Ecole Polytechnique Federale de Lausanne

ORAL PRESENTATION

for the Degree of

Docteur es Sciences

by

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Indo-Swiss Collaboration in Biotechnology

ISCB

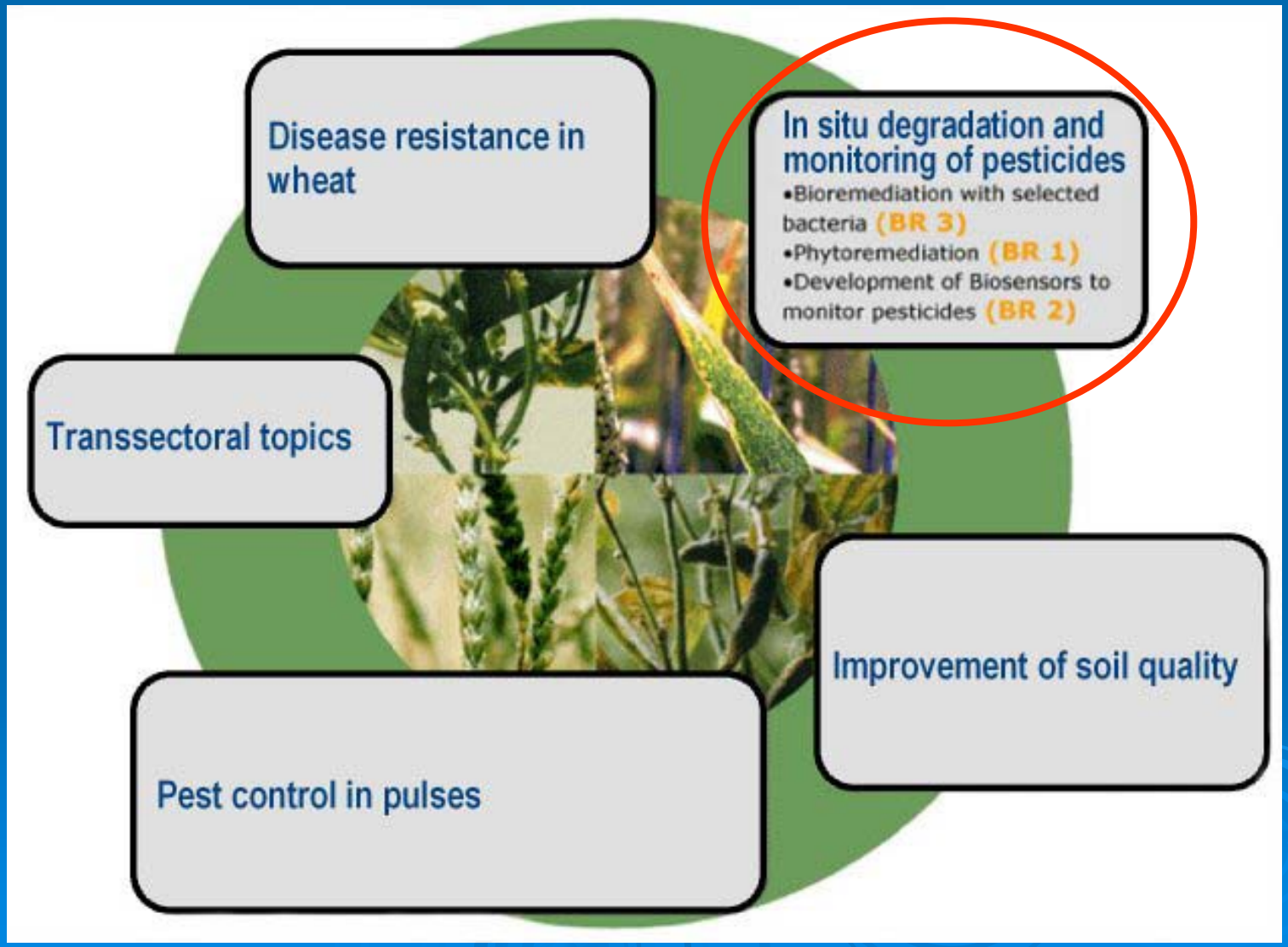
EPFL

ÉCOLE POLYTECHNIQUE FÉDÉRALE DE LAUSANNE

SSIE

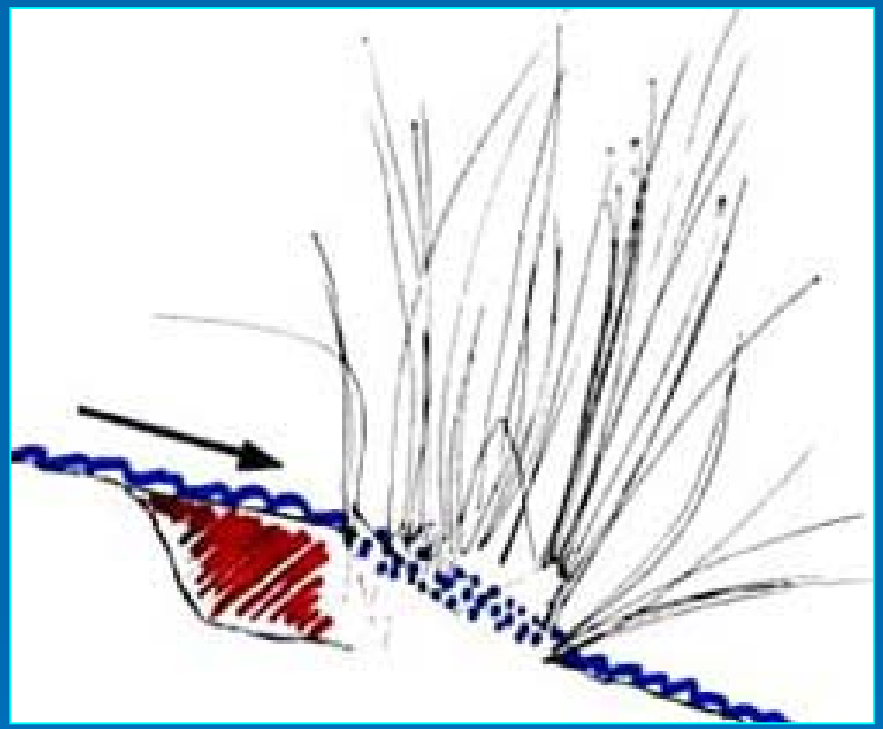
A phytoremediation approach to remove pesticides (atrazine and lindane) from contaminated environment

Thesis as a part of ISCB projects



Modified from http://www.biotech.biol.ethz.ch/india/

A phytoremediation approach to remove pesticides (atrazine and lindane) from contaminated environment



Old vetiver hedges for a new job?

Buffer zones and water quality protection

Sylvie Marcacci

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Laboratory for Environmental Biotechnology (LBE) EPFL

Lausanne, the 20th of February 2008

Old vetiver hedges for new job?

Introduction

What can be done to reduce atrazine in drink water in tropical and sub-tropical countries?

Laboratory results

What is the fate of atrazine in vetiver plants?

Perspectives

Vetiver as tool for water protection?

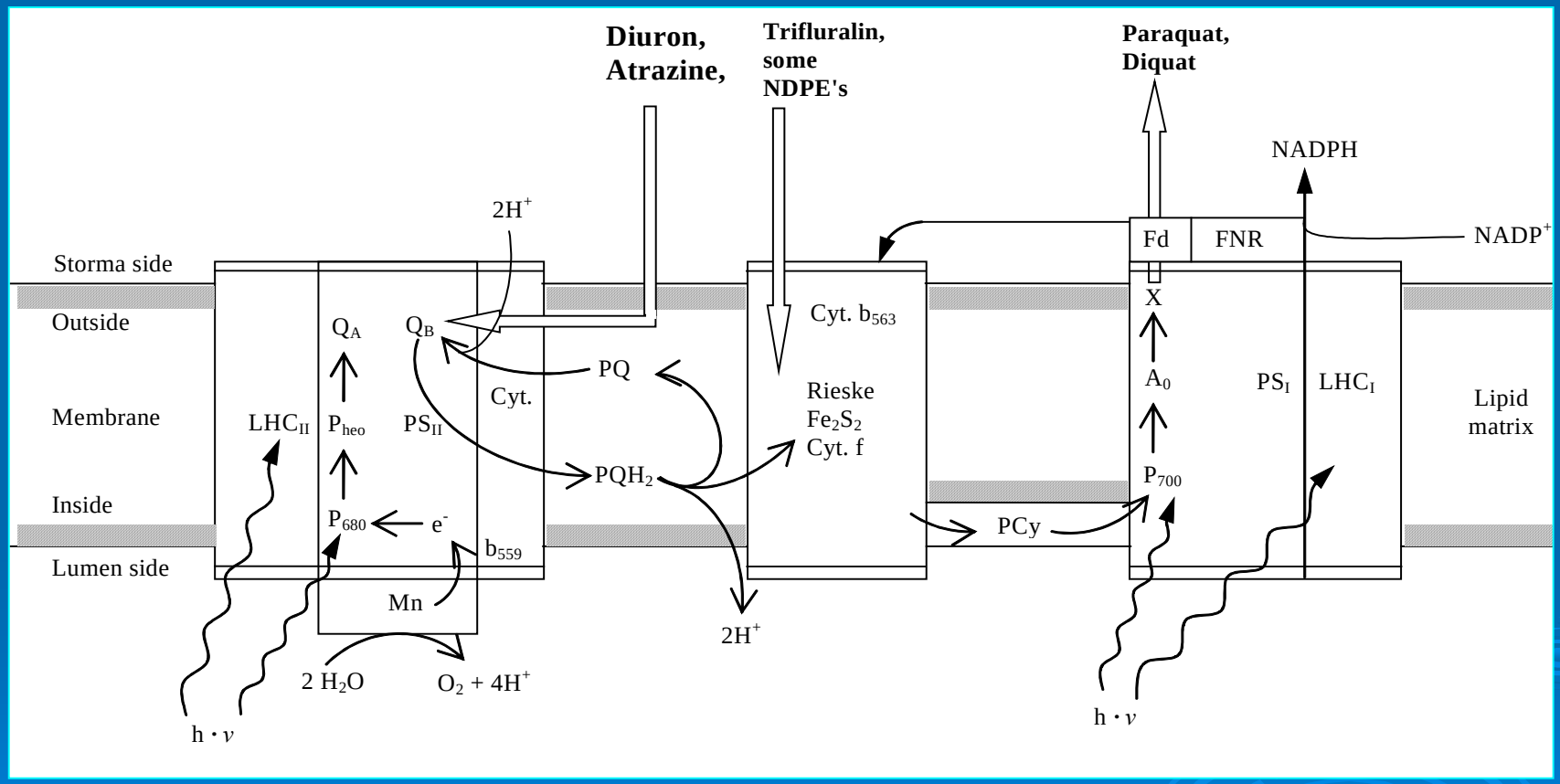
Atrazine is a worldwide used selective systemic herbicide

