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Degradation of benzo[a]pyrene in an experimentally contaminated paddy soil by vetiver grass (*Vetiveria zizanioides*)

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Abstract

A pot experiment was conducted to study the effect of growing vetiver grass on the biodegradation of benzo[a]pyrene (B[a]P) under glasshouse conditions. Plant biomass, microbial biomass C and degradation of B[a]P were determined. B[a]P disappeared faster in the plant treatments than in unplanted controls. Disappearance of B[a]P was accompanied by an increase in soil microbial biomass C. Vetiver grass may promote the biodegradation of B[a]P under flooded conditions by plant roots by stimulating the microbial biomass. Microbial biomass was the main factor affecting dissipation of B[a]P under flooded conditions.

Introduction

Vetiver grass (*Vetiveria zizanioides*) is one of the most widely distributed grass species in south and southeast Asia where it has been grown for centuries for its aromatic oil, roof thatching and fodder for livestock. The plant has stiff erect stems and a fast growing extensive root system (upto 3 m deep after growth for 12 months). Currently, vetiver grass is being used worldwide as a low-cost, low technology and effective means of soil and water conservation and land stabilization (Dalton *et al.* 1996). In the last few decades it has been found that this plant can be used for cleaning up heavy metals from contaminated soils and wastewaters (Truong & Baker 1998; Xia & Wu 2002).

Polycyclic aromatic hydrocarbons (PAHs) are important pollutants. They occur as common constituents of petroleum, coal tar and shale oil, but are most frequently formed by incomplete combustion of fossil fuels (Pothuluri & Cerniglia 1994; Maila & Thomas 2002). PAHs are present in

urban and rural areas and are found in high concentrations on many contaminated sites, and because some of them have mutagenic and carcinogenic properties, there is considerable interest in the development of methods to remediate PAH-contaminated soils.

The prospect of using vegetation to enhance the degradation of organic contaminants in soil systems is an attractive cost-effective alternative to traditional engineering approaches (Yoshitomi & Shann 2001). Plants can enhance the remediation of soils containing organic pollutants by various processes (Shimp *et al.* 1993; Cunningham *et al.* 1996). The roots of alfalfa and other plant species have been shown to be effective in the removal of several aromatic compounds, including PAHs (Ferro *et al.* 1997; Schwab *et al.* 1998; Pradhan *et al.* 1998; Flocco *et al.* 2002). To study the interaction of PAHs with vetiver grass, a hydroponic culture system has been employed, and results indicate that the grass can promote B[a]P degradation by root absorption, accumulation and transfer (Li *et al.* unpublished).

Plants can promote degradation of PAHs by stimulating the microbial biomass (Binet *et al.* 2001). The microbial metabolism of PAHs containing up to three rings (naphthalene, phenanthrene, anthracene, and fluorene) has been studied extensively (Grifoll *et al.* 1995). However, until recently there has been a paucity of information on the bacterial degradation of the larger, more recalcitrant, high molecular weight PAHs containing four or more fused benzene rings (Juhasz & Naidu 2000).

Moisture is a critical parameter for degradation of PAHs (Loehr *et al.* 1992). In the past decade, many advances have been made in the understanding of the anaerobic degradation of monocyclic hydrocarbons, but research on multiple-ring compounds has been rare. Although important information concerning the biodegradation of PAHs has been accumulating, it is still too early to formulate a protocol for the rational design of a PAH bioremediation system (Shigeaki, 1997). At present, targets for clean up of PAH contamination are not well defined.

The objective of the present study was to investigate whether or not vetiver grass can promote the disappearance of B[a]P from soil. In addition, the possible role of the soil microbial biomass C in the remediation process was investigated.

Materials and methods

Contaminated soil

A paddy soil of pH (in H₂O) 6.96 and with 5.93% organic matter (OM) content from Changshu Agricultural Zoology Experimental Station, Academia Sinica, not previously exposed to B[a]P, was air dried and passed through a 2-mm steel sieve. Five gram of soil were soaked in acetone containing B[a]P (purity >97%, Sigma Chemical Co.). The acetone was evaporated and the treated soil was diluted by mixing with uncontaminated soil to obtain a final concentration of B[a]P of 100 mg kg⁻¹ soil. After adjusting the soil moisture content to 60% WHC or flooded conditions with water the soil was stored in a growth chamber for 12 days before it was used for the pot experiment.

Experimental design

Vetiver (*Vetiveria zizanioides*) seedlings were transplanted in pots containing 2.5 kg of the agricultural soil either amended with B[a]P or unamended. There were six treatments: (1) soil at 60% WHC and planted; (2) soil at 60% WHC with B[a]P and planted; (3) soil at 60% WHC with B[a]P and unplanted; (4)–(6) flooded soil with the same three combinations of planting and B[a]P. There were four replicates, giving a total of 24 pots in a fully randomized design in a greenhouse at the Institute of Soil Science, Chinese Academy of Sciences, Nanjing. Plants were grown under glasshouse conditions during April–June in 2002, and harvested 60 days after transplanting. Plants were harvested by separating into roots and shoots and the biomass of each part was determined after oven drying at < 70 °C. Soil microbial biomass C was determined on sub-samples of soil from the pots.

