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Full Length Research Paper

In vitro effects of metals and pesticides on dehydrogenase activity in microbial community of cowpea (*Vigna unguiculata*) rhizoplane

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Effects of heavy metals and pesticides on cowpea (*Vigna unquiculata*) rhizoplane microbial community were assessed *in vitro* via dehydrogenase activity. The microbial community was exposed to various concentrations of heavy metals and pesticides in a nutrient broth-glucose-2,3,5-triphenyl chloride (TTC) medium. At 0.2 mM, iron and cadmium stimulated the dehydrogenase activity of the microbial community. For all the metal ions, there was progressive inhibition with each successive increase in the concentration of metal ion, reaching near 100% at 0.6, 0.8, 1.2, 0.12 and 12 mM for cobalt, cadmium, iron, mercury and nickel, respectively. Between 0.2 and 0.4 mM, zinc sharply inhibited dehydrogenase activity and at concentration above 0.4 mm, inhibition of dehydrogenase activity became less pronounced. The order of toxicity is Hg^{2+} > Co^{2+} > Cd^{2+} > Zn^{2+} > Fe^{2+} > Ni^{2+}. The herbicides Cotrazine (Atrazine 80W) and Northrin®10EC stimulated dehydrogenase activity of the microbial community at 0.2% and inhibited it at higher concentrations. The median inhibitory concentrations (IC_{50}s) of Cotrazine (Atrazine 80W) and Northrin®10EC were 0.552 ± 0.028 and 0.593 ± 0.051%, respectively. The dehydrogenase activity varied significantly ($p < 0.05$) with the type and concentrations of metals or pesticides. The result indicates that the heavy metals and pesticides are potentially toxic to *V. unquiculata* root surface microorganisms. In soil, this toxicity may affect nitrogen fixation processes and by extrapolation affect crop yield.

Key words: Dehydrogenase activity, rhizosplane bacteria, atrazine, cypermethrin, heavy metals, cowpea.

INTRODUCTION

Contamination of agricultural soils with organic and inorganic pollutants results from industrial and domestic wastes, agricultural inputs and several other human activities. These pollutants are usually disseminated in soil by repeated flooding. Optimization of agricultural resources for improved and sustainable agriculture involves the use of pesticides. Various herbicides and insecticides have been used to control unwanted weeds and insects. Atrazine (2-chloro-4-ethylamino-6-isopropyl-amino-1,3,5-triazine) is a member of s-triazine group of herbicides. It is used mainly for pre- and post-emergence control of annual grasses and broad-leafed weeds in many commercial crops. Cypermethrin, the active agent in Northrin®10EC, is a synthetic pyrethroid insecticide that has high insecticidal activity and adequate stability in air and light (Kaufman et al., 1981). Cypermethrin is relatively non-persistent in soils with typical half-life of 2 - 4 weeks in sandy soils (Chapman and Harris, 1981). Increased Cypermethrin persistence was observed in soils with high organic matter, high clay content, reduced microbial activity and anaerobic conditions (Chapman et al., 1981).

Application of pesticides to agricultural soils may affect soil biological activity in a variety of ways (Shetty and Magu, 1997, 1998). Herbicides may have negative effects on the growth of rhizobia (Clark and Mahanty, 1991; Mårtensson, 1992; Singh and Wright, 2002) and influence nodulation and biological nitrogen fixation in legumes. Also, it has been shown that metals influence microorganisms by adversely affecting their growth, morphology and biochemical activities resulting in decreased bio-

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Table 1. Physico-chemical properties of soil.

Parameter/unit	Value
pH (H_2O)	4.89
Organic matter (%)	1.754
Organic carbon (%)	1.018
Nitrogen (mg/kg soil)	740
Phosphorus (mg/kg soil)	130
Metals (mg/kg soil)	
Calcium	16432.8
Magnesium	6805.4
Potassium	6412.1
Sodium	5517.6
Nickel	0.010
Cobalt	0.005
Zinc	52.700
Iron	82.600
Mercury	0.000
Cadmium	0.042

mass and diversity (Hattori, 1992; Frostegard et al., 1993; Roane and Kellogg, 1996).

