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Bioremedial Degradation of Some Herbicides by Indigenous White Rot Fungus, *Lentinus subnudus*

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Abstract: A study was undertaken to investigate the efficacy of a white rot fungus (*Lentinus subnudus*), which is indigenous to Nigeria, in degrading the herbicides: Atrazine, heptachlor and metolachlor in herbicide polluted soil. Screening, in soil extract agar, of the white rot fungus for tolerance to atrazine, metolachlor and heptachlor individually and in combination under two different temperature regimes suggested high tolerance of these pollutants by *L. subnudus*. A liquid culture study was conducted with soil extract broth for pesticide degradation to avoid the technical limitations of examining pesticide degradation in soil extract agar. The fungus was able to grow in soil extract broth and degraded mixture of pesticides. About 94% degradation of metolachlor and heptachlor was observed, while about 78% of the atrazine was degraded after 25 days. This indicates high efficacy of use of this fungus as bioremedy to soil pollution by these herbicides.

Key words: Bioremediation, herbicide, white rot fungus, soil extract agar

INTRODUCTION

Recently, there has been increasing interest by researchers in the use of micro-organisms (fungi or bacteria), which may be either naturally occurring or introduced to degrade pollutants in the environment. This application of microorganisms for effective biodegradation of contaminants is called bioremediation. The goal of bioremediation is to at least, reduce pollutant levels to undetectable, non-toxic or acceptable levels i.e., within limits set by regulatory agencies (Pointing, 2001) or ideally completely mineralize organo-pollutants to carbon dioxide. From environmental point of view this total mineralization is desirable as it represents complete detoxification (Gan and Koskinen, 1998).

The use of herbicides enormously increases agricultural productivity. On the other hand, they are potentially hazardous to the environment. The expansion of agricultural and industrial activities in Nigeria has led to increased use of herbicides. The consequence of this situation is increased pollution of soil and ground water with herbicides. When the concentration of an herbicide, its metabolite or by-products is significantly excessive, remediation is necessary to avoid migration to a more sensitive area of the environment.

Contamination with herbicides can lead to pollution of surface water and ground water (Juhler *et al*., 2001). It can also lead to reduction in biodiversity and depression in the activities of soil heterotrophic bacteria and fungi (Ahmed *et al*., 1998). A major source of herbicide contamination is the inadequate management practices specifically involving the on-farm handling of pesticides.

Currently, bioremediation conducted on a commercial scale utilizes bacteria with only few attempts to use white rot fungi. White rot fungi however, offer advantages over bacteria in the diversity of compounds they are able to oxidize (Pointing, 2001). These organisms are robust organisms and are generally more tolerant to high concentration of polluting chemicals than bacteria (Evans and Hedger, 2001). Therefore, white rot fungi represent a prospective tool in environmental bioremediation.

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White rot fungi have been demonstrated to be capable of transforming and/or mineralizing individual pesticides in the soil. It was reported that *Phanerochaete chrysosporium* gave complete transformation of alachlor within 56 days of treatment (McFarland *et al*., 1996). Reddy and Matthew (2001) also showed that *P. chrysosporium* degraded DDT, lindane and atrazine. *Pleurotus oestreatus* has been tested for lindane degradation and was found to effectively reduce its concentrations from 345 to 30 mg L^{-1}, within 45 days, in a branch-scale test (Sasek, 2003). The efficacy of using *L. subnudus* for bioremediation of oil-polluted soil was reported by Adenipekun and Fasidi (2005).

These organisms have been described as good bioremediation agents because of their robust mass and tolerance to several pollutants. Very few studies have examined the degradation of pesticides with white rot fungi. It is therefore important to examine the ability of an indigenous white rot fungus (*L. subdunus*) in the degradation of some common herbicides.

MATERIALS AND METHODS

This study was conducted in July 2006 in University of Port Harcourt, Nigeria.

