potential of reduction of DCPIP with a spectrophotometer, or by measuring increased fluorescence emission when electron transport is inhibited by herbicides with a chlorophyll fluorometer (179).

To explore possible chloroplastic resistance of vetiver to atrazine, it was relevant to study light reactions and the effect of atrazine and diuron (DCMU) on isolated thylacoids. By so, plant metabolism occurring in plant cytosol such as conjugation, and hydroxylation was avoided, as well as dealkylation by the action of P 450 located in endoplasmic reticulum. Choice of method was done on the basis of available spectrophotometer in the laboratory. Study of Hill reaction was found simple to be used, since Hill reagent DCPIP was easily available. Study of inhibition of O_2 release was not done, neither fluorescence study.

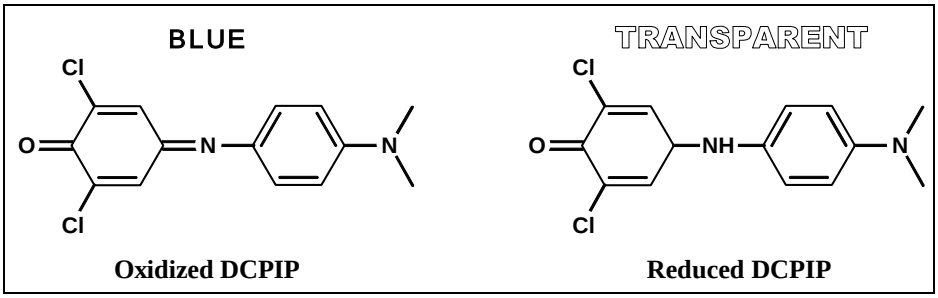


Figure 5.1 Hill acceptor DCPIP

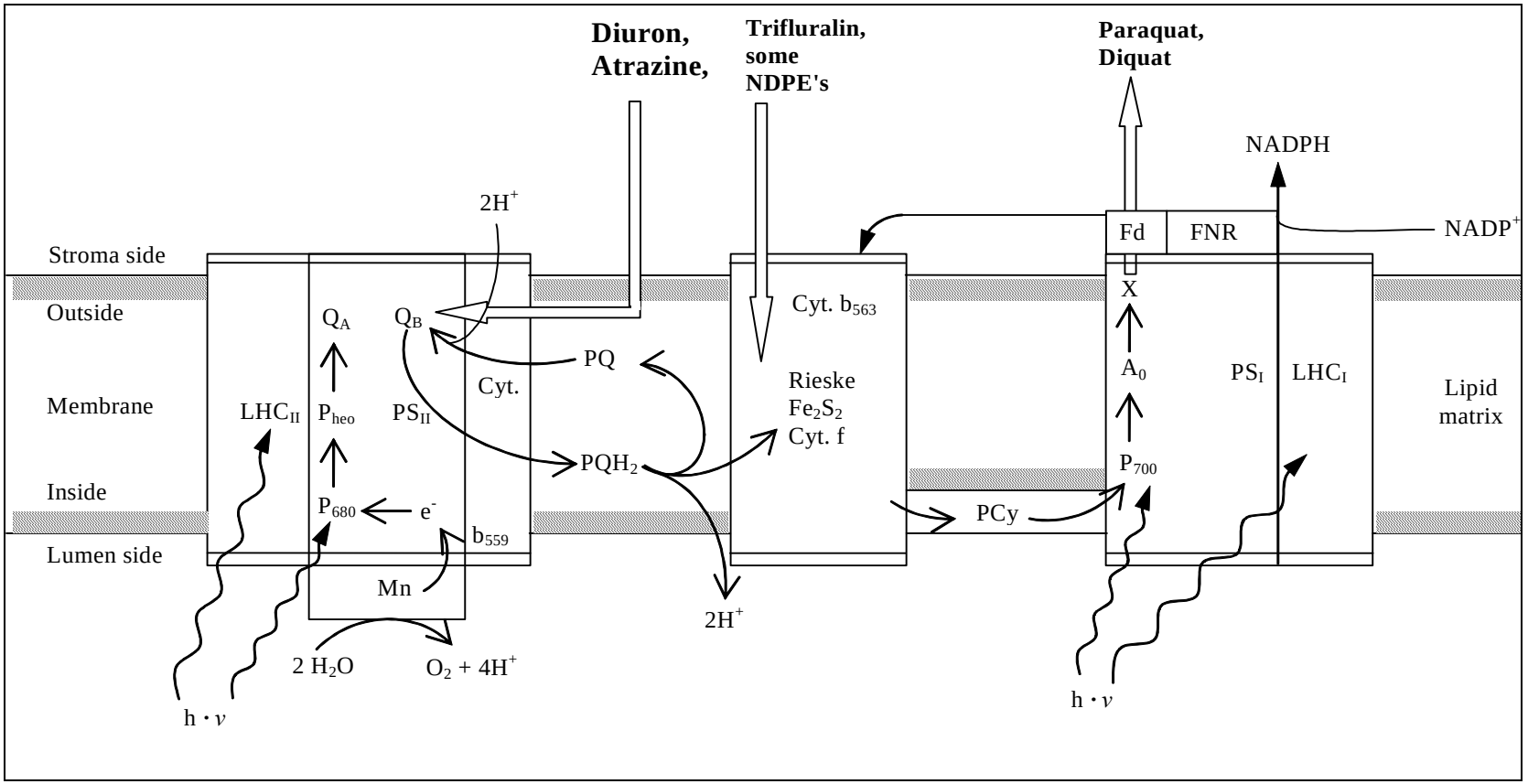


Figure 5.2 Inhibition of electron transport chain by atrazine and diuron

(Modified from Devine et al. (55))

5.2 Material and methods

Vetiver cultivation

Vetiver slips were received in February 2002 from Portugal, provided by M. Pease[3]. The plants originated from an importation made by himself from Zimbabwe in 1998 and further cultivated in Portugal until 2002. The name of the variety is "Vallonia". There is a commonality of genotype between "Sunshine" in the USA, "Vallonia" in South Africa, "Monto" in Australia and "Guiyang" in China. These varieties share about the same genotype and are used generally throughout the world for soil erosion control. Thus, the present work will have a relevance to similar plants grown in many other countries (personal communication, M. Pease).

Sixty slips were received wrapped into newsprint paper from Portugal in February 2002, each slip selected with 3 or 4 tillers. Half of the pool was planted in organic soil (**Figure 5.3**) and the other half was rooted directly in hydroponics (**Figure 5.4**). As Luwasa® was a cheaper ready-nutrient medium, it was used for hydroponic plant maintenance, and when experiments were run, Hoagland solution was used. Plants grown in organic soils were used as a nursery. Vegetative reproduction (**Figures 5.5 5.6, and 5.7**) allowed obtaining new plants for nursery, small plants for autoradiography, and new plants for hydroponic experiments.

Plants were maintained in glasshouse supplemented with sodium lamps (Son-T Agro, 80% IR + 20% UV) under the following conditions: minimal temperature was 20°C during day time and 18.5 °C during night time; minimal humidity was 65% during day time and 45% during night time.

Amongst 60 slips received from Portugal, only 4 of them never rooted again and died. Vetiver slips could be rooted and grown directly in hydroponic solution: after 6 months, vetivers grown in hydroponics were of comparable size as those grown in organic soil. Vetiver in hydroponics supplemented with available ready nutrient solutions Luwasa® or with Hoagland full strength exhibited the same appearance in term of estimated biomass and leaf colour.

[3] Mr Michael Pease, Coordinator for the European and Mediterranean Vetiver Network (EMVN), Lagos, Portugal

Figure 5.3 Vetiver plant before splitting

Figure 5.4 General view of plants in hydroponices

Figure 5.5 Splitting of tillers

Figure 5.6 Slips obtained form splitting

Figure 5.7 New leaves after 1 week

Tested herbicides

The inhibition of photoystem II by herbicides atrazine and DCMU is due to a reversible non covalent bond of the herbicide on the action site of protein D1. In case of atrazine resistance, the inhibitory activity of atrazine is decreased 1000 times, whereas DCMU is still inhibiting strongly electron transport (122). This suggests that although these 2 herbicides are both inhibitors of photosystem II, they act differently on it. This is confirmed by the identification of one point mutation of plant resistant to atrazine, namely the replacement of one serine by glycine in position 264 of D1 protein.

This part of the work aimed to address the possible chloroplastic resistance of vetiver. In case of sensitivity of vetiver thylacoids towards atrazine, they should be equally sensitive towards DCMU. In case of resistance of vetiver thylacoids towards atrazine, and if it is assumed that chloroplastic resistance in vetiver is also due to the change of one amino acid in D1 protein, a clear different effect of atrazine and DCMU should be observed (no decreased reduction of DCPIP and decreased reduction of DCPIP respectively). Therefore, DCMU herbicide was studied for a comparison with atrazine effect on vetiver thylacoids.

Plant preparation

Thylacoids were extracted from pea (*Pisum sativum* L., variety “pois nain”, Caillars Ltd, France) and vetiver. Pea was taken as a known sensitive plant towards atrazine (negative control). No positive control was used (atrazine resistance due to chloroplastic resistance).

As pea was grown from seeds, and vetiver was multiplied vegetatively, phenologic states of both species were not comparable. Nevertheless, to obtain the best possible comparison, vetiver leaves of 3 mature vetiver plants were cut above 15 [cm] soil pot level, and new leaves (2 and 8 weeks old) were collected. Pea leaves were collected after 2 and 8 weeks following seeding.

Thylacoids preparation

Thylacoids extraction and Hill reaction measurements were based on Prof. Kessler protocol[4]. Fresh leaves of vetiver (250 [g]) and pea (25 [g]) were separately finely chopped at 4°C with a blender (Waring) in 650 [mL] and 70 [mL] of buffer A respectively (25 mM HEPES-KOH buffer pH 7.6 containing 0.33 M sorbitol, 30 mM KCl, 5 mM NaCl, 2 mM EDTA-Na_2, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 0.5 mM K_2HPO_4, 5 mM ascorbate, 4 mM cysteine). After straining through 4 layers of muslin, the homogenate was centrifuged at 1088 g, 4°C, for 3 min. Pellets were suspended in 20 [mL] of buffer B (25 mM HEPES-KOH buffer pH 7.6 containing 30 mM KCl, 5 mM NaCl, 2 mM EDTA-Na_2, 1 mM $MgCl_2$, and 1mM $MnCl_2$). After 4 min on ice allowing lysis of chloroplasts, the solution was centrifuged under the same conditions mentioned above. Pellets were then suspended gently in a minimal volume of buffer B. Extracted thylacoids were adjusted to 1 absorbance unit at 600 [nm] (estimation of total chlorophylls) with a spectrophotometer (U-2001, Hitachi), and by dilution with buffer B, allowing comparison between the two tested species. Thylacoid preparation is summarized in **Figure 5.8.**

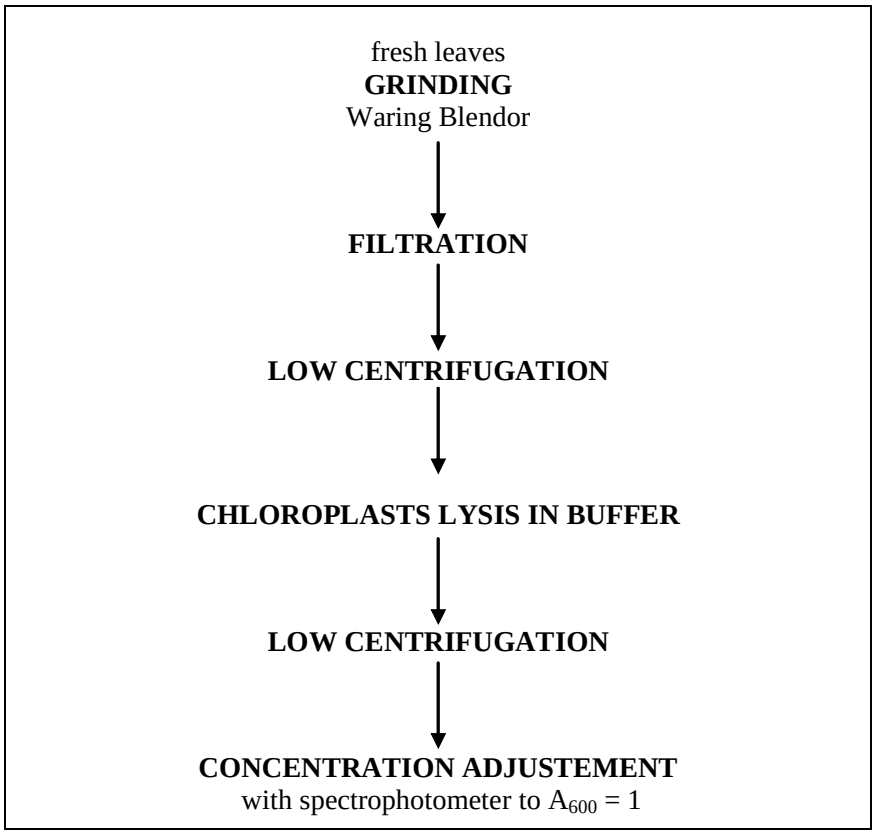


Figure 5.8 Thylacoids preparation

[4] Prof. Felix Kessler, Plant Physiology Department, University of Neuchâtel, Neuchâtel, Switzerland

Test of inhibition of photosystem II by atrazine

Thylacoids suspension (500 [µL]) was tested at room temperature with 200 [µL] of 0.6 mM DCPIP (Sigma), 3 [mL] of buffer B, X [µL] of 1 mM atrazine dissolved in acetone, and Y [µL] H_2O in order to obtain a final testing volume of 4 [ml]. Final tested concentrations of herbicides atrazine and DCMU were 0.05, 0.5, 5, and 50 µM. Test tubes were lighted with a conventional lamp of 250 W. To avoid excess heating of the tested solutions, a glass window was used between the lamp and test tubes.

Controls were the following: (i) DCPIP in buffer representing 100% of oxidized DCPIP or 0% reduced DCPIP; (ii) reduction of DCPIP in the presence of boiled thylacoids; (iii) incubation in the dark; (iiii) incubation of DCPIP with thylacoids representing the optimal conditions for Hill reaction or maximal possible reduction of DCPIP under our selected conditions.

Hill reaction was run for 15 min in presence or absence of atrazine and diuron (**Figure 5.9**). After incubation, 1 [ml] of solution was centrifuged in Eppendorf tubes to remove thylacoids, and absorbance was read at $\lambda =$ 600 [nm] with a spectrophotometer. Results were expressed as a percentage of reduced DCPIP.

5.3 Results

Extraction rate of thylacoids in vetiver was lower than pea, due to the high fiber content of vetiver. To obtain sufficient quantity of functional thylacoids, a higher amount of fresh biomass was used, 250 [g] for vetiver as compared to 25 [g] for pea.

Controls of vetiver and pea extracts without herbicides showed a good ability to reduce DCPIP, showing that the used protocol ended with functional thylacoids. Controls of boiled thylacoids and incubation in the dark showed no reduction potential, proving that it was depending on functional thylacoids and on light (**Figure 5.9**).

Inhibition of thylacoids was increasing with herbicide concentration (atrazine and DCMU), and was similar for pea and vetiver. Hill reaction was inhibited with 5 and 50 [µM] herbicide concentration, independently of leaf age of vetiver and pea (Tables 5.1 and 5.2). This incubation time was the maximum usable, since thylacoids were loosing slowly their viability after 20 min under artificial lighting. After 15 min incubation, reduction of DCPIP in the presence of thylacoids was not complete:

between 50 to 60 % of DCPIP was reduced. DEA and DIA showed a markedly attenuated herbicidal activity on vetiver and pea thylacoids, as compared to atrazine (**Table 5.3**). The effect of DEA and DIA was not significantly different.

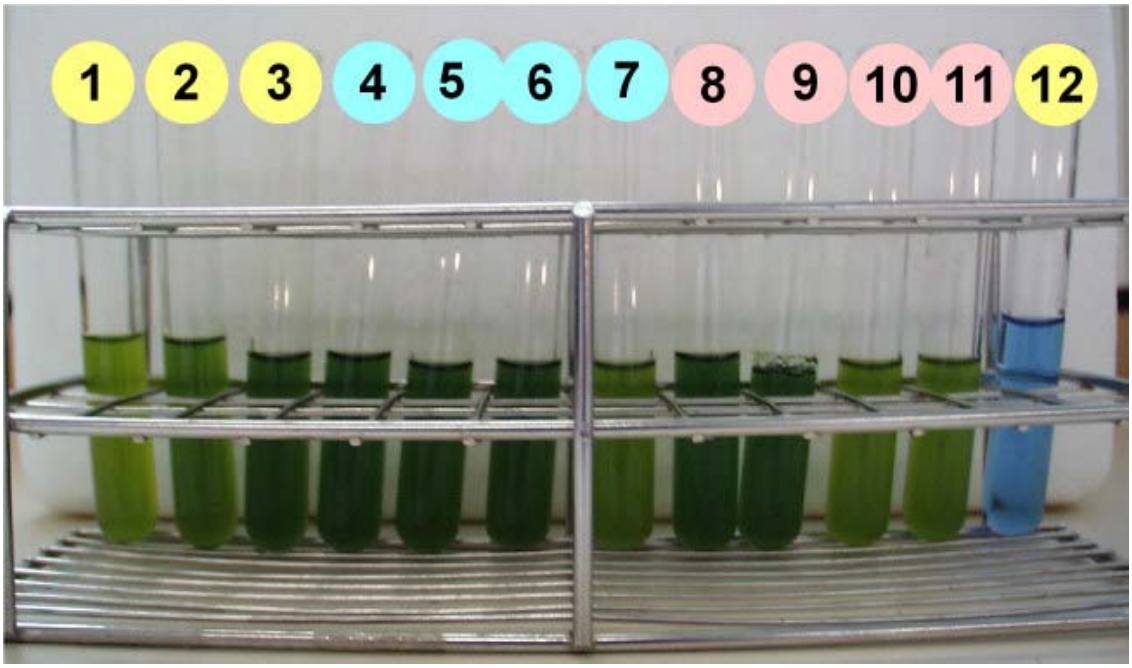


Figure 5.9 Hill reaction in presence of atrazine and diuron

CONTROLS (yellow)	DIURON (blue)	ATRAZINE (pink)
1 = 100% reduced DCPIP	4 = 50 µM	8 = 50 µM
2 = dark incubation	5 = 5 µM	9 = 5 µM
3 = boiled thylacoids	6 = 0.5 µM	10 = 0.5 µM
12 =100 % oxidized DCPIP	7 = 0.05 µM	11 = 0.05 µM

Table 5.1 Hill reaction in the presence of thylacoids extracted from 2 weeks old leaves together with atrazine and diuron
Results of a typical experiment are expressed as a percentage of reduced DCPIP after 15 min incubation

		Controls				Herbicide concentration [µM]			
		Herbicide + DCPIP	Boiled thylacoids	Dark incubation	Maximum reduced DCPIP	0.05	0.5	5	50
Atrazine	Pea	0	0	1	60	60	55	5	1
	Vetiver	0	1	0	55	53	48	2	3
Diuron	Pea	0	0	1	60	59	57	8	1
	Vetiver	0	1	0	55	54	48	2	0

Table 5.2 Hill reaction in the presence of thylacoids extracted from 2 months old leaves together with atrazine and diuron
Results of a typical experiment are expressed as a percentage of reduced DCPIP after 15 min incubation

		Controls				Herbicide concentration [µM]			
		Herbicide + DCPIP	Boiled thylacoids	Dark incubation	Maximum reduced DCPIP	0.05	0.5	5	50
Atrazine	Pea	1	4	5	52	55	54	3	3
	Vetiver	1	3	0	52	46	37	4	3
Diuron	Pea	0	0	1	52	58	57	1	1
	Vetiver	0	1	0	51	47	44	2	0

Table 5.3 Hill reaction in the presence of thylacoids extracted from 2 weeks old leaves together with dealkylates of atrazine, DEA and DIA
Results are expressed as a percentage of reduced DCPIP after 15 min incubation

		Controls				Herbicide concentration [µM]			
		Herbicide + DCPIP	**Boiled thylacoids**	**Dark incubation**	**Maximum reduced DCPIP**	**0.05**	**0.5**	**5**	**50**
Vetiver	**ATR**	0	2	2	50	55	52	3	3
	DEA	0	2	2	55	55	54	54	42
	DIA	0	0	3	50	51	52	52	41
Pea	**ATR**	0	1	1	57	55	46	9	2
	DEA	0	2	2	58	57	54	53	39
	DIA	0	1	1	58	55	54	55	40

5.4 Discussion

The study of inhibition of thylacoids with a Hill acceptor indicated that vetiver thylacoids were sensitive towards atrazine and DCMU. It was not possible to calculate the exact I_{50} (concentration of herbicide required for 50% inhibition of the Hill reaction) with the few tested different concentrations. In sensitive species and biotypes, I_{50} was found to be 0.63 µM for chloroplasts of *Chlorella pyrenoidosa* (196), 0.2 µM for wheat (103), 0.2 µM for pea (24), and 10 µM for *Chenopodium album* (161). In contrast, resistant biotypes of *Setaria viridis* had I_{50} of 195 µM compared to 0.2 µM for a sensitive biotype (51).

However, an estimation of I_{50} was possible: I_{50} was between 0.5 and 5 µM for atrazine and DCMU with the 2 tested species, right in the same range as literature citations above. This estimation of I_{50} in our experiment tends to show that the selected negative control, pea, was actually sensitive to atrazine, like expected. Even if the comparison between pea and vetiver is not directly possible, because of the lack of a normalization of results (protein quantification of thylacoids extracts of pea and

vetiver after adjustment at A_{600} = 1 prior the *in vitro* test, showed that content was slightly higher in vetiver thylacoids extracts and than pea extracts), vetiver tested was clearly sensitive to atrazine.

Most of the time, chloroplastic resistance is acquired with time by a selection pressure. Several publications attest that different biotypes of *Setaria spp*. showed acquired resistance linked to chloroplastic resistance (51, 67, 68, 179), as well as *Polygonum lapathifolium* (52). It is not known if vetiver plants imported from Zimbabwe and Portugal were treated with atrazine over long period of time. The observation that atrazine inhibited vetiver thylacoids indicate that the imported slips were probably not continuously treated with atrazine and/or did not developed chloroplastic resistance. This conclusion is only valid for the present tested biotype. It is not excluded that vetiver cultivated for oil extraction developed chloroplastic resistance, since atrazine treatment was recommended to enhance oil production (118).

As vetiver chloroplasts were sensitive to atrazine, and as vetiver was shown to be resistant to atrazine, it seemed likely that vetiver tolerance was due to the metabolism of the herbicide. This hypothesis could have been checked in vetiver entire leaves by following the O_2 release or fluorescence: unchanged response of these two parameters in the presence of atrazine could have confirmed the presence of rapid and efficient metabolism preventing atrazine to reach the target D1 protein (75, 179). Cull et al. (47) measured photosynthetic activity of vetiver grass shoots with a pulse-amplitude modulated fluorometer (PAM) 27 days afer the application of 2 [mg/L] application of atrazine or diuron. Fluorescence of vetiver leaves was not affected by application of diuron and atrazine, suggesting strongly that plant metabolism was taken place rapidly and efficiently.

6 Evaluation of tolerance by chemical metabolization

6.1 Introduction

It is of first interest to evaluate possible tolerance of vetiver to atrazine due to hydroxylation by hydroxamic acid derivatives: hydroxylated atrazine results in non phytotoxic compound and is therefore of great value for phytoremediation. It was first established that benzoxazinones hydroxylate simazine (33, 34, 71). Later, it was shown by Raveton et al. (135) that hydroxamic acid class of benzoxazinones hydroxylate atrazine. Hydroxylation is the pre-eminent metabolic pathway in maize seedlings (39, 135). Interestingly, benzoxazinones are not only present in plants, but also in maize (64, 120), wheat and rye (8) exudates, possibly enhancing iron uptake by plant thanks to these natural chelators. In maize, the enzymatic release of DIMBOA after wounding is complete within half an hour. In intact plant cells, cyclic hydroxamic acids are sequestered and stabilized as glucosides in the vacuole. In response to tissue damage or pathogen attack, the more toxic aglucones are released by vacuolar β-glucosidases (121). The half life of DIMBOA in the exudate of injured maize cells is about 24 hours (158). This suggests that amongst grasses containing benzoxazinones, these exudated compounds may play a role in atrazine disappearance from soil, and that phytoremediation probably does not only rely on the classical uptake of contaminants followed by phytotransformation.

Under *in vitro* experimental conditions, Raveton et al. (135) showed that a benzoxazinones mixture (10 mM) extracted from corn plantlets is able to transform 91% of atrazine (6 mM) in hydroxy atrazine within 24 hours at 25°C. Such a concentration of 10 mM benzoxazinones is a high value compared to 6 mM atrazine, but without this amount, the reaction does not occur. Though, the apparent concentration of total benzoxazinones in maize plantlets is between 10 and 20 mM (8, 39), and the *in vitro* experimental ratio atrazine/benzoxazinones used in the test seems close to the *in vivo* ratio in treated maize seedlings. The reaction is temperature dependent, and the pH value of the incubation medium greatly influences the hydroxylation of atrazine. No hydroxylation is detected at pH 9, hydroxylation occurs at pH 7, but at pH 5.5 maximum hydroxylation is performed. Solvents acetone and ethanol inhibit hydroxylation: 80% of acetone or ethanol in test solution result in 6 and 8% hydroxyatrazine only, respectively (134). ^{14}C- atrazine is always provided

with solvent by manufacturers and for this reason, *in vitro* test of hydroxylation of atrazine always contains an organic solvent to different percentage depending on the used source of radio-labelled atrazine.

In order to maximize the chance to detect benzoxazinones and their hydroxylating activities, tested conditions were the same as those defined by Raveton et al. (134, 135), except that the concentration of putative benzoxazinones was increased to 20 mM. Assays were incubated for 24 hours at 25°C with 6 mM atrazine at pH 5.5.

In the present work, detection of hydroxamic acids derivatives DIMBOA and DIBOA and their glucosylated forms was performed (**Figure 6.1**), since vetiver is a *Poaceae* and that hydroxamic acid class of benzoxazinones hydroxylate atrazine *in vitro* (135). Other classes of benzoxazinones were not explored.

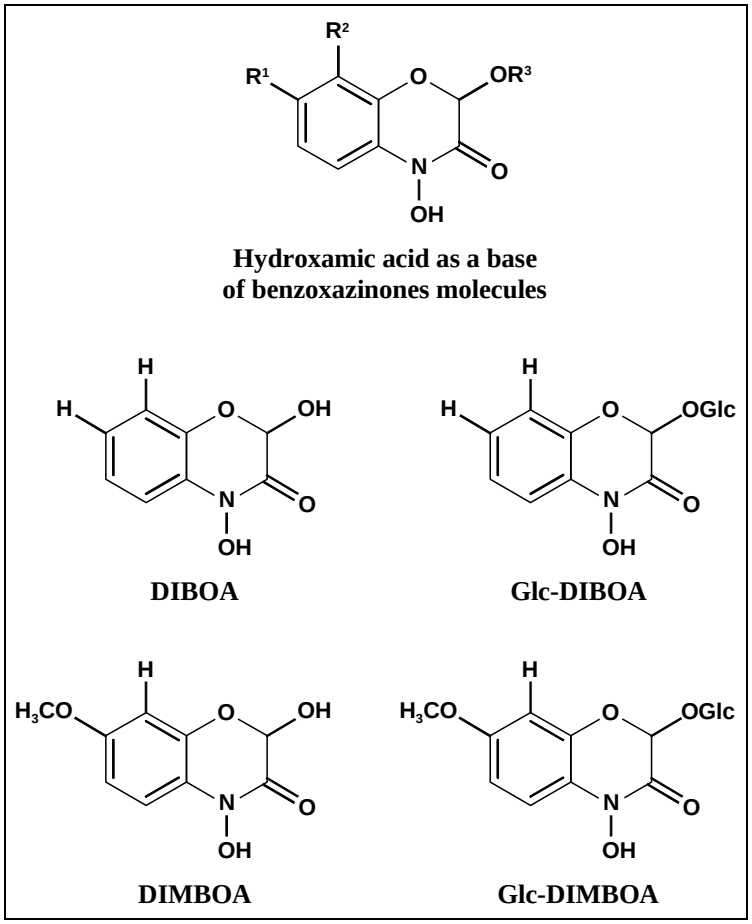


Figure 6.1 Hydroxamic acid as a base of benzoxazinones molecules
Glc = glucose

6.2 Material and methods

Benzoxazinones identification and *in vitro* test of hydroxylation of atrazine was based on Dr M. Raveton protocol[5] and publications (39, 134).