Maize, sorghum, sugar cane

coffee, asparagus, vines, fruit orchards, pineapples, oil palms, roses, forestry

• World pesticides production	2.5 mio tons/year
• ATR world production	70’000 tons/year
• ATR USA production	40’000 tons/year
• ATR Switzerland use	35 tons/year
• ATR India production	1000 tons/year

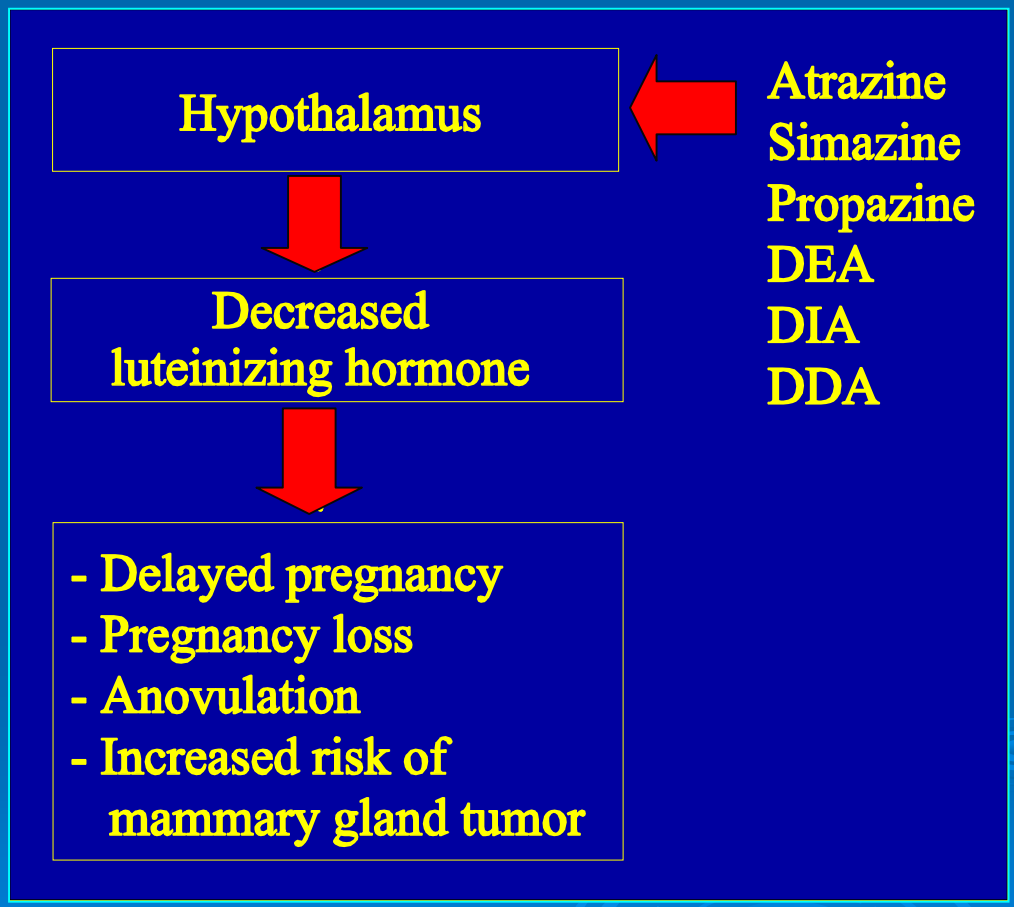
Atrazine inhibits electron flux in photosystem II in plants…



… but acts also on non target organisms causing environmental and health problems

- **Photosynthesis inhibition in algae[1]**
- **Crop rotation problems[2]**
- **Feminization of male frogs[3]**

[1] Stratton, 1984
[2] Delmonte et al., 1997
[3] Hayes T et al., 2002



Modified from the U.S. EPA report, 2002

Atrazine is found above admissible concentrations in drink water …

Countries	MCL per pesticide [ppb]	MCL per total pesticides [ppb]	Occurrence of atrazine in water
Switzerland[1]	0.1	0.5	**All lakes**
Europe[2]	0.1	0.5	**France: 43% of population drinks contaminated water**
USA[3]	3	?	**24 contaminated = 10.5 mio persons concerned!**
WHO[2]	2	?	

No conventional water treatment eliminates atrazine!

[1] *Gerecke et al, 2002*
[2] *http://assoc.wanadoo.fr/erb/colqP3.htm*
[3] *US EPA, 2002*

…which contamination could be avoided by source limitation

Micro pollutants

=

Low concentration of xenobiotics exhibiting toxicity

End of pipe treatment

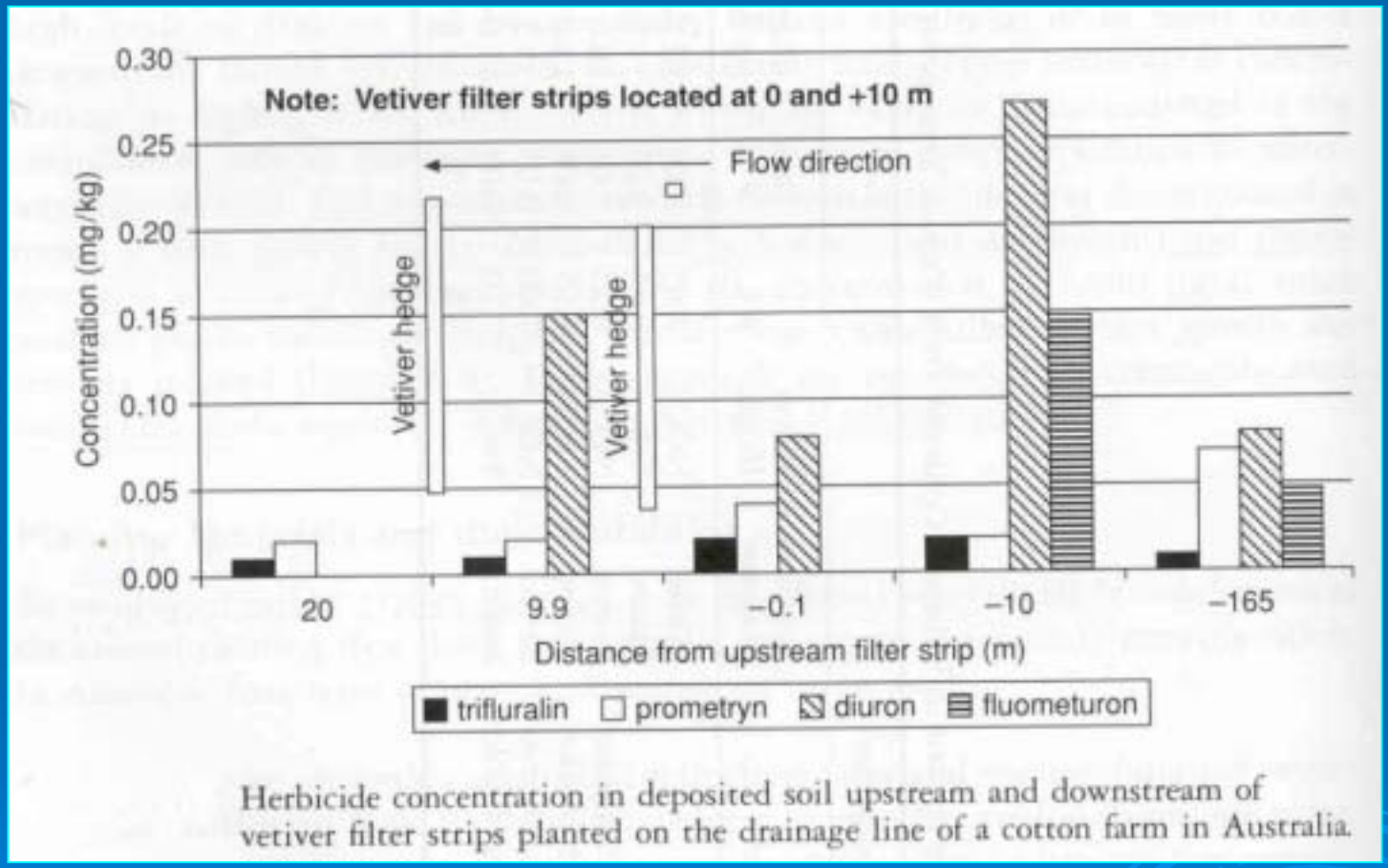
- Oxydation
- Nanofiltration
- Activated charcoal
- LLE (liquid/liquid extraction)
- Perstraction?
- Oil bodies?
- Photocatalysis?

Source limitation

GAP (Good Agricultural Practices, FAO)

Phytoremediation?

Vetiver hedges reduce non-point pollution of several herbicides … so what about atrazine?



Herbicide concentration in deposited soil upstream and downstream of vetiver filter strips planted on the drainage line of a cotton farm in Australia.

In Maffei, Truong et al, 2002

Old vetiver hedges for new job?

Introduction

What can be done to reduce atrazine in drink water in tropical and sub-tropical countries?

Laboratory results

What is the fate of atrazine in vetiver plants?

Perspectives

1. Is vetiver taking up atrazine?
2. What is the fate of atrazine in vetiver?
3. What is the limit of the use of hydroponic system?

