The Possible Role of Hydroxylation in the Detoxification of Atrazine in Mature Vetiver (*Chrysopogon zizanioides* Nash) Grown in Hydroponics

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The resistance mechanism of vetiver (*Chrysopogon zizanioides*) to atrazine was investigated to evaluate its potential for phytoremediation of environment contaminated with the herbicide. Plants known to metabolise atrazine rely on hydroxylation mediated by benzoxazinones, conjugation catalyzed by glutathione-*S*-transferases and dealkylation probably mediated by cytochromes P450. All three possibilities were explored in mature vetiver grown in hydroponics during this research project. Here we report on the chemical role of benzoxazinones in the transformation of atrazine.

Fresh vetiver roots and leaves were cut to extract and study their content in benzoxazinones known to hydroxylate atrazine, such as 2,4-dihydroxy-2*H*-1,4-benzoxazin-3(4*H*)-one (DIBOA), 2,4-dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one (DIMBOA) and their mono- and di-glucosylated forms. Identification of benzoxazinones was performed by thin layer chromatography (TLC) and comparison of retention factors (Rf) and UV spectra with standards: although some products exhibited the same Rf as standards, UV spectra were different. Furthermore, *in vitro* hydroxylation of atrazine could not be detected in the presence of vetiver extracts. Finally, vetiver organs exposed to [^{14}C]-atrazine did not produce any significant amount of hydroxylated products, such as hydroxyatrazine (HATR), hydroxydeethylatrazine (HDEA), and hydroxy-deisopropylatrazine (HDIA). Altogether, these metabolic features suggest that hydroxylation was not a major metabolic pathway of atrazine in vetiver.

Key words: Vetiver, Benzoxazinones, Atrazine, Hydroxylation

Introduction

For phytoremediation purpose, plants must be resistant to the target compound to be removed. Preliminary experiments revealed that vetiver is resistant to 20 ppm atrazine for at least 6 weeks, even with a maximum bioavailability created by the use of a hydroponic system (Marcacci, 2004). Atrazine resistance could be due to chloroplastic resistance, dilution of the herbicide into plant biomass, sequestration of atrazine before it reaches its target site in leaves, and plant metabolism. It has been shown that vetiver thylacoids are sensitive to atrazine, excluding therefore chloroplastic resistance (Marcacci, 2004).

Plants known to metabolise atrazine rely on (i) hydroxylation mediated by benzoxazinones, (ii) conjugation catalyzed by glutathione-*S*-transferases (GST) and (iii) dealkylation probably mediated by cytochromes P450. Tolerance of atrazine can be due to the high intensity of one metabolic pathway, *e.g.* conjugation in sorghum (Lamoureux *et al.*, 1970), or by the involvement of several metabolic pathways, like in maize (Shimabukuro *et al.*, 1970). In contrast, the lack of metabolism explains the sensitivity of species like wheat and soybean, whereas dealkylation alone confers intermediate tolerance of atrazine in pea.

Therefore, all three metabolic pathways have been explored in vetiver to understand its resistance to atrazine and to evaluate its potential for phytoremediation (Marcacci, 2004). The aim of the present work was to detect the putative presence of benzoxazinones in vetiver grown in hydroponics, and to establish their possible role in the detoxification of atrazine.

Natural benzoxazinones play a major role in the defense of cereals against insects (Niemeyer, 1988;

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Niemeyer *et al.*, 1989), fungi and bacteria (Niemeyer, 1988), in the chelation of Fe^{3+} (Pethö, 1992a, b), in allelopathic effects (Barnes and Putnam, 1987; Nair *et al.*, 1990) and in the detoxification of herbicides (Hamilton, 1964; Niemeyer, 1988; Raveton *et al.*, 1997a). The family of benzoxazinones is divided in several classes, namely the cyclic hydroxamic acids, lactams, methyl derivatives, and benzoxazolinones. Benzoxazinones are predominantly present in the Poaceae family including the genera *Aegilops*, *Arundo*, *Chusquea*, *Coix*, *Elymus*, *Secale* (rye), *Tripsacum*, *Triticale*, *Triticum* (wheat), and *Zea mays* (maize). In contrast, they are not found in *Avena* (oat), *Hordeum* (barley), or *Oriza* (rice) (Niemeyer, 1988).