B[a]P extraction

The following procedure, adapted from Song (1995), was used to extract and analyze B[a]P in plant tissue and in soil after sampling at 60 days.

Plant extraction

A 0.5-g sub-sample of ground dry plant material was placed in 15 ml methanol and extracted for 30 min by ultrasonic agitation. The sample mixture was centrifuged at 2000 rpm for 10 min followed by collection of the supernatant in a conical flask. A further 15 ml methanol were added and the extraction process was repeated. The supernatants from both extractions were combined in a conical flask and filtered through anhydrous sodium sulfate. The filtrate was the plant (either shoot or root) B[a]P extract.

Soil extraction

In accordance with Binet *et al.* (2001), the root density in the planted pots was very high and all of the soil was therefore considered to be rhizosphere soil. The soil from planted (rhizosphere soil) and unplanted pots (non-rhizosphere soil) was carefully collected. Soil samples were air dried in the dark and passed through a 2-mm steel sieve. Five g soil were mixed with 20 ml dichloromethane

and extracted for 30 min by ultrasonic agitation. Samples were centrifuged at 2000 rpm for 5 min. The supernatant was the soil B[a]P extract.

Measurement

Method for B[a]P

The methanol was removed from 10 ml of soil or plant B[a]P extract in a rotary evaporator until dry. Cyclohexane (2 ml) was added and the mixture was vigorously shaken with a vortex mixer for 1 min. A 0.5-ml aliquot of the slurry was passed through a silica gel column. The column was washed with dichloromethane: hexane (1:1 v/v) and the first 1 ml of liquid was discarded. The following 2 ml of liquid was collected and dried with N₂ gas. Acetonitrile (2 ml) was added and shaken with a vortex mixer and then injected directly into the HPLC.

Chromatographic conditions

Solvents that constituted the mobile phase were A (cyclohexane) and B (water). The flow rate and the column temperature were set at 0.5 ml min⁻¹ and 30 °C, respectively. Other parameters adopted were as follows: injection volume, 40 µl; detection wavelength, 290 nm.

Soil microbial biomass C

Soil microbial biomass C was determined using the chloroform fumigation-extraction method as described by Vance *et al.* (1987).

Statistical analysis

The data were subjected to analysis of variance and means were compared using least significant difference at the 5% level.

Results

Plant biomass

Table 1 shows the mean biomass of vetiver grass in each treatment. There was a significant decrease in shoot and root biomass with addition of B[a]P ($p < 0.01$). However, no difference was found between the two soil moisture treatments in the B[a]P treatments. Shoot and root biomass of plants in the uncontaminated soil were greater

Table 1. Shoot and root dry matter yield (g pot⁻¹) of vetiver grass in soil at 60% WHC and in flooded soil with or without B[a]P.

Soil moisture regime	B[a]P	Shoots	Roots
60% WHC	Present	23.6 b	18.5 a
Flooded	Present	32.8 c	17.3 a
60% WHC	Absent	20.6 a	20.0 b
Flooded	Absent	40.0 d	24.9 c

Values are means of four replicates. Within each column, means followed by the same letter are significantly different by LSD at the 5% level.

than those in contaminated soil. The behavior of root and shoot biomass showed that the presence of contaminants in the soil impaired plant yield and the impairment was different between the two soil moisture levels. Shoot biomass in the flooded treatment was significantly higher than in aerated soil with or without contamination but no marked influence was detected on root biomass between the two soil moisture levels in contaminated soil.

Plant B[a]P concentration

Shoot and root concentrations of B[a]P are presented in Table 2. Compared with the controls, root and shoot B[a]P concentrations were greatly increased when the soil was contaminated with B[a]P. Moreover, no difference was found between flooded and aerated soil moisture levels in the controls. Shoot B[a]P concentrations under flooding conditions with added B[a]P were high compared with the lower soil moisture level with added B[a]P. However, root B[a]P concentrations showed a different trend from the shoot B[a]P results and this may be related to transport effects in the plants. B[a]P was detected in the shoots of plants grown in flooded and control treatments in

Table 2. B[a]P concentrations in vetiver grass in soil at 60% WHC and in flooded soil with or without B[a]P.

Soil moisture regime	B[a]P	Shoots	Roots	Root/Shoot
60% WHC	Present	0.053 a	2.60 a	49
Flooded	Present	0.106 b	1.81 b	17
60% WHC	Absent	0.053 c	ND	0
Flooded	Absent	0.018 c	ND	0

Values are means of four replicates. Within each column, means followed by the same letter are significantly different by LSD at the 5% level. ND, not detected (below detection limit).

uncontaminated and this may be related to the weak volatility of B[a]P and plant absorption of B[a]P that volatilized to the atmosphere and thus accumulated in the plant tissues.