Vetiver is a giant grass producing dense roots and leaves…

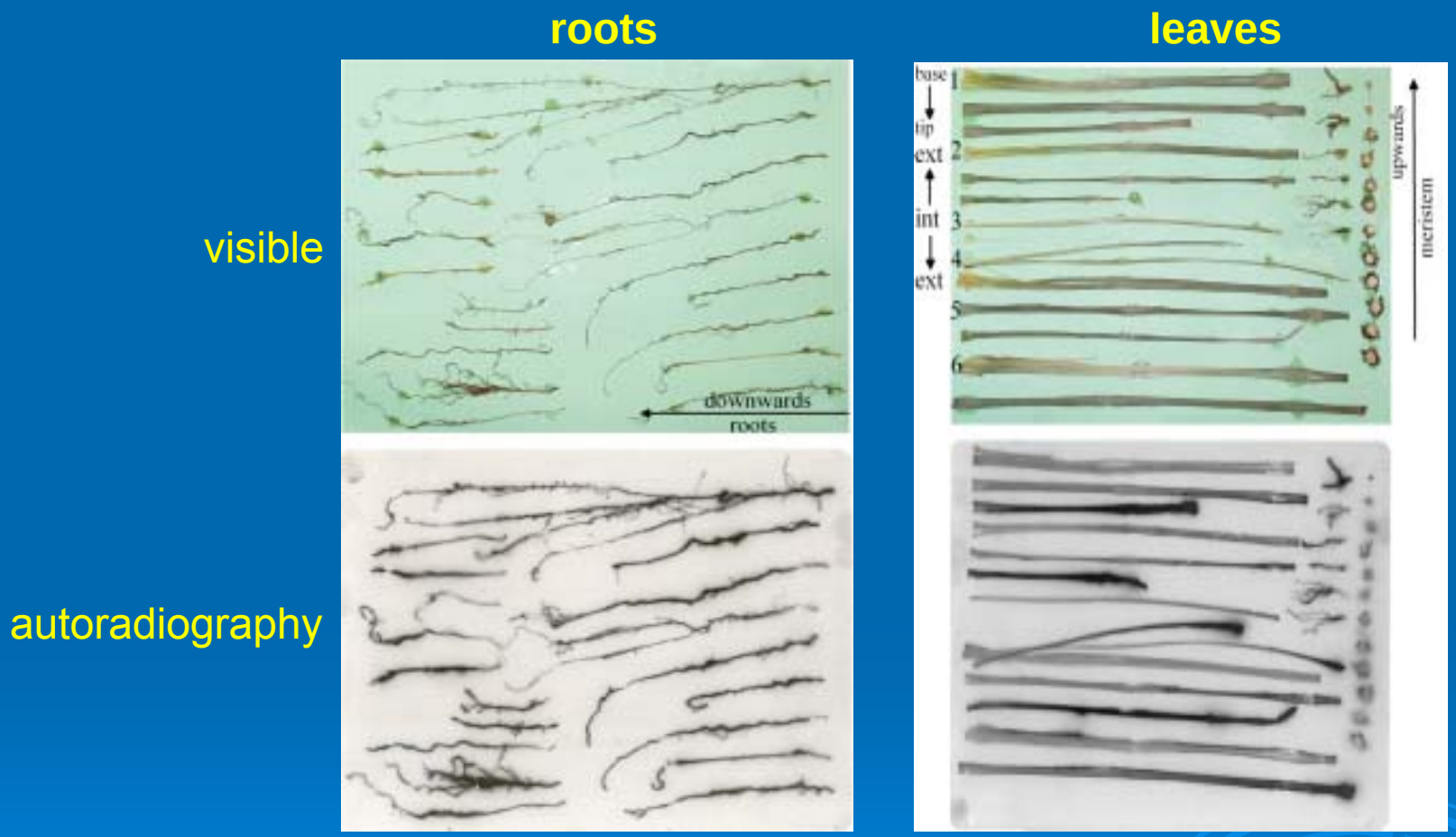
Vetiver taxonomy	
Family	*Poaceae*
Subfamily	*Andropogoneae*
Genera	*Chrysopogon*
Species	*zizanioides*
Related Genera	
Sorghum	

…which was studied in hydroponics together with radiolabelled or cold atrazine

Hydroponics a simplification strategy!

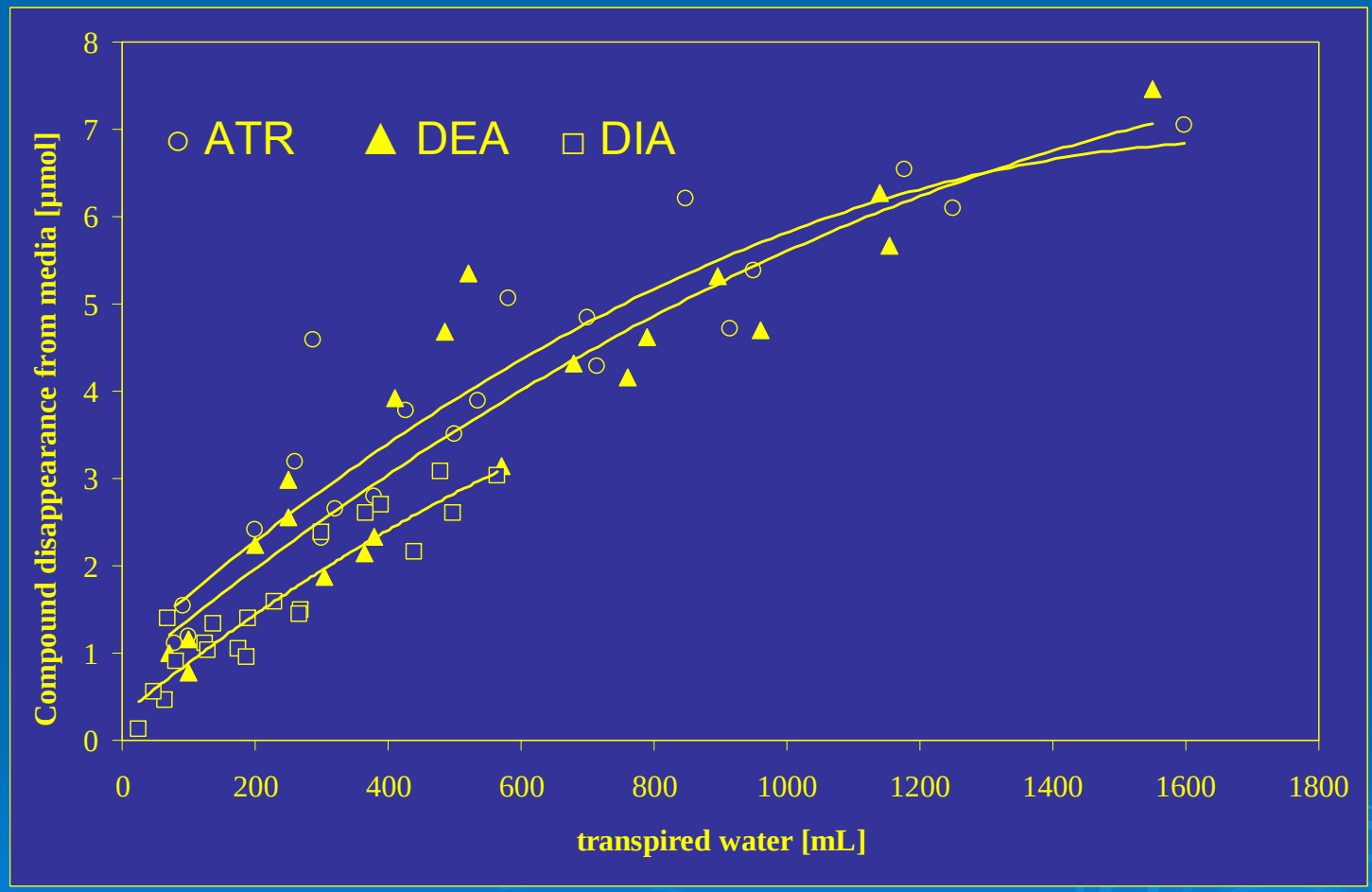
Localization of atrazine in plants	**Autoradiography**
Quantification of atrazine equivalents in plants	**Scintillation counter**
Study of plant metabolism	**TLC and radioactive plate analyzer**
Disappearance of atrazine from hydroponics	**HPLC**
In vitro enzymatic activity on atrazine	**HPLC**
Log $K_{oil/water}$ determination	**HPLC**

Is vetiver taking up atrazine?



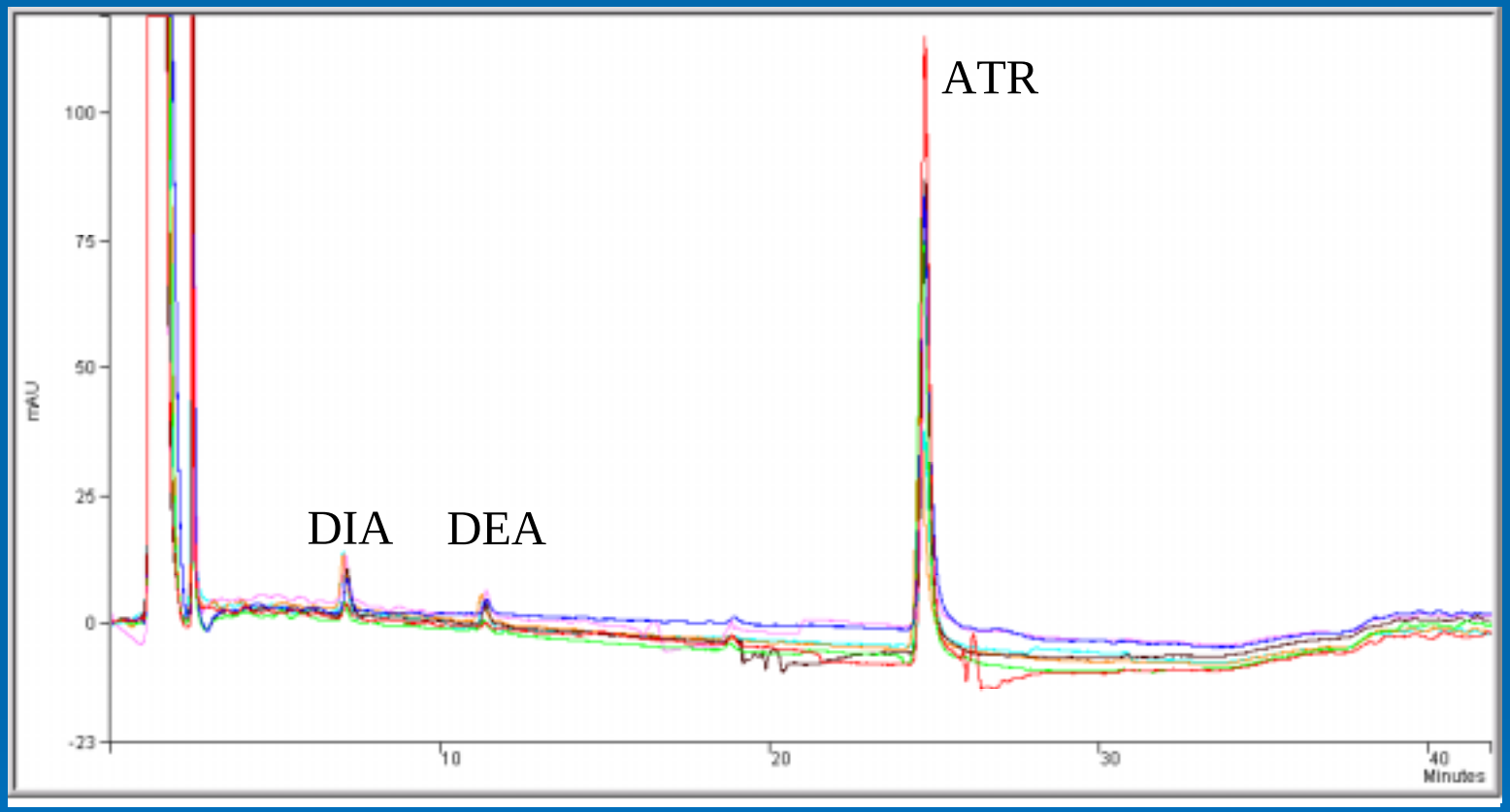
Atrazine is taken up by roots, translocated to leaves and accumulated at the tip of leaves

How is vetiver taking up atrazine and dealkylates?



Atrazine, DEA and DIA are passively taken up by vetiver

Beside uptake of dealkylates capacities, is vetiver itself a dealkylates producer in the medium?



Chromatogram of hydroponic solution at days 2, 5, 7, 9, 13, 16 and 19 days

DEA and DIA net concentration in the medium is believed to be driven by in and out passive diffusion

Is there any chloroplastic resistance in vetiver?