Vetiver (*Vetiveria* or *Chrysopogon zizanioides*) belongs to the Poaceae family, subfamily Panicoideae, tribe *Andropogonae* and subtribe *Sorghina*, and the genus includes ten species (Bertea and Camusso, 2002). It is closely related to the genus *Sorghum.* Interestingly, *Sorghum bicolor* (sorghum) is subject to contradictory information: two authors have reported the absence of benzoxazinones in sorghum (Hamilton, 1964; Shimabukuro, 1967), whereas a review mentions that other authors have detected benzoxazinones in this plant (Niemeyer, 1988).

It was first established that benzoxazinones hydroxylate simazine (Castelfranco *et al.*, 1961; Castelfranco and Brown, 1962; Hamilton, 1964). Later, it was shown (Raveton *et al.*, 1997a) that hydroxamic acid belongs to class of benzoxazinones, like 2,4-dihydroxy-2*H*-1,4-benzoxazin-3(4*H*)-one (DIBOA), 2,4-dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one (DIMBOA) and their glucosylated forms which hydroxylate atrazine (Fig. 1). Chemical transformation of atrazine into hydroxyatrazine (HATR) has been well studied in whole maize plant (Castelfranco *et al.*, 1961; Raveton, 1996). The replacement of the chlorine atom by a hydroxy group results in non-phytotoxic metabolites, explaining the tolerance of atrazine in plants producing benzoxazinones. Under *in vitro* experimental conditions, a mixture of 10 μM benzoxazinones extracted from maize plantlets is able to transform 90% of atrazine (6 μM) into hydroxyatrazine within 24 h (Raveton *et al.*, 1997a). These results obtained *in vitro* have been correlated with the massive presence of hydroxyatrazine metabolites *in vivo* in maize plantlets, such as HATR, hydroxy-diethylatrazine (HDEA), and hydroxy-diisopropylatrazine (HDIA) (Raveton *et al.*, 1997b).

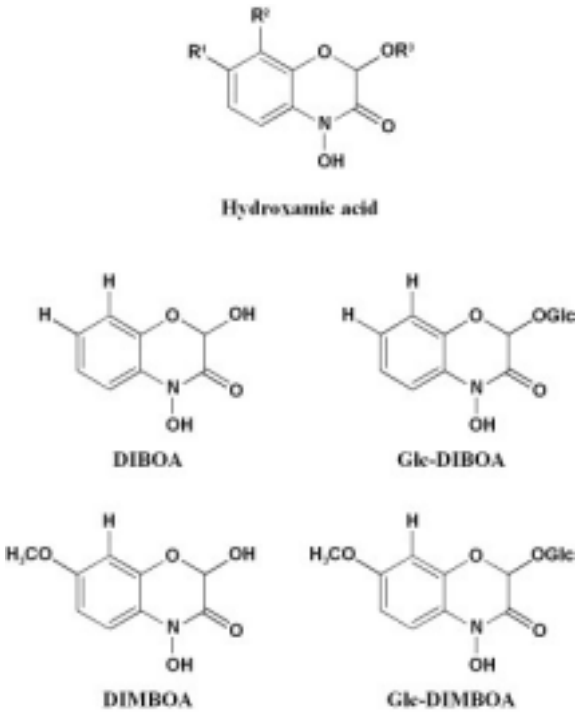

Fig. 1. Hydroxamic acid as the basic structure of benzoxazinones (Glc = glucose).

Within cereals, DIMBOA is the main hydroxamic acid occurring in wheat and maize, whereas DIBOA is the major form in rye (Niemeyer, 1988). Both are present at relatively high contents, up to 1 mg g^{-1} fresh weight (Sicker *et al.*, 2000). 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 released. Interestingly, benzoxazinones are not only present in plants, but also in exudates from maize (Friebe *et al.*, 1998; Pethö, 1992a), wheat and rye (Argandona and Corcuera, 1985). In maize, the enzymatic release and exudation of DIMBOA after wounding is complete within half an hour (Pethö, 1992b). This suggests that amongst grasses containing benzoxazinones, these exudated compounds may play a role in atrazine detoxification directly in soil, and that phytoremediation probably does not rely only on the classical uptake of contaminants followed by phytotransformation.