Putative benzoxazinones from 55 [g] fresh mass of leaves and roots were extracted separately with acetone followed by acetone/water 80/20, v/v. Plants were 8-months old and were grown in hydroponics. Extracts were partially purified with petrol ether (B.P. 40-60 °C). The obtained extracts were partitioned with ethyl acetate, in order to separate apolar benzoxazinones (DIMBOA and DIBOA) from polar benzoxazinones (mono and diglucosylated DIMBOA and DIBOA). The phase was evaporated and the extracts redissolved in pure ethanol. The volume of the aqueous-acetonic phase was reduced with the help of butanol, and dissolved at the end with a minimal volume of ethanol and water 70/20, v/v.

Ethyl acetate extracts and water-acetonic extracts transferred into ethanol and ethanol/water respectively were loaded on thin layer chromatography (TLC) silica-gel plates (60_{F254}, Merck) and developed with ethyl acetate/formic acid/acetic acid/H_2O 40/2/2/4, v/v/v/v. Pure DIMBOA was used as a standard, and was obtained from M. Raveton[1] (description of DIMBOA standard purification in publication (135)). The movement of the analytes was expressed by retardation factors, Rf such as:

$$Rf = \frac{distance\ movement\ by\ the\ analyte\ \ from\ the\ origin}{distance\ movement\ by\ solvent\ \ from\ the\ origin}$$

Obtained Rf were compared with those obtained by M. Raveton (134). The extracts were then further loaded (1.2 mL) on TLC plates and obtained separated products were further scrapped, eluted with minimal volume of ethanol, and centrifuged at 13'000 g for 1 min in Eppendorf tubes to remove any trace of silica. UV spectra between 200 and 400 [nm] were further studied and compared to existing published benzoxazinone spectra (39, 134).

[5] Dr Muriel Raveton, Laboratory for Xenobiotics and Environmental Perturbations, University Joseph Fourier, Grenoble, France

Benzoxazinones quantification in partially purified extracts was also determined spectrophotometrically (using ε_{262} = 8000 M^{-1} cm^{-1}) in order to prepare *in vitro* test of putative hydroxylation of atrazine. A summary of benzoxazinones extraction is shown in **Figure 6.2**.

In vitro hydroxylation of atrazine

Previously obtained extracts were concentrated to 20 mM of putative benzoxazinones with the help of a Speed vacuum device (Savant). Extracts were tested for their ability to hydroxylate atrazine. Test solution contained 200 [µL] of 20 mM extracted putative benzoxazinones, 800 [µL] 0.1 M phosphate buffer pH 5.6, and 14 [µL] of 5 mM [^{14}C]-atrazine 1440 [MBq mL^{-1}]. Final ethanol concentration was 20%. Test solutions were incubated at room temperature under agitation for 24 hours, then frozen at – 20°C until being analyzed. After thawing by hands, samples were extracted twice with 2 [mL] ether diethyl to collect unreacted ^{14}C-atrazine. The ether diethyl phases were further evaporated until dryness and dissolved in 5 [mL] ethanol. Aqueous phases were also evaporated to remove all traces of ether diethyl, and 4 [mL] of water was then added to the test solution. Finally, 10 [mL] of scintillation liquid were added to ethanolic and aqueous samples (Ready Safe™ for organic samples and Ready Safe™ Beckman for aqueous samples respectively). The radioactivity of samples was measured with a scintillation counter (Wallac, Winspectral). A summary of benzoxazinones extraction is shown in **Figure 6.3**.

Controls were the following: (1) atrazine together with phosphate buffer (spontaneous hydroxylation) (2) standard DIMBOA in the same conditions as tested extracts (positive control) and (3) atrazine rate extraction by ether diethyl .

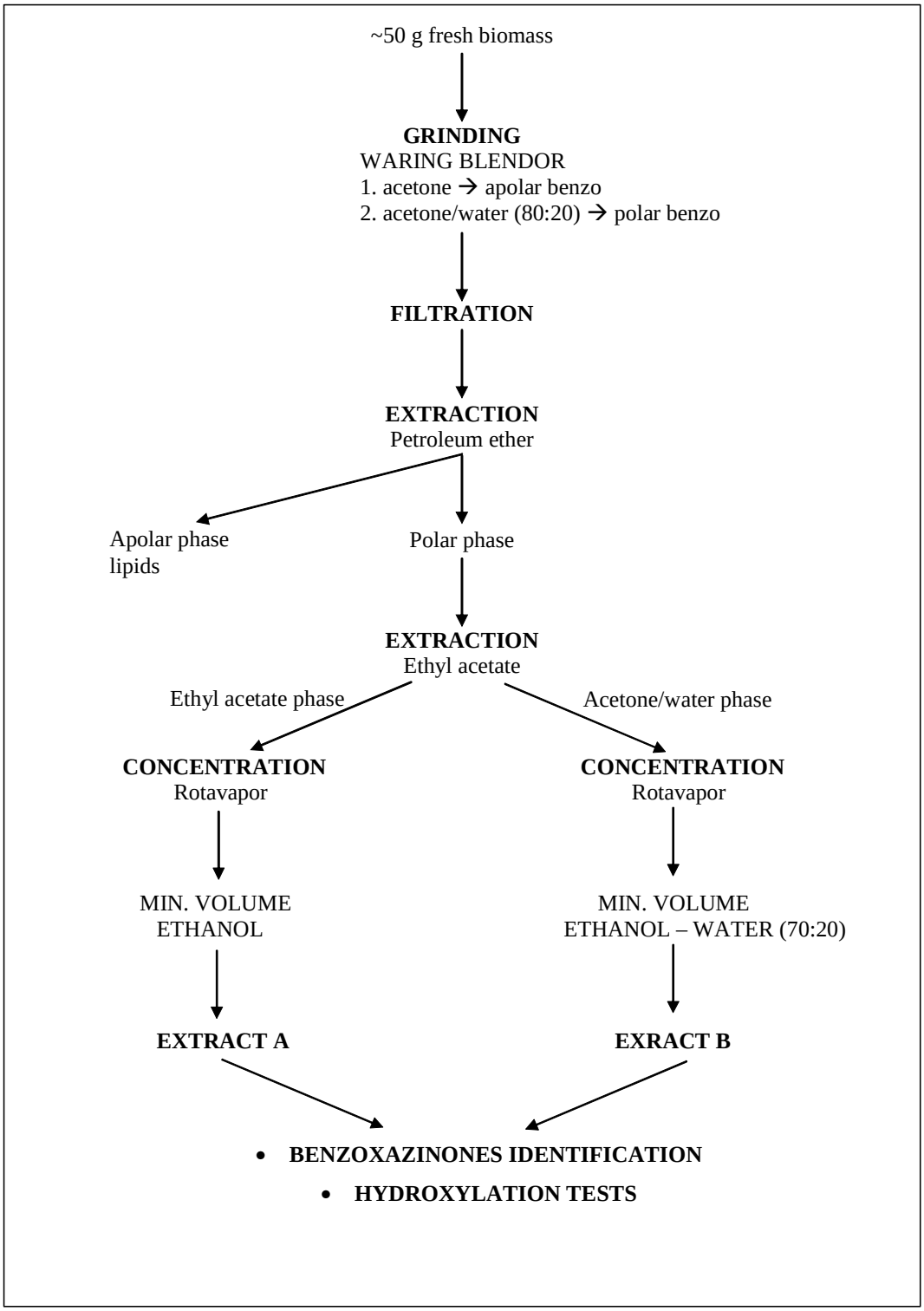


Figure 6.2 Benzoxazinone extraction

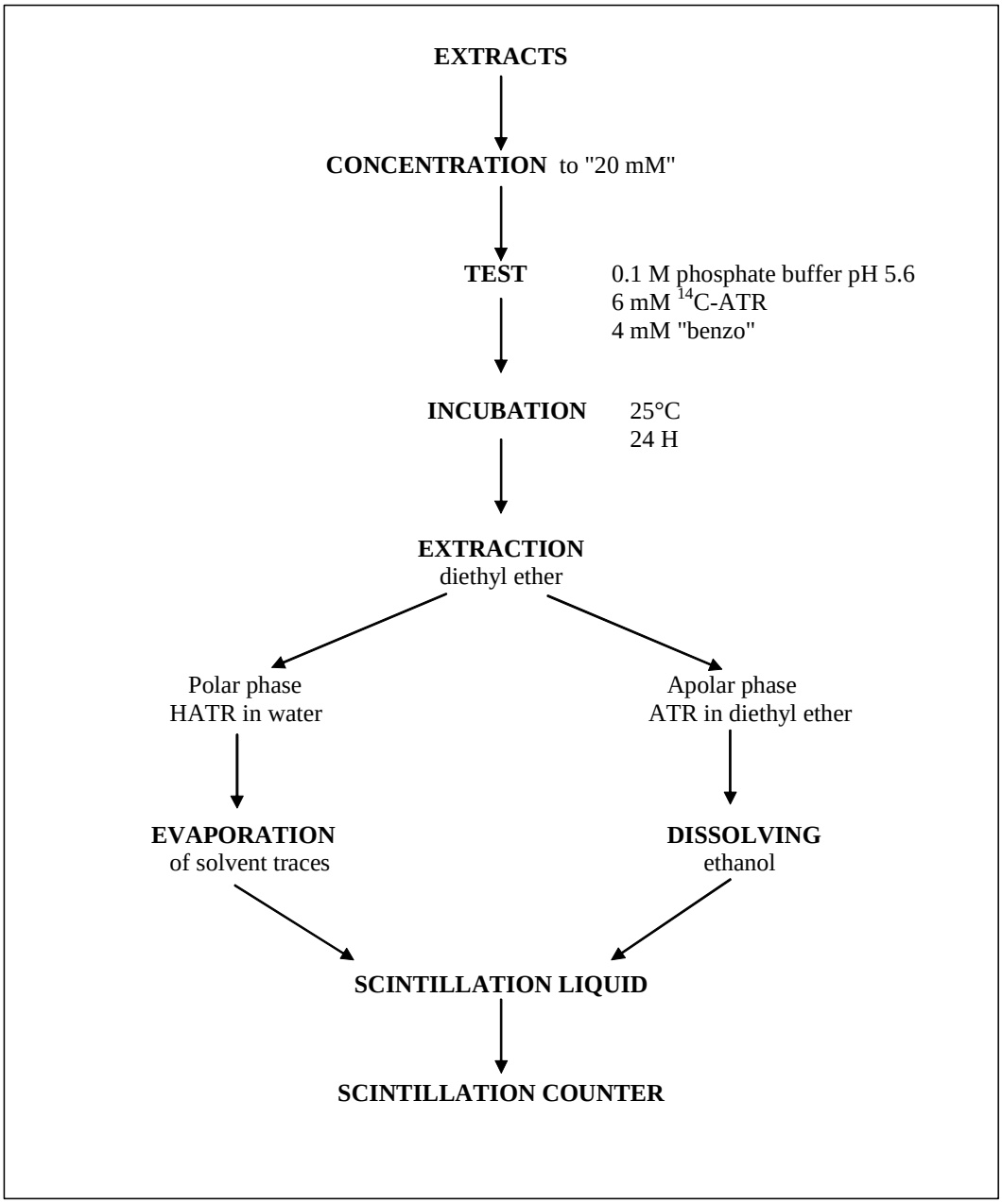


Figure 6.3 *In vitro* hydroxylation test of atrazine

6.3 Results

The acetate ethyl extract of leaves exhibited clear bands with Rf which might correspond to DIMBOA (Rf = 0.90) and to DIBOA (Rf = 0.83). The aqueous-acetonic extract of leaves was found to have a product Rf = 0.83 which might correspond to DIBOA. Nevertheless, as the solvent used for extraction was water, it seemed unlikely that this band could be DIBOA; the latter being mainly extracted by ethyl acetate. The aqueous extract of leaves exhibited a product remaining at the origin, which could correspond to diglucosylated benzoxazinones (**Figure 6.4 and Table 6.1**).

The acetate ethyl extract of roots exhibited weak bands with Rf corresponding to DIMBOA (Rf = 0.88), and DIBOA (Rf = 0.81). A product remaining at the origin was unlikely corresponding to diglucosylated benzoxazinones, as the solvent used was ethyl acetate. The aqueous-acetonic extract of roots was found to have products at Rf = 0.21 and Rf = 0.17 which might correspond to monoglucosylated benzoxazinones. A strong band remaining at the origin could also correspond to diglucosylated benzoxazinones (**Figure 6.4 and Table 6.1**).

Ethyl acetate and aqueous extract of leaves were therefore massively (1.2 mL) reloaded on TLC plates. Separated products were scrapped and their UV spectra further studied, except the aqueous extract product with Rf = 0.83 for the reason mentioned above. Aqueous extract of roots was also massively (1.2 mL) reloaded on TLC plate, scrapped and eluted to study their UV spectra. UV spectra of products of ethyl acetate root extract were not studied as product with Rf = 0.90 and 0.83 were too weak, and product Rf = 0 was unlikely diglucosylated benzoxazinone.

None of the spectra of products previously separated by TLC exhibited typical pattern of benzoxazinones (**Figures 6.5 and 6.6**). Spectra of purified products obtained by scratching TLC plate were lacking the characteristic pattern of benzoxazinones UV spectrum with maximum absorbance at 200-215 [nm] and 265 [nm]. No bathochromic effect was observed after the addition of $AlCl_3$, as described by Raveton (134).

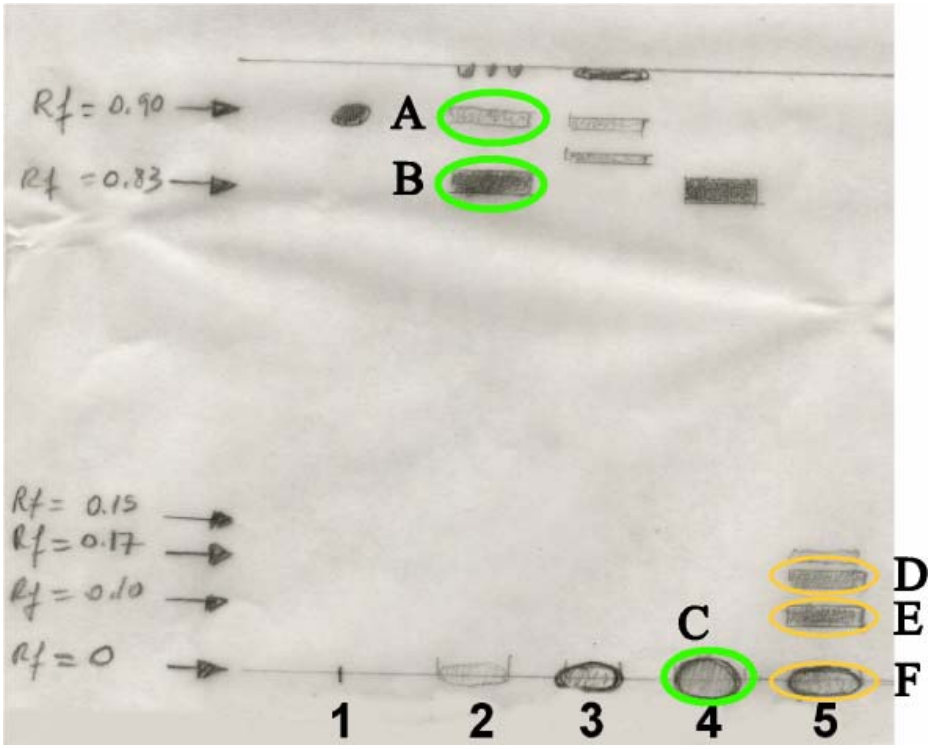


Figure 6.4 Trial of benzoxazinones detection in vetiver

TLC of vetiver leaf and root extracts with developing solvent ethyl acetate/formic acid/acetic acid/H_2O 20/1/1/2, v/v/v/v. Track 1 DIMBOA standard, track 2 ethyl acetate extract of leaves, track 3 ethyl acetate extract of roots, track 4 aqueous extract of leaves, track 5 aqueous extract of root. Circled products were further scrapped and eluted and their spectra studied (see also Figures 6.5 and 6.6).

Table 6.1 Rf standards migration on TLC plate
Developing system ethyl acetate/formic acid/acetic acid/H_2O 20/1/1/2, v/v/v/v described by Raveton M, 1996 (134)

Rf	Corresponding benzoxazinone	Solvent extractor
0.88	DIMBOA	ethyl acetate
0.81	DIBOA	ethyl acetate
0.17	Glc-DIMBOA, Glc-DIBOA	water
0	diGlc-DIMBOA	water

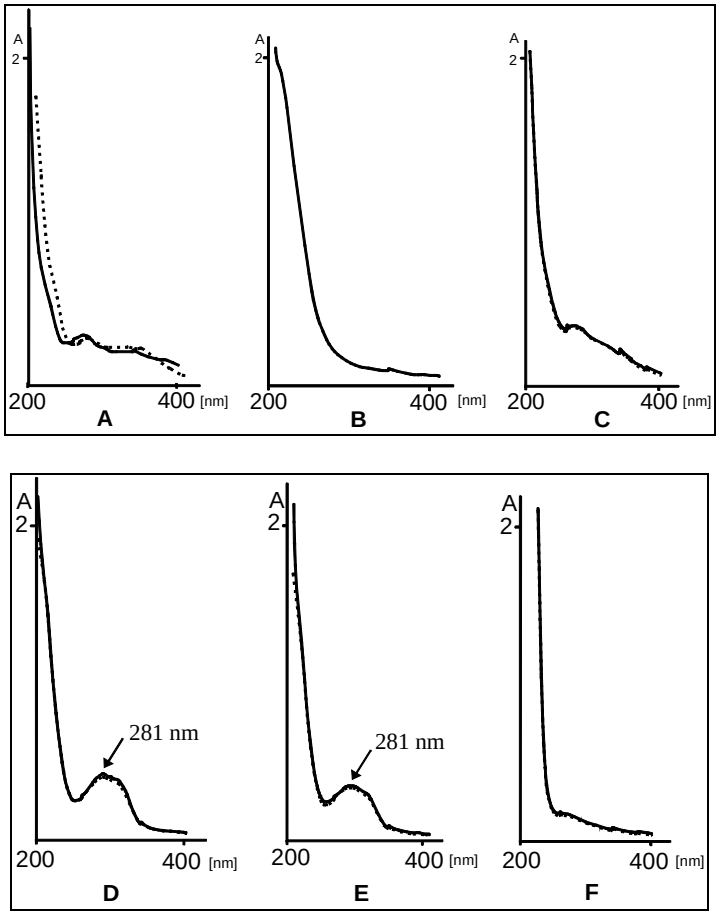


Figure 6.5 Spectrum of products obtained from vetiver leaves
(A, B, C) and roots **(D, E, F)**
(A) ethyl acetate extract Rf = 0.90 (B) ethyl acetate extract Rf = 0.83 (C) aqueous extract Rf = 0. (D) aqueous extract Rf = 0.21 (E) aqueous extract Rf = 0.17 (F) aqueous extract Rf = 0. Dashed lines represent the tested product with adjunction of $AlCl_3$

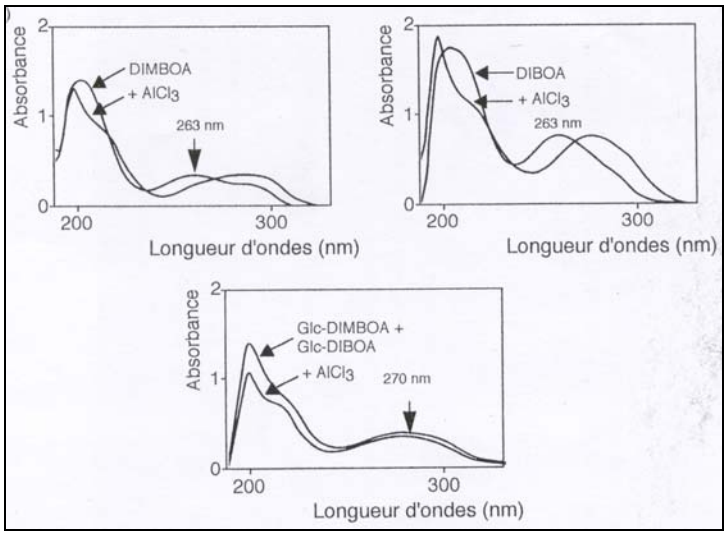


Figure 6.6 Reference spectra of benzoxazinones by Raveton (134)

Each extract was though quantified for its benzoxazinones content by taking absorbance value at 281 [nm] instead of 265 [nm]. An approximation was made using the known molar extinction coefficient of DIMBOA at λ = 262 nm (ε_{262} = 8000 [M^{-1} cm^{-1}]). Knowing that extracts were not pure, it was assumed that the background of the extracts could shift the peak from 265 [nm] to 281 [nm], or completely hiding the peak at 265 [nm]. By so, it was possible to test hydroxylation potential of atrazine by the extract.

No activity of vetiver leaf and root extracts towards atrazine was detected (**Table 6.2**). If hydroxylation of atrazine would have occurred, low amount of radioactivity should have been detected in the aqueous test solution after ether diethyl extraction of atrazine. It was not the case. Moreover, the percentage found in the aqueous phase of the tested extracts was right in the percentage of extraction rate of atrazine by ether diethyl (control 3) (92.2 % and 91.6 % respectively). Atrazine alone in buffer (control 2) was in the same range of extraction rate of atrazine by ether diethyl (control 3), showing that under our tested conditions, spontaneous hydroxylation was negligible. Finally, the assay was considered as valid since positive control, atrazine together with pure DIMBOA, was highly hydroxylated (60%).

Table 6.2 *In vitro* test of hydroxylation of atrazine

Percentage of radioactivity extracted by ether diethyl (= remaining intact atrazine) of a test solution containing atrazine and putative benzoxazinones. Control 1 was incubation ofatrazine in buffer to check spontaneous hydroxylation, control 2 was done with atrazine and standard DIMBOA and control 3 was rate of atrazine extraction from aqueous buffer with ether diethyl solvent

Leaves		**Roots**		**Control 1**	**Control 2**	**Control 3**
Ethyl acetate extract	**Aqueous acetonic extract**	**Ethyl acetate extract**	**Aqueous acetonic extract**	**Spontaneous hydroxylation**	**Positive control**	**Extraction rate of atrazine**
92.4 %	91.8 %	92.5 %	90.6 %	93.8 %	40 %	92.2 %

6.4 Discussion

In vitro, the standard DIMBOA could hydroxylate 60% of atrazine with 20% ethanol present in the assay, showing that the tested conditions were correct to detect any hydroxylation of atrazine by vetiver extracts. Hydroxylation was in the range as expected, as compared with Raveton (134) who showed that the presence of an organic solvent (ethanol or acetone) was responsible for decreased hydroxylation in the reaction medium: 50% ethanol resulted in 30% hydroxylated atrazine.

In the present study, no special treatment was done to preserve glucosylated-DIMBOA and DIBOA. Because it was shown by Raveton et al. (135) that DIMBOA, DIBOA and their glucosylated derivatives were able of hydroxylating atrazine, it was not found necessary to undergo special treatment to preserve glucosylated DIMBOA and DIBOA. In the case of positive detection of benzoxazinones and hydroxylating activities, it could have been interesting to know the respective contribution of non glucosylated and glucosylated benzoxazinones to hydroxylation of atrazine; special care to preserve glucosylated DIMBOA and DIBOA could have been done, to avoid production of aglucones by active endogenous glucosidases when aqueous extracts are prepared from plant material.

As shown by Virtuanen and Wahlroos (176) and Pethö (120, 121), DIMBOA and DIBOA are transformed by heating into 1,3-benzoxazin-2-one (BOA) and 7-metoxy-1,3-benzoxazin-2-one (MBOA) respectively. These derivatives are unable to hydroxylate atrazine (134, 135). In contrast, the glucosylated derivatives remain resistant to heat degradation, even for 15 min at 100°C. Thanks to separation of non polar benzoxazinones (DIMBOA and DIBOA) and polar benzoxazinones (glucosylated DIMBOA and DIBOA) by partition with ethyl acetate, it was possible to avoid the formation of BOA and MBOA. The concentration of ethyl acetate containing putatives heat sensitive DIMBOA and DIBOA was done with a Rotavapor, without heating because of high volatility of the solvent. Concentration of the acetonic-water phase containing putative glucosylated benzoxazinones was done with butanol and by heating at 40°C. It was therefore concluded that the non hydroxylation of atrazine by vetiver extracts was unlikely due to degraded benzoxazinones, but could be rather explained by the absence or low amount of benzoxazinones in vetiver.

Maximization of detection of benzoxazinones was done, strengthening the statement that these secondary metabolites were not present in our vetiver extracts. Despite of a high biomass used for benzoxazinones extraction, 55 [g] fresh mass, no confirmation of their existence and activity could be done. As a comparison, 10 [g] of fresh leaves of maize give much more intense spots on TLC plates, as benzoxazinones are massively produced as secondary metabolites in maize (Prof Michel Tissut, personal communication[6]). Only 10 [g] of maize leaves contain 241 [µmol g^{-1}] of DIMBOA and roots contain 128 [µmol g^{-1}] (134). Moreover, none of the putative benzoxazinones exhibited a characteristic peak at 265 nm and UV spectra of partial purified products did not fit to those obtained by Raveton (134).

Leaf and root sampling were done on a plant grown hydroponically for 8 months. It is possible that under these conditions, benzoxazinones are no longer found in the plant. Cherifi et al. (39) showed that apparent concentration in maize leaves decreases after one week from 20 mM to 6 mM, with a stabilization at this apparent concentration until week 4. Root apparent concentration of total benzoxazinones is stable at around 4 mM for 4 weeks, but benzoxazinones maize content was not assessed later on. From these data, it can be anyway concluded that benzoxazinones from hydroxamic acid class are much more abundant in young than in old plants. Friebe et al. (64) pointed out that the concentration of benzoxazinones in plants is highly dependent not only on plant age, but also on environmental growth conditions: increasing levels of hydroxamic acids are caused by light and water deficiencies. Cherifi et al. (39) on the other hand could not observe a clear dependence of benzoxazinone accumulation on light.

In summary, although some Rf of products separated by TLC were similar to benzoxazinones, their UV spectra study did not confirm the detection of benzoxazinones. Moreover, the addition of $AlCl_3$ did not result in bathochromic shift, and finally no hydroxylating activity on atrazine by vetiver extracts was observed. Because maximization of benzoxazinones detection was done, it could be concluded that benzoxazinones are not playing a major role in metabolization of atrazine, and if present in vetiver, they are not produced as high amount as in maize. Hydroxylation

[6] Prof. Michel Tissut, Laboratory for Xenobiotics and Environmental Perturbations, Université Joseph Fourier, Grenoble, France

mediated by benzoxazinones should therefore be low or nil in vetiver, at least in 8 months-old plant.

Nevertheless, these conclusions are valid for the tested plant age, 8 months, and for a plant grown in hydroponics, since benzoxazinones seem to depend on environmental conditions and on plant age. It is also not excluded that vetiver could contain other classes of benzoxazinones, different from hydroxamic acid derivatives class. It is not known if lactams, methyl derivatives, and benzoxazolinones classes could have been extracted by the present protocol, and if they are capable of hydroxylating atrazine or other herbicides.

Interestingly, Sicker et al. (158) cited studies where cultivated barley lost benzoxazinones, whereas wild barley was found to contain these secondary plant compounds. This might occur during agriculture breeding from wild barley. It is not exluded that cultivated vetiver biotype selected for soil erosion control also lost benzoxazinones.