Soil B[a]P concentrations

The extractable concentration of B[a]P in pre-treated soil was 96 mg kg^{-1} . Soil B[a]P concentrations after 60 days of cultivation are shown in Figure 1. The concentration of extractable B[a]P in pre-treated soil in unplanted pots was significantly higher than in the unplanted pots. There was no significant difference between aerated soil and flooded soil in the rate of disappearance of added B[a]P in planted pots, but the rate was lower in aerated soil (64.6 mg kg^{-1}) than in flooded soil (74.8 mg kg^{-1}) in unplanted pots. Thus, the decreasing trend with different water regimes may result from the effects of microorganisms in the soil.

Soil microbial biomass C

Microbial biomass C in the soil at harvest showed significant treatment effects as shown in Figure 2. In comparison to unplanted pots, microbial biomass carbon (C) increased sharply in soils of planted pots. The influence of B[a]P on biomass C of the vetiver rhizosphere changed according to soil moisture regime. Biomass C was significantly higher in aerated than in flooded conditions when the plants were not exposed to B[a]P but was higher in flooded conditions than aerated conditions when the plants were exposed to added B[a]P.

Fig. 1. Mean B[a]P concentration in soil with different water regimes. (bars: standard error, $n=4$). ND, (not detected, below the detection limit). B, B[a]P added and unplanted; BP, B[a]P added and planted; P, no B[a]P added and planted.

Fig. 2. Effect of B[a]P on microbial biomass C under different soil water regimes. Data are means of 4 replicates. Bars: Standard Error. For key to treatments, see Figure 1.

Discussion

Phytoremediation is the use of green plants to detoxify or render harmless environmental pollutants. Although the growth index was severely affected, vetiver grass was capable of withstanding a soil B[a]P concentration of 100 mg kg^{-1} and surviving to the end of the assay. In addition, no symptoms of contamination were detected in the plants by simple observation. Further research would be required to elucidate the mechanisms involved in the removal of B[a]P from soil by vetiver grass.

Shoot concentrations of B[a]P $< 0.2 \text{ mg kg}^{-1}$ were detected but $1.8\text{--}2.6 \text{ mg kg}^{-1}$ B[a]P was detected in roots. The translocation of B[a]P from roots to shoots appeared to be weak and this may be related to a detoxification process to protect the shoots from damage and may also be related to low solubility of B[a]P.

Measured root B[a]P may have been located inside or may have represented the pollutant adhering to root surfaces. The experiment did not discriminate between these but the data show that vetiver roots enhanced the removal of B[a]P from the contaminated soil, especially under flooded conditions.

Many tolerant plants are capable of partially degrading pollutants or supporting a healthy rhizosphere capable of degradation. We studied the fate of B[a]P in the rhizosphere with a natural

aerated water regime and under flooded conditions. Vetiver grass accelerated the dissipation of B[a]P under both water regimes. A statistically significant loss of B[a]P was evident in plant-free soil under both moisture treatments, and up to 25% (flooded) or 35% (aerated) of the B[a]P disappeared from the soil. In contrast, the rate of disappearance of B[a]P was more rapid (51–57%) in the presence of vetiver plants. This strongly suggests that the roots of vetiver grass may be responsible for the disappearance of B[a]P from the soil, with little effect of soil water regime on phytoremediation of the contaminated soil.

It has been shown that plants may contribute to the dissipation of PAHs by an increase in microbial numbers (Binet *et al.* 2001). In our experiment we showed that the decrease in B[a]P concentration in the rhizosphere was associated with an increase in microbial biomass C. The accumulation of B[a]P by plants was significantly greater in the aerated water regime than in the flooded conditions. The disappearance of B[a]P from the rhizosphere soil of flooded and aerated treatments was not significantly different. The results indicate that microbial activity may have contributed to the degradation of B[a]P. The soil microbial biomass may have a significant direct effect on B[a]P dissipation, especially under flooded conditions. In addition to the effects of microbial biomass on degradation of B[a]P, there may exist another pathway related to B[a]P dissipation in aerated soils and this needs further investigation.

Conclusions

Vetiver grass promoted the dissipation of B[a]P in soil. The study of Xia & Wu (2001) also showed that vetiver grass could remediate metal-contaminated mining soil by its strong roots and high biomass. Vetiver may therefore be useful for the phytoremediation of heavy metals and organic pollutants in soils. Results indicate that *V. zizanioides* may be useful for phytoremediation of B[a]P contaminated sites.

The influence of the water regime of the contaminated soil may not be restricted to the phytoremediation of B[a]P by vetiver grass, but also the microbial biomass of rhizosphere soil. The effect of water logging on bioremediation of B[a]P is

perhaps due to changes in aerobic versus anaerobic microbial activity and the microbial species present.

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