Typical Hill reaction in presence of vetiver thylacoids and atrazine

Percentage of reduced DCPIP (%)							
Maximum reduced DCPIP	Effect of atrazine (concentration in µM)				Controls		
- ATR	0.05	0.5	5	50	- thylacoids	+ boiled thylacoids	+ dark incubation
60	60	55	5	1	0	0	1

Vetiver chloroplasts are sensitive to atrazine!

Is vetiver resistance due to plant metabolism? (A) An *in vitro* approach

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
874 ± 68	863 ± 42	901 ± 54	839 ± 52	0.36 ± 0.06	0.42 ± 0.02	n/d	n/d

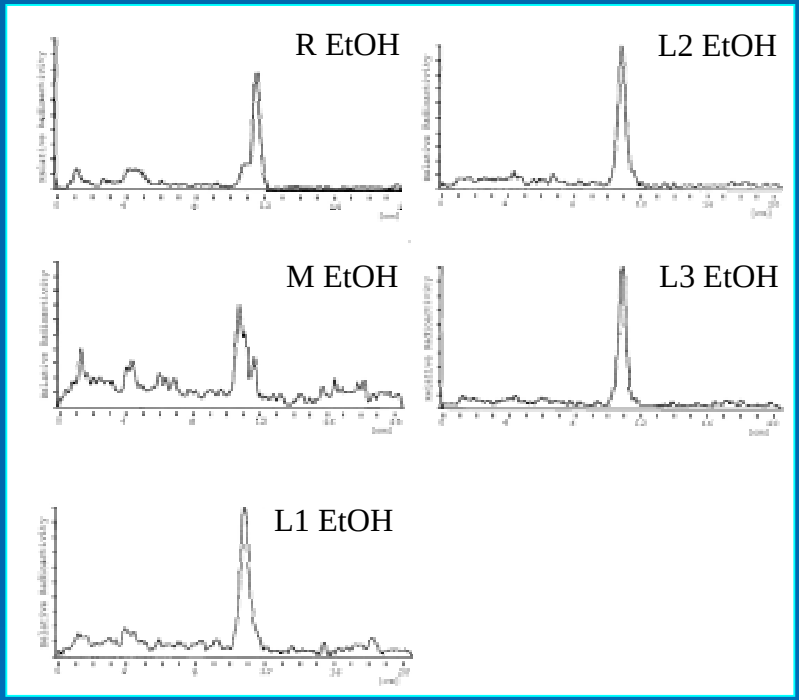
[1] Values refer to the mean of triplicates determinations of 1 experiment

[2] Values refer to the mean of triplicates of 3 independant experiments

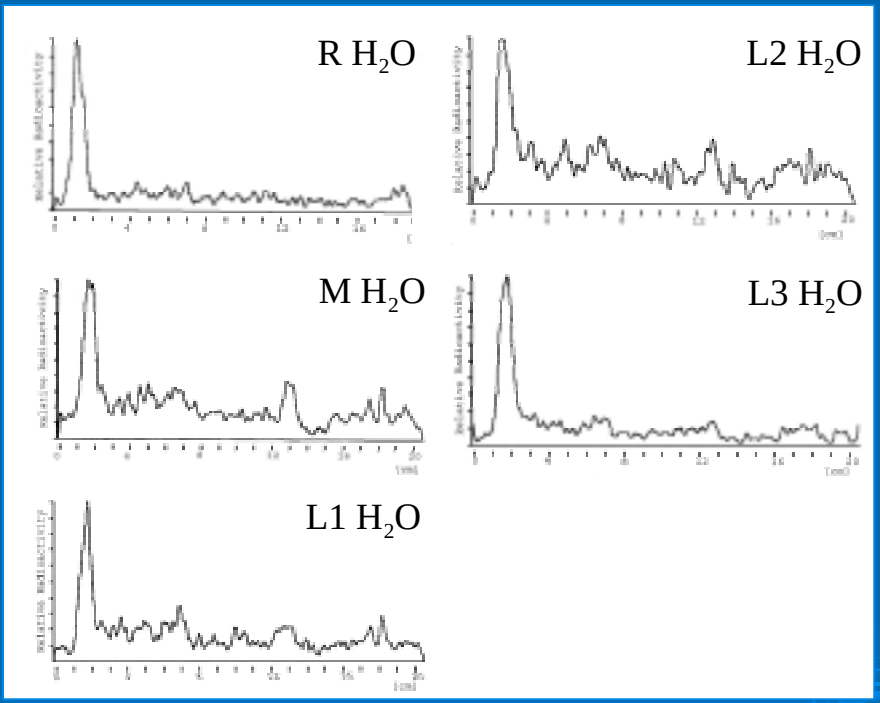
Vetiver GSTs can conjugate atrazine with glutathione

Is vetiver resistance due to plant metabolism? (B) Study of fate of atrazine in entire plant

Ethanolic extracts



Aqueous extracts

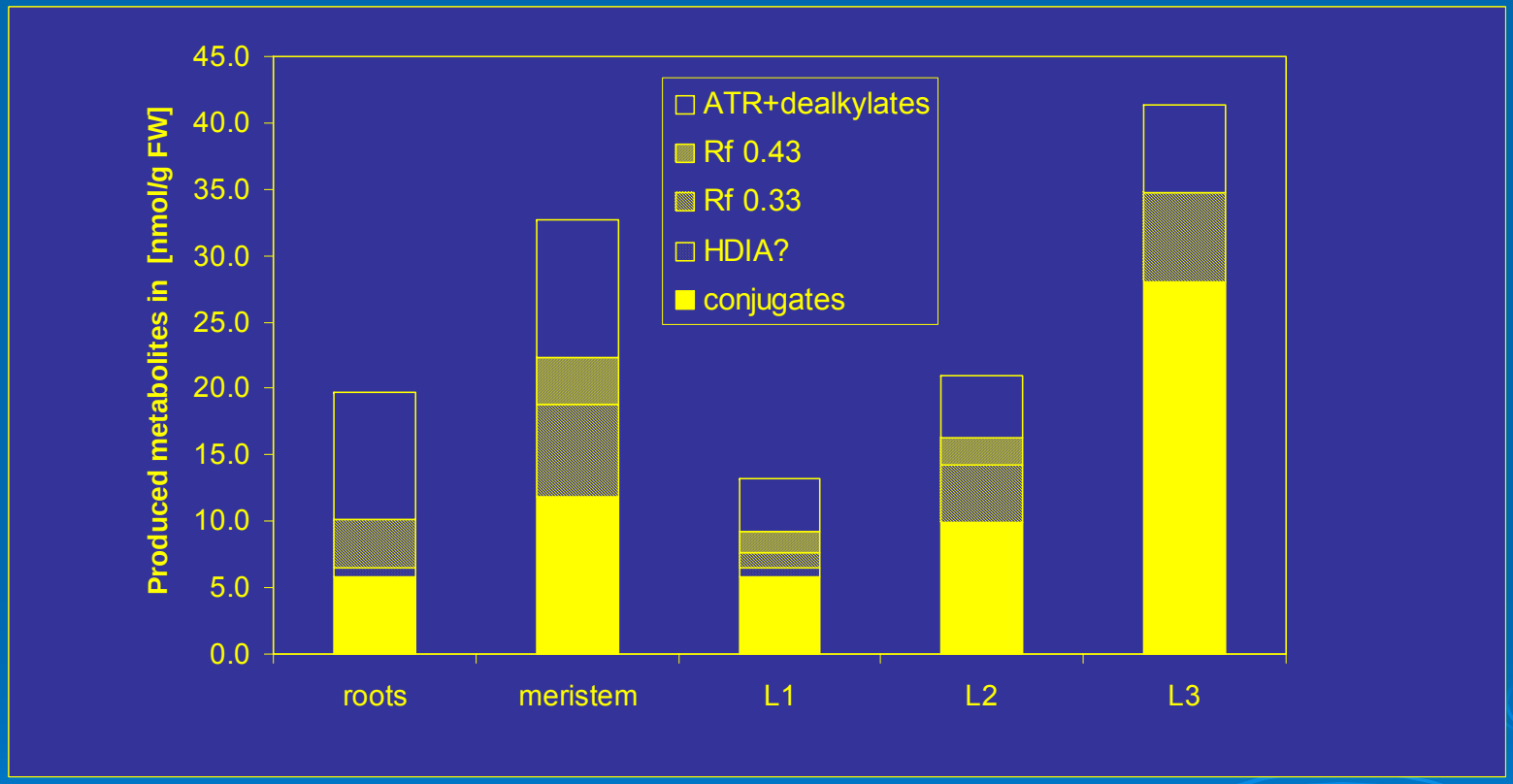


Scan of vetiver extracts loaded on TLC

R roots M meristem **L1** basal leaves **L2** median leaves **L3** distal leaves

All plant parts are able to produce a polar compound product presumably being conjugates of atrazine

Is vetiver resistance due to plant metabolism?
(C) Study of fate of atrazine in entire plant



Leaves produced the most important part of conjugates

Are roots comparable when grown in hydroponics or in soil?

Hydrophobic content from vetiver roots grown in hydroponics or in soil for 1 year
Results are expressed as a percentage of fresh biomass

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

Concentration factor of atrazine equivalents in vetiver roots grown in soil

	Concentration factor
< 1mm	0.8
> 1mm	4.0

Partition of atrazine between water and vetiver oil

Log K water/oil (ATR) ≥ 2.4

Surconcentration of atrazine is best explained by high hydrophobic content in vetiver roots grown in soil

What is the fate of atrazine in vetiver plants?

1. Vetiver uptake of atrazine is passive and highest removal of the compound is achieved with highest transpiration rate
2. Vetiver is a dealkylates producer, but also contribute to their removal from the medium according to passive diffusion law
3. Vetiver resistance to atrazine is best explained by conjugation
4. Root sequestration of atrazine could also explain atrazine resistance of vetiver grown in soil (hydroponics limitation)

Old vetiver hedges for new job?

Introduction

Vetiver hedges are good putative candidates for atrazine run-off control from agricultural fields

Laboratory results

What is the fate of atrazine in vetiver plants?

Perspectives

Vetiver as a tool for water protection?

From plant physiology to phytoremediation!