Measurement of the metabolic activity of a microbial community gives a more immediate and realistic indication of the effects of toxicants on the community as it is found in nature (Barnhart and Vestal, 1983). Dehydrogenase activity in soil is among the several biological parameters commonly used to assess the side effect of chemicals on microorganisms.

In soil, metals and pesticides are adsorbed or form complexes with organic and inorganic matter, reducing chemical mobility. Their toxicity in a polluted soil therefore depends on the concentration speciated in soil water. It therefore becomes necessary to establish the toxic levels of these substances as a benchmark in the management of pesticides and metal contamination of agricultural soils. In this work, the toxicity of metals and pesticides was determined *in vitro* on the microbial community extracted from rhizoplane of cowpea (*Vigna unguiculata*) via aqueous phase dehydrogenase activity assay.

MATERIALS AND METHODS

Heavy metals and herbicides

The heavy metal ions, Hg^{2+}, Co^{2+}, Cd^{2+}, Zn^{2+}, $Fe^{2+}$and Ni^{2+} were used as $HgCl_2$, $CoCl_2$, $CdCl_2$, $ZnSO_4$, $FeSO_4.7H_2O$ and $NiSO_4$, respectively. The herbicides are Cotrazine (Atrazine 80W) manufactured by Nantong Foreign Trade Mehco, Shanghai China and Northrin®10EC, a formulated cypermethrin (100 g/l) manufactured by Danyang Agrochemicals Company Limited, Jiangsu China. The herbicides were purchased from a local dealer's store in Lagos, Nigeria.

Growth of cowpea and source of microbial community

The cowpea plant (*Vigna unguiculata*) was grown to maturity in an arable loamy soil. The full-grown plant was uprooted and the soil particles that attached to the roots were shaken off. The nodule-rich roots were aseptically cut and placed in 300 ml sterile physiological saline contained in 500 ml Erlenmeyer flask and shaken vigorously to extract the root surface organisms. The resultant suspension of microorganisms served as the microbial community that was used as inoculum in the study. The physico-chemical characteristics of the soil are shown in Table 1.

Enumeration of microorganisms

The bacterial population of the microbial community extracted from the root surface of *V. unguiculata* was enumerated on nutrient agar plates. The fungal population was enumerated on potato dextrose agar supplemented with 50 µg/ml each of tetracycline and streptomycin. Incubation was at room temperature (28 ± 2°C) for 24 h (bacteria) and 72 h (fungi).

Determination of TTC-reactive microbial population

Plates with discrete colonies of bacteria and fungi were selected from the ones used for counts and the colonies sprayed with aqueous solution (0.4%) of 2,3,5-Triphenyl chloride (TTC). The plates were further incubated in the dark at room temperature for 3 h (bacteria) and 24 h (fungi). The number of red colonies (indicating those that could reduce TTC to red formazan) were counted and expressed as a percentage of the population.