7 *In vitro* atrazine conjugation by vetiver extracts

7.1 Introduction

The relative rate of herbicide detoxification between tolerant crops and sensitive weed species is frequently cited as a major determinant in herbicide selectivity (76). A well established example is the correlation between the relative rates of detoxification of the chloro-*s*-triazine herbicide atrazine by glutathione conjugation in sensitive and tolerant plant crops. Hatton et al. (76) were interested in identifying the range of GST activities toward herbicides and their associated weed grass species and in determining the role of these enzymes in herbicide selectivity. They developed an assay suitable for determining GST activities toward a variety of herbicide substrates, including atrazine. Conditions of saturating substrate concentrations were used to allow a comparison between species. They correlated specific GSTs activity (pkats per mg of protein) with the observed selectivity of herbicides and rate of metabolism in detached maize leaves sprayed with atrazine.

In contrast, in whole maize seedlings where atrazine penetrated through the roots, it was shown by Cherifi et al. (39), that this species metabolizes atrazine via the classical three pathways hydroxylation, conjugation and dealkylation, with a clear pre-eminence of namely hydroxylation. Instead of measuring specific activities of GSTs of a plant sample, they obtained an acetone powder of entire leaves and/or root biomass. They could express GST activities in nmol of conjugate per plantlet. By so, a clear contribution of conjugation could be estimated and correlated with detected conjugates in entire plants. By total quantification of benzoxazinones, total hydroxylation could be calculated and directly compared to conjugation.

It seems that in the case of pre-emergence treatment, i.e. penetration of atrazine through roots, which corresponds to a situation of phytoremediation, and in the case of the presence of high amount of benzoxazinones leading to hydroxylation, no correlation between specific activities and tolerance is possible.

Nevertheless, we decided to reproduce the same conditions as Hatton et al. (76), especially because the authors worked with sorghum species, known to tolerate atrazine thanks to conjugation (91-93, 150). As vetiver is related to this species, direct comparison of GST specific activities is relevant to assess the possible ability of

vetiver extracts to conjugate atrazine. As *in vitro* hydroxylation and benzoxazinone detection were negative (chapter 10), the GST specific activities could explain vetiver tolerance to atrazine. Correlation of specific activities with *in vivo* tests in detached organs and entire plants is described in the next chapters of the present thesis. But unlike Hatton et al. (76), correlation with *in vivo* experiment was done in entire plant treated from the roots.

The global GST activity can be shown when using a substrate such as CDNB: indeed the standard experimental assay for GST activity uses 1-chloro-2,4-dinitrobenzene (CDNB), a model for most, but not all GSTs. Conjugation of CDNB with GSH (by chlorosubstitution) results in a change of absorbance of the compound at 340 nm, providing a simple spectrophotometric assay. This substrate was used in the present experiments in order to evaluate extraction of GSTs and the stability of extracts.

7.2 Material and methods

Plant material

Vetiver plants were grown hydroponically or in organic soil for 1 year in glasshouse (see chapter 5 for culture conditions). New leaves ("young" leaves) of 4-5 weeks were collected from plants grown in soil, as well as the tip of leaves of 8-12 months ("old" leaves). Plant roots grown hydroponically were divided into unsuberized, white "young" roots and suberized, brown, "old" roots.

To validate the protocol in use for testing GSTs activities in vetiver, *Zea mais* (LG 2185, Limagrin, obtained from Samen Mauser Ltd, Switzerland) was grown from seeds for 5 weeks in quartz sand watered with nutritive solution Luwasa®.

Collected material of vetiver and maize plants were immediately used for GSTs extraction.

Plant extraction of GSTs

Plant extraction of GSTs was based on Dr P. Schröder protocol[7]. Ten [g] of leaf or root tissues were ground separately into powder with a pestle and mortar using liquid nitrogen. The powder was thawed gently at 4°C in 100 [mL] of 0.1 M phosphate

[7] Dr Peter Schröder, Institute of Soil Ecology, Forschungzentrum für Umwelt and Gesundheit, Neuherberg, Germany

buffer pH 7.8 containing 5 mM EDTA (Fluka), 1% PVP K30 (BDH), 5 mM DTE (Sigma) and IGEPAL CA-630 (ICN). The next extraction steps were all performed at 4°C: the homogenate was then centrifuged at 39’000 g for 30 min. To the supernatant was added 40% ammonium sulphate and it was again centrifuged under the same conditions. To the supernatant was then added ammonium sulphate to obtain 80% saturation. The protein pellet was collected by centrifugation and it was then desalted in phosphate buffer (2 mM pH 6.8) by using Sephadex G-25 columns (Pharmacia PD10). Protein content was determined using the Bio Rad Lowry assay using bovine serum albumine (BSA) as a standard. Prior testing conjugation activities, extracts were adjusted to 10 mg protein per mL, frozen in liquid nitrogen, and stored at -80 °C until use. Summary of GSTs extraction steps are shown in **Figure 7.1**.

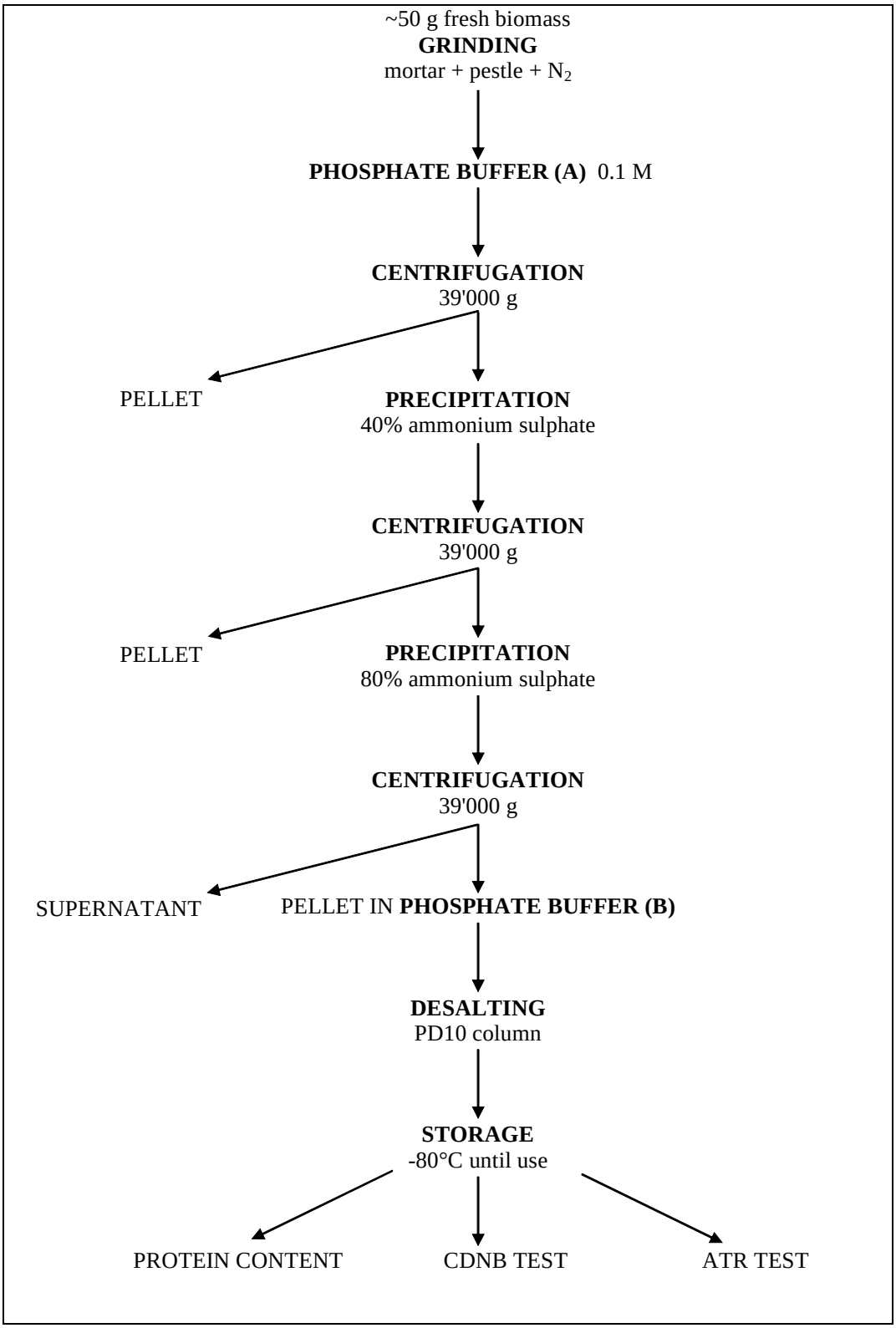


Figure 7.1 Vetiver GSTs extraction steps

Assay of vetiver GSTs activity on CDNB

Assay of vetiver GSTs activity on CDNB was based on Dr P. Schröder protocol[1]. After extraction and concentration steps, as well as before and after freezing, GSTs activity was checked using the substrate 1-chloro-2,4-dinitrobenzene (CDNB, Merck). The CDNB conjugate formation was followed for 5 min at 340 [nm] with a spectrophotometer (U-2001, Hitachi). GSTs activity toward the herbicide substrate was determined by mixing 30 [µL] of enzyme extracts with 20 [µL] of 30 mM CDNB dissolved in acetone, 10 [µL] 60 mM GSH (Sigma) dissolved in 0.1 M phosphate buffer pH 6.4, and 540 [µL] 0.1 M phosphate buffer pH 6.4. Specific activities were calculated using ε_{340} = 9.6 [mM^{-1} cm^{-1}], according to following formula:

$$specific\ activity\ \left[pmol\,\sec^{-1}mg^{-1}\right] = \left[pkats\ \ mg^{-1}\right] = \frac{\Delta A}{\Delta t} * \frac{V_{tot}}{\varepsilon * d * m * ne^{-} * V_s} \quad (16)$$

A/ t = change of absorbance per sec

ε = extinction coefficient [mM^{-1} cm^{-1}]

d = path length of the cuvette 1 [cm]

ne^- =number of electrons involved in the reaction

V_{tot} = total volume in the cuvette [mL]

V_s =volume of sample added in the cuvette [mL]

Assay of vetiver GSTs activity on atrazine

Assay of vetiver GST activity on atrazine was based on publication of Hatton et al. (76). Atrazine conjugation test consisted of 210 [µL] of the extract, 17.5 [µL] of 10 mM atrazine dissolved in acetone, 35 [µL] of 10 mM GSH in 0.1 M phosphate buffer pH 6.8, and 87.5 [µL] 0.1 M phosphate buffer pH 6.8. The mixture was incubated at room temperature for 10, 30 and 60 min. The reaction was then terminated by the addition of 10 [µL] of 0.6 M hydrochloric acid. The precipitated proteins were removed by centrifugation (12'000 g, 5 min).

ATR-GS detection with HPLC

Conjugated atrazine detection was based on publication (76). 250 [µL] of the supernatant obtained as described previously was injected on a C_{18} HPLC column (Spherisorb Octadecyl 250 x 4.6 mm, 5 µm diameter particle, Macherey-Nagel). Elution conditions were those applied by Hatton et al. (76): equilibration was done for 12 min. with solvent A + solvent B (95 + 5 by volume), where solvent A was water + phosphoric acid (99 + 1 by volume) and solvent B acetonitrile. The column was then eluted at 0.8 [mL min^{-1}] with a two step gradient from solvent A + solvent B (95 + 5 by volume) at time 0 to solvent A + solvent B (90 + 10 by volume) at 5 min and then to solvent A+ solvent B (43 + 57 by volume) at 28 min. The eluant was monitored for UV absorbance at 264 nm and 220 nm. After each run, the column was washed with acetonitrile for 15 min to remove any trace of atrazine, and re-equilibrated for 12 min.

Controls consisted of (i) omitting GSH from the incubation to correct for material which might be co-eluted with ATR-GS; (ii) omitting the enzyme, to correct for the non enzymatic rate of GSH conjugation (spontaneous conjugation). Retention time and spectra of peaks exhibiting increasing areas were compared to ATR-GS standard.

ATR-GS standard preparation

Attempts to use chemical synthesis described in (46, 76) failed, therefore enzymatic synthesis was chosen to obtain ATR-GS standard. 210 [µL] of 30 [mg mL^{-1}] equine liver purified GSTs (Sigma, 70 units mg^{-1} solid) were used together with 17.5 [µL] of 10 µM atrazine, 35 [µL] 10 mM GSH in 0.1 M phosphate buffer pH 6.8, and 87.5 [µL] 0.1M phosphate buffer pH 6.8. The reaction was ended by the addition of 10 [µL] 0.6 M hydrochloric acid after 5, 10, 20, and 30 min. Precipitated proteins were removed by centrifugation (12’000 g, 5 min). Quantification of formed ATR-GS was then done by conversion of quantity of disappeared atrazine, and allowed to obtain a calibration curve of ATR-GS.

7.3 Results

Activities of maize extracts

Specific activity of leaf extract of maize with CDNB was almost 3 times higher than found by Hatton et al. (76), 912 and 383 [pkats mg^{-1} protein] respectively (**Table 7.1**). In contrast, specific activity of maize extracts on atrazine was somewhat lower than described by the same author, 0.85 in the present work and 1.03 [pkats mg^{-1} protein] respectively. These differences could be attributed to the different maize varieties tested. No significant loss of GST activities was observed after deep freeze and thawing of the extracts. The observed differences of specific activities were considered as minor, and it was assumed that the protocol was useful to assess GST activities of vetiver.

Table 7.1 GSTs activity toward CDNB and atrazine in desalted extracts of 5 weeks old leaves of maize

Specific activity (± SD) [pkats mg^{-1} protein][1]		
	CDNB	**Atrazine**
Before freezing	912 ± 55	0.78 ± 0.04
After thawing	801 ± 68	0.85 ± 0.09

[1] Values refer to the mean of triplicates determinations of 1 experiment

Activities of vetiver extracts

Specific vetiver GSTs activities on CDNB were high when applying extraction and assay conditions used for maize (**Table 7.2**). Five weeks old leaves of vetiver and maize exhibited similar specific activities on CDNB (compare **Tables 7.1 and 7.2**). Specific activities on CDNB were slightly lower in 8-month old leaves compared to 5-week old leaves (**Table 7.2**). Roots were found to conjugate CDNB in the same range as leaves, but were found slightly higher in young roots than old roots. Specific activities were not affected by freezing and thawing procedure.

Vetiver GSTs activities were not detected in young and old vetiver roots, whereas ATR-GS was detected in 8-month and 5-week old leaves (**Table 7.2**). Typical elution profile of atrazine and ATR-GS are shown in chromatograms of **Figures 7.2 and 7.3**. Specific activities for atrazine were up to 2500 times lower than specific activities for CDNB, considering vetiver leaves.

Table 7.2 GSTs activity toward CDNB and atrazine in desalted extracts of vetiver

Specific activity (± SD) [pkats mg^{-1} protein]								
	CDNB [1]				**Atrazine[2]**			
	young leaves	**old leaves**	**young roots**	**old roots**	**young leaves**	**old leaves**	**young roots**	**old roots**
Before freezing	924 ± 55	824 ± 64	868 ± 35	843 ± 36	n/a	n/a[3]	n/a	n/a
After thawing	874 ± 68	863 ± 42	901 ± 54	839± 52	0.36± 0.06	0.42± 0.02	n/d[4]	n/d

[1] Values refer to the mean of triplicates determinations of 1 experiment
[2] Values refer to the mean of triplicates determinations of 3 independent experiments
[3] n/a: not available
[4] n/d: not detected

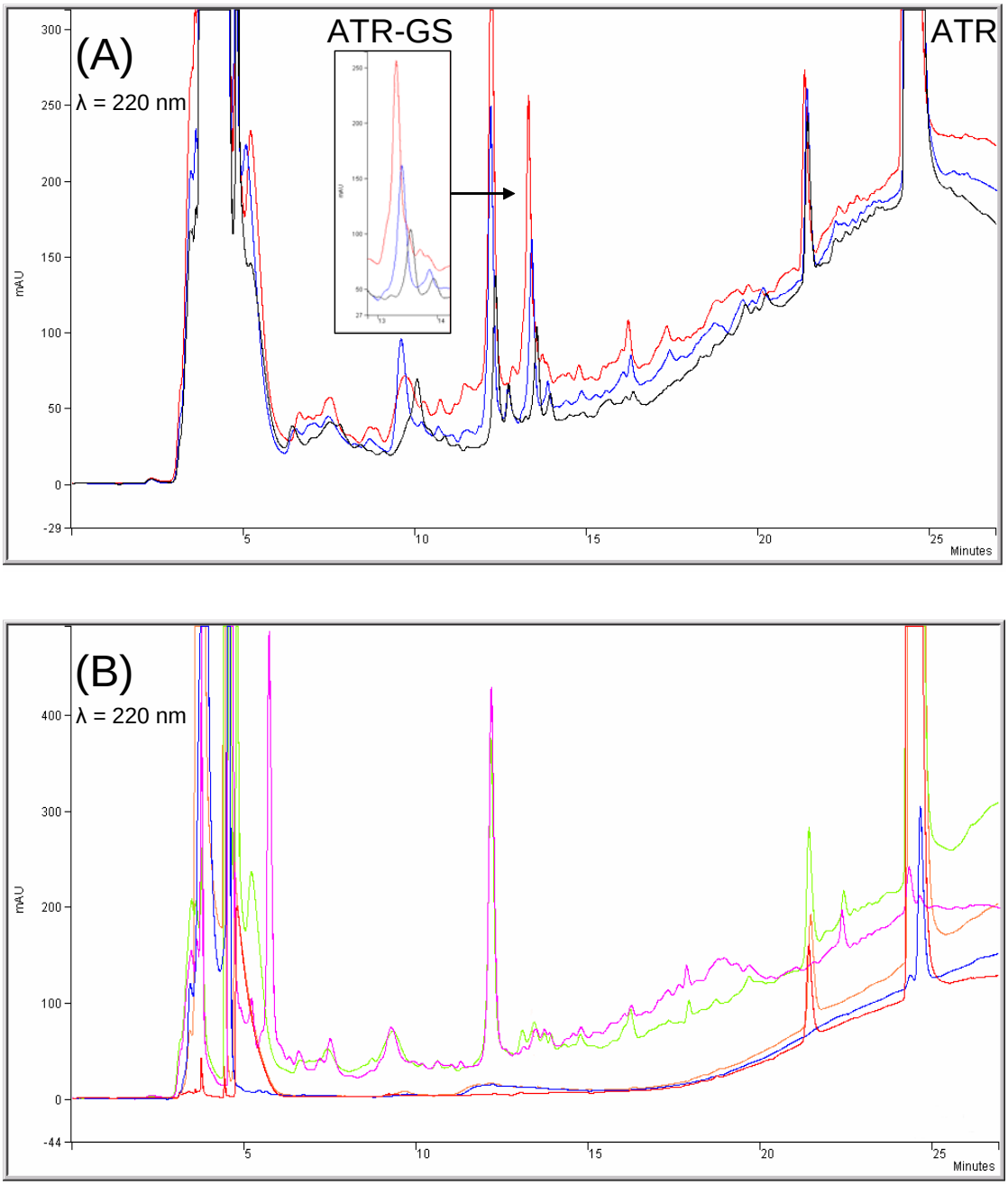
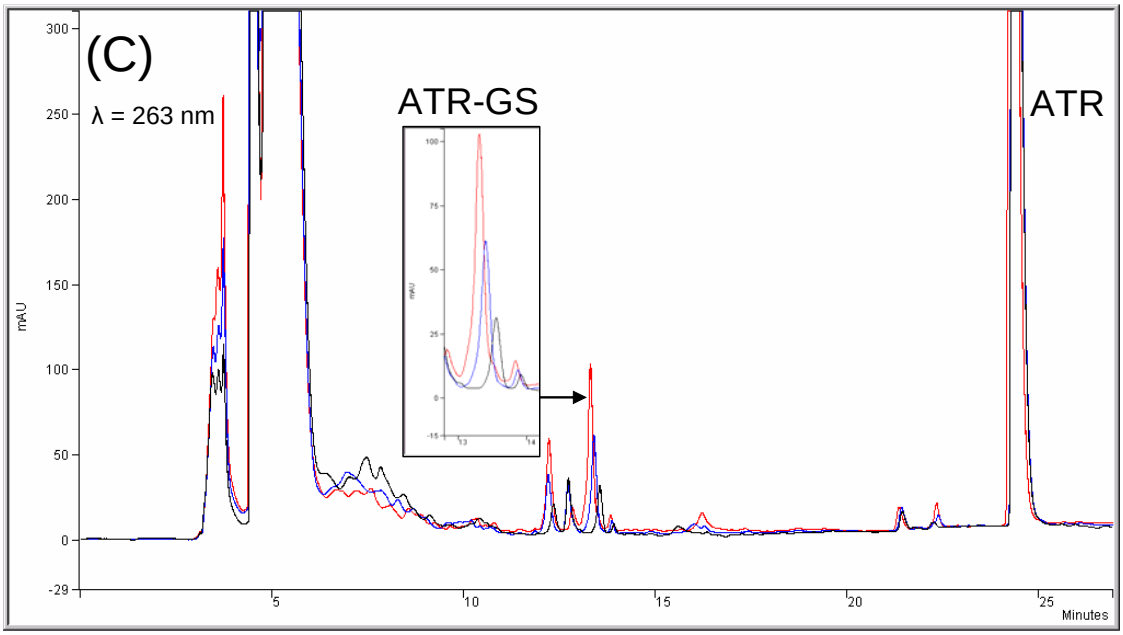


Figure 7.2 Chromatograms of Old leaves extract with atrazine and GSH at 10, 30, 60 min

(A) chromatogram at 220 nm, 10 min (black), 30 min (blue), and 60 min (red)
(B) corresponding controls at 220 nm at 60 min incubation: atrazine alone (red), GSH alone (blue), extract alone (pink), atrazine with GSH (orange), atrazine and extract (green)



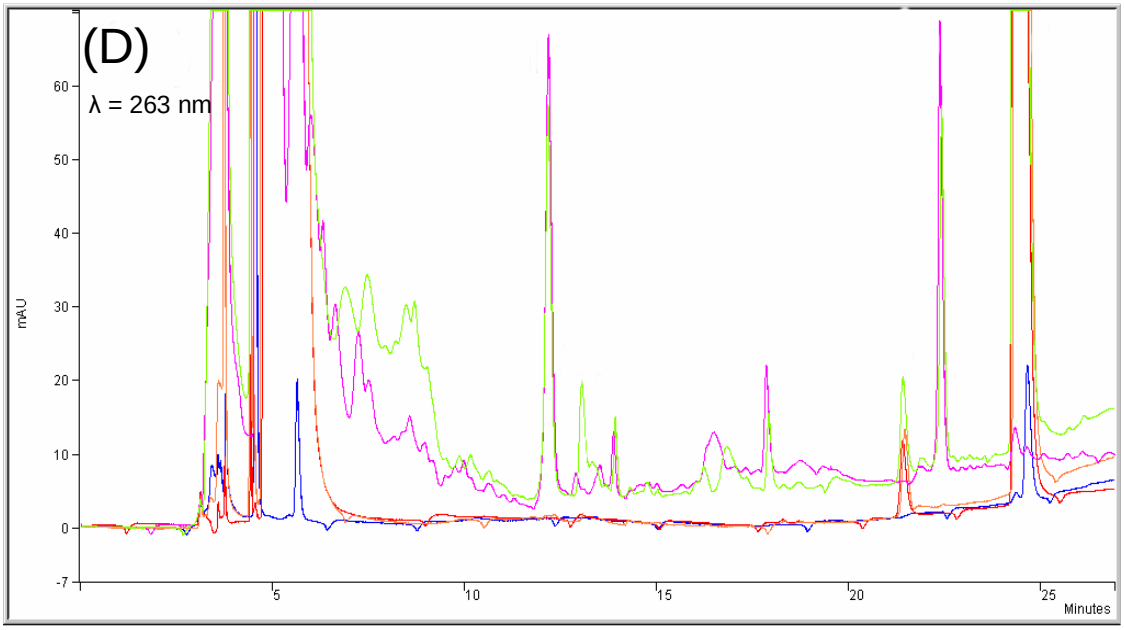


Figure 7.3 Chromatograms of old leaves extract with atrazine and GSH at 10, 30, 60 min

(C) chromatogram at 263 nm, 10 min (red), 30 min (blue), and 60 min(pink)
(D) corresponding controls at 263 nm at 60 min incubation: atrazine alone (red), GSH alone (blue), extract alone (pink), atrazine with GSH (orange), atrazine and extract (green)

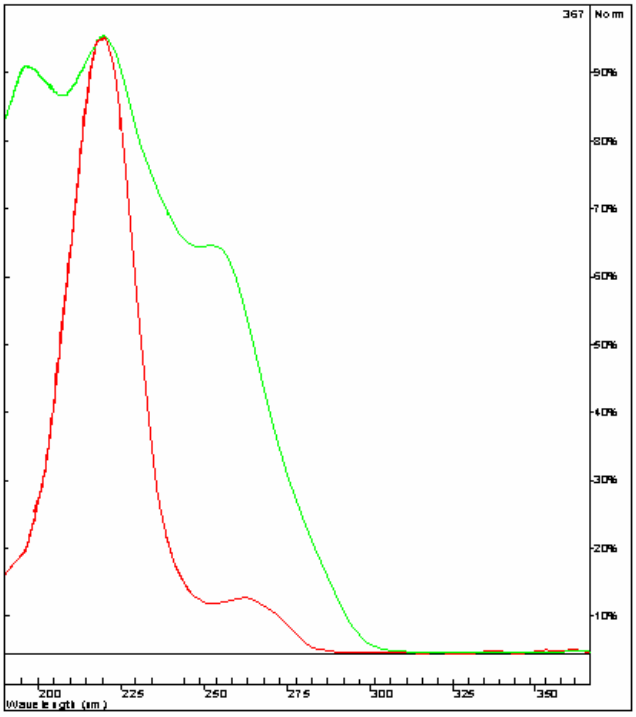


Figure 7.4 Normalized UV spectra of atrazine

Atrazine = red
Conjugated atrazine standard = green

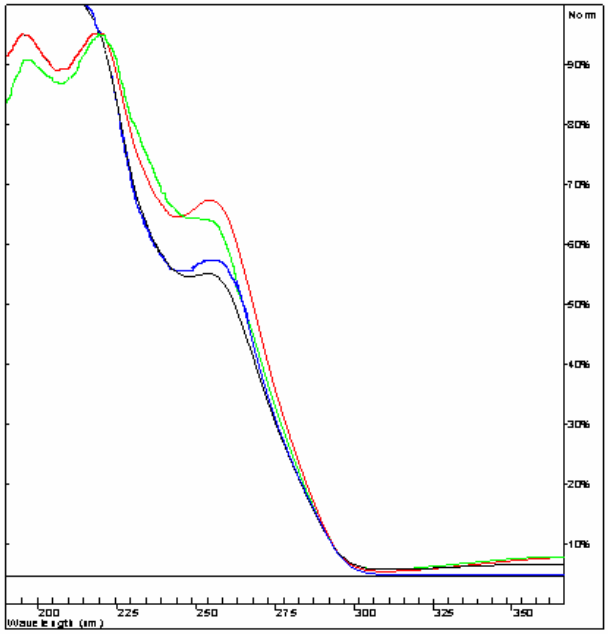


Figure 7.5 Normalized UV spectra of *in vitro* formed ATR-GS product

Incubation time of 10 min (black), 30 min (blue), 60 min (red). Conjugated atrazine standard (green). Analyzed products from extracts from old leaves, with retention time of 13.5 min

Spectra of the ATR-GS standard exhibited increased absorbance at 263 nm and between 180 and 220 nm compared to atrazine (**Figure 7.4**). Spectra of the formed products in the assays after 10, 30, 60 min were very similar to the ATR-GS standard (**Figure 7.5**). Moreover, absorbance was greater at 220 nm than 263, in agreement with the spectra of ATR-GS with a maximum absorbance at 220 nm higher than the shoulder at 263 nm. For all these reasons, the formed product at 13.5 min was identified as conjugated atrazine. Crude enzyme preparation from maize and vetiver were stable for 3 hours at room temperature, and for at least 7 hours at 4°C (longer time period was not assessed).