Atrazine resistance study leads to…

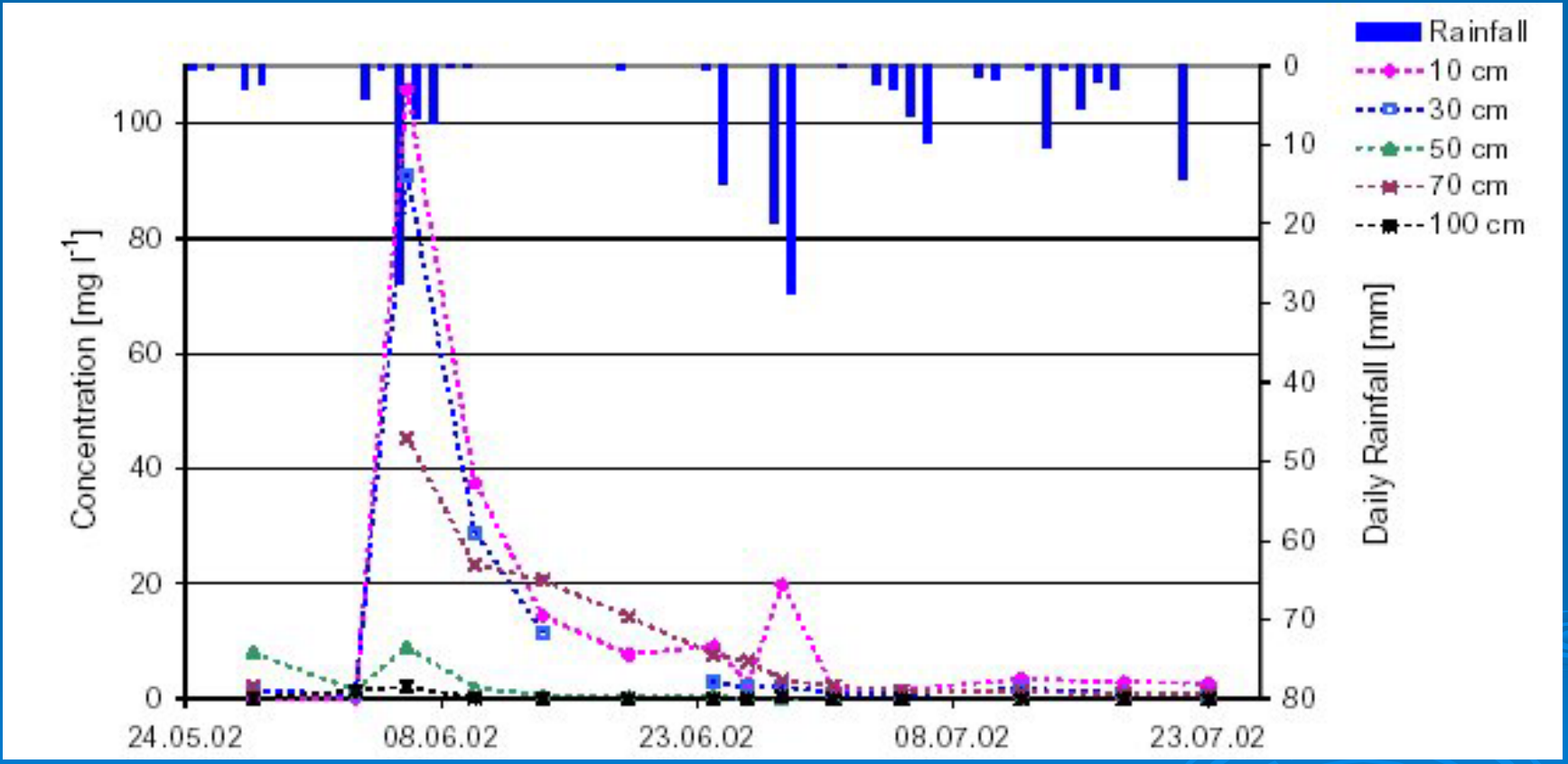
- Chloroplastic resistance? NO
- Enzymatic metabolization? YES
- Root sequestration? YES

… risk assessment and phytoremediation!

1. Metabolic resistance due to conjugaison means detoxification
2. Sequestration in roots means incompatiblity with oil production
3. Resistance to atrazine means possible establisment of vetiver in atrazine contaminated areas
4. Removal of 9 µmol/L or 2 mg/L atrazine correspond to:

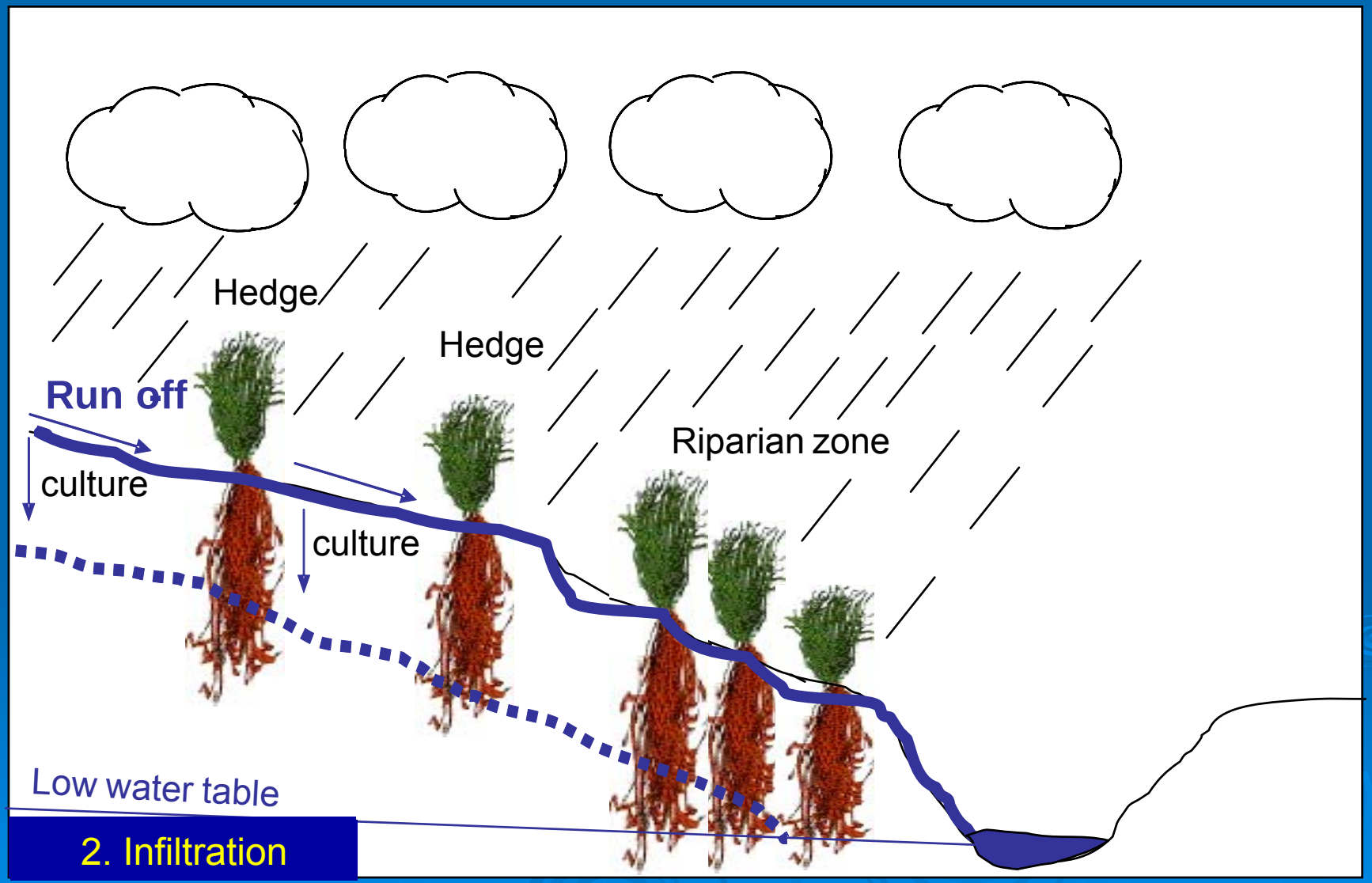
- 1 [kg] leaves
- 75% humidity
- 10h day 14h night
- ~ 500 [mL/24h]
- 15 hours uptake

The highest concentration of atrazine following treatment is found with first rain event…

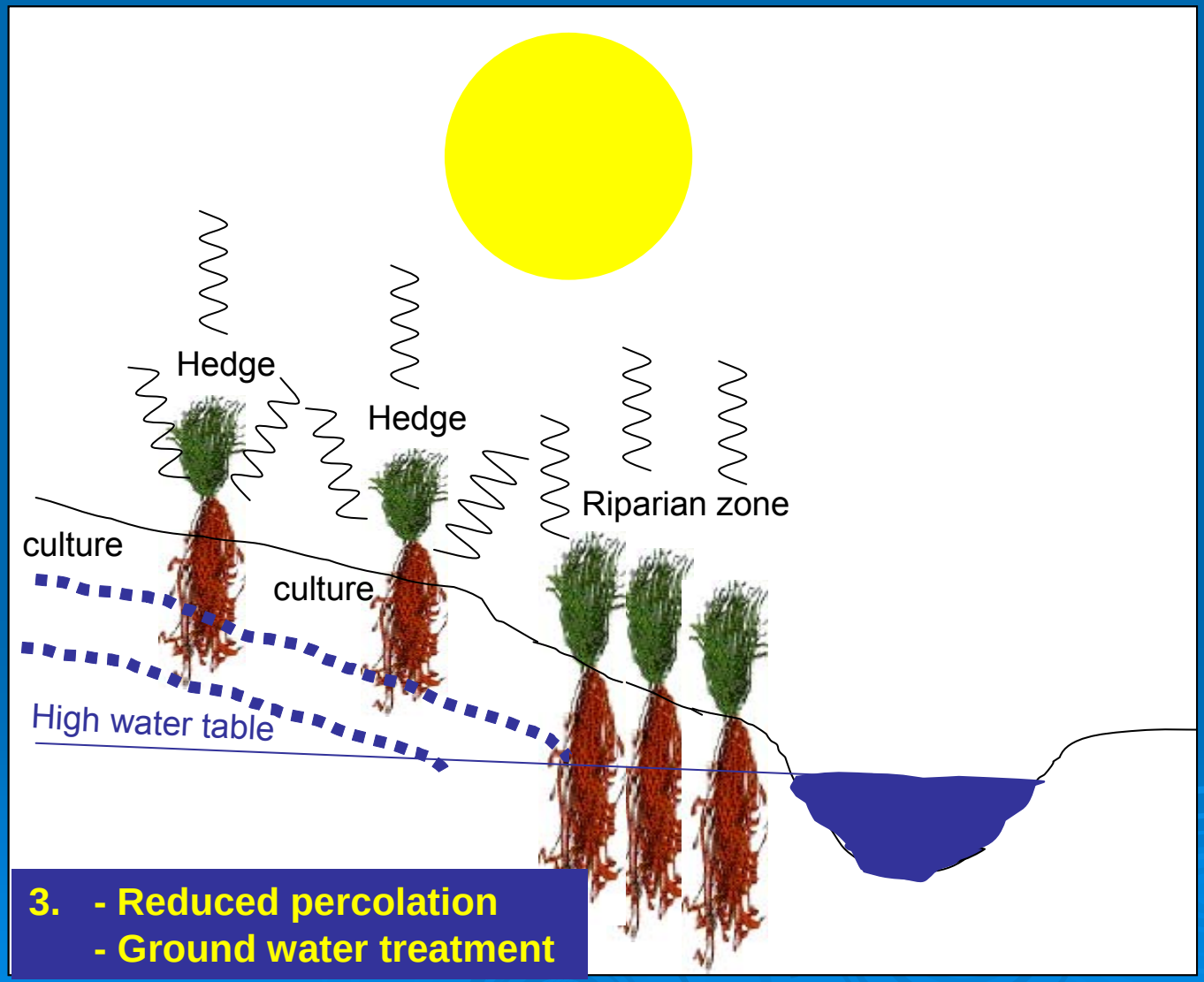


From Meiwirth, 2003

… corresponding to nil transpiration of vetiver plants!