In this context, it was of great interest to evaluate the potential involvement of hydroxylation re-

actions in the resistance of vetiver to atrazine. The possible presence of DIMBOA and DIBOA and of their glucosylated forms was investigated by extraction of vetiver roots and shoots followed by partial purification, study of UV spectra of obtained products, chelation reaction, as well as by *in vitro* and *in vivo* hydroxylation of atrazine.

Materials and Methods

Plant material and growth conditions

Vetiver slips were received in February 2002 from Mr M. Pease, co-ordinator of the European and Mediterranean Vetiver Network, Lagos, Portugal. The plants (variety "Vallonia") originated from an importation made by himself from Zimbabwe in 1998 and further cultivated in Portugal. The varieties "Sunshine" in the USA, "Vallonia" in South Africa, "Monto" in Australia and "Guiyang" in China share almost the same genotype and are used generally throughout the world for soil erosion control (Bertea and Camusso, 2002; National Research Council, 1993). Thus, the present work has a relevance to similar plants grown in many countries.

Slips were developed for 8 months in hydroponics with nutrient solution Luwasa®. The plants were maintained in glasshouse supplemented with sodium lamps (Philips Son-T Agro 400, 400 W m^{-2}, 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.

Detection of hydroxamic acids from vetiver organs

The identification of benzoxazinones and the hydroxylation of atrazine *in vitro* were done according to Raveton (1996) and Cherifi *et al.* (2001). Putative benzoxazinones from 55 g fresh mass of leaf or root were extracted separately with acetone followed by acetone/water (80:20, v/v). Extracts were partially purified with petroleum ether (B.P. 40–60 °C). The obtained extracts were partitioned with ethyl acetate, to separate apolar benzoxazinones (DIMBOA and DIBOA) from polar benzoxazinones (mono- and di-glucosylated DIMBOA and DIBOA). The phase was evaporated and the extracts redissolved in pure ethanol. The volume of the aqueous-acetonic phase was reduced with butanol, and finally dissolved in 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). DIMBOA was purified and used as a standard, as described by Raveton *et al.* (1997a).

The retention factors (Rf) were compared to those obtained by Raveton (1996). Extracts were then loaded (1.2 ml) on TLC plates and separated products were scrapped, eluted with a minimal volume of ethanol, and centrifuged at 13,000 × *g* for 1 min in Eppendorf tubes to remove any trace of silica. The UV spectra of separated products were measured between 400 and 200 nm and compared to existing published benzoxazinone spectra (Cherifi *et al.*, 2001; Raveton, 1996).

The quantification of benzoxazinones in partially purified extracts was also done spectrophotometrically (using ε_{262} = 8,000 M^{-1} cm^{-1}) to prepare *in vitro* test of atrazine hydroxylation.

In vitro atrazine hydroxylation

In order to maximize the detection of benzoxazinones and of their hydroxylating activities, conditions were the same as those defined by Raveton (1996) and Raveton *et al.* (1997a), except that extracts were concentrated to 20 mM of putative benzoxazinones with the help of a Speed vacuum device (Savant). The extracts were then 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, 1,440 MBq ml^{-1}. The final ethanol content was 20%. Test solutions were incubated at room temperature under agitation for 24 h, then frozen at – 20 °C before analysis. After thawing by hand, samples were extracted twice with 2 ml diethyl ether to collect unreacted [^{14}C]-atrazine. The diethyl ether phase was further evaporated until dryness, then dissolved in 5 ml ethanol. The aqueous phase was also evaporated to remove totally diethyl ether, then 4 ml of water were added to the test solution. Finally, 10 ml of scintillation liquid were added to ethanolic and aqueous samples (Beckman, Ready Safe™ for organic and aqueous samples, respectively). The radioactivity of samples was measured with a scintillation counter (Wallac, Winspectral). Controls were as follows: (1) atrazine

with phosphate buffer (spontaneous hydroxylation), (2) standard DIMBOA under the same conditions as tested extracts (positive control), and (3) atrazine extraction rate by diethyl ether.