Dehydrogenase activity assay

Dehydrogenase activity was determined using 2,3,5-triphenyltetrazolium chloride as the artificial electron acceptor, which is reduced to the red-coloured triphenylformazan (TPF). The dehydrogenase activity of the microbial community was determined in 3-ml volume of nutrient broth-glucose-TTC medium supplemented with varying concentrations of metal ions or pesticides in separate screw-capped test tubes. Portions (0.3 ml) of suspension of the microbial community were inoculated into triplicate glass tubes containing 2.5 ml of phthalate-buffered (pH 7) nutrient broth-glucose medium amended with heavy metal ions or pesticides and preincubated on a rotary incubator at room temperature (28 ± 2°C) for 30 min. Thereafter, 0.2 ml of 0.4% (w/v) TTC in deionized distilled water was added to each tube to obtain final concentration ranges of 0.02 – 0.12 mM (Hg^{2+}), 0.2 – 1.2 mM (Co^{2+}, Cd^{2+}, Fe^{2+} and Zn^{2+}), 2 – 12 mM (Ni^{2+}) and 0.2 – 1.6 % (pesticides) in different test tubes. The final concentrations of nutrient broth, glucose and TTC in the medium were 2, 2 and 0.267 mg/ml respectively. The controls consisted of inoculated media without heavy metal ions and pesticides. The reaction mixtures were further incubated statically in the dark at room temperature (28 ± 2°C) for 24 h. The TPF produced was extracted in 4 ml of ethylacetate and determined spectrophotometrically at 460 nm (λmax). The amount of formazan produced was determined from a standard dose-response curve [0-20 mg/l TPF (Sigma) in ethylacetate; R^2 = 0.998]. Dehydrogenase activity was expressed as micrograms of TPF formed per milliliter of suspension of the microbial community per hour.

Heavy metal and pesticide inhibitions or stimulations of dehydrogenase activity was calculated relative to the control. The IC_{50} of the metal ions and pesticides, which is the inhibitory concentration of toxicant required to reduce 50% of the dehydrogenase activity, were determined based on correlation coefficients (R^2) obtained from direct %inhibition-dose plot, logarithmic (log_{10}%inhibition-dose plot) or gamma parameter (Γ) (gamma parameter-dose plots) transformations of the % inhibition.

Table 2. Fungal and bacterial counts and TTC-reactive fraction in the microbial community of *Vigna unquiculata* rhizoplane.

Group	Count (cfu/ml)	TTC-reaction		
		Number of colonies sprayed	Number of red colonies	Red colonies (%)
Fungi	3.7×10^4	306	21	6.86
Bacteria	5.3×10^7	632	612	96.84

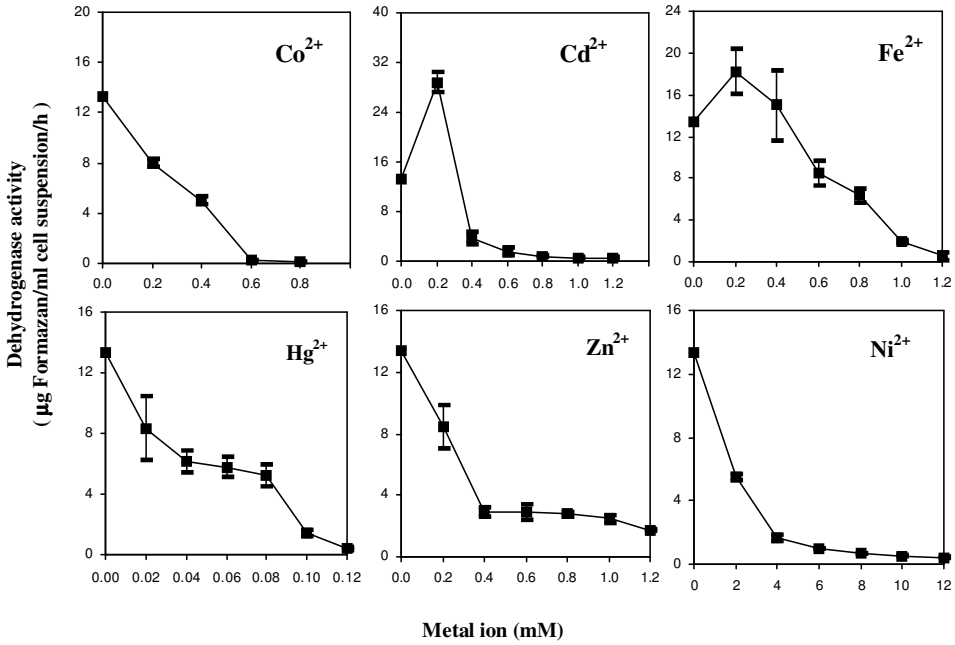

Figure 1. Effects of metals on dehydrogenase activity of microbial community of *Vigna unguiculata* rhizosplane. Data represents mean ± standard deviation (n=3). Some standard deviations are within data points.

$$\Gamma = \frac{\%\ \text{Inhibition}}{100\ -\ \%\ \text{Inhibition}}$$

Statistical analyses

Data generated were subjected to multiple factor analysis of variance (2-Way ANOVA).