Transpiration could have the main role especially in riparian zone?



3. - Reduced percolation
- Ground water treatment

Vetiver plant: an international network for soil erosion control…

World Bank program

1950 Fiji

1960 India

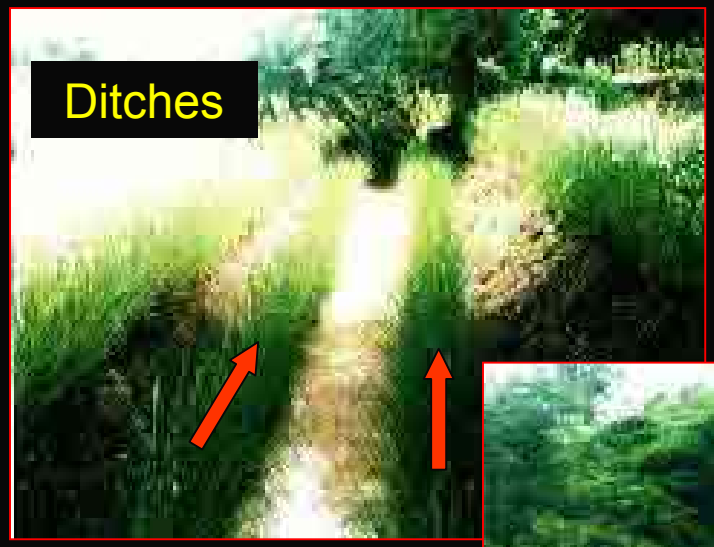
1989 Vetiver Network

2002 25 countries!

Introduction | Results | Perspectives

From http://www.vetiver.org/

…resulting in strategical ecological network for the control of non-point source pollution of atrazine?!

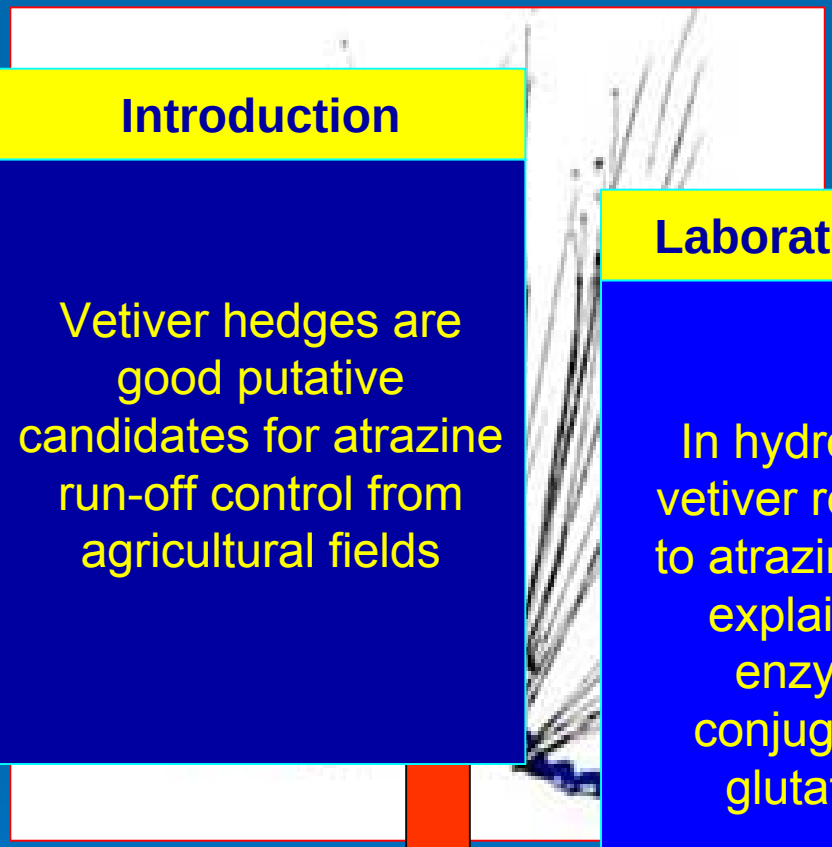


Vetiver as buffer zones for water protection in tropical, sub-tropical and Mediterranean countries

- Non invasive plant
- Large ecological tolerance
- Large pollution tolerance
- High root biomass
- Useful aerial biomass
- Easy obtainable plants due to the vetiver network
- Different ecotypes for for oil production and soil erosion control

From http://www.vetiver.org

Old vetiver hedges for new job?



Introduction

Vetiver hedges are good putative candidates for atrazine run-off control from agricultural fields

Laboratory results

In hydroponics, vetiver resistance to atrazine is best explained by enzymatic conjugation to glutathione

Perspectives

Vetiver as a tool for water protection?

1. What is plant uptake and detoxification versus oil seq
2. What is plant uptake versus micro-organisms activity?

Acknowledgments

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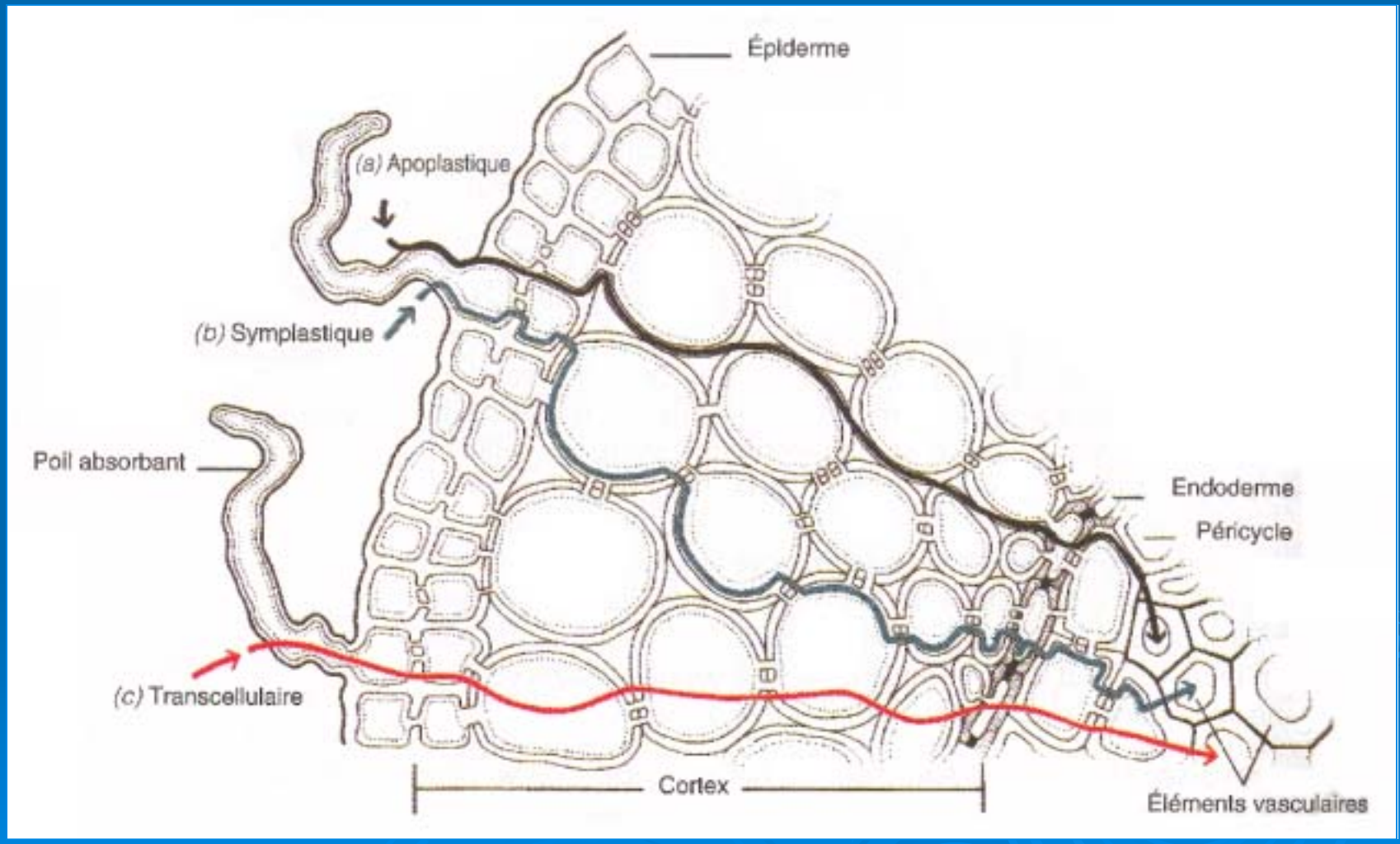
  LGCB (Laboratoire de Génie Chimique et Biologique)

  EPFL, Switzerland

What remains to be tested

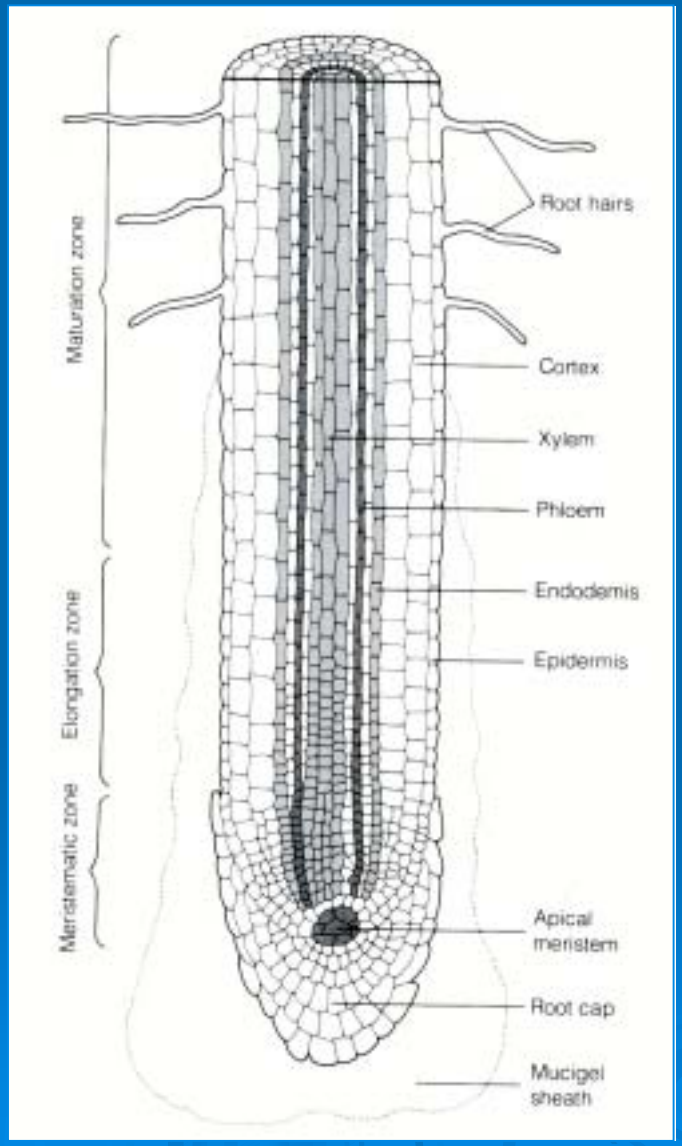
1. Log Kow $_{water/oil}$ of DEA, DIA
2. Conjugation of DIA, DEA, DDA *in vitro* tests
3. Root concentration of ^{14}C-DEA, and ^{14}C-DIA
4. Induced GSTs
5. Enhanced metabolization thanks to safeners
6. Key enzymes for dealkylation (P450?)
7. Atrazine remobilization from oil
8. Field case studies (hedges dimensions)
9. Triazines (simazine, propazine)
10. Chloroacetanilides (alachlor, metolachlor, propachlor)

