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Verification of *Aspergillus Niger* as a Myco-remediation Agent of Lambda-Cyhalothrin and Associated Heavy Metals in *Lactuca Sativa* (L.) Leaf

***SANYAOLU, ADENIYI AKEEM ADETOLA**

**Department of Botany and Ecological Studies, Faculty of Science, University of Uyo. P.M.B. 1017 Uyo, Akwa Ibom State, Nigeria.*
**Email: adeniyisanyaolu@uniuyo.edu.ng*
Phone: 08053091616.

ABSTRACT: Pesticide residue constitutes a danger to soil micro and macro fauna and flora and to humans. This study assessed the impact of the application of *Aspergillus niger* as a pesticide remediating agent on Lambda-cyhalothrin and some of its associated heavy metals translocated to the leaves of *Lactuca sativa*. A Complete Randomized Design (CRD) experiment using three Treatments was conducted. Lambda-cyhalothrin was extracted from the Samples using the multiple residue technique and its residue determined using Gas Chromatography with Pulsed Flame Photometric Detector (PFPD). The associated heavy metals were determined using the Atomic Absorption Spectrophotometric method. Data so generated were means from five replicates, and were subjected to a one way analysis of variance - ANOVA with Tukey HSD Test for differences between means at 99% confidence interval ($P \leq 0.01$). The results show that the highest mean value of Lambda-cyhalotrhrin residue in *L. sativa* leaves was found in Treatment A (1.50 mg/kg), a value which was significantly higher ($P \leq 0.01$) than the residue found in Treatments B (1.0 mg/kg) and C(0.02 mg/kg). Mean Lambda-cyhalothrin residue in Treatment A alone was above the Maximum Residue Level (MRL) of 1.0 mg/kg in vegetable set in the legal EU regulation.The application of *A. niger* also precipitated a significant reduction ($P \leq 0.01$) in the level of all the heavy metals evaluated except Arsenic. All the heavy metals evaluated were however below the WHO/FAO safe limit. The result from this study posits *A. niger* as a promising mycoremediation agent of pesticide pollution.

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Pesticide use has evoked grave concerns not only because of potential detrimental effects on wildlife and sensitive ecosystems but also on human health (Power, 2010; Damalas and Eleftherohorinos, 2011). There is a strong connection between pesticide use and heavy metal contamination of the environment and edible plants (Mansour *et al*., 2009, Tariq *et al*., 2016 and Singh *et al*., 2017). Pests contribute significantly to food losses and the control of pests is therefore very central to the attainment of food security at all spatial scales (Iya and Kwaghe, 2007; Al Fed *et al*., 2006).Lambda-cyhalothrin is extensively used in agricultural production to control pests in an effort to reduce or eliminate yield losses and preserve high product quality (Eskenazi *et al*., 2008).

Lambda-cyhalothrin is a manmade organic insecticide belonging to the chemical class of pesticide called pyrethroids (WHO, 1990). Pyrethroids are formulated to mimic (both in structure and function) the naturally occurring pyrethrum (Maund *et al*., 1998). By their nature, most lambda-cyhalothrin show a high degree of toxicity to some organisms because they are intended to kill and thus create some risk of harm (Zidan, 2009; Abdelgadirand and Adam, 2011).
The use of lambda-cyhalothrin has increased 50 folds and 2.5million tons of industrial pesticides are now used annually (Farag *et al*., 2011). This phenomenal increase in use is expected because food security issues, particularly in developing countries, are very high on the International agenda. Although pesticides are manufactured under very strict regulation processes to function with minimal impact on human health and the environment, serious concerns, have nevertheless been raised about health risks resulting from pesticide residues in food (Damalas and Eleftherohorinos, 2011; Eskenazi *et al*., 2008).

Problems associated with pesticide use include recalcitrance and or persistence of its residue in environmental matrices, deposition of associated heavy metals in the environment and on the plants being protected. Other concerns include bioaccumulation and migration through the food chain, culminating in wildlife losses, water quality degradation and illnesses and or fatality to humans (Muthukumaravel, 2013).