RESULTS AND DISCUSSION

The fungal and the bacterial counts of the microbial community extracted from the root surface of cowpea (*V. unguiculata*) were 3.7×10^4 and 5.3×10^7 cfu/ml respectively (Table 2). This indicated that there were more bacteria than fungi in the rhizoplane. Bacteria are usu-ally in symbiotic association with leguminous plants and are the major microorganisms involved in nitrogen fixa-tion. The TTC reactivity assay indicated that 6.86 and 96.83% of fungal and bacterial populations respectively were able to reduce TTC to triphenyl formazan. The lack of red color in some colonies could be attributed to their inability to use TTC as electron acceptor, as different microorganisms have different dehydrogenase system. The lower use of TTC by fungi than bacteria might be as a result of poor diffusibility of this compound into fungal cells. Similar observations of poor TTC reactivity by fungi have been made (Praveen-Kumar, 2003). This showed that the dehydrogenase activity of the microbial community is to a large extent representative of the bacterial population. The effects of the heavy metal ions on the dehydrogenase activities of the rhizoplane microbial community are shown in Figures 1 and 2. Apart from cadmium and iron, which had stimulatory effects on dehydrogenase activities at low concentrations of 0.2 and 0.4 mM respectively, progressive inhibition of dehydrogenase activity were observed with all the metal ions studied. For cobalt, this inhibition is linearly dependent upon the concentration of the metal ion up to 0.6 mM. At 0.8 mM, there was almost total inhibition of dehydrogenase activity. For zinc, at 0.2 and 0.4 mM, sharp inhibitions of dehy-

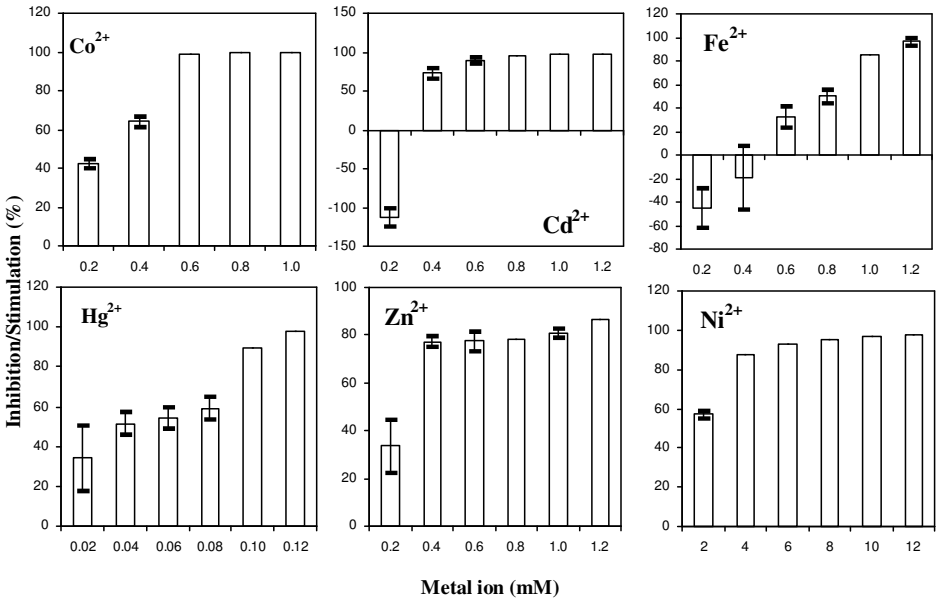

Figure 2. Relative dehydrogenase activity in microbial community of *Vigna inguiculata* rhizosplane in response to different concentrations of metals. Bars indicate standard deviation (n = 3); > 0% = inhibition of dehydrogenase activity; < 0% = stimulation of dehydrogenase activity.