Vetiver leaf metabolism of [^{14}C]-atrazine

To study vetiver metabolism in organs (leaf and root), experimental conditions developed by Cherifi *et al.* (2001), Raveton (1996), and Raveton *et al.* (1997b) were used, including organ extraction, and TLC development solvent system, ethyl acetate/formic acid/acetic acid/H_2O (40:2:2:4, v/v/v/v), previously optimized for the separation of hydroxyatrazine derivatives; dealkylates are found around the migration front together with atrazine, whereas atrazine conjugated to glutathione remains at the origin.

Approximately 1 g of fresh leaves was cut from vetiver plant rooted in soil and transferred in hydroponics 4 weeks before uptake experiment. "Young" leaves (4-week-old) were sampled, as well as "old" leaves grown in hydroponics for 7–12 months. 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 (Hoagland Basal Salt, Sigma) spiked with 56 μl of 5 mM [^{14}C]-atrazine, 1,440 MBq ml^{-1}, corresponding to 70 M atrazine final concentration. The lower half of the leaf segments was immersed, allowing the upper part to transpire. Parafilm was used to limit the loss of water from the system other than transpiration by leaves. The leaves were incubated for 72 h 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^{-1}) in water to prevent any rapid efflux of atrazine and metabolites. Organs were then crushed first in a minimal volume of ethanol, then in water, together with sand of Fontainebleau. Pellets were counted to quantify the extraction rate of radioactivity. Samples were bleached with sodium hypochlorite to avoid quenching when measuring with the scintillation counter (Wallac).

Aqueous samples were concentrated with a Speedvacuum system (Savant) and ethanolic extracts were concentrated with a rotavapor to reach at least 1,000 dpm/100 μl, the minimum radioactivity detectable with the 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 were then loaded on a TLC plate (60_{F254}) and developed with solvent system ethyl acetate/formic acid/acetic acid/H_2O (40:2:2:4, v/v/v/v) able to separate hydroxylated compounds of atrazine.

Vetiver root metabolism of [^{14}C]-atrazine

Plant roots were divided into (a) roots smaller than 1 mm ("young" roots); (b) roots larger than 1 mm diameter ("old" roots); (c) boiled roots smaller than 1 mm ("young boiled" roots); (d) boiled roots larger than 1 mm diameter ("old boiled" roots). This latter condition was found useful to study metabolism when enzymes were inactivated. 1 g of fresh roots was totally immersed in 10 ml Hoagland solution spiked with 140 μl of 5 mM [^{14}C]-atrazine, 1,440 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 h at room temperature under agitation.

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

Results

Detection of hydroxamic acids from vetiver organs

The results obtained with vetiver extracts are presented in Fig. 2. Leaf (tracks 2 and 4) and root (tracks 3 and 5) extracts loaded on a TLC plate contained several products which might correspond to benzoxazinones: the ethyl acetate extract

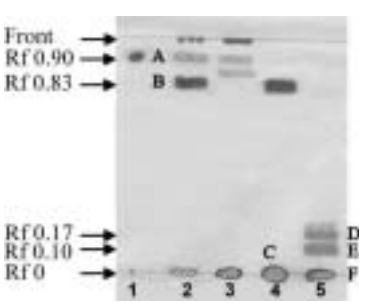

Fig. 2. TLC of vetiver leaf and root extracts for benzoxazinones detection. 1, Standard DIMBOA; 2–5, vetiver extracts; 2, ethyl acetate extract of leaves; 3, ethyl acetate extract of roots; 4, aqueous extract of leaves; 5, aqueous extract of root; A, B, C, D, E, F, putative benzoxazinones; A, DIMBOA; B, DIBOA; D, E, Glc-DIMBOA and Glc-DIBOA; C, F, diGlc-DIMBOA.

of leaves exhibited clear bands with Rf which might be DIMBOA (Rf= 0.90, product A) and DIBOA (Rf = 0.83, product B). The aqueous-acetonic extract of leaves also contained a product at Rf = 0.83, which might correspond to DIBOA as well. Nevertheless, as the solvent used for extraction was water, it seemed unlikely that this band could be DIBOA, mainly extracted by ethyl acetate. The aqueous extract of leaves exhibited a product remaining at the origin (Rf = 0, product C), which could be diglucosylated benzoxazinones.