**Email: adeniyisanyaolu@uniuyo.edu.ng*

Accumulation of heavy metals in arable land may result in soil contamination and increase heavy metals uptake by crops and thus adversely affect food quality and food safety (Ho and Tai, 1988). It is therefore imperative to monitor food quality, and to consistently strive at improving food quality given that plant uptake is one of the main pathways through which heavy metals and pesticide residues enter the food chain. Within this framework, this research sets out to verify the potential of *Aspergillus niger* as a remediation agent on lambda-cyhalothrin residue and its associated heavy metals uptake under good agricultural practice (GAP) in *Lactuca sativa* leaves.
The objective of this study is to determine the level of heavy metal burden in leaves of *L. sativa* and compare Lambda-cyhalothrin residue levels in *Lactuca sativa* leaves grown under *Aspergillus niger* bioaugmented soil relative to a non-bioaugmented soil.

MATERIALS AND METHODS

*Isolation And Sub-Culturing of Aspergillus Niger***:** The soil from which *A. niger* was isolated was collected from a refuse dump in Shomolu market, Lagos State, Nigeria (N 06° 31' 38", E 003° 22' 22").Serial dilution of up to 10^5 was done using 1g of soil in 10ml of sterile distilled water. Using a sterile pipette, 1 drop each of the serially diluted soil-water solution was dropped into a sterile petri dish to which a freshly prepared sterile Potato Dextrose Agar (PDA) supplemented with a capsule of chloramphenicol (500mg BP to 500ml of freshly prepared sterile PDA) and 2 drops of lactic acids (to each Petri dish before it solidifies) was added. Afterwards, the plates were sealed with masking tape and incubated at a temperature of 28-31^{O}C for 48 hours or more depending on the rate of growth of each plate. To identify this fungus, a small portion of the pure isolate was teased with a sterile inoculating loop into 2 drops of lacto phenol in cotton-blue on a clean slide and a cover slip was placed on it. This was examined under a light microscope. Identification of fungus was done using morphological parameters {that is , the examination of the size, shape ,colour, spore formation and the number of days for the fungus to reach maximum diameter (9cm) of the Petri dish and the texture of fungal growth} as described by Bryce (1992). Two hundred plates of *Aspergillus niger* were sub cultured from the pure stock cultures that were thus isolated from this soil.

Collection of Soil Sample and Application of Treatments: This study involved collection of top soil from two different places in 15 buckets altogether. The first set of soil sample was collected from Irrigation Centre in Tejuosho Area, Lagos-Nigeria (N 06^0 $30^{'}$ $04.3^{"}$, E $003^0 22^{'} 06.6^{"}$). Here, farming activities has been taking place for over 3 decades non-stop, prior to this time when soil sample was collected from there. The soil samples from this location were placed in 10 buckets named Treatments A and B of five buckets each. The second set of soil sample was collected from Yaba College of Technology Staff Quarters, Lagos-Nigeria -N 06^0 $31^{'}$ $0.75^{"}$,E 003^0 $22^{'}$ $38.8^{"}$- (a location with no history of farming activities). This set of Soil Sample was put in the last 5 buckets (Treatment C). *L. sativa* seedlings from the nursery were transplanted into each of the fifteen different Treatment buckets, and Lambda-cyhalothrin was applied to the *L. sativa* plants of Treatments A and B only (at the recommended rate) as often as was necessary until the plants were harvested. In addition spores of *Aspergillus niger* that were cultured in the laboratory were aseptically transferred into each of the soil of the five buckets of Treatment B only (at the rate of forty plates/bucket) and worked into the soil up to a depth of about 15cm just before the *L. sativa* seedlings were transplanted into the buckets.

The three Treatments applied in this experiment were as outlined below: *Treatment A*: Soil (with the history of use of Lambda-cyhalothrin and other agricultural inputs) + application of lambda-cyhalotrin (at recommended rate) + *L. sativa* plants.
Treatment B: Soil (with the history of use of Lambda-cyhalothrin and other agricultural inputs) + application of lambda-cyhalothrin (at recommended rate) + Spores of *A. niger* (40 plates/pot) + *L. sativa* plants.
Treatment C (Control Treatment): Soil (with no history of use of Lambda-cyhalothrin nor any other agricultural input) + *L. sativa* plants.