7.4 Discussion

It was assumed that vetiver GSTs had a pH optimum of 6.8, especially because vetiver is a species close to sorghum and belongs also to the same family as maize, *Poaceae*. GSTs from maize and sorghum have an optimal pH at pH = 6.8. GSTs activities on metolachlor, alachlor and fluorodifen from *Zea mays, Setaria faberi* (75), *Abutilon theophrasti, Digitaria sanguinalis, Echinochloa crus-galli, Panicum miliaceum,* and *Sorgum bicolor* (76) species have also an optimal pH at 6.8. The standard assay conditions were not optimized for vetiver and activity values reported have to be considered as minimal values.

Different soluble fractions were not tested for their potentiality of conjugation of atrazine: only the soluble fraction with ammonium sulphate 40-80% was tested. It was *assumed* that major activities were taken place in this fraction, like shown by different authors (39, 46, 75, 76). Conjugation of atrazine was assumed to be linked to soluble GSTs, and microsomal fraction was not explored.

For unknown reasons, attempts to chemically synthesize ATR-GS according to (46, 76) failed. Enzymatic synthesis was chosen to obtain ATR-GS standard. Purified equine liver GST enzyme in the presence of atrazine and glutathione produced glutathione conjugate of atrazine. This is not very surprising, since mammals and plants share similar detoxification pathways. As a comparison, Crayford and Hutson (46) obtained a standard of glutathione conjugate of atrazine enzymatically with a soluble fraction of rat homogenate precipitated with 65-85% ammonium sulphate in contact with atrazine and GSH.

Spectrum of biologically synthesized ATR-GS was consistent with the one described by Lamoureux et al. (91) who tried to identify major metabolite of atrazine in sorghum. They studied atrazine, simazine and propazine and found that UV spectra were nearly identical, indicating that minor variations in the alkylamino side chains do not cause a significant change in the ultraviolet absorption of light. The presence of CH_3S group instead of chlorine in the 2 position of triazine ring (ametryne) increased absorbance between 235 and 250 nm, with a slight shoulder at 235 nm. The increased absorbance was found in the present study beween 230 and 300 nm, with a shoulder at 263 nm. This difference could come from different solvent used (this information is lacking in the cited article). Nevertheless, the described pattern is very similar, despite of this "shift". The increased absorbance between 180 and 220 nm is due to peptidic bonds of glutathione. The UV spectrum of ATR-GS is more or less the addition of ametryn and glutathione, as checked with a spectrophotometer (data not shown).

With this *in vitro* test, it was concluded that the product formed at the retention time 13.5 min was ATR-GS. Nevertheless, literature reported that glutathione conjugate of atrazine can be further metabolized to γ-glutamylcysteine, L-cysteine, *N*-acetyl-L-cysteine and lanthionine conjugates (91-93). The metabolite γ-glutamylcysteine has only one amino acid less than glutathione conjugated atrazine, and should have a very similar UV spectrum and physical properties to ATR-GS. HPLC elution conditions were not optimized to separate glutathione, γ-glutamylcysteine, L-cysteine, *N*-acetyl-L-cysteine, and lanthionine conjugates. We should thus consider the possible presence of several conjugates of atrazine rather than glutathione conjugate only.

GSTs specific activities towards CDNB were higher than atrazine, in maize and in vetiver. Different authors found that specific activities of maize and sorghum were between 500 to 1500 times higher in the case of CDNB than atrazine (39, 75, 76). In the case of the genus *Setaria spp,* specific activities are similar for CDNB and atrazine (179). In vetiver, GSTs activity towards CDNB was 2300 times higher than atrazine. From this observation, it is evident that GSTs specific activities towards CDNB and atrazine can not be correlated.

This is reinforced by the fact that GSTs specific activities towards CDNB were of the same magnitude into vetiver leaves and roots, whereas no conjugates of atrazine could be detected into vetiver roots. As extracts were all adjusted to 10 mg protein per

mL before use for the enzymatic assay, we suggest that GSTs acting on CDNB and on atrazine are different and/or expressed in lower amount in roots than leaves.

Conjugation of atrazine was found in roots of entire vetiver plants (chapter 9), showing that with long time exposure, conjugation is detectable in vetiver roots. The test should have been run with higher concentration of root proteins to overcome HPLC detection and quantification limit. Induction of GSTs by safeners could also have been performed to enhance chances of detection of conjugates in vetiver roots.

GST specific activities on atrazine of young and old leaf extracts were found slightly higher than the one occurring in sorghum, as described by Hatton et al. (76) (0.36 and 0.25 [pkats mg^{-1} protein] respectively). Shimabukuro et al. (151) found GST specific activities in different corn biotypes leaves in the range of 0.45 to 0.91 [pkats mg^{-1} protein] Comparison with these articles could be considered as valid, because assays used substrate concentrations in excess of the reported Km values for all substrates (39, 76).

The *in vitro* conjugation of atrazine by vetiver leaf extracts was shown and GSTs specific activity on atrazine was found in the same range as sorghum leaves, suggesting that vetiver was able to metabolize atrazine in same the magnitude as sorghum, which is a species tolerant to atrazine. It is commonly admitted that during plant aging, less enzyme activities are recorded. GSTs of vetiver leaves of 8-month old, had slightly higher specific activities than 5-week old leaves, also showing GSTs important endogenous role. Interestingy, Lamoureux and Rusness (90) observed also that both GST and GSH decreased in corn (*Zea mays*) and increase in giant fotail (*Setaria faberii*) as tissues matured. For phytoremediation, this was interesting to know that conjugation potentiality was kept during plant aging. *In vitro* conjugation of atrazine by vetiver leaf extracts was found encouraging, and further identification of major metabolites of atrazine *in vivo* was therefore carried out.

8 Fate of ^{14}C-atrazine in excised vetiver organs

8.1 Introduction

Several authors worked with model systems and radio-labelled pesticides to optimize detection of metabolism and better understand plant resistance. Studies used leaf discs (91), excised leaves by immersing the cut end in solution containing the herbicide (92, 155), entire young seedlings totally immersed in solution (136), and cell cultures (60).

In corn leaf discs, it was found that major atrazine metabolite was glutathione conjugated atrazine (91). In excised leaves of sugarcane, maize and sorghum, 73% of atrazine was transformed into soluble compounds identified as conjugates (92). Shimabukuro et al. (155) observed that excised leaves of maize treated with ^{14}C-atrazine transformed within 30 hours 99% of the given atrazine. Conjugated atrazine was 82%, hydroxyatrazine 2.7%, and dealkylates 12.2%. In contrast to these studies, Raveton et al. (136) showed that immersed young seedlings of maize metabolize 95% of atrazine within 72 hours into hydroxy derivatives. Cherifi et al. (39) showed that maize seedlings treated from roots, transformed atrazine mainly in hydroxy derivatives in roots and leaves. Conjugation mainly occurs in the aerial parts and is effective only after 1 week cultivation.

In the case of maize, a clear difference of contribution of each metabolic pathway is observed in excised leaves and leaves of root-treated plant, and depends also on plant age and maize variety.

In contrast, in sorghum, the primary route of metabolism appears to be independent of the way of entry into the plant. The initial metabolic step is conjugation with glutathione, followed by conversion to lanthionine atrazine conjugate (92). The metabolites of atrazine produced by short term treatment of sorghum leaf disks are the same as those produced by root-treated sorghum plants, indicating that atrazine metabolism in sorghum leaf sections qualitatively and quantitatively approximates atrazine metabolism in intact plants (91).

Studying atrazine in excised vetiver organs was interesting for the present work, since we wanted to know if vetiver was acting rather like maize or sorghum. *In vitro* test of hydroxylation of atrazine was negative (chapter 6), whereas *in vitro* conjugation of atrazine was positive (chapter 8). Conjugation of atrazine was expected *in vivo*,

knowing that comparison of excised organs with entire plant would allow an actual quantification of plant metabolites.

To study vetiver metabolism in organs, experimental conditions developed by Raveton et al. (39, 134, 136) were used, including organ extraction, and TLC development solvent system. The ethyl acetate/formic acid/acetic acid/H_2O (40/2/2/4, v/v/v/v) solvent system is a good compromise to detect the presence or absence of the 3 classical classes of atrazine metabolites, dealkylates (around the front), hydroxylates (middle of migration), and conjugates (remaining at the origin). Originally, this solvent system was optimized for separation of hydroxyatrazine derivatives; separation of dealkylates is possible, but all types of conjugates remain at the origin.

To study dealkylates, a more apolar TLC development solvent system was used. Polar conjugates were not studied in details, keeping in mind that although tolerant species corn, sugar cane and sorghum are quite similar in their ability to convert atrazine to water soluble metabolites, notable differences are detected in the relative concentrations of the glutathione, cysteine, and lanthionine conjugates (92, 93).

The question rose if atrazine would accumulate in oil produced by vetiver roots. Mackay (98) pointed out that physical-chemical factors influence bioconcentration factors of organic solutes. Direct proportional relationship exists between bioconcentration factor and octanol-water partition coefficient. Log K_{ow}(ATR) is 2.5 and solubility is relatively low (33 mg/L), suggesting that atrazine would possibly accumulate in vetiver oil.

Vetiver oil is extremely complex, containing more than 300 components. It consists mainly of bicyclic and tricyclic sesquiterpenoids (hydrocarbons, alcohols, ketones, aldehydes, acids) (35, 50, 174), but also monoterpenoids and phenols have been detected. Typical representatives of the vetiver oil are (+)-α-vetivone, (-)-β-vetivone, khusinol and khusilal (97). In young roots no oil is detected and only in about six-month old roots, the oil appears in the form of oil drops mainly in the first cortical layer outside the endodermis. In older roots, cells in the cortical parenchyma are lysed forming lysigenic lacunae, which are filled with essential oil, resulting in oil ducts (172, 173, 177). Harvest of roots takes place at 15-24 months (184). The yield of oil is up 3% dry DW (50) and depends not only on the vetiver type cultivated, but also on

the climate, soil, frequent cutting of the grass, harvesting time, methods and time of distillation.

8.2 Material and methods

Exposure of vetiver leaves to ^{14}C-atrazine

Approximately precisely 1 [g] of fresh leaves was cut from vetiver plant rooted in soil and transferred in hydroponics 4 weeks before uptake experiment. New formed "young" leaves of 4 weeks were sampled. "Old" tested material was collected on the tip of green dark leaves (about 7-12 months old). The collected material was cut in segments of 3 [cm] length and put vertically into a glass vessel filled with 4 [mL] of Hoagland solution spiked with 56 [µL] of [^{14}C]-atrazine 5 mM, 1440 [MBq mL^{-1}], corresponding to 70 µM atrazine final concentration. The lower halves of the leaf segments were immersed, allowing the upper part to transpire (**Figure 8.1**). Parafilm around leaves parcel and vessel limited losses of the system other than transpiration of the leaves. Leaves were incubated for 72 hours at room temperature under agitation. Control consisted of 1[g] fresh leaves incubated with Hoagland solution without atrazine.

At the end of the assay, leaves were carefully washed with concentrated non-radioactive atrazine (25 [mg/L]) in water to prevent any rapid efflux of atrazine and its metabolites. Organs were then crushed first in a minimal volume of ethanol and then in water, together with sand of Fontainebleau. Pellets were counted to quantify extraction rate of radioactivity. Samples were bleached with sodium hypochlorite to avoid quenching when counting with the scintillation counter (Wallac, Winspectral).

Aqueous samples were concentrated with a Speedvacuum system (Savant) and ethanolic extracts were concentrated with Rotavapor to obtain sufficient radioactivity (1000 [dpm/100 µL]) to be detected with a TLC linear reader (LB 213, Berthold Analyzer). Extracts were re-dissolved in a minimal volume of their respective solvent, ethanol or water. 80 [µL] of each extract was then loaded on a TLC plate (60_{F254}, Merck) and developed with solvent system A (ethyl acetate/formic acid/acetic acid/H_2O 40/2/2/4, v/v/v/v) able to separate hydroxylated compounds of atrazine.

A second TLC plate was loaded and developed with solvent system B (petrol ether/chloroform/acetone 40/10/10, v/v/v) useful to separate dealkylates.

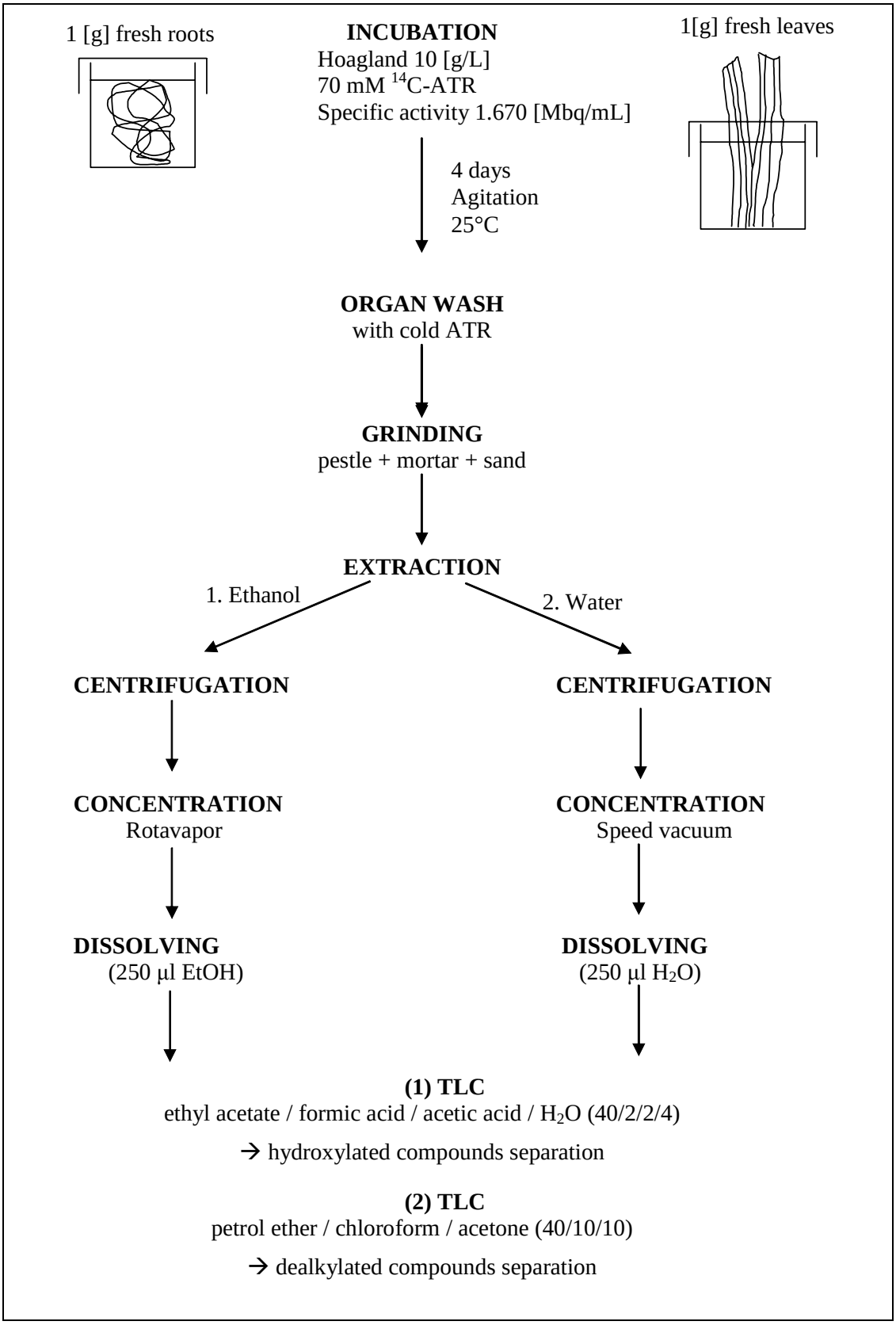


Figure 8.1 Metabolization of ^{14}C-atrazine in vetiver excised organs

Vetiver roots exposure to ^{14}C-atrazine

Approximately precisely 1 [g] of fresh roots was collected from vetiver plant rooted in soil and transferred into hydroponic system 4 weeks before experiment. Plant roots were divided into (a) roots smaller than 1 [mm], “young” roots (b) roots larger that 1 [mm] diameter “old” roots, (c) boiled and non-boiled “young” and “old” roots. This latter case was found useful to study metabolism when enzymes are inactivated. Root diameter was not taken into account for sampling. 1 [g] of fresh roots was totally immersed in 10 [mL] Hoagland solution (Hoagland Basal Salt, Sigma) spiked with 140 [µL] of [^{14}C]-atrazine 5 mM, 1440 [MBq mL^{-1}], corresponding to 70 µM atrazine final concentration. Containing vessel was maintained totally closed with parafilm to prevent any evaporation. Roots were incubated for 72 hours at room temperature under agitation.

Extraction and analysis were performed according to the procedure described for leaves exposed to ^{14}C-atrazine.

Lipid extraction from vetiver roots

Lipid extraction was performed following Muriel Raveton protocol (134). Plant roots grown hydroponically for 1 year were divided into unsuberized (white) and suberized (brown) roots. Amongst these 2 categories, young roots smaller that 1 [mm] diameter were separated from roots larger than 1 [mm]. All plants grown in soil were suberized, and were therefore simply separated into roots smaller than 1 [mm] and larger that 1 [mm] diameter.

Approximately precisely 10 [g] of each category of roots were sampled and crushed in hydroacetonic phase acetone/water 80/20, v/v (3 X 50 mL). Extraction was performed until total disappearance of stock pigments. After straining the acetonic-water phase with 4 layers of muslin, and glass prefilter (Millipore), it was further re-extracted with petrol ether (B.P. 40-60 °C) (3 X 30 mL). The volume of ether phase was reduced with a Rotavapor, and further rinsed with water to remove any precipitate. Ether was then totally evaporated with a nitrogen flux, and subsequent obtained lipids were weighted.

In vitro atrazine partition in vetiver oil

Vetiver oil was kindly provided by Givaudan Ltd[8]. This oil was described to come from Haitian vetiver plants, and was filtered and dried by the manufacturer. In order to mimic as much as possible lipid and water proportion of a cell, 5 % of oil was used in order to determine log $K_{oil/water}$ (ATR). Atrazine tested concentration was 28 [mg/L]. Log $K_{oil/water}$ (ATR) partition into vetiver oil was calculated using atrazine disappearance from the aqueous compartment with HPLC (Agilent 1100, Hewlett Packard) at 220 nm. Aqueous phase was filtered at 0.2 [µm] (Sarstedt) prior injection in HPLC. 5 [µL] of aqueous phase was injected on column Supelcosyl octadecyl reversed phase (LC-18-T, 125 x 4 mm, Supelco). Column was equilibrated for 5 min with 70% acetonitrile and 30% water at 1 [mL/min], and column was eluted under the same conditions for 5 min. Log K_{ow} value was calculated with the following formula:

$$\frac{C_{equilibrium(ATR)}}{C_{initial(ATR)}} = \frac{V_{tot\ H_2 0}}{V_{tot\ H_2 0} + K_{oil\ /\ water} * V_{oil}}$$

C = concentration
V = volume

In order to compare log $K_{oil/water}$ (ATR) with log K_{ow}(ATR), the same conditions were used for experimental determination of log K_{ow}(ATR). The obtained log K_{ow}(ATR) was then compared to log K_{ow}(ATR) published in literature (134, 137, 166). Because the experimental determination of log K_{ow}(ATR) was similar to published values, it was assumed that equilibrium was also reached for atrazine between water and vetiver oil, allowing calculation of log $K_{oil/water}$ (ATR).

[8] Contact person: Mr Gandillon, Technical Unit, Givaudan Ltd, Geneva, Switzerland

8.3 Results

Penetrated radioactivity and concentration of atrazine equivalent in plant organs

Recovery of radioactivity of vetiver organs incubated 4 days with 14C-atrazine was 80%. Penetrated radioactivity was higher in leaves than roots, because leaves transpired the total volume of the solution (**Table 8.1**). Radioactivity was higher in the pool of old roots than in young roots. Water movement in leaves was the main way of entrance of atrazine, unlike roots totally immerged where partition and diffusion phenomenon's were dominant. To confirm that penetrated radioactivity was higher in old roots than young ones, a real concentration factor was calculated for roots, by using the approximation that 1 [g] of roots was representing 1 [mL] (**Table 8.2**). Old roots over-concentrated more atrazine equivalents than youg roots. Young boiled roots concentrated less radioactivity than non boiled roots. The reverse situation was observed in the case of old roots, where boiled organ accumulated more radioactivity than non boiled roots. Despite of this discrepancy, the same range of over-concentration of atrazine equivalents was observed suggesting simply that sampled roots differed in oil content.

It was confirmed that old roots were concentrating more atrazine equivalents than young ones. This different ability of roots to accumulate atrazine was best explained by the lipid content of roots (**Table 8.3**). Interestingly, roots grown in soil for 8 months, then transferred for 4 weeks in hydroponics contained more lipids than those grown in hydroponics for 8 months. On the other hand, lipid content seemed not to be related with root diameter. Difference of lipid content between roots from hydroponics and from soil may be related to the suberization of roots (young and old roots all with brown colour) higher in plants grown in organic soil than in water. Beside colour difference, roots grown in water were smooth and roots grown in soil where stiff. It is believed that these two macroscopic observations are due to different suberization of the roots grown in hydroponics or in organic soil.

Partition of atrazine in vetiver oil

Calculation of log $K_{oil/water}$ (ATR) was only possible with high concentrations of atrazine (28 and 14 [mg/L] atrazine were used), because surprisingly, oil diffused in water, giving a strong background on HPLC chromatograms. Background was subtracted to atrazine peak in order to calculate disappearance of atrazine from the

aqueous medium. Log K_{ow} (ATR) experimental determination was 2.5, in agreement with literature. Therefore it was assumed that under tested conditions, equilibrium of atrazine was reached between aqueous and octanol phases, and so should atrazine between aqueous phase and vetiver oil. Log $K_{oil/water}$ (ATR) could be calculated and resulted in a value of 2.41, showing that vetiver oil accumulated atrazine. Knowing Log K_{ow} of dealkylates, it was possible to calculate their respective log $K_{oil/water}$, assuming that linear relationship was true between log K_{ow} and log $K_{oil/water}$ (**Table 8.4**). Log $K_{octanol/water}$ and log $K_{oil/water}$ of dealkylates were similar, as are Log $K_{octanol/water}$ and log $K_{oil/water}$ of atrazine.

Table 8.1 Percentage of penetrated radioactivity in vetiver organs

Organ	% Penetrated radioactivity
Young roots	8.0
Young boiled roots	5.7
Old roots	18.7
Old boiled roots	31.4
Young leaves	49.4
Old leaves	62.0

Table 8.2 Concentration factor of atrazine equivalents in vetiver roots

	Concentration factor
Young roots	0.8
Young boiled roots	0.6
Old roots	3.0
Old boiled roots	4.0

Table 8.3 Lipid content from vetiver roots grown in hydroponics or earth for 1 year

Results are expressed as a percentage of fresh biomass.

Hydroponics				Soil	
White roots		Brown roots		Brown roots	
<1 mm	>1 mm	<1 mm	>1 mm	<1 mm	>1 mm
0.3	0.4	0.3	0.4	3.8	4.2

Table 8.4 Log $K_{oil/water}$ of atrazine and dealkylates

	log $K_{octano/water}$	**Log $K_{oil/water}$**
ATR	2.5[a]-2.4[b]	2.41[c]
DEA	1.7	1.63[d]
DIA	1.38	1.33[d]
DDA	0.78	0.75[d]

[a] data from (137, 166)
[b] data from (134)
[c] experimental data obtained in the present work
[d] deduced log K value from log $K(ATR)_{oil/water}$

Metabolism of atrazine in vetiver organs

Autoradiography of TLC plates of ethanolic extracts revealed that atrazine treatment solution before experiment contained other product traces with Rf 0.85 (DDA), Rf 0.72 (non identified product), Rf 0.32 (HATR) and Rf 0.27 (HDEA) (**Figure 8.2 (A)**). However, the main product of treatment solution was atrazine and therefore it was considered that experiment was valid. Quantification of products formed by vetiver extracts with same Rf of "impurities" as treatment solution was obtained by subtraction of "impurities" of treatment solution.

Autoradiography revealed the presence of dealkylates DIA, DEA, DDA and HATR in all tested extracts as shown in **Figures 8.2 (A) and (B)**. But when subtraction of background metabolites from treatment solution was done, these products were less than 1% of metabolites. Vetiver young and old leaf extracts exhibited product

remaining at the origin of TLC plate developed with solvent A, corresponding to conjugates. Vetiver root extracts did not show any product at origin of the migration.

Boiled and non boiled roots exhibited the same pattern (**Figure 8.2 (A)**) suggesting that observed traces of HATR and dealkylates were mainly coming from the treatment solution. After subtraction of this background, remaining HATR in boiled and non boiled roots was probably due to spontaneous hydroxylation. Remaining radioactivity of dealkylates in boiled roots could possibly come from bacterial activities in treatment solution. No conjugates were detected after 72 hours organ incubation with atrazine.

Segregation of metabolites should have occurred by using ethanol and water solvents for organ extraction. Ethanol extracts should contain atrazine and dealkylates, whereas water phase should contain hydroxy derivatives and conjugates. Inadvertently, the first aqueous extraction was mixed with ethanolic extracts, explaining why aqueous extracts were poorly loaded in radioactivity. Therefore, aqueous extracts autoradiographic pellicles and scans were fade and are not shown.

Autoradiographic pellicles were exposed for 2 months to radioactive TLC plates, revealing any minor formed product. However, quantification of each produced metabolite was done with a TLC linear reader. This device helped also to have a better picture of vetiver metabolic potentiality, because of lower sensitivity than autoradiographic pellicles. Only major products formed were detected (**Figure 8.3**). However, resolution near the front region was lost and atrazine and dealkylates should be grouped.