Table 3. Median inhibitory concentrations of metal ions against microbial community of *Vigna unquiculata* rhizoplane.

Toxicant	IC_{50}
Metal ions (mM)	
Cobalt (Co^{2+})	0.239 ± 0.031
Cadmium (Cd^{2+})	0.256 ± 0.019
Zinc (Zn^{2+})	0.257 ± 0.075
Nickel (Ni^{2+})	2.122 ± 0.245
Iron (Fe^{2+})	0.825 ± 0.118
Mercury (Hg^{2+})	0.043 ± 0.024
Pesticides (%)	
Cotrazine (Atrazine 80W)	0.552 ± 0.028
Northrin®10EC	0.593 ± 0.051

drogenase activity were observed. However, at higher concentrations, the relative inhibition became less pronounced. Like zinc, nickel sharply inhibited dehydrogenase activity at 2 and 4 mM. The inhibitory effect became less severe at concentrations greater than 4 mM. This reduced relative inhibition of dehydrogenase activity at high concentrations could be ascribed to saturation of active sites in the microbial cells. For mercury, the dehydrogenase activity decreased with increasing concentrations. Mercury is known to be highly toxic to microorganisms for its affinity to thiols groups in proteins and their lipophilic tendencies. Mercury is not essential for biological functions and is a strong inhibitor of microbial metabolism even at low concentration (Gadd, 1993; Ross, 1975).

The toxicity thresholds of the metal ions against the microbial community are shown in Table 3. Mercury having the lowest IC_{50} of 0.043 ± 0.024 is the most toxic while nickel having the highest IC_{50} of 2.122 ± 0.245 is the least toxic metal. The 2-way ANOVA showed that the dehydrogenase activity varied significantly ($p < 0.05$) with metal type and the concentrations. The order of toxicity is $Hg^{2+} > Co^{2+} > Cd^{2+} > Zn^{2+} > Fe^{2+} > Ni^{2+}$.

The herbicides Cotrazine (Atrazine 80W) and Northrin®10EC stimulated dehydrogenase activity of the microbial community at low concentrations (0.2%) and inhibited it at high concentrations. The observed inhibition of dehydrogenase activity at high concentrations (> 0.2%) (Figure 3) is consistent with the reported toxic effect of atrazine at high concentration (Dzantor and Felsot, 1991). The median inhibitory concentrations (IC_{50}) of Cotrazine (Atrazine 80W) and Northrin®10EC were 0.552 ± 0.028 and 0.593 ± 0.051%, respectively. The dehydrogenase activity varied significantly ($p < 0.05$) with the pesticide type concentrations. Atrazine and cypermethrin have been reported to affect microbial processes in soil (Dzantor and Felsot, 1991; Tu, 1991; Accinelli et al., 2002; Hu et al., 2005; Gundi et al., 2005). Respiratory activity in soil was stimulated with the application of 50 mg atrazine/kg of soil (Hu et al., 2005) (Figure 4). Accinelli et al. (2002) and Gundi et al. (2005) also reported the stimulation of soil dehydrogenase activity and respiration by cypermethrin and atrazine at low concentrations. The stimulatory effect could be attributed to the use of the pesticides as source of electron and energy. Initial rise in microbial counts, attributable to temporal mineralization