Water path in roots



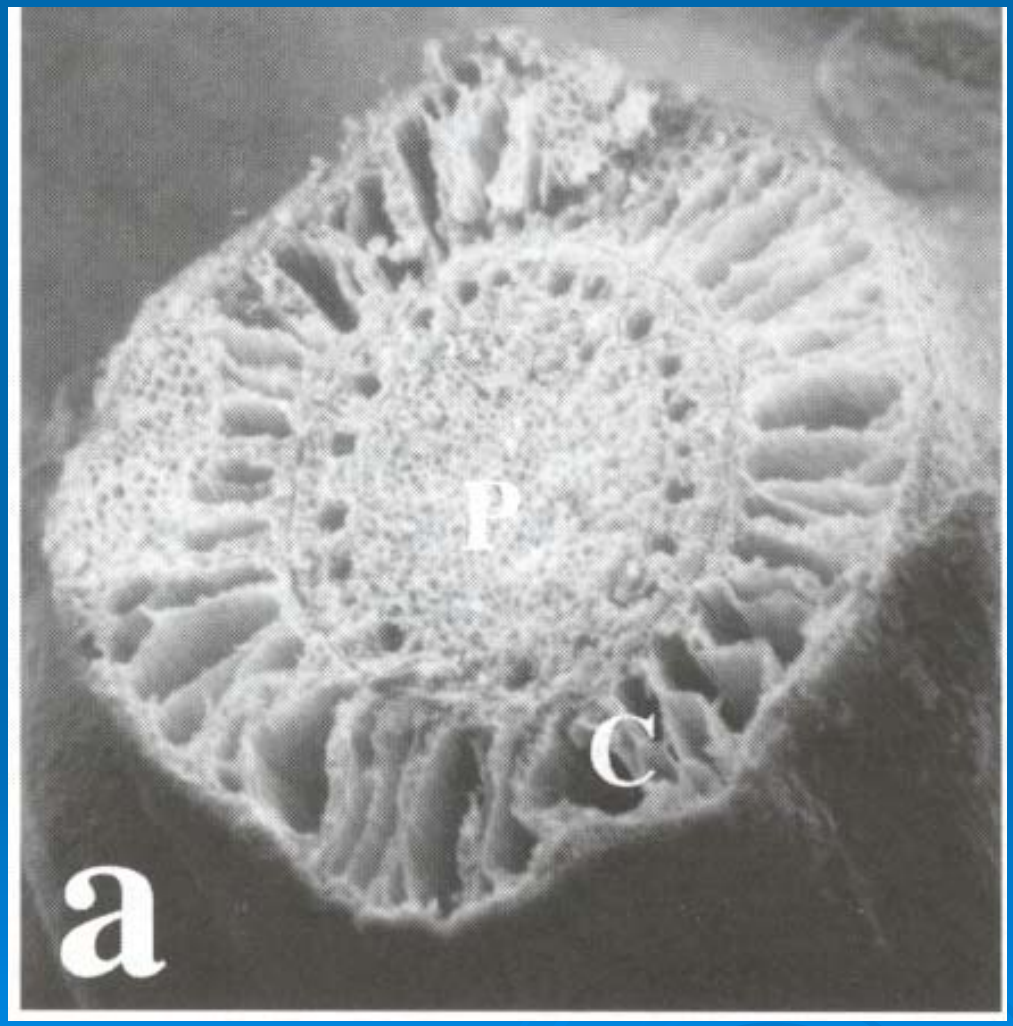
From Raven et al. 1999

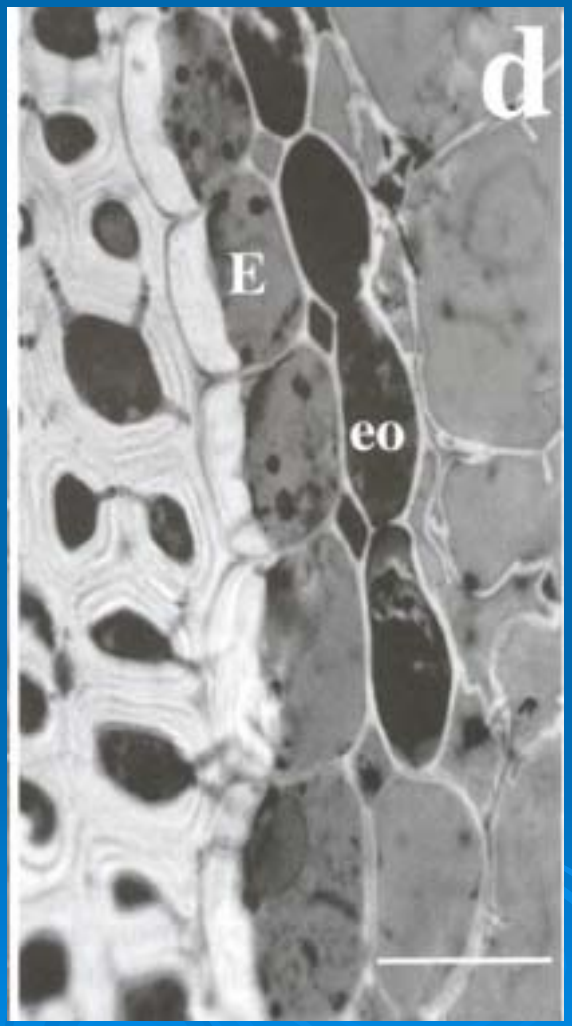
Root tip and water absorption



From Taiz and Zeiger, 1991

Vetiver oil localization





From Maffei M, 2002

In vitro test for $K_{oil/water}$

Test solution

- 5% oil v/v
- 28 [mg/L]
- agitation 350 rpm
- 1H30 incubation
- injection on HPLC

Control

Without atrazine (backround)

$$\frac{C_{équilibrium(ATR)}}{C_{initial(ATR)}} = \frac{V_{aqtot}}{V_{aqtot} + K_{water/oil(ATR)} * V_{oil}}$$

Formulas

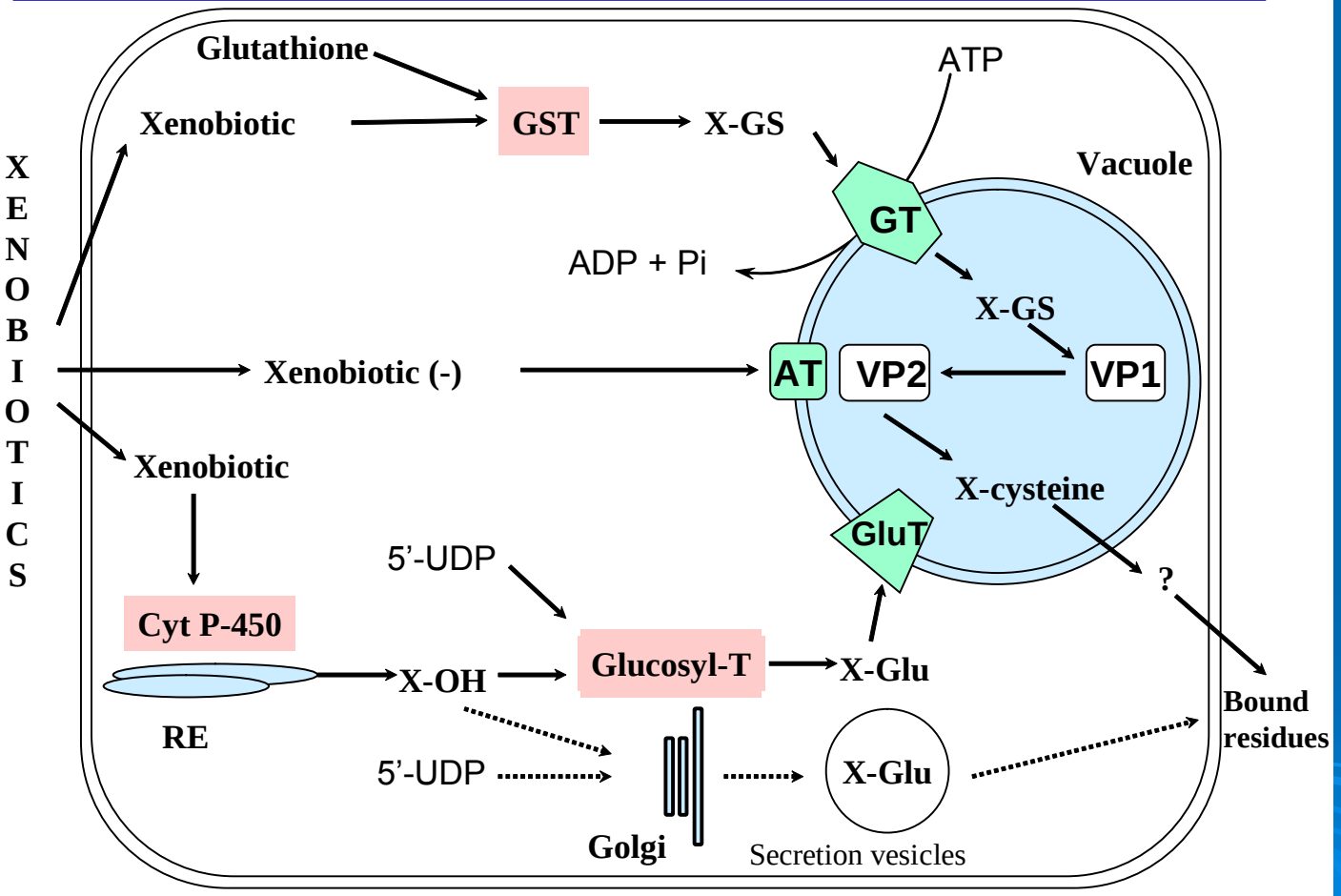
$$\text{Specific activity } [\text{pmol.sec}^{-1}.\text{mg}^{-1}] = \frac{\Delta A}{\Delta t} * \frac{V_t}{\varepsilon * d * m * ne^- * V_s}$$

$$\frac{C_{équilibrium(ATR)}}{C_{initial(ATR)}} = \frac{V_{aqtot}}{V_{aqtot} + K_{water/oil(ATR)} * V_{oil}}$$