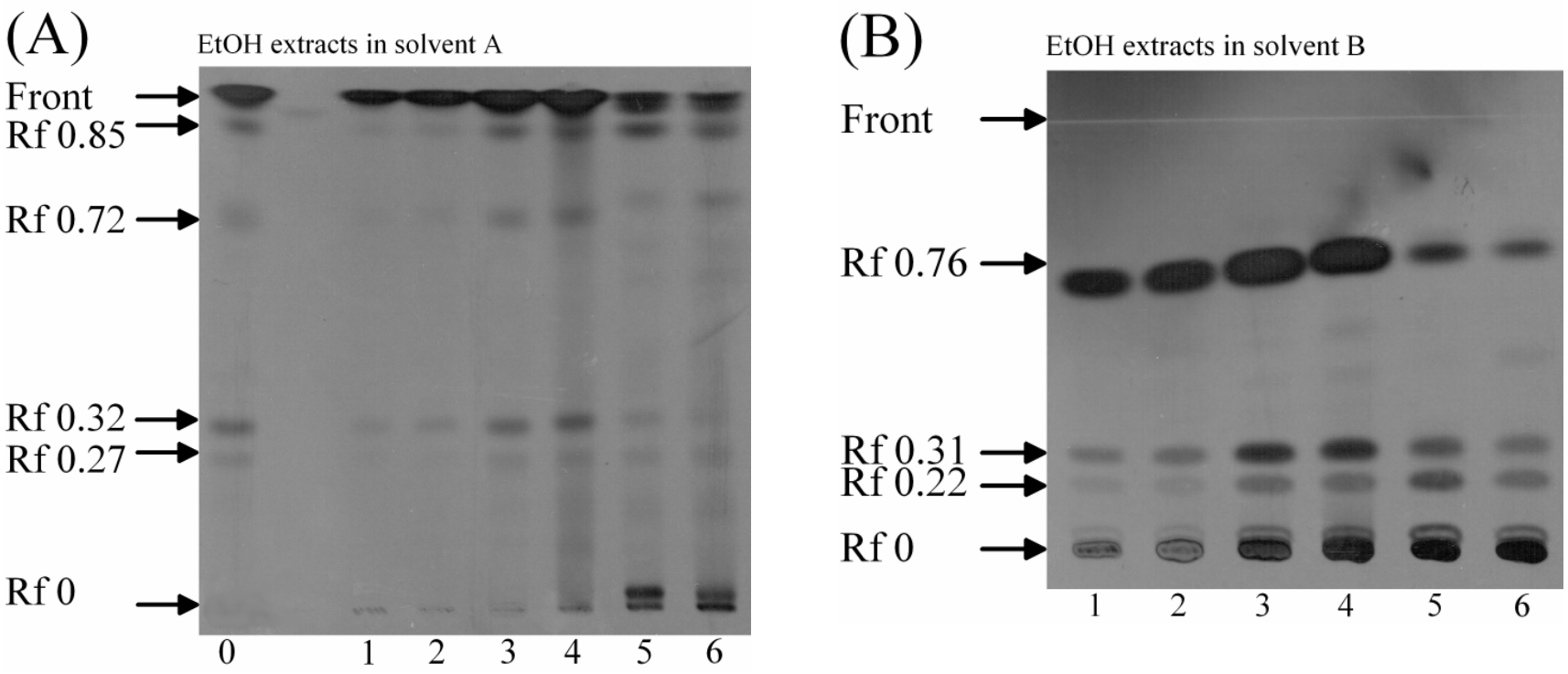


Figure 8.2 Autoradiographic pellicles of ethanolic extracts of vetiver roots and leaves loaded on TLC developed in solvent A and B

(A) Development in solvent A, (B) Development in solvent B

Track O treatment solution before experiment, **track 1** young roots, **track 2** young boiled roots, **track 3** old roots, **track 4** old boiled roots, **track 5** young leaves, **track 6** old leaves

(A) Rf 1 = ATR, Rf 0.85 = DDA, Rf 0.72 = ?, Rf 0.32 = HATR, Rf 0.27 = HDEA, Rf 0 = conjugates
(B) Rf 0.76 = ATR, Rf 0.31 = DIA, Rf 0.22 = DEA, Rf = = DDA, HATR, and conjugates

TLC developed in solvent A was used for calculation of percentage of metabolites in excised plant organs. Leaves of 7-12 months old ("old" leaves) were found to conjugate atrazine in the same range as 4-week old leaves ("young" leaves) (**Figure 9.3**).

Conjugates formation expressed in nmol per fresh biomass per hour showed that old leaves had the best potentiality of producing putative atrazine conjugates under the tested conditions (**Table 8.5**).

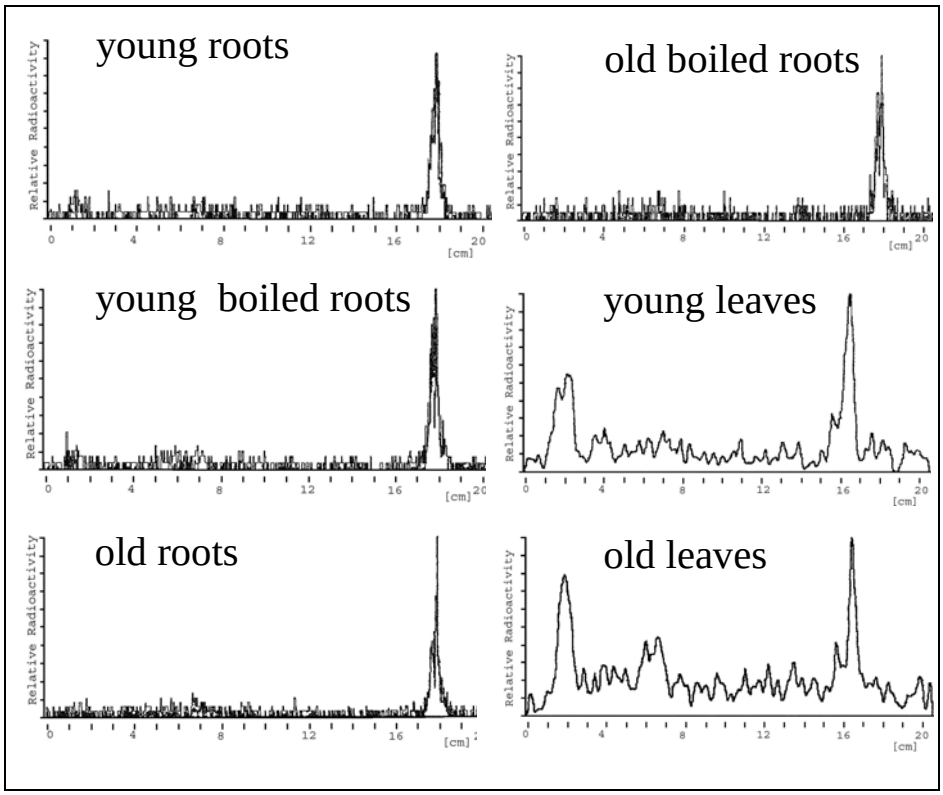


Figure 8.3 Radioactive scan of ethanolic extracts of vetiver roots and leaves

Table 8.5 Main metabolites in excised vetiver organs exposed to ^{14}C-atrazine

	Rf 1 % of ATR + dealkylates	**Rf O % of conjugates**	**Conjugates [nmol g^{-1} h^{-1}]**
Young roots	100	0	0
Young boiled roots	100	0	0
Old roots	100	0	0
Old boiled roots	100	0	0
Young leaves	53.7	46.3	0.41
Old leaves	41.5	58.5	2.64

8.4 Discussion

Roots were able to accumulate atrazine thanks to their lipid content. Lipid content was different between plants rooted and grown in hydroponics, and plant rooted in organic soil for 8 months. It is not known if these latter roots transferred for 4 weeks in hydroponics before contact with ^{14}C-atrazine "lost" already part of their lipids. It is clear that vetiver roots grown in hydroponics are not comparable to roots grown in organic soil. This is in agreement with Virmani and Datta (175) who mentioned that soil with low clay content improves quality of oil, but simultaneously decreases oil yield. In sandy pond and river bank, the decrease of oil is so extreme that distillation of vetiver roots becomes uneconomical. River bank could be possibly assimilated to hydroponics situation.

Atrazine metabolization study was performed with roots grown in organic soil and subsequently transferred to hydroponics 4 weeks before being sampled and put in contact with atrazine. Interestingly, in this type of roots, no significant metabolization was observed: dealkylation and hydroxylation were negligible and no conjugates were detected, suggesting that partition in lipids and vetiver oil could be important and unable enzymes to reach atrazine. *In vitro* partition of atrazine in vetiver oil was demonstrated and supports the latter hypothesis. From 8 months old, roots are full of oil in the whole cortex, suggesting a role of "sponge" of vetiver roots toward atrazine and dealkylates. Hydroxy derivatives produced in the soil, with log K_{ow} much smaller than atrazine and dealkylates (134) are unlikely accumulated in oily vetiver roots. It is not known if with time, remobilization of atrazine from vetiver oil of roots to aqueous compartment and to whole plant could occur.

The partition of atrazine in vetiver oil was tested *in vitro*, and further work should be done with extracted vetiver root oil from fields treated with atrazine to see if atrazine accumulation in oil is a physiological reality. However, the *in vitro* test is of great importance for oil producers who should be careful with atrazine use and more generally with all hydrophobic pesticides.

Dealkylation occurred, but at a very slow rate in all tested extracts, young roots and leaves, old roots and leaves, and boiled and non boiled roots. Dealkylation in boiled roots is surprising, since it is enzyme mediated. A possible explanation is that boiling was insufficient to destroy dealkylating enzyme activities. Hydroxylation also occurred in all tested organ extracts, but was negligible, in agreement with non

detection of benzoxazinones in vetiver extracts (chapter 6). Leaves had the highest potentiality to transform atrazine into non harmful conjugates, with higher transformation in leaves of 7-12 weeks than leaves of 4 weeks. This observation is in agreement with *in vitro* higher GST activities on atrazine in 8-12 months leaves than in 4-5 weeks old leaves (chapter 7).

The absence of hydroxy metabolites and conjugates in vetiver root extracts is not due to their disappearance in the surrounding medium. After 72 hours, this latter did not contain hydroxy derivatives in higher concentration than before experiment. This is not surprising, since Raveton et al. (136) showed that hydroxy derivatives were highly accumulated in corn seedlings but were almost unable to diffuse into the fresh medium. As log K_{ow} of hydroxyatrazine is close to 1.5, it is not high enough to prevent permeation through plant cell membranes, therefore Raveton et al. (136) proposed that ionization of the hydroxy group might induce a repulsion of the product by the electronegatively charged membranes, or that hydroxyatrazine binds tightly to cellular protein as it binds to soil component. Shimabukuro (150) showed that corn and sorghum absorbed radiola-belled hydroxyatrazine, but it was not readily translocated from root to shoots as reported for atrazine, strengthening the hypothesis of segregation of hydroxy derivatives in cell roots.

Conjugates were also not observed in treatment solution after 72 hours exposure to vetiver organs. This is in agreement with Shimabukuro et al. (155) who observed that some radioactivity leaked out of sorghum leaf discs into surrounding buffer solution. Only unchanged atrazine was detected, suggesting differences in permeability of the cell membrane to the highly lipophilic atrazine and its hydrophilic metabolite GS-atrazine. At the pH of cytosol (around 7.4), the glutathione conjugate would have a negative charge. This statement is based on the pKa valued of glutathione of 2.12 (carboxyl), 3.59 (carboxyl) and 8.75 (NH_3^+) (43). Consequently membrane transport by simple diffusion would not be possible. The energy barrier for moving the anion onto the low dielectric of the lipid bilayer would be very large.

Vetiver leaf segments tolerate atrazine thanks to its conjugation; the conversion of atrazine to water soluble metabolites in excised segment leaves of vetiver was 58%, a very high value, as compared to excised leaves of sensitive species: 17% in oat, 15.2% in pea, 14.4% in wheat, 3.83% in soybean, 1.2% in carrots and 1.2% in lettuce. In excised leaves of tolerant species, conjugates represent 70% of atrazine metabolites

in sugarcane, 75% in corn, 72% in sorghum and 37% in barley (92). Moreover, the atrazine concentration in leaf tissue of vetiver was actually higher than required to inhibit photosynthesis. After 72 hours, atrazine equivalents increased to 0.49 [nmol/g FW] in young leaves and 1.87 [nmol/g FW] in old leaves. Assuming that 1 [g] of leaf tissue equals 1[mL] of water, atrazine equivalents were 35 µM and 134 µM in young and old leaves respectively. Atrazine I_{50} values for inhibition of photosynthesis by thylacoids of vetiver was between 0.5 and 5 µM (chapter 5). Therefore, atrazine concentrations in the leaves were 7 to 27 fold greater than the I_{50} values for photosynthetic inhibition. On the other hand, the conclusion could also be that apparent concentration of atrazine in leaves was not high enough to inhibit photosynthesis: Raveton et al. (136) showed that cells of *Acer platanus* accumulate atrazine twice as compared to the external medium, thanks to partition in cellular lipids. The calculated apparent concentration in cell water may have been overestimated, because of atrazine partition in leaf lipids. Moreover, Van Asche (168) described that death of plant is only obtained for a total inhibition of photosynthesis for more than one week, but the experiment was run for 72 hours, without obtaining vetiver leaves necrosis.

The remaining question is if conjugation is a physiological reality in entire plants, since roots of 8-month old vetiver accumulated atrazine, ending maybe in the sequestration of atrazine.

9 Fate of ^{14}C-atrazine in vetiver entire plant

9.1 Introduction

Mass-balance studies of herbicide dissipation are usually done with radio-labelled herbicides which are applied to foliage or soil under controlled environmental conditions. It allows measuring the total amount of chemical and its metabolites that move or are degraded over a specific time period. The results of these studies are used to aid in the registration of environmentally safe products and in the removal of unsafe ones from the market. Many parameters that are measured are used to develop models useful in predicting the ultimate behaviour and fate of the chemicals in plants, mammals, and in diverse soil types under different climatic conditions.

It was observed that generally, phytoremediation studies with organic compounds measured the disappearance of the parent compound from the surrounding medium of the plant. Without the help of radioactivity, understanding the fate and distribution of the parent compound and/or its metabolites in the plant is almost impossible. Indeed, in the present thesis, all attempts to localize and quantify atrazine in the plant without the help of radio-labelled compound failed. The first problem was to assess the extraction rate of atrazine from the plant matrix. Radio-labelled atrazine allows counting how much radioactivity is recovered after plant exposure, as compared to the initial applied quantity, whereas without radio-labelled atrazine, it is impossible to quantify how much remains in the matrix and/or is lost during extraction procedure. Nevertheless, detection of atrazine and its metabolites DEA, DIA and HATR with HPLC was tried, based on Berg et al. publication (15). But preliminary experiments revealed that solid phase extraction (SPE) developed for purification of atrazine, DEA, DIA and HATR in natural water was not applicable to the compounds in plant matrix; indeed, strong co-elution of plant matrix from the SPE column occurred together with atrazine and metabolites. Peaks of analytes were therefore not visible on HPLC chromatograms, because of strong background noise. Moreover, DIA recovery after SPE was not reproducible.

To our knowledge, the study of fate of atrazine in plants without radioactivity was never done. Thanks to radio-labelled atrazine, many authors could study metabolism of atrazine in plants: according to Gray et al. (68), it seems that dealkylation as a major pathway is restricted to dicotyledonous plant species such as *Pisum sativum*

(pea) (149, 152), *Gossypium hirsutum* (cotton) (154), *Chenopodium album* (lambs quarter) (81), and *Populus nigra* (poplar tree) (27, 28, 110). Conjugation as a major pathway seems to occur in monocotyledons species *Sorghum bicolor* (sorghum) (91, 150, 156), *Zea mays* (maize) (76), *Panicum miliaceaum* and *Echinochloa crus–galli* (76), *Setaria faber*, *S. viridis*, *S. glauca*, and *S. adherens* (51, 67), *Andropogon gerardii*, *Panicum virgatum*, *Sorghastrum nutans*, *Bouteloua curtipendula* (183), *Panicum lapathifolium* (52) and also to a lesser extent in dicotyledons *Abutilon theophrasti* (velvet leaf) (127), and *Polygonum lapathifolium* (52). Radioactivity allowed Jensen et al. (82) to test 40 grass species metabolism, leading to the conclusion that conjugation is a major pathway in all tested *Panicoideae* subfamily species, all tolerant to atrazine. They even concluded that tolerance to atrazine could be used as a method for differentiating between the “panicoid” and “festucoid” type of photosynthesis in *Poacea* family. Hydroxylation as a major transformation of atrazine seems only restricted to monocotyledon plants containing benzoxazinones, such as maize (39, 135) and wheat (153).

Thus, based on this literature, atrazine metabolism in vetiver is expected to be dealkylation and conjugation. Laboratory results supported this assumption, where conjugation was detected *in vitro* (chapter 7) and *in vivo* in vetiver organs together with dealkylation (chapter 8).

Determination of respective contribution of dealkylation and conjugation metabolic pathways of atrazine is of interest, first from a scientific standpoint to understand vetiver tolerance to atrazine, and secondly for the future use of vetiver for phytoremediation.

The choice of concentration of atrazine in hydroponics was done carefully: Raveton (134) cited that after the first rainfall following 1 [kg/hectare] atrazine application, the peak of concentration of the compound in runoff water can reach 2 [mg/L] (about 8 µM). After application of 0.39 [kg/hectare] formulated atrazine (90% active ingredient), Meiwirth (106) detected 3.3 [mg/L] atrazine in soil solution near the surface of an alluvial soil. Therefore, a realistic 2 [mg/L] concentration of atrazine was chosen to be tested with vetiver plant, since agricultural runoff is the primary target for phytoremediation as developed and explained in chapters 3 and 4.

9.2 Material and methods

Plant preparation

Plants were prepared to fit to the autoradiography pellicles size (approximately A4 size) and to minimize the use of radioactive atrazine. Plants were prepared as small as possible according to the following procedure: leaves of one vetiver plant were cut 15 [cm] above soil level. The plant was then planted out and split into parts until obtaining 1 or 2 tillers. Roots were cut to 8 [cm] lengths from the meristematic region. The obtained small plants were rooted individually in organic soil for 2 months. Plant survival was 50 %: only plants with large enough meristematic region survived. Plants with new leaves were then transferred into Erlenmeyers of 100 [mL] and grown in hydroponics for 1 month. Hydroponic medium was supplemented with Luwasa® at the dilution recommended by the manufacturer. Intermittent air sparging was used to aerate the solution and avoid hypoxic conditions. Six plants of approximately the same biomass were chosen for the present experiment. These plants were adapted 3 days before exposure to atrazine in a growth chamber at 28°C, 75% humidity, 10h day and 14h night. Before atrazine exposure, dried and bent leaves were cut, dead meristematic regions without leaves and damaged roots were eliminated. Roots were washed carefully with distilled water and remaining soil particles were removed as much as possible to avoid further heavy contamination of solution treatment by micro-organisms.

Plant exposure to atrazine

Five plants were exposed for 19 days to 50 [mL] containing 8 µM final concentration of ^{14}C-atrazine 417 [Bq/mL] supplemented with nutrient solution Luwasa®. Uniformly ring labelled atrazine source in ethanol was 7 X 10^5 [Bq µmol^{-1}] (95 % pure, Sigma). Controls were (i) plant without atrazine (plant fitness); (ii) pot without plant (water loss of the system). No sparging was used to aerate the hydroponic solution in order to avoid possible stripping of radioactivity. Roots were dipped in only half of their total length, allowing the upper part to be “air oxygenated”. To keep a constant concentration of atrazine, treatment solution was changed at day 5 and 12. In between, treatment solution concentration was followed by counting radioactivity with a scintillation counter. Entire fresh biomass was measured before and after plant

treatment. At days 5 and 20, one plant was taken for autoradiography, and at day 5, 12 and 20, one plant was taken for extraction.

Autoradiography

Plant roots were rapidly washed in 2 X 500 [mL] of concentrated non radioactive atrazine (25 [mg/L] in water) to avoid rapid efflux of atrazine. Plants were carefully dissected, freshly glued on hard paper, and air dried for 1 week. Plant organs were exposed for a fortnight to autoradiography pellicles (Direct Exposure, Kodak).

Plant extraction

At day 5, and 20, plants were rapidly washed into 2 X 500 [mL] of concentrated cold atrazine (25 [mg/L]) to avoid rapid efflux of atrazine. Each organ (roots, meristematic region and leaves) were separated and weighted. Leaves were separated into 3 parts: L1 corresponding to the first 5 [cm] from the meristematic region, L2 corresponding to the next 5 cm, and L3 corresponding to upper part of leaves. These plant organs were frozen at -20°C until use. Plant extraction was done by grinding organs together with sand and ethanol, until total disappearance of plant pigments. The ethanolic fraction was further concentrated with a Rotavapor. Following this concentration step, precipitates were dissolved by washing the ethanolic phase with a minimum volume of EtOH/H_2O, followed by a minimum volume of water. The non dissolved remaining pellet was removed by centrifugation. Plant organs were further extracted with water, and the obtained aqueous phase was further concentrated with the help of butanol in a Rotavapor. Final concentration step was obtained by the use of a Speed-vacuum device (Savant). Aqueous phases were then centrifuged to remove non dissolved material. Extracts were concentrated to reach a minimum of 1000 [dpm/100 µL] radioactivity for metabolites identification with TLC linear reader.

Mass balance of radioactivity

100 [µL] of aqueous extracts were counted after addition of 4.9 [mL] water and 5 [mL] scintillation liquid (Ready Safe scintillation liquid for aqueous samples, Beckman) with a scintillation counter (Wallac, Winspectral). Hundred [µL] of ethanolic extracts of roots and meristematic region were counted after addition of 4.9 [mL] of ethanol and 5 [mL] of scintillation liquid (Ready Safe scintillation liquid for organic samples, Beckman). To avoid quenching effects, minimum volumes of

ethanolic leaf extracts (20 [µL]) were counted, and before counting, samples were bleached with 3 drops of sodium hypochlorite.

Metabolites identification

100 [µL] of extracts were loaded on 1 [cm] of TLC plate ($60_{F254,}$ Merck), and allowed to migrate in solvent system ethyl acetate/formic acid/acetic acid/water 40/2/2/4, v/v/v/v). Plates were further read with a TLC liner (LB 213 Analyzer, Berthold).

Lipid extraction from vetiver roots

Lipid extraction was performed according to M. Raveton protocol (134). Roots of control plant without atrazine were crushed in hydroacetonic phase acetone/water 80/20, v/v (3 X 50 mL). Extraction was performed until total disappearance of stock pigments. After straining the acetonic-water phase with 4 layers of muslin, and glass prefilter (Millipore), it was further re-extracted with petrol ether (B.P. 40-60 °C) (3 X 30 mL). The volume of ether phase was reduced with a Rotavapor, and further rinsed with water to remove any precipitate. Ether was then totally evaporated with a nitrogen flux, and subsequent obtained lipids were weighted.

9.3 Results

Experiment monitoring

The plant biomass increased only by 2% within the 20 days of experimentation, and was thus considered as negligible and not taken into account for further calculations. Tested plant control with atrazine did not exhibit any leaf chlorosis and their biomass increased in the same range as the control without atrazine. Maximum variation of atrazine concentration in treatment solution at days 0, 4, 5, 8, 12, 14, and 20 was 15%. Water loss in the control without plant was 5.4% after 20 days, indicating that plant transpiration was responsible for water disappearance from plant vessel.

Autoradiography

Vetiver plants were able of taking atrazine up and to translocate it and/or atrazine equivalents from roots to shoots. At day 5, radioactivity was localized mainly in root tips which were in contact with treatment solution. Some radioactivity was also found in leaves, with an accumulation in their tips. After 20 days, this pattern was the same, but with more radioactivity everywhere and above all with a clear accumulation at the tip of the leaves (**Figures 9.1, 9.2, 9.3, and 9.4**). This observation was also true when plants were extracted and counted with scintillation counter.

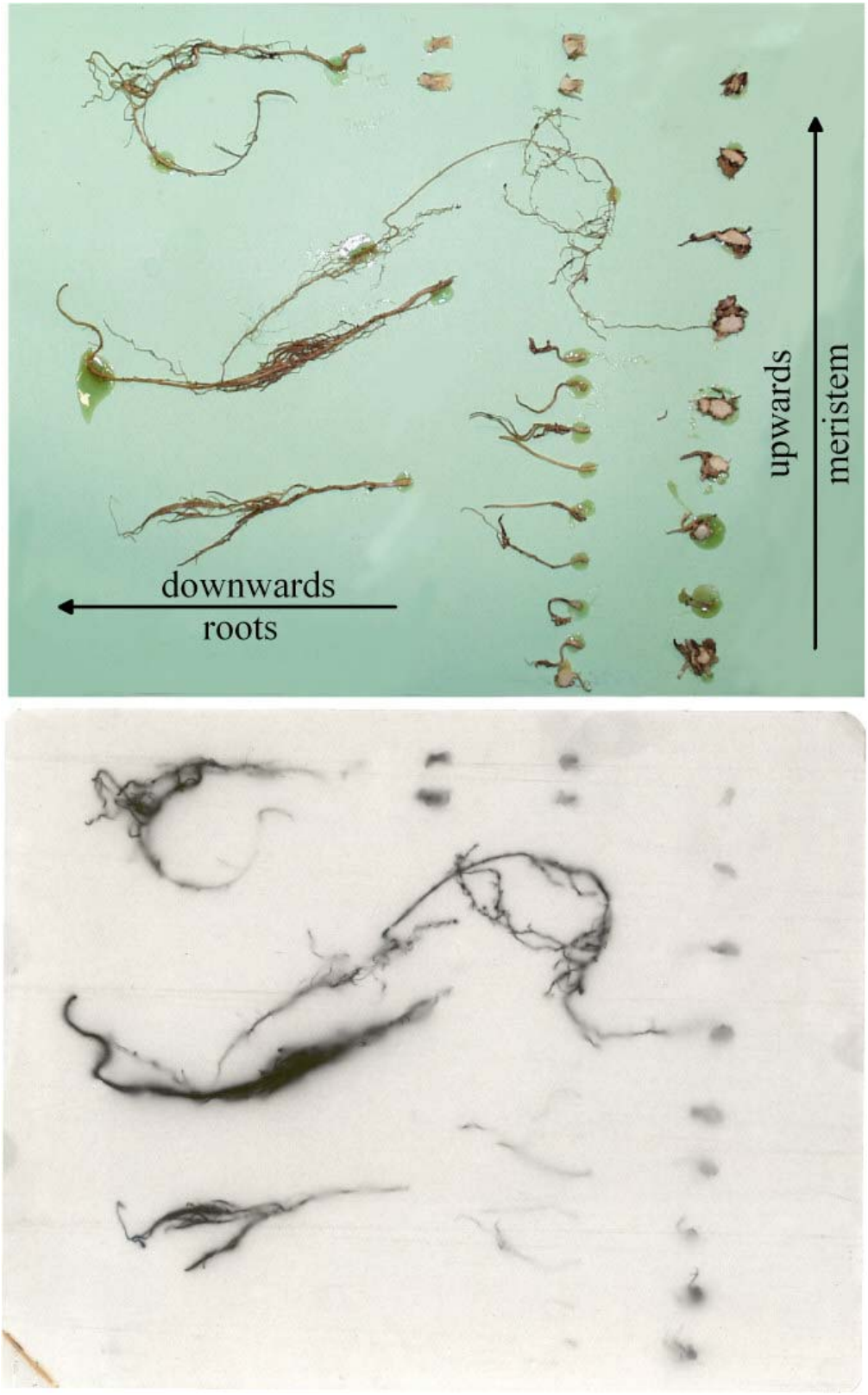


Figure 9.1 Autoradiography of roots and meristem of vetiver plant exposed 5 days to ^{14}C-atrazine

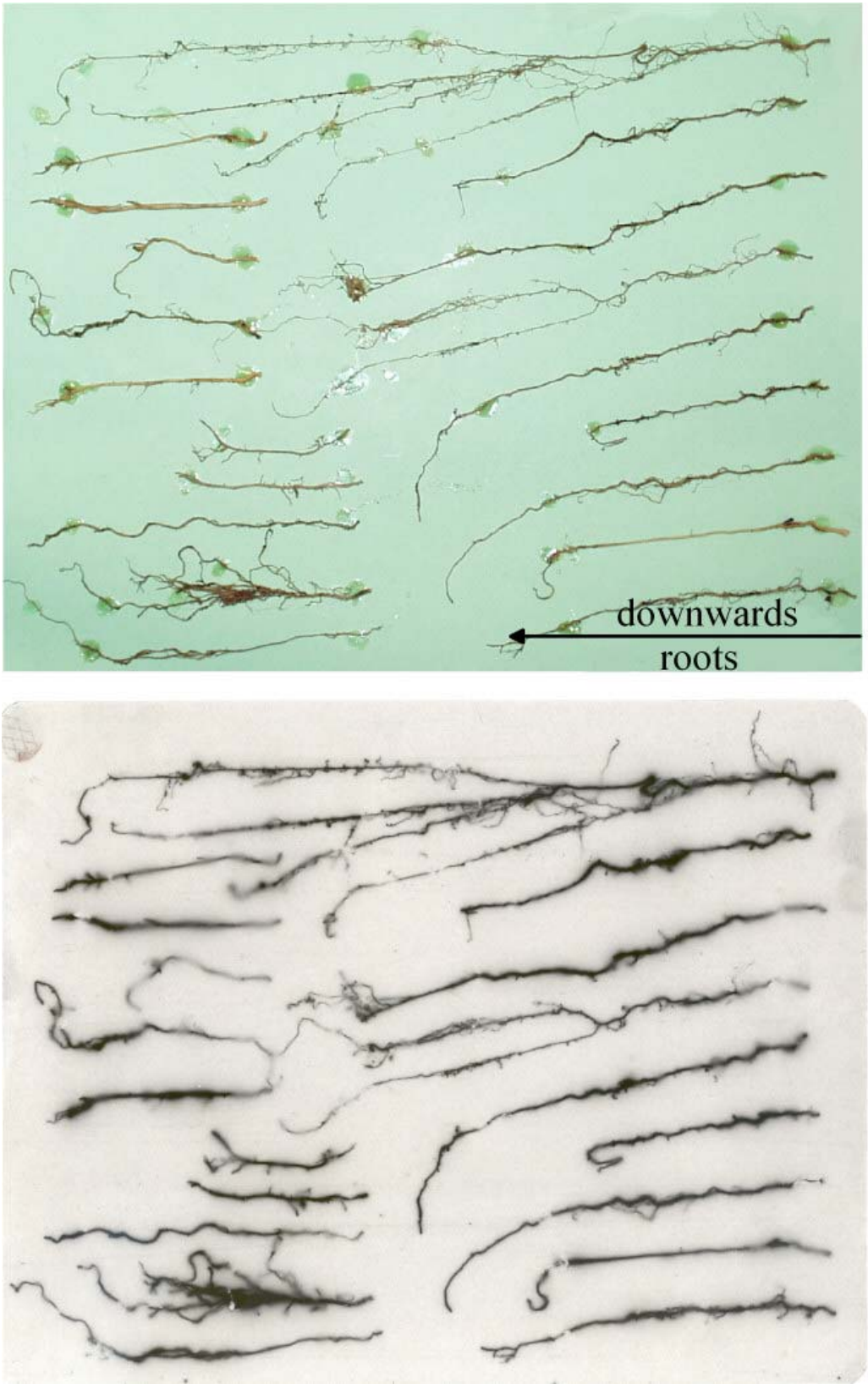


Figure 9.2 Autoradiography of roots and meristem of vetiver plant exposed 5 days to ^{14}C-atrazine