*Extraction and Determination of Lambda-cyhalothrin Residue in L. Sativa Leaves***:** The extraction and the analysis of Lambda-cyhalothrin residue was carried out by following amodified approach of Luke and Doose (1984) for multiple residue extraction, while its determination was followed using the organophosphorus pesticide residues Manual of Analytical Methods for the Analysis of Pesticides Q38.These test methods cover the Capillary Gas Chromatographic determination of various pesticides, including some of their degradation products and related compounds.

Samples of the freshly harvested *L. sativa* leaves were kept in less than 4 degree centigrade for 24 hours until analysis. Fifty gram of this sample was weighed into a borosilicate container. Twenty gram of dried aluminium oxide was added along with 25ml of deionised water

and 280ml of the acetonitrile. The mixture was blended for about 2minutes, after which it was filtered through the suction to recover the filtrate. Two fifty mills of the filtrate were decanted after the addition of the surrogate standard solution to the sample and later transferred to the extracting bottle that was cocked with TFE-flourocarbon which was extracted by the addition of 100ml of petroleum ether, 10ml of sodium chloride saturated solution along with 500ml of deionised water. This procedure was repeated twice after the recovery of the organic layer in the separating bottle and the extracts of the first and second extraction combined. The extract was washed twice with 100ml of the deionised water.

The combined extract was dried by pouring through a drying column containing a 10-cm column of anhydrous sodium sulphate (previously rinsed with methylene chloride), and the filtrate which was concentrated in the concentrator flask was rinsed with a stream of nitrogen. The wall of the concentrator flask was rinsed with Methyl tert-butyl ether (MTBE) as to bring the final volume of the extract to 5.0ml.

The clean-up of the concentrated extract was done by diluting with MTBE and later concentrated to 2ml followed by packing the column with florisil. Gas Chromatography with Pulsed Flame Photometric Detector (PFPD) with the following conditions for the analysis as stated below was used:

Analysis of Lambda-cyhalothrin using GC: The condition of the HP 5890/6890 Powered with HP Chem Station Rev. A 09. 01 [1206] Software

Injection Temperature	Split Injection
Split ratio:	20:1
Carrier Gas	Hydrogen
Flow Rate	1.0ml/min
Inlet Temperature	250^{0}C
Column Type	HP 5MS
Column Dimensions	30m x 0.25mm x 0.25µm
Oven Program:	Initial @ 80^{0}C for 1minutes First Ramp @ 12^{0}C/min to 250^{0}C Second Ramp @ 15^{0}C/min to 310^{0}C constant at 1mins
Detector:	PFPD
Director Temperature:	320^{0}C
Hydrogen Pressure:	22psi
Compressed Air:	28psi

Heavy Metal Analysis: The heavy metal content in the *Lactuca sativa* leaves were determined using the Atomic Absorption Spectrophotometer (AAS) Perkin-Elmer atomic model Analyst 300 machine using an air-acetylene fuel rich flame with hollow cathode lamps. A slightly modified method (as detailed below) according to AOAC (2000) was adopted for all the heavy metals tested.

Procedure: Two grammes (2.0g) of sample was weighed and incinerated to a white ash at 550^{o}C in a muffle furnace for three hours and thereafter cooled at room temperature in a desiccator. The ash was washed into 250 ml beaker with 30 ml of concentrated trioxonitrate (v) acid (HNO_3) to solubilize the ash.

The sample solution was evaporated to dryness on hot plate magnetic stirrer placed in a fume cupboard and the residue was further heated for 30 minutes. Thereafter, the sample was dissolved in 40 ml of concentrated hydrochloric acid (HCl) and digested for about 2 hours on hot plate magnetic stirrer. One ml (1.0ml) of diluted hydrochloric acid solution was further added to the sample and boiled for about 1 hour, then filtered while hot using Whatman No. 4 filter paper, and washed with HCl solution and the solution was made up to a volume of 100ml with distilled water. The sample solution was aspirated into the instrument after all the necessary set up and standardization procedures had been made.