Fungal Inoculant

The fungal isolate used throughout this study was the white rot fungus, *L. subnudus*, provided by Dr. Mike Ayeni, IITA Ibadan.

Media and Substrates

A soil extract medium was used in this study. This medium was prepared with a sandy loam soil from botanical garden of University of Port Harcourt, Nigeria containing: 71.78 sand 15.09% silt, 11.40% clay, 5.06% organic matter, 80.3 mg kg^{-1} soil extractable phosphorous, 4.7 mg kg^{-1} soil nitrate-N, 0.7 mg kg^{-1} soil ammonium-N, organic matter: furnace 5.10%, titration 1.65% and pH of 6.03 (analyzed by Petroleum Chemistry (PC) laboratory Shell, Port Harcourt, Nigeria).

Soil extract was prepared by using 200 g of untreated field moist soil from botanical garden of University of Port Harcourt, Nigeria in 400 mL of tap water. The soil/water mixture was autoclaved for 30 min, centrifuged at 2400 g for 20 min and filtered through Whatman No. 1 filter paper, using a vacuum pump. The soil extract was a liquid broth. For solid medium experiments, 2% technical agar was added to the liquid medium.

Analytical Grades of the Herbicides

Atrazine, heptachlor and metolachlor were bought from a pesticide shop in Port Harcourt, Nigeria. Stock standard solutions were prepared by dissolving to analytical standard in methanol and storing in amber bottles at 4°C. Working standard solutions were obtained by dilution with acetonitrile. In this study, xenobiotic concentrations were expressed in mg L^{-1} soil (for liquid and agar studies) or mg kg^{-1} soil (for soil studies).

Investigation of the Tolerance of the White Rot Fungus, *L. subnudus*, to Herbicides *in vitro*

One approach to this study was to assess the ability of the white rot fungus to grow in soil extract agar supplemented with atrazine, heptachlor and metalochlor individually and in mixtures. The soil extract agar used as the culture medium was modified by separate addition of atrazine, heptachlor and metolachlor in 0, 5, 10 and 20 mg L^{-1} concentrations. The media were thoroughly mixed and poured into 9 cm Petri dishes. Each of the Petri dishes containing the media was centrally inoculated with 4 mm agar plug taken from the margin of a growing colony of the fungus. The treatments used in this experiment were: control (without addition of herbicide), atrazine, heptachlor and metolachlor treated separately at 5, 10 and 20 mg L^{-1} (total concentration). This experiment was carried out at

15 and 25°C. Growth was measured regularly for up to 25 days. The percentage growth inhibition was calculated for the 0, 5, 10 and 20 mg L^{-1} treatments. Growth of the fungus in the presence of mixture of the herbicides was assessed.

Soil extract agar was modified by the addition of atrazine, heptachlor and metolachlor as a mixture of herbicides in the range 0-20 mg L^{-1} by addition to the molten agar and inoculated with the colony of the test-isolate as described in the experiment above. Growth inhibition was also measured as described above. Calculation of the fungal growth rate was carried out by taking colony diametric measurements frequently throughout the incubation period. Two readings, at right angles to each other, were taken. The data obtained were then processed.

Investigation of Herbicides Degradation Rates in Soil Extract Liquid Broth

Soil extract liquid broths used in this study were supplemented with a mixture of atrazine, metolachlor and heptachlor to give final concentrations of 0, 5, 10, 15 and 30 mg L^{-1} and final volume of 100 mL in 250 mL Erlenmeyer flasks. Four plugs of actively growing mycelia were inoculated in each flask at 27±1°C, for 25 days with constant agitation at 150 rpm. All treatments were carried out in triplicate.

Sampling and Dry Weight Determination

After the incubation period, the mycelium was filtered through Whatman No. 1 filter papers and the biomass was determined by drying for 48 h at 80°C. The fresh filtrate was frozen at -20°C and used later for herbicide quantification.