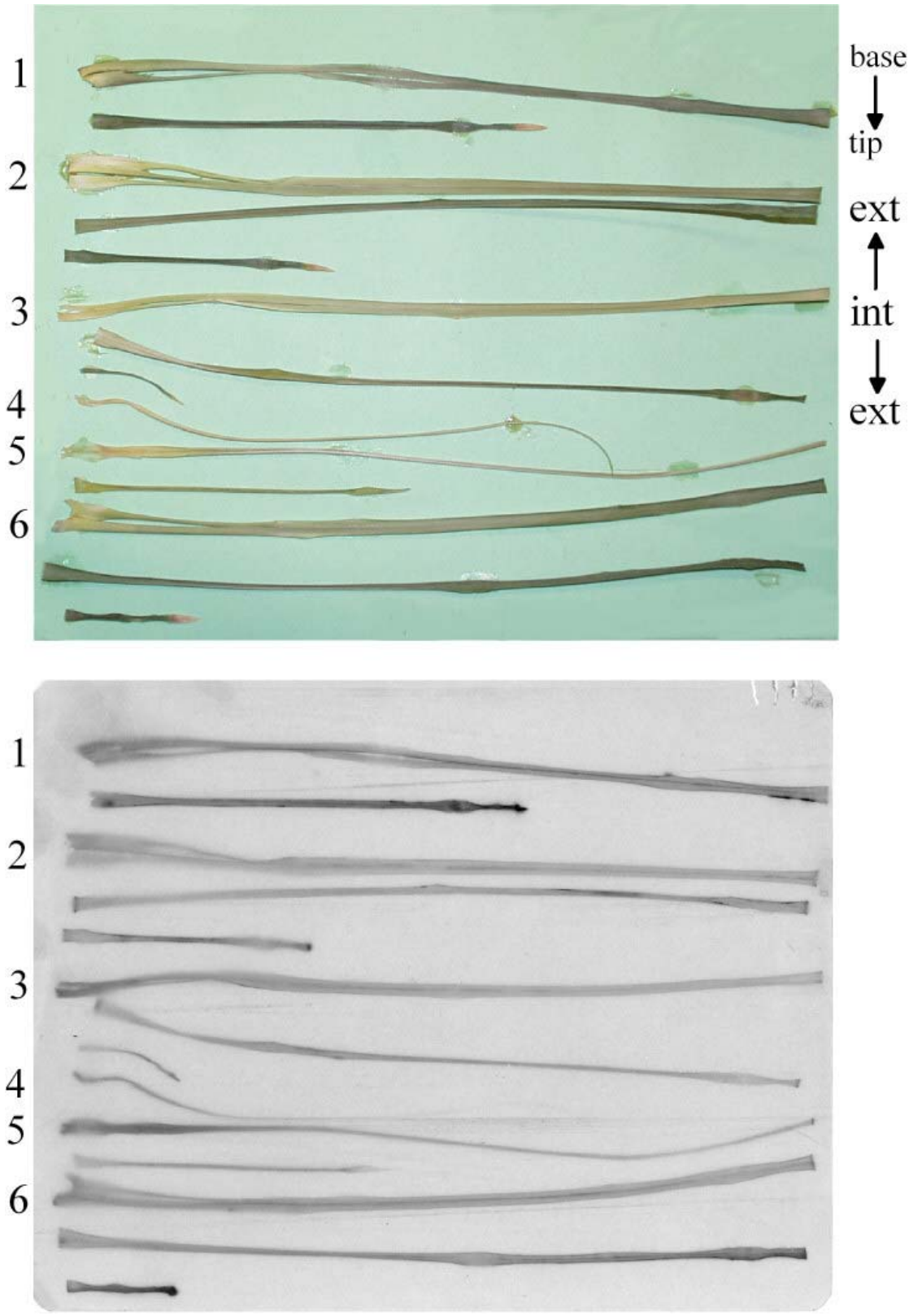


Figure 9.3 Autoradiography of leaves from vetiver plant exposed 5 days to ^{14}C-atrazine

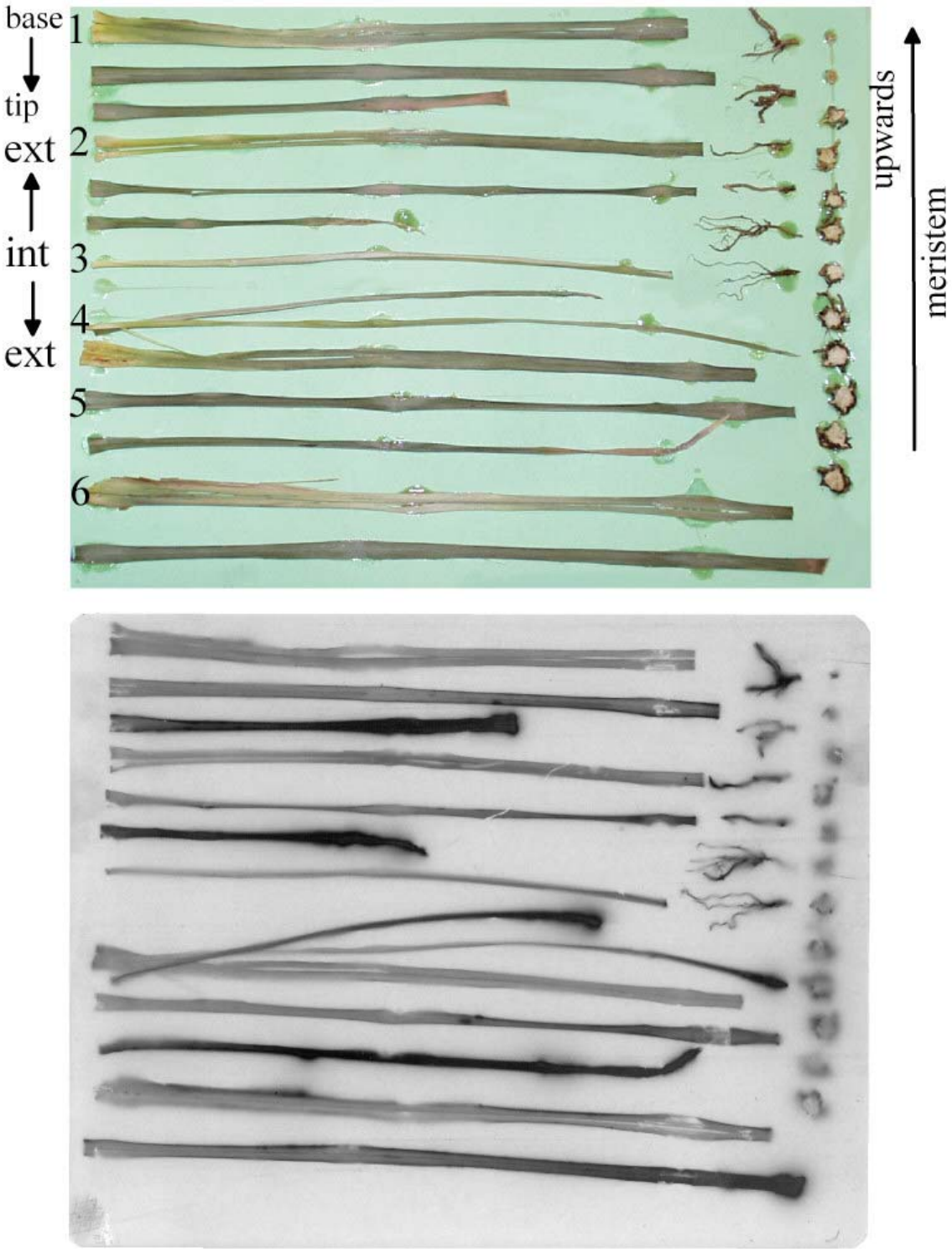


Figure 9.4 Autoradiography of leaves from vetiver plant exposed 20 days to ^{14}C-atrazine

Plant metabolization

Radioactivity was found in all parts of vetiver plants. Maximum radioactivity was present in leaves (**Table 9.1**). About half of radioactivity was found in hydroponic solution and rinsing solution of roots and half of radioactivity was found in plants. The ratio of radioactivity remaining in treatment solution was not similar between plants sampled at day 5 and 20. This was probably due to higher root biomass of plant sampled at day 20, and to a possible different handling of the plants when taken them out of vessel to be analyzed.

Table 9.1 Balance of counted radioactivity in percentage of initial radioactivity in [dpm]

	Plant			Hydroponic solution		
	roots	**meristem**	**leaves**	**solution**	**rinsing**	**total**
Day 5	13.10	6.30	28.10	48.7	9.80	106
Day 20	10.6	5.3	24.1	23	22	85

By expressing atrazine equivalents in nmols per [g] of fresh biomass, it was observed at day 5 that radioactivity was almost evenly distributed in leaves, but at day 20 atrazine equivalents were found especially in L3, the distal part of leaves from meristem (**Figure 9.7 (A)**). Only half of roots were immersed in treatment solution and for calculation of atrazine equivalents, total root biomass was used. This did not allow a real comparison in atrazine equivalents for leaves and roots exposed for different periods to atrazine. For the same reason, root concentration factor was not calculated.

Metabolization study was done by loading vetiver aqueous extracts on TLC, which allowed the identification of polar product(s) at Rf = 0 that was (were) assumed to be conjugate(s) (**Figure 9.5**). Vetiver ethanolic extracts showed intact atrazine probably together with dealkylates (**Figure 9.6**). Since autoradiography of TLC plate was not done, separation of atrazine and dealkylates was not possible with the Berthod TLC reader liner (see chapter 12). Products at Rf 0.17, 0.33 and 0.43 were also detected, but their quantification was only approximative, since their peaks were poorly

resolved. Nevertheless, they were taken into account. The product at Rf 0.17 could be HDIA, but the two others could not be identified by comparison with Rf of standards.

Conjugates were found in roots, meristematic region, the first 5 cm of leaves (L1), next 5 (L2), and the tip of leaves (L3). The maximum conjugates production was observed at day 20, at the tip of the leaves (**Figure 9.7 (B) (C)**).

Percentage of metabolites compared to total penetrated radioactivity into plants were 49.5% of conjugates, 1.2 % of HDIA, 20.8% of unidentified products and 28.5% of atrazine + dealkylates at day 20.

Treatment solutions from days 0 to 5, and from 5 to 12 days were not analyzed by TLC. Treatment solutions from days 12 to 20 showed 15% of product at Rf 0.43.

Atrazine disappearance from medium was linearly correlated with transpired water at day 5 (**Figure 9.8**).

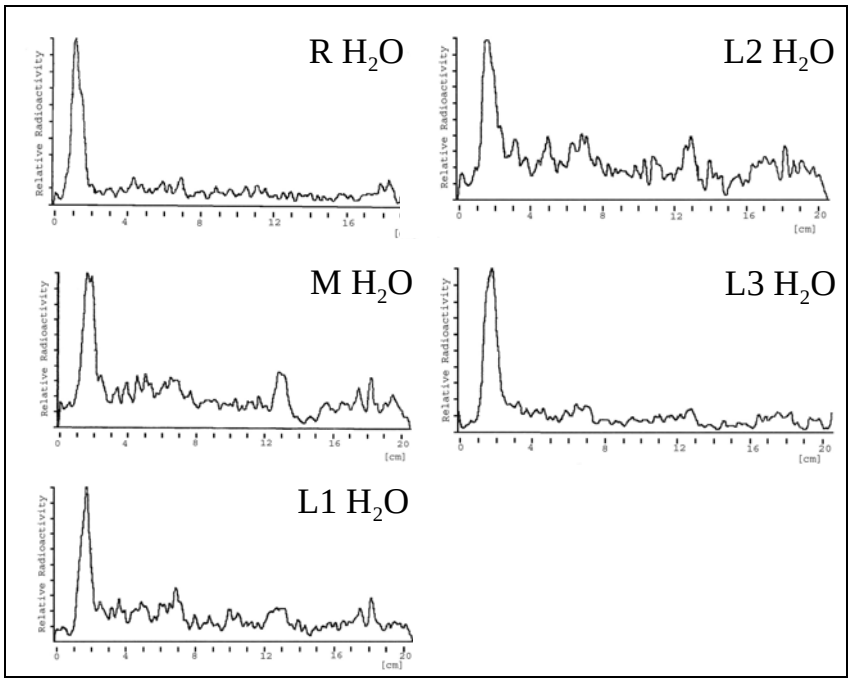


Figure 9.5 Scan of aqueous extracts of vetiver T=20 days

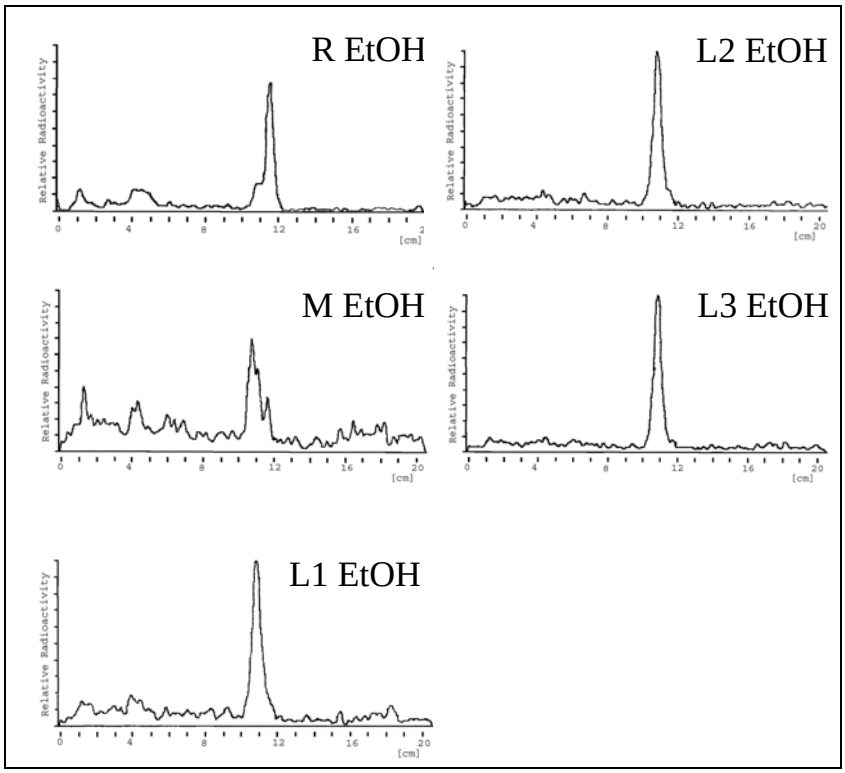
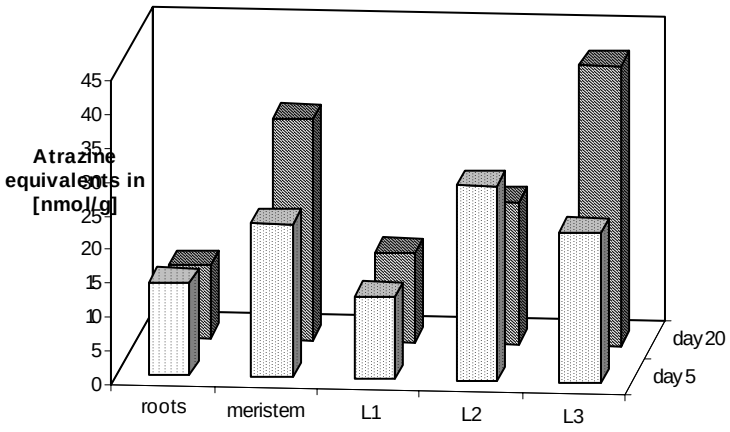
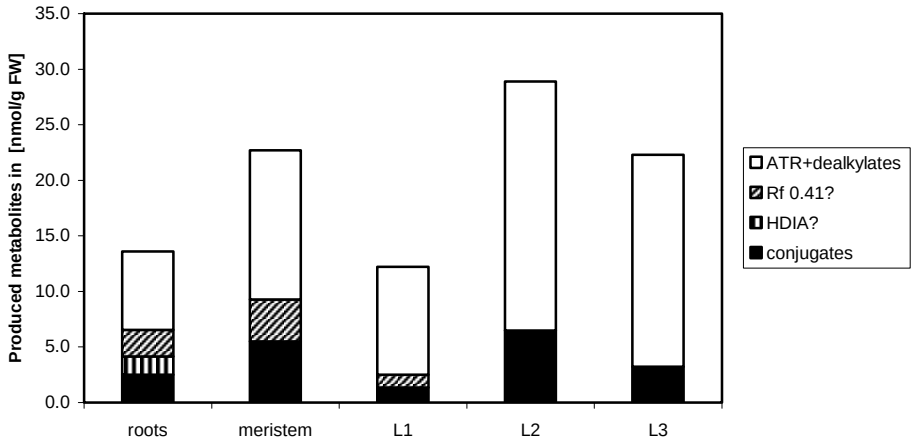


Figure 9.6 Scan of ethanolic extracts of vetiver T=20 days





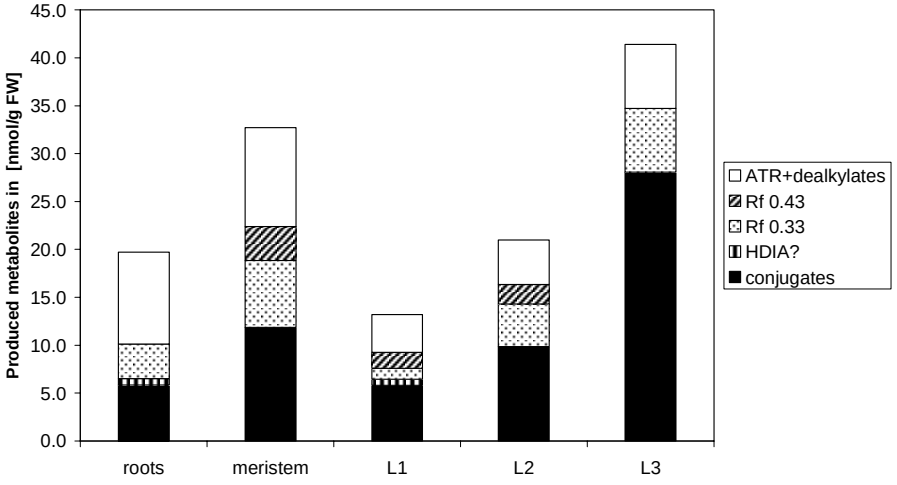


Figure 9.7 (A) Radioactivity distribution in vetiver organs (B) Metabolites distribution in vetiver organs at day 5 (C) Metabolites distribution in vetiver organs at day 20

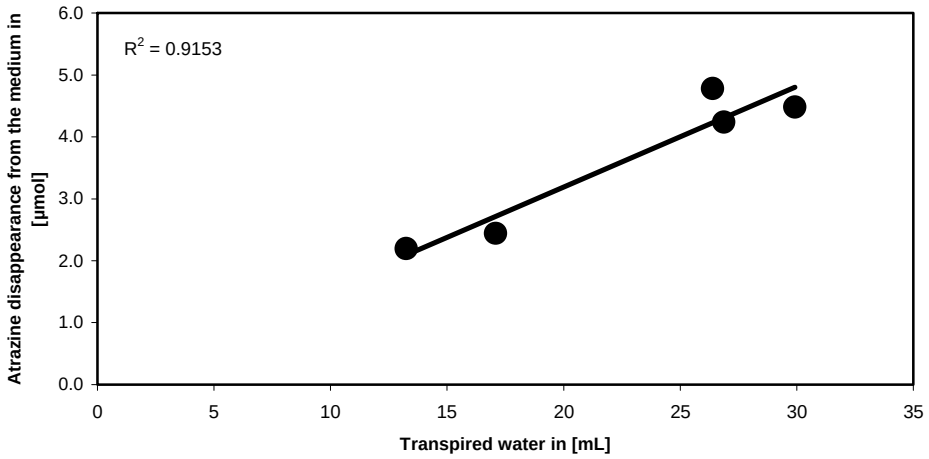


Figure 9.8 Relationship between atrazine disappearance from medium and transpired water at day 5

9.4 Discussion

It was shown that atrazine disappearance from the medium was linearly dependent on water transpired by plants, suggesting that atrazine volatilization, micro-organisms action, and root absorption/adsorption/sequestration/partition in lipids were negligible. Moreover, good recovery of radioactivity tends also to show that atrazine volatilization was negligible, in agreement with low Henry's law constant 6.2 X 10^{-6} [atm L mol^{-1}] and low vapor pressure of 2.78 10^{-7} [mm Hg]. The hydroponic system was therefore suitable for plant metabolism and plant uptake studies.

Almost 20% of metabolites were not identified. Their peaks were not found in excised vetiver organs (chapter 8), suggesting that excised organs did not exactly represent the metabolism of entire plant. Nevertheless, the highest metabolic pathway in both excised vetiver organs and entire plant was conjugation.

After 5 days, conjugates were found in roots, unlike *in vitro* assays where GSTs activities were neither detected in vetiver roots (chapter 7), nor in excised vetiver roots exposed to ^{14}C-atrazine (chapter 8). *In vitro* test of GST activities were run for 1 hour, whereas detection of conjugates in excised vetiver roots was performed after 72 hours. The comparison with *in vitro* GST activities suggested that with longer time exposure to atrazine, ATR-GS could be detected. Conjugates detection in root could be also a function of root age and in which medium they were grown. Lipid content of root of the plant control was 0.5% fresh biomass, whereas lipid content was about 4%

in excised roots grown for 8 months in organic soil (chapter 8). Oil drops are produced in the cortical cell layer, but later, oil is spread in the whole root cortex, in the lysigenic space, part of the apoplast. Sequestration of atrazine in such oil loaded roots could be very important, preventing atrazine to reach symplast where GSTs could conjugate it with glutathione. Different oil content could explain that in one case (excised vetiver roots, chapter 8) conjugates were not detected, whereas conjugates were produced in roots of entire plant (present chapter). In other words, oil would be able to trap atrazine and to subtract it to enzymatic action.

About half of the radioactivity in the plant remained in roots. As a comparison, Shimabukuro (149) reported that in ^{14}C-atrazine treated plants under 40% humidity, most of the radioactivity (72%) is present in the shoots of soybean, pea, wheat and sorghum except corn. Radioactivity remaining in the roots of corn is 64%, where rapid hydroxylation of atrazine occurs and from which further metabolite translocation is drastically reduced. So, even under transpiring conditions, hydroxylation is fairly rapid and important in maize; if this metabolization process was important in vetiver, high percentage of radioactivity in roots should have been observed. This was not the case, in agreement with benzoxazinones detection, *in vitro* hydroxylation of atrazine (chapter 6) and metabolism in excised organs (chapter 8).

It seems that vetiver tolerates atrazine thanks to conjugation. Shimabukuro (149) pointed out that a striking difference between the resistant species sorghum and corn, and other sensitive species is the higher percentage of water soluble metabolites (conjugates and/or hydroxylates) present in the resistant plants (between 35%-40% in sorghum and maize, and maximum 15% in sensitive species). In the case of vetiver, almost 50% of radioactivity was found in conjugates. Moreover, no necrosis of leaves was observed in the tip of leaves where radioactivity was mainly accumulated, confirming the statement that an active metabolism took place to transform atrazine. Finally, the atrazine concentration in leaf tissue of vetiver was actually higher than required to inhibit photosynthesis. At day 5, atrazine equivalents increased to 12.2 [nmol/g fresh weight], 28.9 and 22.3 in L1, L2 and L3 respectively. At day 20, atrazine equivalents increased to 13.2, 21 and 41.4 [nmol/g FW] in L1, L2 and L3 respectively. Assuming that 1 [g] of leaf tissue equals 1 [mL] of water, atrazine concentrations in vetiver leaves would have been between 12 and 29 µM at day 5, and between 13 and 41 µM at day 20. Atrazine I_{50} values for inhibition of photosynthesis

by thylacoids of vetiver was between 0.5 and 5 µM (chapter 5). Therefore, atrazine concentrations in the leaves were at least 2.5 fold greater than the I_{50} values for photosynthetic inhibition.

Considering that vetiver hedges are planted around fields where atrazine application occurs only once or several times in the year, and that first rain is washing out atrazine (106), exposure of vetiver plant to atrazine is not continuous. Interestingly, some authors exposed plants to ^{14}C- atrazine for a few days, rinsed roots, and put them back in a new medium without herbicide (51, 93, 134, 149). Lamoureux et al. (93) observed that translocation of atrazine from the roots to the shoots of sorghum occurs very rapidly and is nearly complete 3 days after the source of atrazine to the roots is removed. De Prado et al. (51) showed that when *Setaria viridis* and *S. faberi* are removed from herbicide and placed in water for 24 and 48 hours, almost all radioactivity is found in shoots (between 81.8 and 89.2% after 48 hours in water), and only a small amount is found in the nutrient solution. More important for phytoremediation, Shimabukuro (149) observed almost total disappearance of atrazine in sorghum treated for 48 hours, followed by 336 hours in fresh medium without atrazine. From these comments, it is clear that hydroxylation due to benzoxazinones is complete in maize roots within a few hours, whereas atrazine conjugation in leaves is clearly a fonction of days, because of translocation from roots to leaves.

In conclusion, conjugation was the main metabolization pathway of atrazine in entire vetiver plant. Under moderate transpiring conditions, and in young plants with low lipid content, there was no atrazine accumulation in roots, but rather translocation of atrazine and accumulation in leaf tips, where major conjugation occurred. Old big vetiver plants grown in organic soil were not tested, because of the limited size of autoradiographic pellicles and also to limit use of radioactivity. Behaviour of vetiver under saturating humidity conditions was not explored, but it is suspected that under these conditions, atrazine could remain at least partially in roots.

10 Dealkylates uptake by vetiver compared to atrazine

10.1 Introduction

In soils, several studies indicate that atrazine is degraded to DEA, DIA and DDA, or a combination of these metabolites by the action of micro-organisms (22). As DEA is the major metabolite formed after atrazine application, and the main product entering aquifers, Adams and Thurman (2), as well as Schiavon (144) even concluded that this metabolite is a good indicator of atrazine transport through the soil. The proportion of atrazine metabolites was DEA (26%), DIA (10%), and HATR (9%) in Mersie and Seyvold study (107). Panshin et al. (117) observed that after atrazine application, deethylatrazine was the dominant degradation product detected in the first year and didealkylatrazine was the dominant degradation product the second year. *Rhodococcus* sp produces 69% of metabolite DEA and 25% DIA. (170). *Pseudomonas* (13) and *Nocardia* (66) cultures, were reported to be able of degrading atrazine, predominantly by dealkylation of the side chains.