Sample and standard solutions were aspirated and the main signal responses were recorded at each element's respective wavelength. The concentration of each element was thereafter calculated as follows:

$$Conc. = \frac{Conc\ Std\ x\ Abs\ Sample\ 100\ x\ DF}{Abs.Std\ x\ Wt\ Sample}$$

Conc. Std = Conc. of standard; Abs Sample= Absorbance of sample; Abs Std = Absorbance of standard; Wt sample = weight of sample; DF = Dilution factor

Experimental Design and Statistical Analysis of Data:The experiment was set up in a Complete Randomized Design (CRD) pattern of five replicates per Treatment. Samples collected from each of the five replicates were bulked together and thereafter divided again into five samples that were taken into the laboratory for analysis.

Data were collected on the amount of Lambda-cyhalothrin and heavy metal residues in the leaves of the *L. sativa* plants. Data for each parameter were the mean value from five replicates.

Data so obtained were subjected to a one way analysis of variance - ANOVA with Tukey HSD Test for differences between means at 99% confidence interval ($P \leq 0.01$) using Statplus 2009 professional 5.8.4 statistical software.

RESULT AND DISCUSSION

Lambda-cyhalothrin residue in L. sativa leaves: The results for the above stated parameter as presented in Table 1 show that the highest mean level of Lambda-cyhalothrin residue in the leaves was found in Treatment A (1.50 mg/kg), a value which was significantly higher (P≤0.01) than the value found in Treatment B (1.0 mg/kg). The Lambda-cyhalothrin residue for Treatment C (.02 mg/kg) was significantly lower (P≤0.01) than what was obtained for Treatments A and B.

Table 1: Mean value of lambda-cyhalothrin residue in *L. sativa* leaves for each Treatment

Treatment	residue (mg/kg)
A	1.5^{c}
B	1.0^{b}
C	0.02^{a}

Treatment mean values bearing different superscripts are significantly different at P ≤ 0 .01

Heavy metal residue in L. sativa leaves: The results as presented in Figure 1 show that the mean value of Pb in Treatment A was highest (0.298mg/kg). This was significantly higher (P≤0.01) than what was obtained for Treatment B (0.221mg/kg). In addition Treatment B had a value which was significantly higher (P≤0.01) than Treatment C (0.093mg/kg).As shown in Figure 1 below, the highest mean value 0.133 mg/kg of arsenic was obtained for Treatment B. Treatment A had the lowest mean value of (0.073mg/kg) compared to the other two Treatments. Treatment C however recorded a mean value of 0.078mg/kg for arsenic. There was a significant difference (P≤0.01) among the three Treatment means. Treatment A had the highest mean value of 0.232mg/kg, a value which was significantly higher (P≤0.01) than what was obtained for Treatment B (0.174mg/kg). In addition Treatment B had a value which was significantly higher (P≤0.01) than Treatment C (0.131mg/kg). Cadmium in Treatment A had the highest mean value of 0.055mg/kg, a value which was significantly higher (P≤0.01) than what was obtained for both Treatments B and Treatment C which had the same mean values of 0.024mg/kg (Figure 1).

Traces left by pesticides on treated products are otherwise referred to as residues. In other word, they are deposits of pesticide degradation product, its metabolite or the active ingredient present after its deliberate application or inadvertent spillage in a place or on a product. Maximum residue level (MRL) on the other hand is the highest level of a pesticide residue that is legally tolerated in or on food or feed when pesticides are applied in tandem with GAP (EC, 2005). Mankind, for several good reasons, has been keenly interested in monitoring and keeping to as low as reasonably possible (ALARP) the pesticide and associated heavy metal residues associated with the seeming inevitable use of pesticides on agricultural produce and food.