Quantification of Herbicide Concentration in Soil Extract Broth

Samples were filtered through 0.2 μm filter (Fisher, FDP-466-OO1C) and diluted with acetonitrile (75% sample: 25% acetonitrile) prior to injection in the HPLC system. HPLC quantification of all three herbicides was performed with a Gilson HPLC system equipped with a UV detector (117 UV detector, Gilson), Gilson 401C Dilutor, Gilson 231XL sampling injector, Gilson 306 Pump and Gilson 81 1C Dynamic Mixer, equipped with Altima C18 5 pm column (4×250 ×4.6 mm). The column was operated at ambient temperature with a flow rate of 1.5 mL min^{-1} and an injection volume of 50 μL.

An isocratic mobile phase system was established using acetonitrile: Water at a ratio of 70:30. The HPLC-UV detector was monitored at 215 nm. The HPLC method used in this study was adapted from a method to quantify dieldrin described by Eliassy (1997). The HPLC method used enabled the separation and quantification of atrazine, metolachlor and heptachlor in a single HPLC run of 20 mm.

Statistical Analysis

Comparison between means was carried out using ANOVA followed by Tukey Multiple Comparisons test.

RESULTS

In vitro tolerance of white rot isolate to individual and mixtures of herbicides at two different temperature regimes.

The results of the assessment of white rot isolate to herbicides in soil extract agar individually and as a mixture under the two different temperature regimes were shown in Table 1 and 2. The test isolate was tolerant to the herbicide treatments. However, the growth rate was highly influenced by temperature and herbicide concentration. A comparison of the effect of these two factors on the growth rate of the test isolate was shown in Table 1 and 2. Table 1 showed the EC_{50} values (herbicide concentration causing 50% growth reduction compared to the control). Table 2 showed the percentage

Table 1: Concentration (mg L^{-1}) of the three herbicides individually and as a mixture, that caused a 50 % reduction in fungal growth (EC_{50}) in soil extract agar

Temp. (°C)	Atrazine EC_{50} (mg L^{-1})	Metolachlor EC_{50} (mg L^{-1})	Heptachlor EC_{50} (mg L^{-1})	Mixture EC_{50} (mg L^{-1})
15	20.0	8.2	NI	9.8
25	11.9	3.8	NI	10.6

NI = No Inhibition

Table 2: Growth inhibition (1%) observed for *L. subnudus* growing in soil extract agar supplemented with different concentrations of the three herbicides individually and as a mixture at different temperature regimes

	Atrazine				Metolachlor			Heptachlor			
Temp. (°C)	0	5	10	20	5	10	20	5	10	20	Mixture
15	NI	15	20	24	NI		NI	0.1	NI	NI	38
25	NI	8	11	14	NI		0.2	NI	NI	NI	30

Temp. = Temperature; NI = No Inhibition

Table 3: Concentration of herbicides remaining in soil extract that was initially supplemented with a mixture of different concentrations of the herbicides after 25 days of incubation with *L. subnudus* at different temperature regimes (± Standard deviation of the mean)

		Remaining Pesticide (%)		
Temp. (°C)	Initial conc. mixture (mg L^{-1})	Atrazine	Metolachlor	Heptachlor
20	5	11.5±3.00	1.5±0.24	9.3±3.09
	10	17.5±5.08	1.8±0.30	9.8±0.56
	15	7.8±4.51	6.8±0.96	9.9±0.95
	30	22.8±1.62	1.9±0.23	5.6±0.40
25	5	18.6±2.40	1.0±0.31	20.1±1.23
	10	17.8±3.31	2.6±0.31	11.1±1.48
	15	14.0±0.59	7.0±0.58	8.8±0.24
	30	27.3±1.39	3.1±0.14	19.6±0.12

growth inhibition observed in different herbicide concentration treatment. The isolate showed lower growth inhibition at 25°C and at 5 mg L^{-1} concentrations of the three herbicides. *Lentinus subnudus* showed better tolerance of metolachlor and heptachlor than atrazine.