In contrast, chemical degradation leading to formation of HATR was predominant in Skipper et al. study, and microbial action negligible (160). In anaerobic environment, hydroxyatrazine is the main formed metabolite (40). Atrazine is depleted mostly through biological transformation in alkaline soil and is degraded in acidic soil mainly through both chemical and microbial transformation (128).

The metabolite hydroxyatrazine is formed by chemical hydrolysis, and the rate increases as soil pH decreases and soil organic carbon increases (107). Hydroxylation is also catalyzed by clay surfaces (86). *Pseudomonas sp*. isolated in Switzerland and Louisiana (53) and *Rhizobium* sp (23) contain atrazine chlorohydrolase genes encoding atrazine hydrolysis to hydroxyatrazine, suggesting that these genes are widespread in nature and contribute also to the formation of hydroxyatrazine in soil, a reaction attributed for a long time exclusively to abiotic process. At the present time, 3 genes of chlorhydrolases have been identified and are designed by *Atz*A, *Atz*B and *Atz*C (147).

Predominance of dealkylation in soils was formally shown by Shapir et Mandelbaum (148): significant atrazine disappearance (50%) was detected in subsurface soil by indigenous micro-organisms in the upper part of soil, but only 1% mineralization was

detected, and dealkylation was the major process involved in degradation. Deeper horizons failed in lowering atrazine concentration by dealkylation. More importantly, it could be observed that the limiting factor of transformation of atrazine is the absence of atrazine-mineralizing micro-organisms, leading to the conclusion that bioaugmentation may be preferable to enhancement of intrinsic atrazine-degrading activity to achieve complete atrazine mineralization. Alternatively, to lower dealkylates in environment, plant uptake and further transformation could be performed.

Sorption is the major process that controls the degradation (both biotic and abiotic) and mobility of a herbicide in soil. In agricultural and wetland soils, DEA is less adsorbed than atrazine and HATR is more strongly bound to the soil matrix than DEA or DIA (107). In other words, adsorption coefficient decreased in the order HATR, atrazine, DIA, DEA. More hydroxyatrazine would adsorb on the soil or sediment at pH between 4.4 and 4.7 because the majority of hydroxyatrazine would be protonated at pH lower than its pKa of 5.1 (171). Ionic adsorption means a strong binding mechanism which decreases the opportunity for desorption and subsequent mobility (88). Hydroxyatrazine adsorbed 6 times more than atrazine to sediments (40).

Dealkylates are the main metabolites of atrazine found in soils. Many organisms do not further metabolize the dealkylated products (22). Because U.S. EPA showed that dealkylates DEA, DIA, DDA and atrazine share common endocrinal toxicity (1), it was found relevant to study plant uptake potentiality to remove dealkylates from soil. Plant uptake of HATR was not investigated in the present study, because to the best of our knowledge, no toxicity of this atrazine derivative was described so far. Moreover, it binds tightly to soil, being unlikely bioavailable for plant uptake. DDA could not be studied, since the analytical method chosen could not separate DDA from injection peak.

The analytical HPLC column and solvent elution system was chosen because of its ability to resolve simultaneously HATR, DEA, and DIA. A special non-endcapped column matrix could resolve HATR, unlike conventional end-capped C_{18} column (15). Plant uptake of DIA and DEA was studied under non constant concentration conditions, because it appears important to try to fit to more real situation, where plant uptake, if occurring, is decreasing the herbicide concentration constantly in the medium.

Results presented in chapter 9 validated the hydroponic system to study plant uptake of ^{14}C-atrazine, and because it was shown that uptake of atrazine was dependent on transpiration, it was possible to foresee the study of DEA, and DIA disappearance from hydroponic medium by comparison with atrazine, without the help of radioactivity.

10.2 Material and methods

Reactors

Vetiver reactors were Erlenmeyers of 1000 [mL] wrapped in aluminium foils to prevent any possible photolysis of tested compounds and growth of algae (**Figure 10.1**)**.** Vetiver plants in hydroponics were supplemented with commercially available ready Hoagland Basal Salt full strength (Sigma). The level of water was checked through a mobile aluminium window (**Figure 10.2**). Aeration and refill pipes were made with a Teflon capillary of 0.5 [mm] internal diameter (Maagtechnic). Plants were watered with nutrient solution every 2-3 days through refill pipe with a syringe of 100 [mL] to 900 [mL], the original volume.

Aeration

Although vetiver has constitutive aerenchymes structure in roots (17), it appeared useful to aerate the medium. A hydrophilic filter of 0.2 [µm] (Sarstedt) was installed between the aquarium pump (ACO-9530, Jun®, Switzerland) and the reactor to avoid contamination from ambient air. It could be observed with the help of an O_2 electrode (Oxy 96, WTW), that plants at 25 °C consumed 70% of dissolved oxygen within 2 hours. Therefore, intermittent sparging was controlled with a timer (20 min every 2 hours).

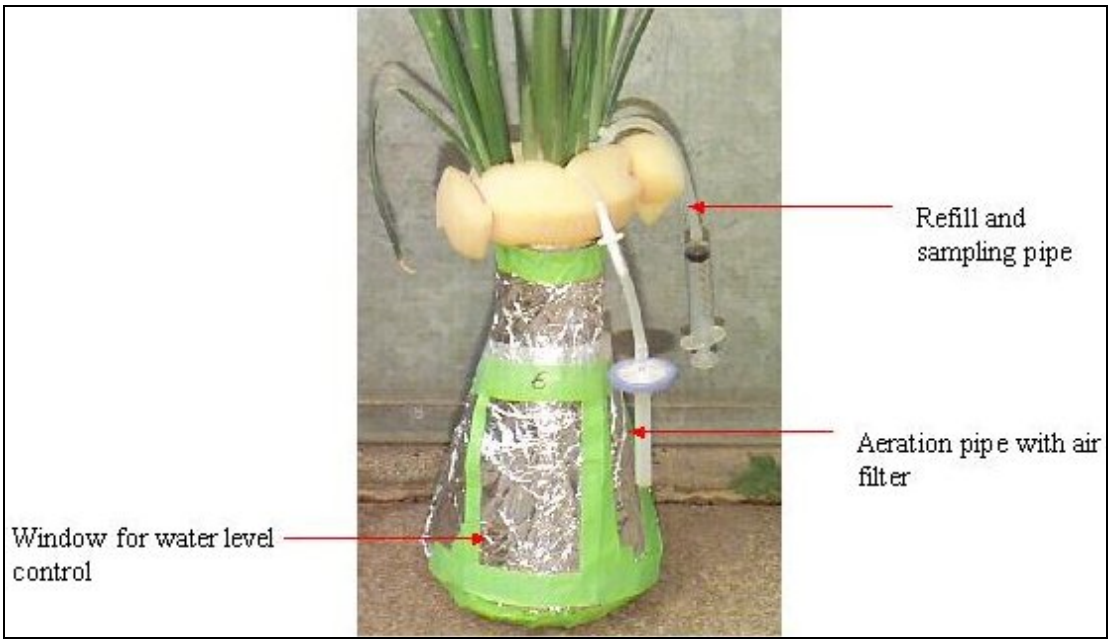


Figure 10.1 Hydroponic system

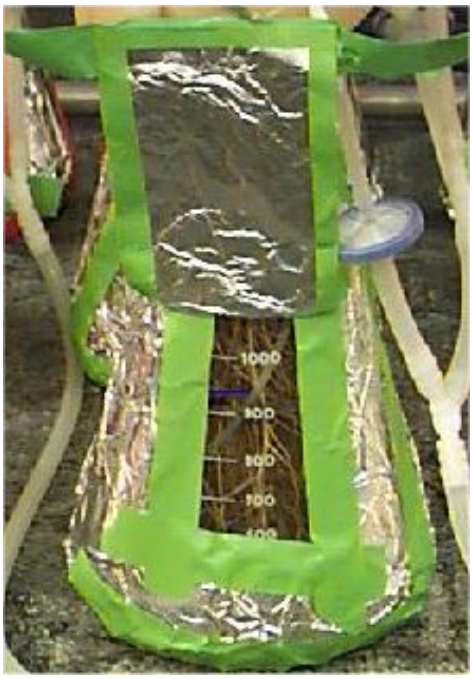


Figure 10.2 Visual water level control

Addition of atrazine, DEA, and DIA to the medium

In order to study experimentally the plant effect on atrazine and dealkylates DEA and DIA, precautions were taken to minimize micro-organisms in hydroponic medium. Therefore, different ways of addition of atrazine to the nutrient medium were tested: spiking the solution with atrazine dissolved in methanol or ethanol, or with atrazine directly dissolved in nutrient medium without any solvent. Final tested concentrations of solvents were 0.2 %, and tested concentration of ATR, DEA, and DIA was 2 ppm (2 mg/L). All hard material was autoclaved before experiments (Erlenmeyers, pipes), and plant roots were washed carefully with distilled water prior experiments. Hoagland solution was autoclaved, and spiking solvent solutions were filtered at 0.2 [µm] (Sarstedt). Plants were therefore the only possible seeders of micro-organisms of the system.

To insure total solubilization of DEA, DIA and ATR, without solvent use, atrazine was prepared as followed: 250 [mg] of atrazine were added to 200 [mL] of water and sonicated 10 min to obtain a saturated solution. The solution was then filtered and DEA, DIA or ATR remaining on the filter was re-used for another 200 [mL] water fraction. This operation was repeated until obtaining 4 [L] of solution. Concentrations of ATR, DEA, and DIA were assessed by HPLC coupled with diode array by comparison with standards dissolved in ethanol. Stock solution was then used diluted to obtain the final concentration needed for hydroponic experiments.

HPLC analysis

HPLC analysis was based on publication of Berg et al. (15). HPLC system Varian was equipped with a gradient pump (Varian 9012), an autosampler (Varian 9100), diode array system (Varian 9065 polychrom) and data acquisition system (Varian, Star). The column was ODS (30) Ultracarb 5, 150 X 4.6 mm (Phenomenex, Torrance, CA). Separation of atrazine, DEA, DIA and HATR was performed using gradient elution at a flow rate of 0.9 [mL/min]. Initial conditions were 15% acetonitrile and 85% of 0.1 mM KH_2PO_4 pH 7.0, isocratic for 1 min, followed by a linear gradient to 70% acetonitrile within 32 min and a postrun of 4 min. After that, the initial conditions were reached within 5 min, and the system equilibrated for another 8 min. The sample volume injection was 250 µL. Absorbance was measured continuously in the range of 200 to 400 [nm] by diode array detection. The peaks were quantified at 220 nm. The

linearity of the HPLC system was tested from 0.2 [mg/L] to 5 [mg/L]. The calibration curves had a coefficient of determination r^2 = 0.991 for atrazine, DEA, DIA and HATR.

10.3 Results

Minimization of micro-organisms in hydroponic solution

Methanol (0.2 %) was found to be a carbon source for micro-organisms, unlike 0.2 % ethanol (acting more like a disinfectant), and solvent free media. After centrifugation of the hydroponic solution spiked with 0.2% of methanol, the obtained pellet was observed under phase contrast microscope 1000 X magnification (Axiolab, Zeiss): a lot of bacteria were present together with a few protozoa. Hydroponic solution was observed to be transparent over 3 months with ethanol or without solvent trace, with some slight yellow colour, and yellow brownish particles coming mainly from dead root cells, together with some micro-organisms. Therefore, to study plant effect on dealkylates over period of 20 days, hydroponic solution was prepared without solvent trace.

DEA, DIA, ATR disappearance from medium

After 20 days, water loss in controls without plant was 3.2% and was therefore considered as negligible. Sampling was done at days 2, 5, 7, 9, 13, 16, 19. Each time 2 [mL] were taken representing less than 2% of tested compound disappearance from starting concentration. ATR, DEA and DIA concentrations decreased linearly with time compared to controls without plants (**Figures 10.3, 10.4, 10.5**). From these figures, it could be deduced that plant contributed to remove ATR, DEA, and DIA from the medium.

Comparison of **Figures 10.3, 10.4, and 10.5** tends to show that plant effect is the greatest on atrazine, followed by DEA and DIA. This ranking however reflects only the choice of plant replicates for each compound tested. It was not randomly made, since plants were grouped per 3 according to their most similar biomass: ATR plant replicates were the largest, followed by the group of plants for uptake of DEA, followed by the last smallest replicates for DIA uptake experiment.

A better representation is the disappeared quantity of herbicide plotted against cumulated transpired water, showing that plants tend to behave similarly towards

ATR and tested dealkylates, at equal transpired water (**Figure 10.6**)**.** Main cause of disappearance of atrazine from the hydroponic system was dependent on transpiring flux of the plant, showing also that micro-organisms influence was negligible. In the present case, relationship was not linear, because tested compounds concentration was not constant: water was refilled to initial level before each sampling. When herbicide was decreasing in the medium, less and less quantity of herbicide was absorbed.

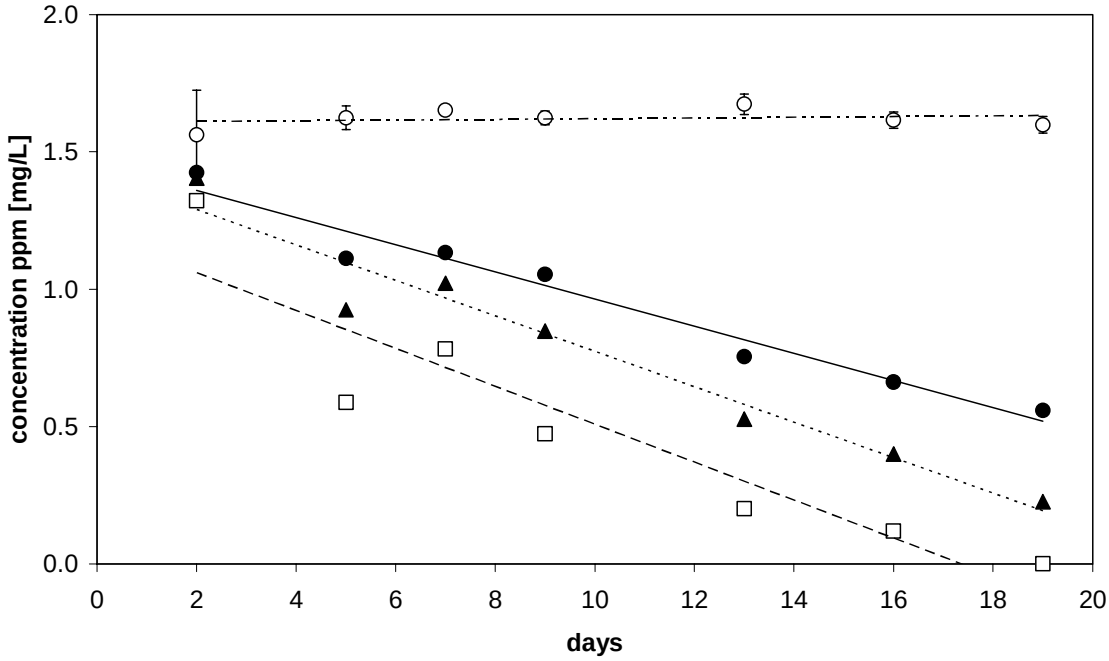


Figure 10.3 Plant effect on ATR concentration in media versus time.

● replicate 1; ▲replicate 2; □ replicate 3; ○ controls without plants. Error bars represent the standard deviation of triplicates

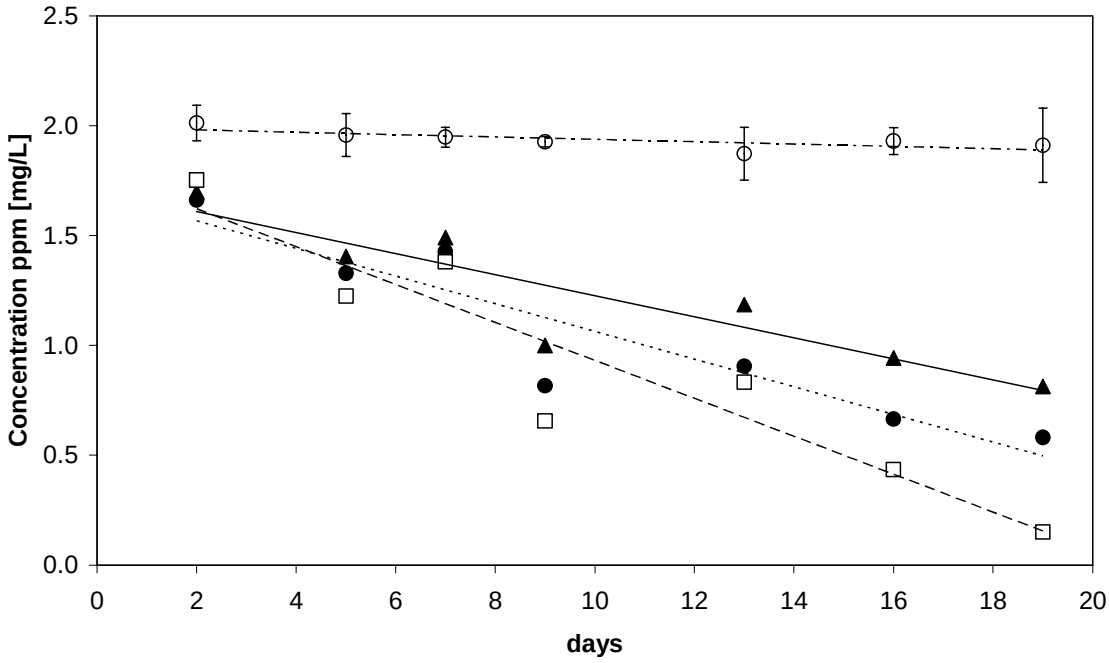


Figure 10.4 Plant effect on DEA concentration in media versus time

● replicate 1; ▲replicate 2; □ replicate 3; ○ controls without plants. Error bars represent the standard deviation of triplicates

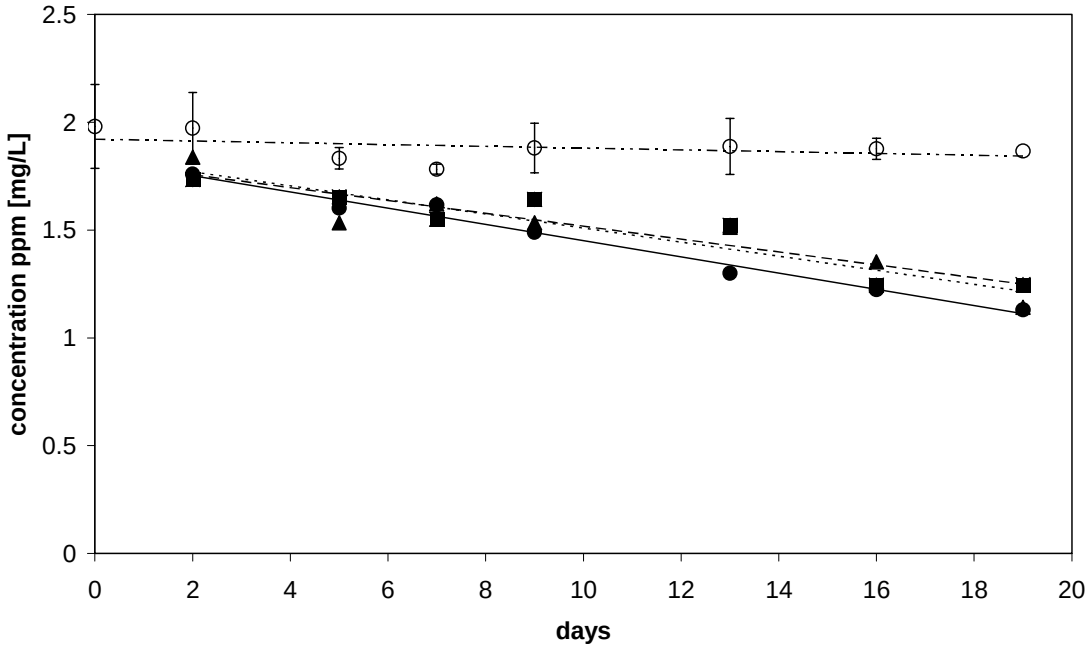


Figure 10.5 Plant effect on DIA concentration in media versus time

● replicate 1; ▲replicate 2; □ replicate 3; ○ controls without plants. Error bars represent the standard deviation of triplicates

After 19 days, uptake of atrazine per litre of transpired water was slightly higher than dealkylates DEA and DIA (**Table 10.1**).

Transpiration is dependent on temperature, humidity, stomata per cm^2 of leaves and foliar surface. The best and easiest correlation of transpiration with one of these variables was obtained with leaf biomass (**Figure 10.7**)**.** In the glasshouse temperature, humidity and light were not constant (only programmed minimal values), but all plants underwent the same variations. Thanks to relation to transpiration and foliar biomass, it was possible to explain that plants with smallest foliar biomass transpired the least, and achieved the lowest uptake of herbicide.

Table 10.1 Qantity of herbicide taken by vetiver plant per quantity of transpired water

Values were calculated with cumulated quantity of herbicide taken by the plant per total transpired water after 20 days exposure to about 10 µM starting concentration of herbicides

Herbicide	[µmol L^{-1}]
ATR	5.2 ± 0.40
DEA	4.9 ± 0.05
DIA	4.8 ± 0.88

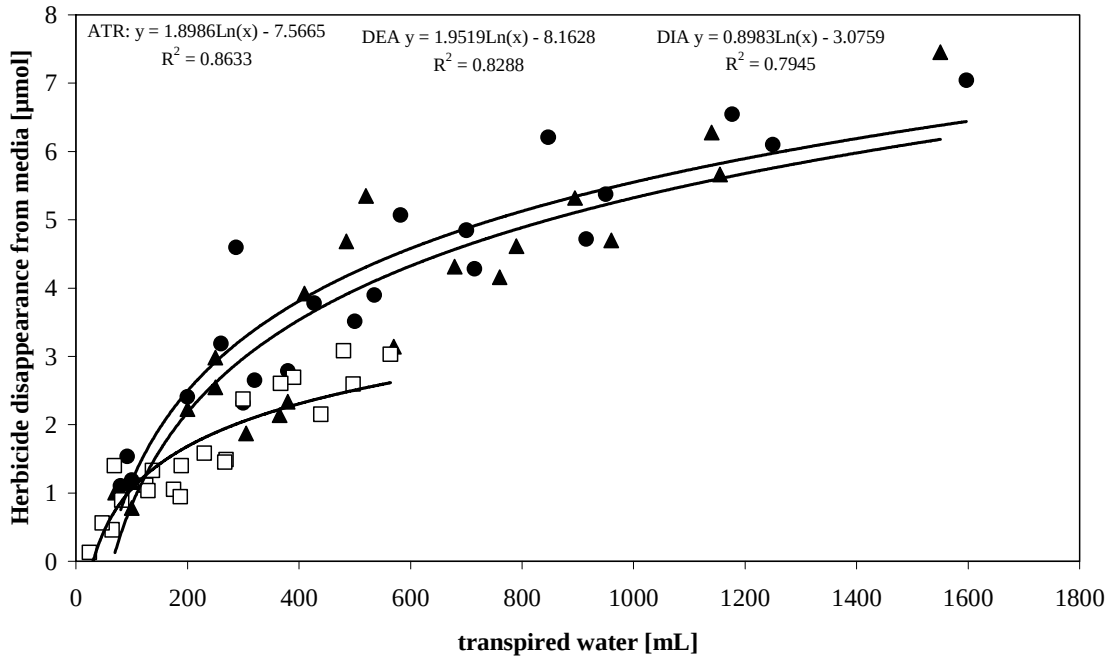


Figure 10.6 Logarithmic correlation of herbicide disappearance of ATR, DEA, and DIA with transpired water

Cumulated disappearance of each herbicide from the media was plotted against cumulated water transpiration at days 2, 5, 7, 9, 13, 16, 19.

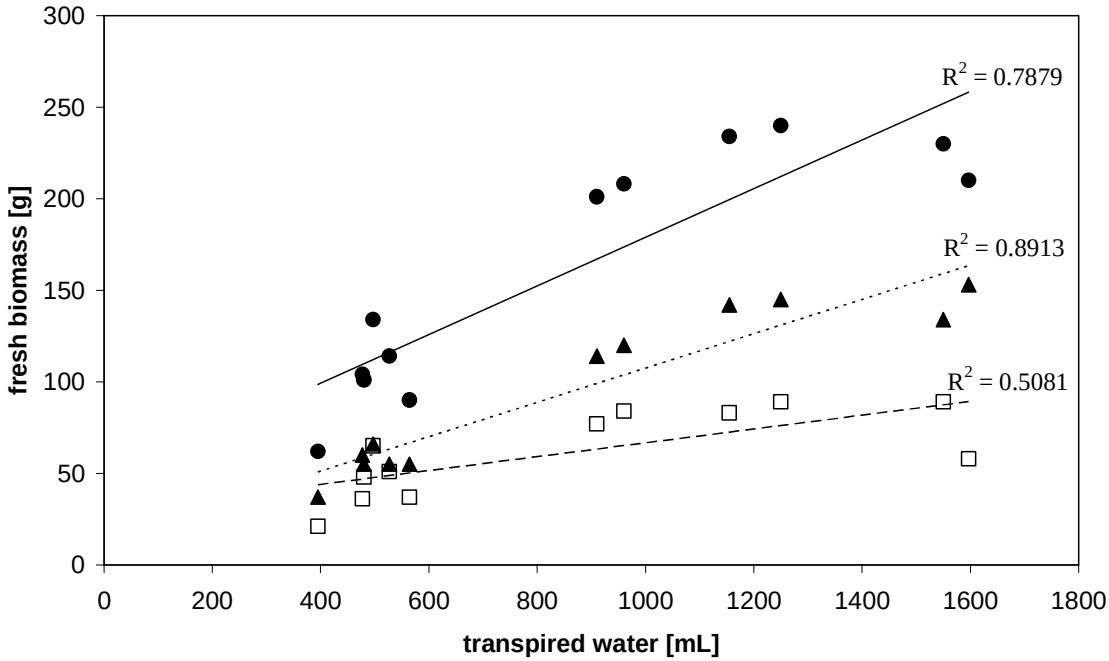


Figure 10.7 Linear correlation of fresh biomass with transpired water.

● fresh biomass of entire plant ▲ fresh biomass of leaves □ fresh biomass of roots

Interestingly, for plants exposed to atrazine, traces of dealkylates DEA and DIA were detected in the medium, identified according to their retention time and UV spectra compared to their respective standards (**Figure 10.8**). Hydroxyatrazine was not detected in the medium, even after 20 days exposure to atrazine. DEA and DIA concentrations in the medium were not a function of time for none of the 3 plant replicates exposed to atrazine.

