ORAL PRESENTATION

for the Degree of

Docteur es Sciences

by

Sylvie Marcacci

Lausanne, March 2003
A phytoremediation approach to remove pesticides (atrazine and lindane) from contaminated environment
Thesis as a part of ISCB projects

- Disease resistance in wheat
- In situ degradation and monitoring of pesticides
  - Bioremediation with selected bacteria (BR 3)
  - Phytoremediation (BR 1)
  - Development of Biosensors to monitor pesticides (BR 2)
- Transsectoral topics
- Pest control in pulses
- Improvement of soil quality

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

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Lausanne, the 20th February 2004
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...

Introduction

Results

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

- Photosynthesis inhibition in algae¹
- Crop rotation problems²
- Feminization of male frogs³

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

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Hypothalamus

Decreased luteinizing hormone

- Delayed pregnancy
- Pregnancy loss
- Anovulation
- Increased risk of mammary gland tumor

Atrazine
Simazine
Propazine
DEA
DIA
DDA

Modified from the U.S. EPA report, 2002
Atrazine is found above admissible concentrations in drink water …

<table>
<thead>
<tr>
<th>Countries</th>
<th>MCL per pesticide [ppb]</th>
<th>MCL per total pesticides [ppb]</th>
<th>Occurrence of atrazine in water</th>
</tr>
</thead>
<tbody>
<tr>
<td>Switzerland(^1)</td>
<td>0.1</td>
<td>0.5</td>
<td>All lakes</td>
</tr>
<tr>
<td>Europe(^2)</td>
<td>0.1</td>
<td>0.5</td>
<td>France: 43% of population drinks contaminated water</td>
</tr>
<tr>
<td>USA(^3)</td>
<td>3</td>
<td>?</td>
<td>24 contaminated = 10.5 mio persons concerned!</td>
</tr>
<tr>
<td>WHO(^2)</td>
<td>2</td>
<td>?</td>
<td></td>
</tr>
</tbody>
</table>

\(^1\)Gerecke et al, 2002  
\(^2\)http://assoc.wanadoo.fr/erb/colqP3.htm  
\(^3\)US EPA, 2002

No conventional water treatment eliminates atrazine!
...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?

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

![Diagram showing Vetiver root system with labeled parts: Tiller, Meristem, slip]
...which was studied in hydroponics together with radiolabelled or cold atrazine

<table>
<thead>
<tr>
<th>Localization of atrazine in plants</th>
<th>Autoradiography</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quantification of atrazine equivalents in plants</td>
<td>Scintillation counter</td>
</tr>
<tr>
<td>Study of plant metabolism</td>
<td>TLC and radioactive plate analyzer</td>
</tr>
<tr>
<td>Disappearance of atrazine from hydroponics</td>
<td>HPLC</td>
</tr>
<tr>
<td><em>In vitro</em> enzymatic activity on atrazine</td>
<td>HPLC</td>
</tr>
<tr>
<td>Log $K_{oil/water}$ determination</td>
<td>HPLC</td>
</tr>
</tbody>
</table>

Hydroponics — a simplification strategy!
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?

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

<table>
<thead>
<tr>
<th>Maximum reduced DCPIP</th>
<th>Effect of atrazine (concentration in µM)</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>- ATR</td>
<td>0.05 0.5 5 50</td>
<td>- thylacoids + boiled thylacoids + dark incubation</td>
</tr>
<tr>
<td>60</td>
<td>60 55 5 1</td>
<td>0 0 1</td>
</tr>
</tbody>
</table>

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**

<table>
<thead>
<tr>
<th></th>
<th>CDNB¹</th>
<th>Atrazine²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Specific activity (± SD) [pkats mg⁻¹ protein]</td>
<td></td>
</tr>
<tr>
<td>Old roots</td>
<td></td>
<td>Old leaves</td>
</tr>
<tr>
<td>Old leaves</td>
<td>874 ± 68</td>
<td>863 ± 42</td>
</tr>
<tr>
<td>Young leaves</td>
<td></td>
<td>Old leaves</td>
</tr>
</tbody>
</table>

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

Results

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.