Interestingly, the growth of the fungus was not inhibited by all the concentrations of metolachlor (Table 2), which suggested that this isolate could easily grow in media supplemented with these herbicides without suffering any toxicity effect. Atrazine was more toxic to the isolate than the other test herbicides.

Degradation of Different Concentrations of Herbicides by White Rot Fungus

The degradation rates of the herbicides by the fungus were affected significantly by temperature regimes and initial concentration of herbicide mixture. This fungus showed significantly less metolachlor degradation in 15 mg L^{-1} treatments, at both temperature regimes ($p<0.001$) Table 3.

For heptachlor, the fungus showed the highest degradation rates in the 30 mg L^{-1} treatment ($p<0.05$) at 20°C and in the 15 mg L^{-1} treatment ($p<0.001$) at 25°C. Regarding atrazine, *L. subnudus* showed the highest significant degradation rates at 15 mg L^{-1} at both temperature regimes ($p<0.05$).

DISCUSSION

The study of fungal growth rates is very important for extrapolation of the potential colonization capacity of the organisms in the field as it provides a good indication of the speed at which a fungus is able to colonize and transverse a substrate. The results observed during the screening experiment at 25°C showed that *L. subnudus* was tolerant to the range of herbicide concentrations used. This conclusion was based on the analysis of the EC_{50} values. The response of the test isolate to the herbicides was influenced by a) herbicide concentration and b) whether the herbicides were mixed or

present individually. Overall, the results showed that *L. subnudus* was more tolerant to metolachlor and heptachlor on a wider range of conditions than to atrazine. The response of the fungus to the mixtures showed similar trend to its response to individual herbicides.

The growth rates may also indicate which species may dominate over a particular substrate. Fast growing species have an advantage over slower species as they can reach and utilize resources before their competitors (Magan and Lacey, 1984; Marin *et al*., 1998a, b). Temperature was considered in this study because it plays a key role in fungal development.

The level of degradation of herbicide was estimated by quantifying the concentration of herbicide remaining in the soil after incubation at 20 and 25°C. The results showed that the treatments with fungal inoculants had higher herbicide degradation than the control. *Lentinus subnudus* degraded metolachlor and heptachlor better than atrazine. The present study on degradation of the mixture of pesticides showed good capacity by *L. subnudus* at both temperature treatments, regardless of the initial concentrations of herbicides between 0 and 30 mg L^{-1}.

Worthy of note is the greater degradation obtained in the treatments with higher initial concentration of herbicides. Tekere *et al*. (2002) reported degradation rates of about 82% for the pesticide lindane by *P. chrysosporium* but the highest degradation rates were achieved when the initial concentrations were as low as 5 and 10 mg L^{-1}. Lower degradation was obtained at initial concentrations of 20 and 40 mg L^{-1}. Few studies have been carried out on the degradation of mixtures of pesticides (Yavad and Reddy, 1993; Bending *et al*., 2002). Yavad and Reddy (1993) described co-mineralisation of a mixture of the pesticides 2,4-D and 2,4,5-T by *P. chrysosporium* wild type and putative peroxidase mutant rich broth, with a small amount (5%) remaining in the mycelial fractions.

Bending *et al*. (2002) showed degradation rates of metalaxyl, atrazine, terbuthylazine and diuron of greater than 86% by white rot fungi in nutrient solution for atrazine and terbuthylazine. Boyle (1995) found bioremediation results to be viable, which may be due to the fungi not always growing well or not always expressing their degradative system in the soil. In addition, the adsorption of pesticide molecules to soil particles may reduce the availability of the compound for microbial attack (Atagana *et al*., 2003).

The results obtained in this study provide valuable data on the ability of *L. subnudus* to colonize the soil and its tolerance and ability to biodegrade some herbicides. The information emanating from this work serves as a sound basis for the exploitation of this species as fungal inoculant in biological remediation processes.

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