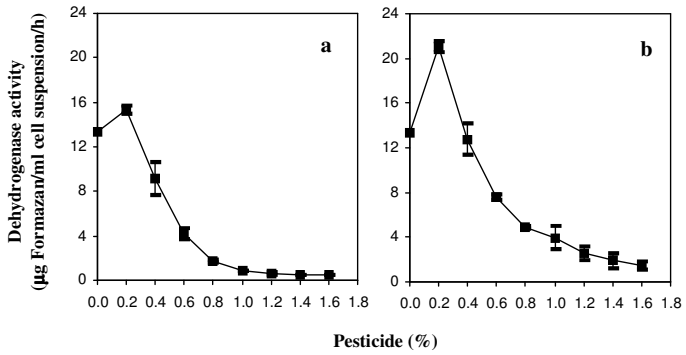

Figure 3. Effect of Cotrazine (a) and Northrin®10EC (b) on dehydrogenase activity of *Vigna unquiculata* rhizoplane microbial community. Means ± Standard deviation (n=3) are indicated by bars.

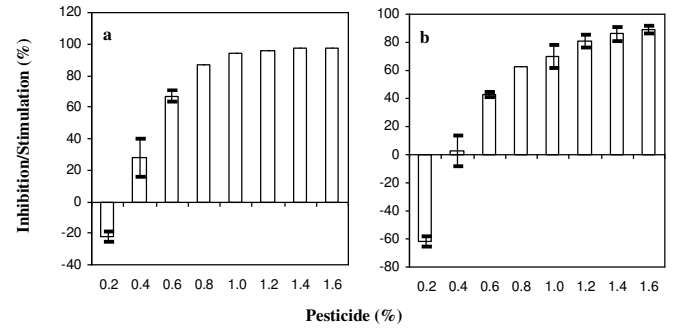

Figure 4. Relative effect of Cotrazine (a) and Northrin®10EC (b) on the dehydrogenase activity of microbial community of *Vigna unguiculata* rhizoplane. Data represents mean ± standard deviation (n=3); > 0% = inhibition of dehydrogenase activity; < 0% = stimulation of dehydrogenase activity.

and use of herbicide as energy source was reported for an arable soil treated with atrazine and metolachor (Ayansina and Oso, 2006). Degradation of atrazine by microorganisms have been reported (Cook, 1987; Mirgain et al., 1993; Radosevich et al., 1995; Cai et al., 2003; Khromonygina et al., 2004). Dehydrogenases are required to catalyze the biological oxidation and dehalogenation of a number of herbicides and other organic compounds (Waarrde et al., 1993; Beller et al., 1996). Mineralization rate of atrazine was reported to be closely correlated with dehydrogenase activity (Lin et al., 2005).

The results reported here for Cotrazine (Atrazine 80W) are based on *in vitro* studies conducted in nutrient broth-glucose medium. It is necessary to know the concentration of this herbicide in the soil where the pesticide is applied in order to predict its effect on the growth of microbial community under field condition. The recommended field application rate for Cotrazine (Atrazine 80W) is 0.5 – 3.0 kg/ha. This rate was converted into mg/l in soil solution by correlating the data of Singh and Wright (2002) obtained as was described by Fletcher (1956). Fletcher (1956) calculated that an application of 1.135 kg of active growth substance per hectare will result in a herbicide concentration of 2.0 – 2.5 mg/l in soil assuming no adsorption, complete solution and a 20% soil moisture content. The application rate corresponds to 0.16 – 1.2 mg/l of Cotrazine (Atrazine 80W) in soil water. This range of concentrations is much lower than the 0.2% (2000 mg/l) that stimulated dehydrogenase activity. Thus, application of the herbicide at field recommended rate is not likely to inhibit the dehydrogenase activity of rhizoplane microrganisms. It is difficult to predict the concentration of cypermethrin in soil since Northrin®10EC is not applied directly to soil.

The results of this study have shown potential toxicity of the heavy metals and pesticides to the root surface microorganisms of *Vigna unguiculata*. Field study may be required to assess the effects of these toxicants under field conditions.

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