<table>
<thead>
<tr>
<th></th>
<th>Hydroponics</th>
<th>Soil</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>White roots</td>
<td>Brown roots</td>
</tr>
<tr>
<td>&lt; 1mm</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>&gt; 1mm</td>
<td>0.4</td>
<td>0.4</td>
</tr>
</tbody>
</table>

Concentration factor of atrazine equivalents in vetiver roots grown in soil

<table>
<thead>
<tr>
<th></th>
<th>Concentration factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 1mm</td>
<td>0.8</td>
</tr>
<tr>
<td>&gt; 1mm</td>
<td>4.0</td>
</tr>
</tbody>
</table>

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!

Results

1. Metabolic resistance due to conjugaison means detoxification
2. Sequestration in roots means incompatibility with oil production
3. Resistance to atrazine means possible establishment 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

Perspectives

Atrazine resistance study leads to…

• Chloroplastic resistance? NO
• Enzymatic metabolism? YES
• Root sequestration? YES

… risk assessment and phytoremediation!
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!

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 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 sequestration?
2. What is plant uptake versus micro-organisms activity?
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  LGCB (Laboratoire de Génie Chimique et Biologique)
  EPFL, Switzerland
What remains to be tested

1. Log $K_{\text{ow}}$ of DEA, DIA
2. Conjugation of DIA, DEA, DDA \textit{in vitro} tests
3. Root concentration of $^{14}$C-DEA, and $^{14}$C-DIA
4. Induced GSTs
5. Enhanced metabolism 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 $K_{o/w}$**

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

\[
\frac{C_{\text{équilibre}(ATR)}}{C_{\text{initial}(ATR)}} = \frac{V_{\text{aqtot}}}{V_{\text{aqtot}} + K_{\text{water/oil}(ATR)} \ast V_{\text{oil}}}
\]

**Control**
Without atrazine (background)
Specific activity \([\text{pmol. sec}^{-1}. \text{mg}^{-1}]\) = \(\frac{\Delta A}{\Delta t} \times \frac{V_t}{\Delta \varepsilon \times d \times m \times n e^{-} \times V_s}\)

\[
\frac{C_{\text{equilibrium}(ATR)}}{C_{\text{initial}(ATR)}} = \frac{V_{\text{aqtot}}}{V_{\text{aqtot}} + K_{\text{water/oil}(ATR)} \times V_{\text{oil}}}
\]
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
- H₂O 0.1 mL

**Controls**
- pea = sensitive plant to ATR
- dark incubation
- boiled thylacoids
- diuron (photosystem inhibitor)
**In vitro GSTs tests**

**CDNB test (spectrophotometer)**
- $\lambda = 340$ nm
- 540 $\mu$L phosphate buffer 0.1 M pH 6.4
- 20 $\mu$L CDNB 30 mM
- 10 $\mu$L GSH 60 mM
- 30 $\mu$L extract 10 [mg/mL]

**Atrazine test (HPLC)**
- $\lambda = 220$ and 265 nm
- 87.5 $\mu$L phosphate buffer 0.1M pH 6.8
- 17.5 $\mu$L ATR 10 mM
- 35 $\mu$L GSH 10 mM
- 210 $\mu$L 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
- 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

From Raveton M, 1996
Vetiver extracts do not hydroxylate atrazine

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

<table>
<thead>
<tr>
<th></th>
<th>Leaves</th>
<th>Roots</th>
<th>Control 1 Spontaneous Hydroxylation</th>
<th>Control 2 Positive Control</th>
<th>Control 3 Extraction Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethyl acetate extract</td>
<td>92.4 %</td>
<td>92.5 %</td>
<td>90.6 %</td>
<td>93.8 %</td>
<td>92.2 %</td>
</tr>
<tr>
<td>Aqueous acetonic extract</td>
<td>91.8 %</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>