The ethyl acetate extract of roots exhibited weak bands with Rf corresponding to DIMBOA (Rf = 0.88), and DIBOA (Rf = 0.81). The product remaining at the origin was unlikely diglucosylated benzoxazinones, as the solvent used was ethyl acetate. The aqueous-acetonic extract of roots was found to contain products at Rf = 0.17 (product D) and Rf = 0.10 (product E) which might be monoglucosylated benzoxazinones. A strong band remaining at the origin (product F) could also correspond to diglucosylated benzoxazinones.

The spectra of purified products (A, B, C, D, E and F in Fig. 2) obtained by scratching the TLC plate did not show the characteristic pattern of benzoxazinones UV spectrum, with a maximum absorbance at 200–215 nm and a peak at 265 nm [results not shown here, but in Marcacci (2004)]. However a significant peak at 281 nm was observed for root extracts only, but not for leaf extracts. Furthermore, no bathochromic effect was observed after the addition of $AlCl_3$, as described by Raveton (1996), indicating the lack of any chelation reaction of the products under investigation.

In vitro atrazine hydroxylation

The indirect detection of benzoxazinones was done by evaluating the ability of vetiver extracts to hydroxylate atrazine. Each extract was quantified for its benzoxazinone content by taking the absorbance value at 281 nm instead of 262–265 nm. Knowing that the extracts were not pure, it was assumed that the background of the extracts could shift the peak to 281 nm, or completely hide the peak at 262–265 nm. It was thus possible to estimate the hydroxylation potential of the extract towards atrazine (Table I).

Results obtained with atrazine in buffer were in the same range as radioactivity remaining in solution after extraction of intact atrazine by diethyl ether, showing that under the test conditions, spontaneous hydroxylation was negligible. The assay was considered as valid, since positive control (atrazine with pure DIMBOA) was highly hydroxylated (60%).

Vetiver leaf and root extracts were thus unable to hydroxylate atrazine. If hydroxylation would have occurred, high amount of radioactivity should have been detected in the aqueous test solution after diethyl ether extraction of atrazine. It was clearly not the case. Moreover, the percentage of radioactivity found in the aqueous phase of leaf and root extracts was in the same range as the percentage of radioactivity remaining after diethyl ether extraction.

Vetiver metabolism of [^{14}C]-atrazine

Around 80% of the radioactivity was recovered from vetiver organs incubated 4 d with [^{14}C]-atrazine and no chlorosis of leaves was observed. The radioactivity and concentration of atrazine equivalent in plant organs were different: 8% in young roots, 5.7% in young boiled roots, 18.7% in old roots, 31.4% in old boiled roots, 49.4% in young leaves and 62.0% in old leaves, explaining the difference of intensity in the different autoradiography extracts.

Autoradiography revealed that hydroxylation of atrazine was not a major metabolic pathway in vetiver (Fig. 3A), although traces of HATR and dealkylates DIA, DEA, DDA were found in all tested extracts. However, negligible amounts of

Table I. *In vitro* test of atrazine hydroxylation. Hydroxylated atrazine in the presence of vetiver extracts. Control 1, spontaneous hydroxylation of atrazine in buffer; control 2, atrazine with standard DIMBOA; control 3, recovered radioactivity after extraction of test solution with diethyl ether solvent to remove intact atrazine.