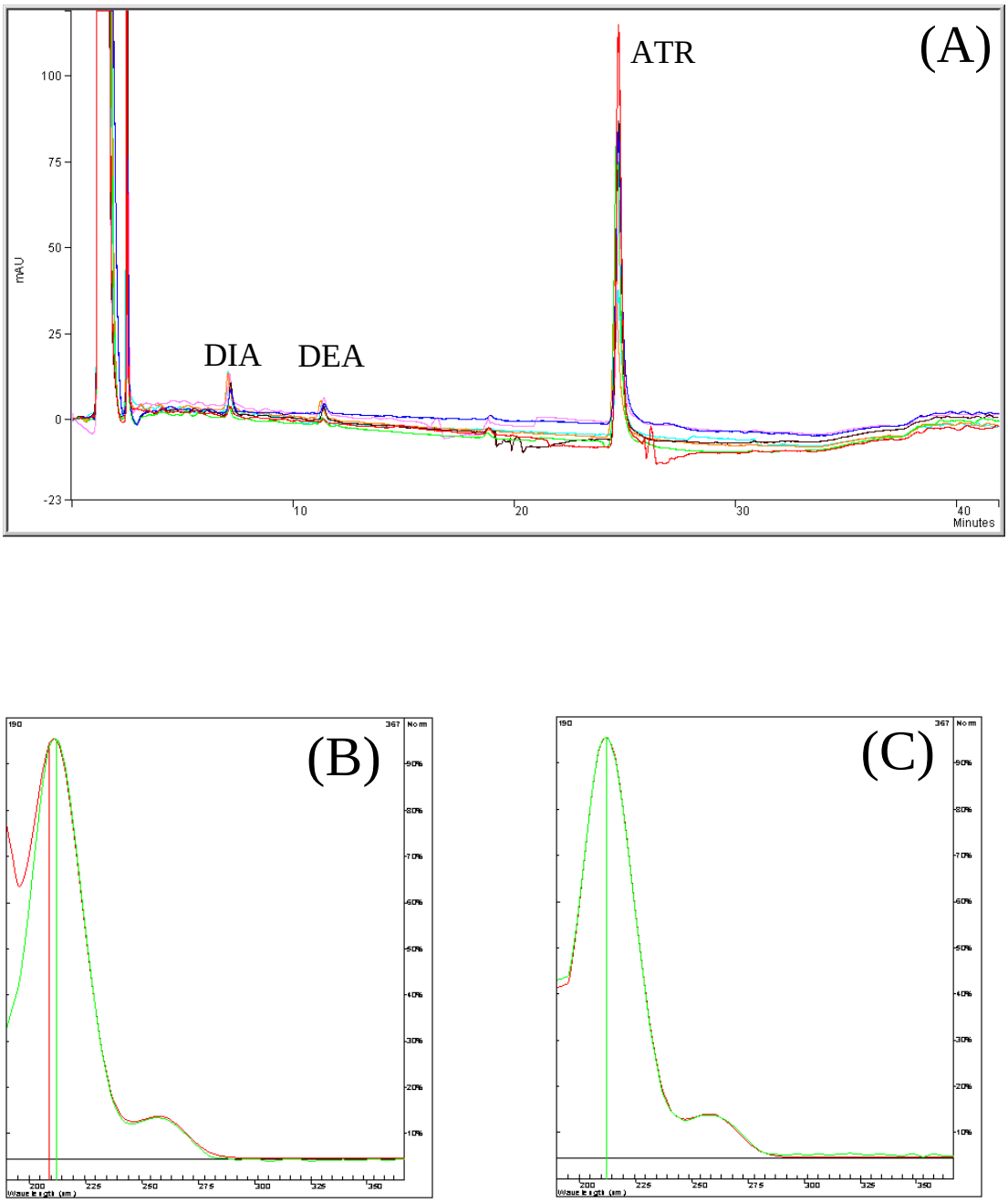


Figure 10.8 Detection of dealkylates of plant exposed to atrazine at day 19

(A) chromatogram of hydroponic solution at days 2, 5, 7, 9, 13, 16, 19
(B) spectrum of formed product at day 19 with retention time 11.49 min compared to spectrum of DIA standard
(C) spectrum of formed product at day 19 with retention time 11.49 min compared to spectrum of DEA standard

10.4 Discussion

Progressively, when concentration of herbicide decreased, less and less quantity of herbicide was absorbed by plants. This observation clearly indicated that uptake was passive, and that concentration between medium and roots equilibrate unceasingly. In our experiment under transpiring conditions, it seemed that adsorption, absorption, diffusion and over-concentration of herbicides into roots were negligible. This is correlated to low amount of lipids (0.3% of fresh biomass) found in vetiver roots rooted in hydroponics. All of them were rooted from dried slips directly in hydroponics for 7 months in *Populus deltoids x nigra.* For understanding relative contribution of over-concentration of herbicide in roots under transpiring conditions, we should have worked ideally with plants grown 3 years in soil later transferred into hydroponics just before plant uptake study. Atrazine uptake appears to be largely a passive process closely associated with the movement of water, as reported by Wilson (189) in *Canna hybrida*, by Raveton (134, 136) in *Zea mais*, in *Populus deltoides nigra* by Burken and Schnoor (27).

Passive uptake of atrazine was shown in cell cultures by McCloskey and Bayer (103) and Raveton et al. (136). In the latter study, passive transfer of ^{14}C-atrazine from the nutrient medium to *Acer platanus* cells is about twice that of medium. This equilibrium is due to a simple partition process; part of the atrazine is dissolved in the cell water and reached the same concentration as in the external medium while the rest is concentrated inside the cellular lipids, resulting in a slight over-concentration of radioactivity. In living immersed maize seedlings, despite of only passive diffusion, high over-concentration is reached due to rapid formation of hydroxy derivatives of atrazine unable to diffuse freely into the external medium.

Although passive diffusion occurred between medium and roots, it seems that roots offered resistance to penetration of ATR, DEA and DIA, since 1 liter of transpired solution did not contribute to the total disappearance of these compounds from the medium. The plant would act like a chromatographic system.

When exposed to atrazine, small quantity of DIA and DEA were detected in the solution. Passive diffusion is assumed to occur as mechanism of plant uptake, then it means that dealkylates can diffuse out of roots, if the external concentration is lower than in the root cells. Together with the correlation of disappearance of atrazine from

the medium with transpiration of the plant, it was deduced that micro-organisms production of dealkylates was negligible in the hydroponic solution, even if dealkylation metabolic pathway is common to micro-organisms and plants. Moreover, if micro-organisms were the only dealkylate producers, mass balance should have been observed between atrazine disappearance and dealkylates appearance in the medium, and accumulation of dealkylates should have been observed with time.

In conclusion, vetiver plants were able to remove ATR, DEA and DIA from hydroponic solution, although being also producers of dealkylates when exposed to atrazine. Dealkylates produced by micro-organisms in soil could thus be taken up by plants with water uptake, as well as dealkylates could diffuse outside root cell membranes, if inside concentration was higher than the surrounding medium. Plant uptake of dealkylates is of great interest, since EPA described them as sharing the same endocrinal effect as the parent compound atrazine.

Conjugation by GSTs of dealkylates and translocation in vetiver was not studied. Hydroxyatrazine undergoes further dealkylation (150) in maize plants, and dealkylates are further conjugated in sorghum plants (93). Nevertheless, log K_{ow} of DIA and DEA of 1.7 and 1.38 respectively indicate more polar compounds that are maybe less mobile than atrazine in the plants. Almost all radioactivity of hydroxyatrazine ^{14}C-treated plants in transpiring conditions remained in roots, indicating that translocation of this relative polar compound (log K_{ow} (HATR) = 0.85 (134, 150)) does not occur (150). Therefore, it is not known if conjugation of dealkylates would be an important metabolic pathway, since conjugation of atrazine is mainly occurring in leaves, after translocation from roots. Translocation of dealkylates is however suspected in vetiver plant, because of their intermediate log K_{ow} between those of HATR and ATR, but it remains to be proved.

11 General discussion

Results showed that the mechanism of resistance to atrazine in vetiver was a reduced availability of the herbicide at the site of action rather than the presence of a less-sensitive site of action.

Photosynthetic electron transport of isolated thylacoids was inhibited by atrazine in vetiver, indicating that the mechanism of resistance to atrazine was not a less-sensitive D1 protein. Photosynthesis inhibition is generally explained by the sensitivity of plant species to the level of triazines. However, isolated chloroplasts from maize are as sensitive to inhibition of the Hill reaction as those from sensitive species (71), showing that metabolization explains maize tolerance to atrazine, as it also seemed to be the case for vetiver. Moreover, no damages of vetiver leaves were observed in the presence of atrazine despite of apparent concentration of atrazine in excised vetiver leaves (chapter 8) and in leaves of entire plant (chapter 9) higher than the concentration of atrazine required for 50% inhibition of the Hill reaction in thylacoids (chapter 5). This is in agreement with Cull et al. publication (47), in which tolerance of vetiver under wetland conditions to atrazine was shown by leaf fluorescence, water use, cumulative leaf area, and dry weight.

Lipid content of roots was found to be correlated with root growth in hydroponics or in soil. Atrazine over-concentration in roots grown in soils was correlated with root lipid content. Roots grown in hydroponics did not over-concentrate atrazine, in agreement with their low lipid content. *In vitro* partition of atrazine in vetiver oil was demonstrated, suggesting strongly that old roots could act as an accumulator of atrazine.

It is believed that generally, hydroxylation of atrazine thanks to benzoxazinones was not a major pathway in vetiver. Hydroxylated products of atrazine were not found in entire plants (chapter 9), in agreement with the lack of benzoxazinones in plant extracts and the absence of *in vitro* hyxdroxylation (chapter 6). Literature mentions that benzoxazinones concentration in plant is a function of plant age, i.e. in maize seed, the concentration is nil, and is maximum in young seedlings (114), decreasing slowly within the first 4 weeks until matured plant (39, 114). One could object that extraction, identification of benzoxazinones and hydroxylation of atrazine *in vitro*

tests were done with 8-months old plants, explaining these negative results. However, the *in vivo* metabolism of atrazine in entire vetiver was done in 4 weeks old tissues, and only traces of hydroxylated compounds were detected, indicating that benzoxazinones were not massively present at any age of vetiver development.

Fate of atrazine in vetiver seems to be rather complex: it is believed to be a combination of several parameters such as age of the plant, root growth in hydroponics or in soil, and transpiring conditions. Due to technical reasons, and lack of time, the only complete picture of fate of atrazine in vetiver is in young plants grown in hydroponics under transpiring conditions, possibly being the ideal case in term of plant detoxification capacity because of rapid and high transformation to non harmful conjugates. On the other hand, the highest atrazine uptake would be obtained by old vetiver plants grown in soil under transpiring conditions, as explained below.

Young or old vetiver grown in hydroponics under transpiring conditions

With vetiver plants grown in hydroponics, root sequestration of atrazine is believed to be nil, because of low lipid and oil content in vetiver, independently of plant age. Lipid content of 4-week old plant was 0.3% fresh weight (FW) (chapter 9), as much as 8-month old roots (chapter 6), showing that lipid content of roots grown in hydroponics is constant during plant aging. The picture of metabolism of atrazine appears to be very similar to *Sorghum* (91-93) and *Panicoidea* subfamily (82) plants in which major metabolization is conjugation in leaves, and dealkylation is low in whole plant.

Stepping of results from different chapters can be performed to understand the fate of atrazine in vetiver grown in hydroponics: *in vitro* conjugation of atrazine (chapter 7), fate of vetiver in excised leaves (chapter 8), fate of atrazine in entire vetiver plant (chapter 9), and dealkylates uptake (chapter 10). However, the fate of atrazine in vetiver excised roots grown in soil (chapter 8) could not be compared to roots studied from entire vetiver plant (chapter 9) because plants were grown in hydroponics.

The global GST activity, as shown when using CDNB as a substrate which is supposed to be ubiquitous, was two thousand times higher than that of GST isoform conjugating atrazine. In other words, GST activities toward atrazine were not correlated with the activities toward CDNB, which was the optimal substrate in vetiver and maize. This observation reinforces previous comments regarding the

limited usefulness of this substrate when attempting to predict GST activities toward herbicides in plant metabolism studies, as also observed by Hatton et al. (76). *In vitro* conjugation of atrazine seemed to be mediated by the action of GSTs, as spontaneous conjugation could not be detected in the control without enzyme extracts, in contrast to the situation reported in sorghum by Lamoureux et al. (93), who found as high as 10 to 20% non enzymatic conjugation. *In vitro* activities of conjugation of CDNB were in the same magnitude in root and leaf extracts, whereas activities on atrazine were only found in leaf extracts after 1 hour incubation (chapter 5). However, the potentiality of conjugation of atrazine was not nil in roots, since longer tested periods (around 5 days) allowed the detection of conjugates in roots of entire vetiver plants (chapter 9).

Observed high concentration of conjugates in the tip of vetiver leaves could therefore be explained by the conjunction of two factors: high conjugation capacity in leaves, together with deposit of atrazine at the end of xylem vessel in plant under transpiring conditions. Because of a negative net charge of conjugated atrazine, it must be poorly redistributed in the plants with transpiration stream once produced, and therefore, the high production of conjugates atrazine at the tip of the leaves would result from a local transformation in the leaf, and not from an accumulation following transport of conjugated atrazine produced by roots. Similarly, in root-treated plants with radio-labelled hydroxyatrazine, radioactivity remains in roots and is not translocated from the roots to shoots (150).

Dealkylation, although present in vetiver, was a minor metabolic pathway of atrazine. Although extracts of vetiver exposed to radio-labelled atrazine (chapter 9) were not loaded on a solvent system able to separate atrazine and dealkylates, it was assumed that dealkylation yield obtained in excised vetiver leaves (chapter 8) was corresponding to the situation in leaves of entire plant: although existing, dealkylation was small. Small amounts of DEA and DIA were detected in the hydroponic medium of plants exposed to atrazine (chapter 10) suggesting that vetiver could be a dealkylate producer in soil. On the other hand, DEA and DIA were shown to be taken up by vetiver in the same range as atrazine, with the transpiration stream of the plant, suggesting a beneficial effect of vetiver to lower atrazine and dealkylates in the environment (chapter 10). However, further work should be done on dealkylates

conjugation, because it would give a sound conclusion about vetiver usefulness to lower these contaminants often detected in soils and water.

Vetiver grown in hydroponics under non transpiring conditions

As shown by Raveton (134), maize seedlings treated with radio-labelled atrazine for 72 hours under 100% atmospheric relative humidity concentrated radioactivity in roots, whereas no radioactivity was found in the aerial part. It can be assumed that under the described conditions, vetiver behaves similarly. Although roots seemed to be less active metabolically, over long period of time, conjugation of atrazine could be non negligible. Similarly, the release of dealkylates in the medium could increase with time. Under heavy rainfall or stream, the equilibrium might never be reached, because of the wash out of dealkylates. If this phenomenon is not too important, release of dealkylates may stop at the equilibrium. Later, under transpiring conditions, the released dealkylates (and dealkylates produced by micro-organisms) may be taken up back in the plant with transpiration stream. Raveton (134) showed also that the efflux of atrazine in the medium is also occurring following plant exposure to atrazine and its transfer in hydroponic medium without atrazine.

Vetiver grown in soil under transpiring conditions

During plant aging, root cortex is more and more loaded in vetiver oil, increasing probably atrazine partition. However, it is also known that roots are a dynamic system: all roots are not of the same age, since roots are continuously renewed. It is also known that nutrient and water absorption is done by young roots, whereas old roots are more an anchorage for the plant. It is possible that old roots over-concentrated atrazine, but thanks to transpiration and water uptake by young roots, this latter phenomenon should be the major cause of atrazine disappearance from the medium, and atrazine partition would be negligible. Remobilization of atrazine from oil to aqueous compartment where main metabolization of atrazine occurs is not known, but is believed to be possible; log $K_{oil/water}$ (ATR) was nearly identical to log K_{ow} (ATR). However, when considering vetiver oil, it is believed that important concentration of atrazine could occur. In other words, the fate of atrazine in vetiver grown in soil under transpiring conditions is believed to be a combination of atrazine temporary (?) sequestration into old roots together with uptake of atrazine by the young roots leading to conjugation into leaves.

Vetiver grown in soil under saturated humidity

Old vetiver grown in soil and under saturated humidity is the situation where partition of atrazine into vetiver oil should be the major uptake phenomenon, for as much that contact time with roots is long enough. Hydrophobic interactions and partition of atrazine in vetiver roots could play a major in water protection by retaining pesticides in runoff water when plant transpiration is nil and rainfall maximum. Neither conjugation, nor dealkylation was detected in excised roots grown in soil, suggesting that the incubation time of roots with atrazine was insufficient to detect metabolites (chapter 8). However, it was observed that these roots were highly able to over-concentrate atrazine because of their lipid and oil content. Only roots grown in soil could over-concentrate atrazine, in contrast to roots grown in hydroponics (chapter 8). The high lipophilic content of roots could also explain the non detection of metabolites, because of partition phenomenon preventing enzymes to transform atrazine.

Consequences of results for oil producers

Pesticides contamination was found in many ginseng supplements in 2001. As ginseng roots are used for making supplements, it is believed that vetiver roots used as herbal medicines could lead consumers to exposure of high pesticides concentration. Chan and al. (36) pointed out that 70-80% of the world population rely on non-conventional medicine mainly of herbal sources in their primary healthcare. Also, in the recent years, there is an increasing popularity of health food, and medicinal products from plants. However, these natural products may be contaminated with excessive or banned pesticides, heavy metals and chemical toxins, enhancing the need of Standard Operating Procedures (SOP) leading to Good Agricultural Practice (GAP), Good Laboratory Practices (GLP), Good Supply Practice (GSP) and Good Manufacturing Practice (GMP). Vetiver extracted oil is mainly used in perfumery and as consequence applied externally with low risks on health. But if roots are prepared as infusions for stomatic, carminative, sudorific, analgesic and headache curative effects, then exposure to high concentration of pesticides could have a global negative effect for health. It is clear that there is a total incompatibility between phytoremediation and medicinal/oil production.

In conclusion, atrazine phytoremediation goals, as defined at the beginning of the present thesis and project were fulfilled: the identification of a plant able to accumulate and/or transform atrazine, identification of metabolites, even if quantification of conjugation and dealkylation under different environmental conditions remains to be understood, and conjugation of dealkylates in vetiver remains to be proven. Hydroponic system was successfully established, but ended with the conclusion that full comprehension of vetiver uptake of atrazine should be performed with soil grown plants. Key enzyme of detoxification was identified, and risk assessment can be pictured: globally, a positive impact of vetiver was observed, as main metabolites are conjugates. However, because of atrazine partition in roots, medicinal use and oil production should be clearly separated. Cellular localization of atrazine and produced metabolites in isolated protoplasts and vacuoles should also be explored.

12 General conclusions

Plant resistance necessary for phytoremediation establishment in atrazine contaminated soil or water was shown. Major metabolism of atrazine in vetiver grown in hydroponics system was conjugation mainly in leaves, a transformation known to be positive for the environment. Cutting moment of leaves for handicrafs, roof cover, cattle feeding should be carefully chosen, at least not just after atrazine application on nude soils and first rainfall. However, hydroponics study revealed some limitation, since production of oil and lipid is only obtained for plants grown in soils. In vetiver grown in soil, partition in roots is believed to occur in old roots, though probably being a minor process in atrazine uptake, since young roots water uptake with transpiration stream and final conjugation into leaves would probably dominate. Although being a slight dealkylate producer, the plant is able to take up DEA and DIA in the transpiration stream. Phylogenetically, vetiver is close to sorghum, a plant described previously to tolerate atrazine thanks to high conjugation capacity. It seems that, as other *Panicoideae* plant subfamily, vetiver follows the same interesting detoxification pathway: conjugation. However, the similarity stops here, since as root oil producer, sequestration of atrazine can possibly occur with unknown remobilization and subsequent metabolization rate in the plant. Hydrophobic interactions may play a key role in retaining pesticides to percolate to groundwater or to runoff in surface water when transpiration is nil. As leaf surface is small compared to phreatophytes, and that best performance of atrazine uptake depends on the volume of transpired water, vetiver is not thought to be very performant for phytoremediation of highly contaminated soil or water. However, due to its high dense root system, it is believed that vetiver is an ideal system against non point pollution of atrazine. Vetiver hedges are a reality against soil erosion, already in place in agricultural fields where atrazine is used. Hydroponic system could be compared to a wetland situation, where vetiver usefulness was shown for removal of atrazine. Therefore, vetiver should play an important ecological role for water protection, which should be integrated in Good Agricultural Practices (GAP).

13 Outlook

13.1 Rhizospheric vetiver studies

To evaluate vetiver hedges efficacy against atrazine runoff, some work should be performed in the field, by measuring atrazine in soil water before and after hedges, at the inlet and outlet of the wetland. Measurement should be performed especially after the first rain following atrazine application, where the performance of phytoremediation should be the most critical, due to saturation of the soil in water, reduced transpiration of plants, and highest water movement through the soil and stream in wetlands. Hedges and wetland planting should be dimensioned, and limits of phytoremediation assessed.

Vetiver's dense, finely structured root system provides an environment that stimulates microbiological processes in the rhizosphere. The respective contribution of rhizosphere versus plant uptake of atrazine should be explored.

There is a growing interest in the use of plants to increase microbial degradation of organic chemicals in soil (phytostimulation). Researchers have noted an increased degradation of pesticides in rhizosphere soil, as compared with unvegetated soils. Indeed, accelerated mineralization of atrazine in maize rhizosphere soil was observed by Piutti et al. (126): the quantification of relative amount of the gene *atz*C, which encodes an enzyme involved in atrazine mineralization, was carried out on soil nucleic acids by using quantitative-competitive PCR assays. It revealed that *atz*C is present at a higher level in the rhizosphere than in bulk soil. Moreover, higher mineralization of atrazine is observed in maize planted soil than bulk soil (100). Atrazine mineralization was also greater in soil collected from *Kochia scoparia* and *Brassica napus* rhizosphere (9). And finally, a high population of atrazine degraders and enhanced rates of atrazine mineralization are also observed in bioaugmented sediments after incubation in flooded mesocosms planted with *Typha latifolia* (cattails) (138).

Knowing the root surface of vetiver, it is not difficult to imagine rhizospheric importance in term of biomass and activity in degradation of pesticides.

13.2 Phytoremediation for other herbicides removal from soils

Studies with other pesticides would be relevant to see if vetiver as a tool against pesticide runoff could be extended. Moreover, atrazine is also used in combination with many other herbicides, such as alachlor, metolachlor, cyanazine, simazine, amitrole + simazine, or diuron + simazine (6, 9). In most pesticide-contaminated agrichemical facilities, atrazine is found in combination with other widely used agricultural chemicals (69). Therefore, remediation strategies must cope with a multiple-contaminated environment.

According to Thurmann and Meyer (165), most used herbicides in the U.S.A. were in 1996, atrazine, metolachlor, and alachlor. In the Midwest of U.S.A. atrazine and metolachlor are frequently present in groundwater (88). Atrazine and alachlor are also frequently detected in groundwater and rivers of many countries (130, 194). There is a critical environmental concern about alachlor and one of its metabolites in environment, 2[2'6'-diethylphenyl)(methoxymethyl)-amino]2-oxoethanesulphonate (ESA) which leaches much more rapidly through the soil than does the parent compound and makes an important contribution to the total organic contaminant load of groundwater in the central U.S.A.

All these herbicides are used for a pre-emergence treatment: Tissut et al. [197] pointed out that persistence of herbicides is linked to their mode of action: only herbicides of post-treatment can be not very remanent. But herbicides of pre-emergence must have an agronomical remanence of several weeks to exert their action: there is a need of days or weeks to exert their phytotoxicity, and to kill weeds which germination is not occurring at the same time. The herbicides are generally retained in the soil thanks to their adsorption on superficial soil horizons, but most of the time, washing of the herbicides occurs with the first rain following application. Besides leaching in the deep soil, there is also a risk of washing of soil particles on sloppy nude soils. Businelli et al. [48] assessed potential danger of groundwater contamination and found that alachlor, atrazine and simazine application should be avoided in sandy soils, and used only in non-irrigating crops.

In other words, pre-emergence herbicides triazines (ametryne, desmetryn, dimethymetryn, terbutryn, atrazine, propazine, cyprazine, simazine, cyanazine) and chloroacetanilides (alachlor, acetochlor, metolachlor, pretilachlor) are massively used

but also detected in groundwater and surface water, except cyanazine which is commonly found in surface water, but rarely in groundwater (165).

Lamoureux et al. (92) mentions that methylthio-*s*-triazines (ametryne, desmetryn, dimethametryn, terbutryn) are not readily metabolized to water soluble metabolites in excised sugarcane leaves and it was shown that the methylthio-*s*-triazines are not substrates for GSTs isolated from corn.

In contrast, plant species that have been shown to readily transport triazines acropetally from roots to leaves include corn, cucumber, spruce, black walnut, yellow poplar, poplar clones, radish seedlings, and barley (189). In most species, plant metabolism of triazines is similar to atrazine (92, 151). Many authors detected GSTs activities on triazines and chloroacetanilides (76, 179), and a positive correlation was found between plant tolerance to chloroacetanilide and triazines herbicides, best explained by conjugation to glutathione mediated by GSTs or not (80).

Moreover, in addition to common detoxification of triazines and chloroacetanilides in plants, atrazine and metolachlor mineralization is greater in soil collected from *Kochia scoparia* and *Brassica napus* rhizosphera (9).

Vetiver was shown to undergo conjugation of atrazine, and therefore it is believed that it is also capable to take up and conjugate chloroacetonilides and other triazines, and not only atrazine in agriculture runoff.

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Curriculum vitÆ

Sylvie Marcacci

Nationality:
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Date of birth: 03.12.72

Professional experiences

11/2000-02/2003

Laboratoire de Biotechnologies Environnementales (LBE), Ecole Polytechnique Fédérale de Lausanne (EPFL), Lausanne, Switzerland

PHD student "A Phytoremediation Approach to Remove Pesticides (atrazine and lindane) from Contaminated Environment".

05- 09/2000

Research and Development unit (R&D), **Serono Ltd**, Fenil-sur-Corsier, Switzerland

Scientific assistant on 5L bioreactors**.** Animal cell culture (CHOs) in bioreactors including optimization, maintenance, building, and control of 5L bioreactors. Contribution to the analysis of results and to their interpretation.

06- 10/1999

Department of Entomology, Station Fédérale de Recherches Agronomiques de Changins (**RAC**), Nyon, Switzerland

Research position for a method development to assess insecticides on San Jose scale *(Quadraspidiotus perniciosi),* leading to a final written report for Novartis Ltd. Knowledge acquired on the main classes of insecticides and their interactions with environment and statistical treatment of results

10/1998- 05/1999

Department of Plant Sciences, **Rhône-Poulenc Ltd**, Ongar, U.K.

Laboratory trainee. Specific project focused on herbicides uptake in isolated protoplasts using dual labelling. This project was a part of the general question on how herbicides are entering plant cells. A final report and oral presentation in English ended the training. Knowledge acquired on the main classes of herbicides.

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12/1996- 04/1998

University of Neuchâtel, Neuchâtel, Switzerland

Different positions in a Swiss University; specific areas and appointments included:

- **Research position** in the Department of Animal Ecology. Method development using cellulosis acetate gels for the analysis of isoenzymes of *Oreina cacaliae*.
- **Laboratory trainee** in the Department of Plant Physiology addressing the cloning and expression of *MRP5* transporter of *Arabidopsis thaliana*.
- **Laboratory work diploma** in the Department of Parasitology. Examination of the CS protein kinetics of *Plasmodium yoelii* in *Anopheles stephensi* and *A. gambiae* (Western Blot, IFAT, confocal microscopy). This project was closing the University studies and lead to a written report and oral presentation. Malaria and general diagnosis in human parasitology were improved by a laboratory training given by the Tropen Institut of Basel, Switzerland.

Temporary jobs

- **02-04/2000**

Teaching of English in the secondary school "Collège de Rolle", Switzerland

- **11/1999-02/2000**

Administrative work in AVS, Clarens, Switzerland

- **05/1997**

Logistical work for the "8th European Congress of Clinical Microbiology and Infectious Diseases", Lausanne, Switzerland

Education

11/2000 - 02/2004	PhD at the Swiss Federal Institute of Technology Lausanne (EPFL)
09/1993 -01/1998	Diploma in Biology at the University of Neuchâtel, Switzerland
09/1988 – 06/1993	Baccalaureate (scientific section), Collège Calvin, Geneva, Switzerland

Other projects and diplomas

- **"Stroke" (2003)**. Mentioned project for the garden contest "Lausanne Jardins 2003", Lausanne, Switzerland. In association with Florian Bach, artist, La Touche Verte, landscape designers, and Hybridées, architects
- **Oboe diploma (1993)**, Conservatoire de Musique de Genève, Switzerland
- **Oboe Third Price (1990)** in the Swiss National Contest "Schweizerischer Jugendmusik-Wettbewerb", Luzern, Switzerland
- **Music theory diploma (1988)**, Conservatoire de Musique de Genève, Switzerland

General information

Languages	French (mother tongue) English (fluent, Cambridge First Certificate) German (scholar knowledge) Italian (oral expression)
Computing skills	Word, Excel, Power Point, Outlook Express, Internet Explorer, Endnote
Hobbies	Theater, guitar, sailing, ornithology, oboe