Vegetative multiplication

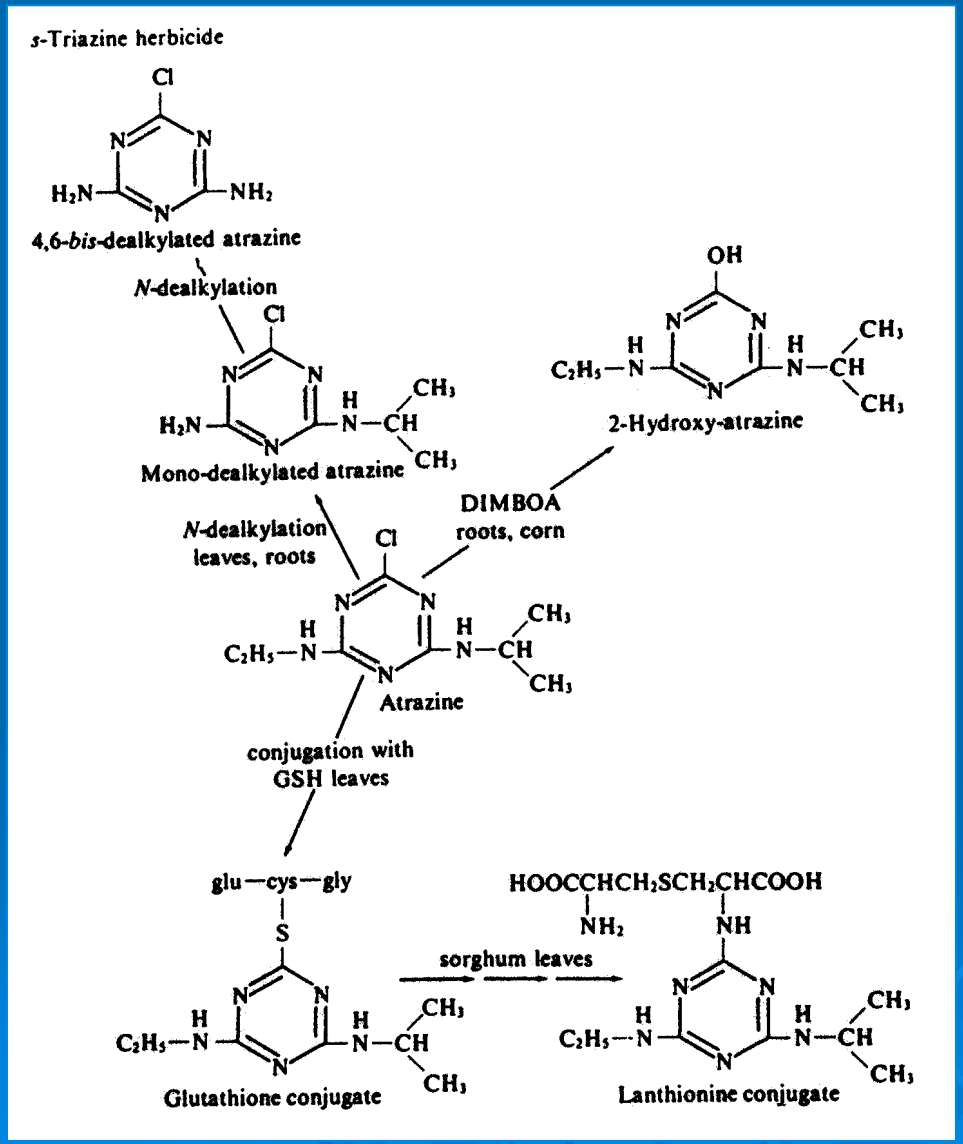
Classical plant detoxification pathways

Detoxification of xenobiotics in plant cells



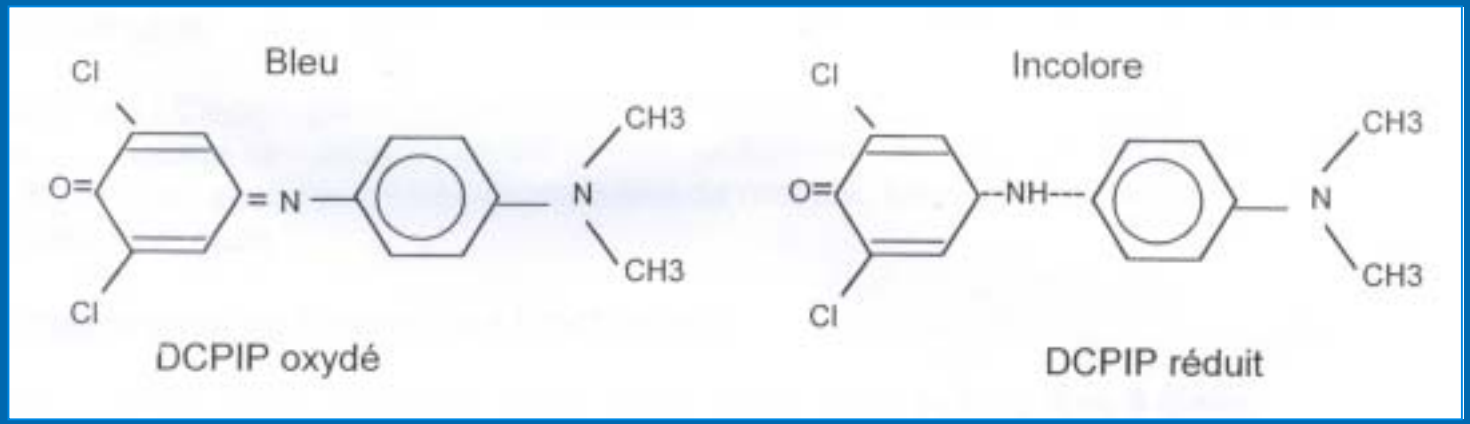
Modified from Coleman *et al.*, 1997

General scheme of atrazine detoxification



From Hatzios KK., and Penner D., 1982

DCPIP structure



Protocol of thylacoids extraction

Test solution

- HEPES Buffer 3 mL
- Thylacoids 0.5 mL
- Atrazine 1 mM x mL
- DCPIP 0.6 mM 0.2 mL
- H_2O 0.1 mL

Controls

- pea = sensitive plant to ATR
- dark incubation
- boiled thylacoids
- diuron (photosystem inhibitor)

In vitro GSTs tests

CDNB test (spectrophotometer)

λ = 340 nm

540 µL phosphate buffer 0.1 M pH 6.4

20 µL CDNB 30 mM

10 µL GSH 60 mM

30 µL extract 10 [mg/mL]

Atrazine test (HPLC)

λ = 220 and 265 nm

87.5 µL phosphate buffer 0.1M pH 6.8

17.5 µL ATR 10 mM

35 µL GSH 10 mM

210 µL extract 10 [mg/mL]

Controls

- Spontaneous conjugation (-extract)
- Other action than GSTs (- GSH)
- Coelution (extract alone)

Some products exhibited similar Rf corresponding to benzoxazinones...

- Rf **0.88** DIMBOA[1]
- Rf **0.81** DIBOA
- Rf **0.17** monoGlc DIMBOA, DIBOA
- Rf **0** diGlc DIMBOA

[1] Raveton M, 1996

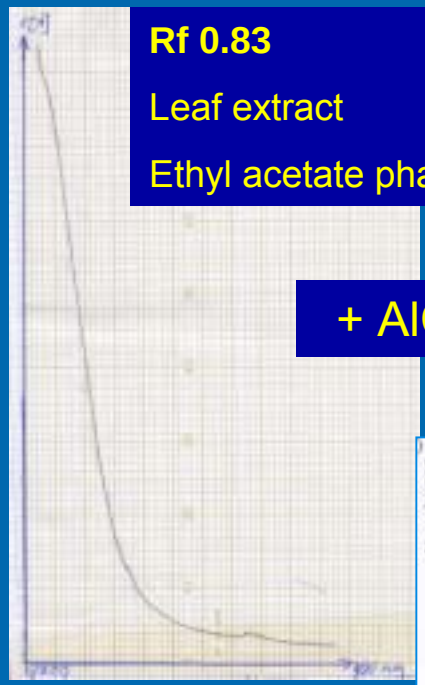
…but UV spectra products were not corresponding to benzoxazinones

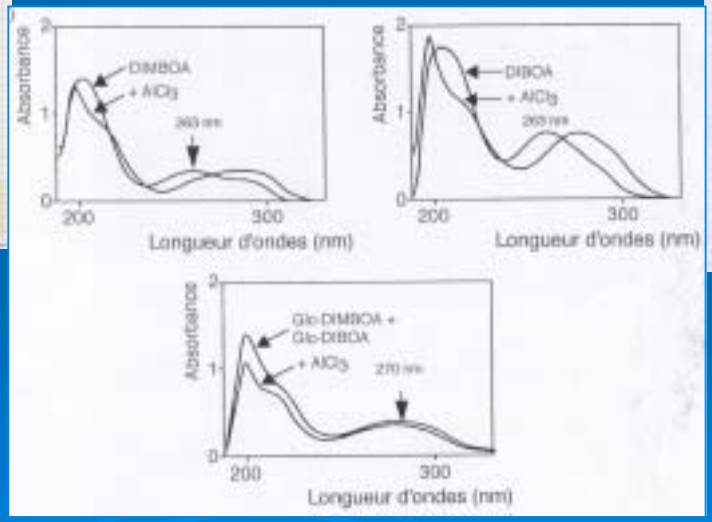
Rf 0.83

Leaf extract

Ethyl acetate phase

+ $AlCl_3$



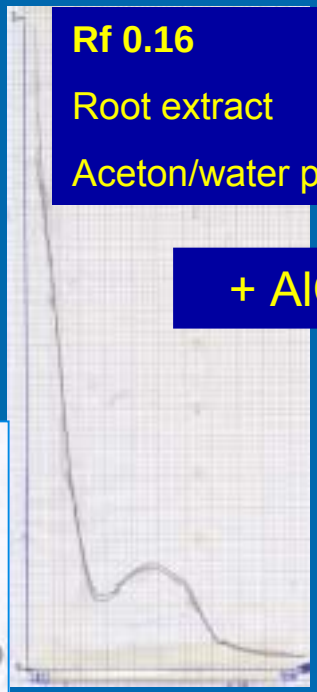


Rf 0.16

Root extract

Aceton/water phase

+ $AlCl_3$



From Raveton M, 1996

Vetiver extracts do not hydroxylate atrazine

Percentage of radioactivity extracted by diethyl ether (= intact atrazine)

Leaves		Roots		Control 1 Spontaneous hydroxylation	Control 2 Positive Control	Control 3 Extraction rate
Ethyl acetate extract	Aqueous acetonic extract	Ethyl acetate extract	Aqueous acetonic extract	Atrazine in buffer	Test with DIMBOA	Atrazine extracted from water
92.4 %	91.8 %	92.5 %	90.6 %	93.8 %	40 %	92.2 %