Hydroxylated atrazine (%)						
Leaves		Roots		Control 1	Control 2	Control 3
Ethyl acetate extract	Aqueous acetonic extract	Ethyl acetate extract	Aqueous acetonic extract	Atrazine in buffer pH 5.6	Atrazine and DIMBOA	Remaining radioactivity after diethyl ether extraction of ATR
7.6	8.2	7.5	9.4	6.2	60	7.8

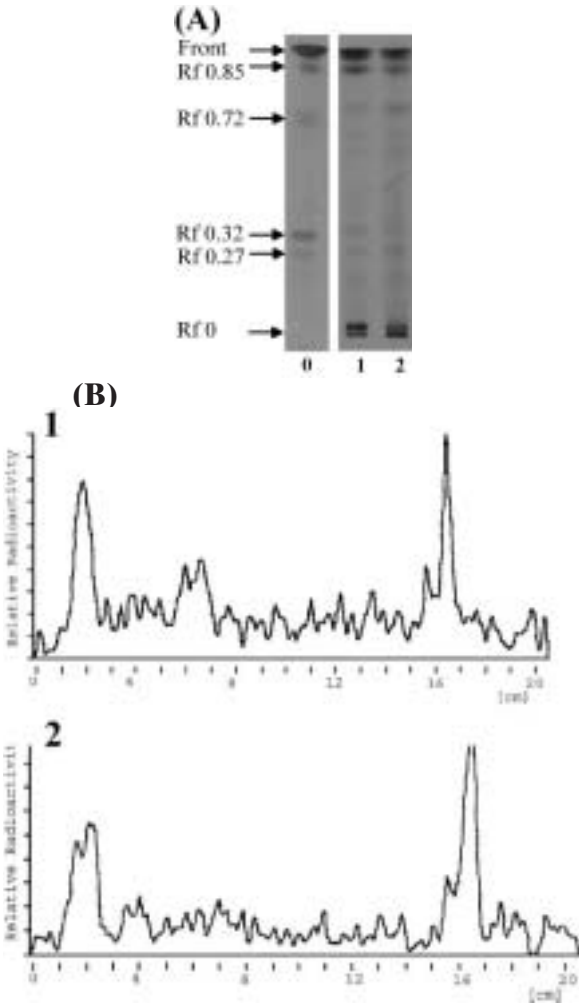

Fig. 3. Autoradiography of ethanolic extracts of vetiver leaves (A) and corresponding scans (B). 0, Treatment solution before experiment; 1, young leaves; 2, old leaves; ATR; Rf 0.85 = DDA; Rf 0.72 = ?; Rf 0.32 = HATR; Rf 0.27 = HDEA; Rf 0 = conjugates.

products with Rf 0.85 (DDA), 0.72 (non identified product), 0.32 (HATR) and 0.27 (HDEA), were also found in the atrazine treatment solution before the experiment, thus explaining traces of dealkylates and hydroxylates found in vetiver extracts; so when subtraction of background metabolites from treatment solution was done, these products represented less than 1% of metabolites. The autoradiography of TLC plates showed that root extracts did not exhibit any metabolization [data not shown here, but in Marcacci (2004)]. Vetiver root extracts showed very few radioactive products at the origin of migration, in contrast to young and old leaf extracts with products remaining at the origin of TLC plate, corresponding to conjugates.

The scan of TLC was used to estimate the percentage of the different metabolites in excised plant organs. "Old" leaf was found to conjugate atrazine in the same range as "young" leaf (46.3 and 58.5%, respectively, see Fig. 3B), whereas root was unable to conjugate atrazine [data not shown here, but in Marcacci (2004)].

Discussion

Although some products separated by TLC had retention factors similar to benzoxazinones, their UV spectra did not confirm the presence of benzoxazinones in vetiver. Moreover, the addition of $AlCl_3$ did not result in bathochromic shift, and no hydroxylating activity of vetiver extracts on atrazine was observed. Finally, no hydroxylates could be detected *in vivo* in vetiver roots and leaves.

The detection of benzoxazinones was however maximized, by using 55 g fresh biomass for benzoxazinones extraction, but their existence and activity could not be confirmed. As a comparison, only 10 g of fresh leaves or roots of maize give much more intense spots on TLC plates, as benzoxazinones are massively produced as secondary metabolites: leaves contain 241 μmol g^{-1} of DIMBOA and roots 128 μmol g^{-1} (Raveton, 1996). Moreover, none of the putative benzoxazinones exhibited a characteristic peak at 265 nm and UV spectra of products purified by TLC in this work did not fit to the known spectra of benzoxazinones (Raveton *et al.*, 1997a; Cherifi *et al.*, 2001).

In vitro, the standard DIMBOA was able to hydroxylate 60% of atrazine with 20% ethanol present in the assay, showing that the conditions used were appropriate to detect any hydroxylation of atrazine by vetiver extracts. Hydroxylation was in the expected range, as compared to the results of Raveton (1996) who showed that the presence of an organic solvent (ethanol or acetone) was responsible for a decreased hydroxylation in the reaction medium: 50% ethanol resulted in 30% hydroxylated atrazine.

By heating, DIMBOA and DIBOA are transformed into 1,3-benzoxazin-2-one (BOA) and 7-methoxy-1,3-benzoxazin-2-one (MBOA), respectively (Virtanen and Wahlroos, 1963). These derivatives are unable to hydroxylate atrazine (Raveton, 1996; Raveton *et al.*, 1997a). In contrast, the

glucosylated derivatives remain resistant to heat degradation, even for 15 min at 100 °C. Due to the 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 the high volatility of the solvent. The 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 would be rather explained by the absence or extremely low amount of benzoxazinones in vetiver.

In vivo, the absence of hydroxylated metabolites and conjugates in vetiver root extracts was not due to their disappearance in the surrounding medium. After 72 h, this latter did not contain a higher concentration of hydroxy derivatives than before the experiment. This is not surprising, since hydroxy derivatives of atrazine highly accumulate in maize seedlings, but are almost unable to diffuse into the fresh medium (Raveton *et al.*, 1997b). As the log K_{ow} of hydroxyatrazine is close to 1.5, it is not high enough to prevent permeation through plant cell membranes; therefore it has been proposed that ionization of the hydroxy group might induce a repulsion of the product by the electronegatively charged membranes (Raveton *et al.*, 1997b). Maize and sorghum absorb radio-labelled hydroxyatrazine, but it is not readily translocated from root to shoot as reported for atrazine, strengthening the hypothesis of segregation of hydroxylated derivatives in root cells (Shimabukuro, 1968).

The concentration of benzoxazinone in plants can change as a function of plant age, *i.e.* in maize seed, the concentration is nil, but it reaches a maximal value in young seedlings, before decreasing slowly within the first 4 weeks until matured plant (Cherifi *et al.*, 2001; Niemeyer, 1988). One could object that extraction, identification of benzoxazinones and *in vitro* hydroxylation of atrazine were done with 8-month-old vetiver, explaining these negative results. However, the *in vivo* metabolism of atrazine in entire vetiver was complete in 4-week-old tissues, and only traces of hydroxylated compounds were detected, indicating that benzoxazinones were not massively present at any stage of vetiver growth and development.

Leaf and root sampling was done on plants grown hydroponically for 8 months. It is possible that under these conditions, benzoxazinones are no longer found in the plant; the concentration of benzoxazinones in plants is highly dependent not only on plant age, but also on environmental conditions: increasing levels of hydroxamic acids can be caused by light and water deficiencies (Friebe *et al.*, 1998). However, a clear dependence of benzoxazinone accumulation on light is not always observed (Cherifi *et al.*, 2001).

We cannot exclude that vetiver contained other classes of benzoxazinones, different from the hydroxamic acid derivative class. It is not known however, if lactam, methyl derivatives and benzoxazolinone classes could have been extracted by the present protocol.

Interestingly, cultivated barley has been reported to lose benzoxazinones, whereas wild barley was found to contain these secondary metabolites (Sicker *et al.*, 2000). This loss of benzoxazinones might have occurred during agriculture breeding from wild barley. The same phenomenon could also occur in selecting and cultivating vetiver for soil erosion control.

In conclusion, hydroxylation mediated by benzoxazinones should therefore be low or nil in vetiver, at least in 8-month-old plant grown in hydroponics. Results presented here clearly indicate that benzoxazinones were not playing a major role, if any, in the detoxification of atrazine by vetiver, in contrast to the situation described for maize.

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