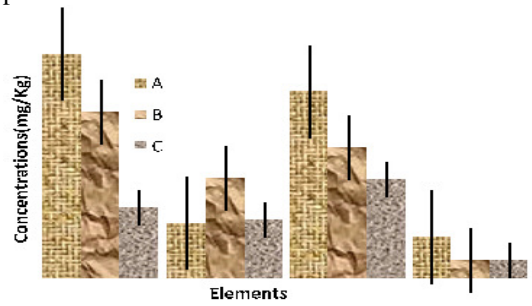

Fig 1: Heavy metal residuesLactuca sativa leaves

In the present study, the amount of Lambda-cyhalothrin residue detected in samples of Treatments B and C were within the maximum residue level(MRL) requirements of 1 mg/kg set in legal EU regulation for lettuce (EC, 2005 and Szpyrka *et al*, 2015). The Lambda-cyhalothrin residue detected in Treatment A was however above this EU MRL regulation for lettuce. That the mean Lambda-cyhalothrin residue was effectively reduced to acceptable MRL in samples of Treatment B relative to those of Treatment A was clearly evident of the effectiveness of the *A. niger* as Lambda-cyhalotrin remediating agent. This result from the present study aligns with the findings of Oliveira *et al*. (2015) who reported *Aspergilli* species such as *A. fumigatus* and *A. terreus* as being capable of degrading the pesticide chlorfenvinphos. In another study, Lone and Wani (2012) reported the ability of *A. niger* and *Trichoderma koningii* at degrading pyrethroids more effectively than some other fungal species such as *Penicillium notatum* and *A. terricola.* In terms of Lambda-cyhalothrin associated heavy metal burden in the *L. sativa* leaves, the Pb, Cr and Cd burdens reported in this workshow that the application of *A. niger* as a mycoremediation agent on Lambda-cyhalotrin caused a significant reduction in themean concentration of all the heavy metals investigated (except As) translocated into the *L. sativa* leaves. The results from this study thus agrees with the findings of Iskandar *et al.*(2011), who reported filamentous fungi as being capable of accumulating significant amount of heavy metals from their environment. In that study, Iskandar *et al.* (2011) singled out *A. niger* in comparison to all the other fungi investigated, as showing the highest uptake capacity for Pb. It should be noted however, that the mean concentration of Pb recorded in the present study for all the Treatment samples was lower than the WHO/FAO safe limit of

0.30mg/kg in vegetables (CODEX STAN 193-1995). Result for Cd from the present study shows that *A. niger* was able to bring about a significant reduction ($p \leq 0.01$) in the mean cadmium concentration in the *L. sativa* leaves, thus pointing to the ability of this fungus to bioaccumulate or biosorp this heavy metal from whatever environmental matrices it is found. This finding correlates with the results of Tsekova *et al*. (2010) and Shoaib *et al*. (2011) who both reported the rapid and efficient ability of *A. niger* cells at removing Cd from single ions solutions. The cadmium concentrations for all the samples were lower than the WHO/FAO safe limit of 0.20mg/kg (CODEX STAN 193-1995). Although the mean concentration of As in all the Treatment samples was lower than the WHO/FAO safe limit of 0.01mg/kg in vegetables (CODEX STAN 193-1995). The result from the present study nevertheless suggests that *A. niger* was not able to bring about a reduction in the mean As concentration in the Treatment samples. Although fungal biosorption of heavy metals has been reported to be dependent on some parameters such as pH, metal ion and biomass concentration, physical or chemical pre-treatment of biomass, and presence of various ligands in solution and to a limited extent on temperature (Kapoor and Viraraghavan, 1995; Maheswari and Murugesan, 2009), none of which was investigated in the present study. The aforementioned notwithstanding, the finding for As in this study appears to be in conflict with reports from similar studies (Kapoor and Viraraghavan, 1995; Maheswari and Murugesan, 2009), for reasons that are yet to be readily obvious. The mean concentration of Cr in Treatment B samples is indicative of the fact that *A. niger* was effective as a mycoremediation agent at bringing about a significant reduction in the amount of Cr translocated into the leaves of *L. sativa*. This finding agrees with Dursun *et al*. (2003) and Zhao *et al*. (2009) where they reported the pollutant removal capacity for Cr by *A. Niger, Pseudomonas aeruginosa* and *Mirococcus yunnanensis* respectively. The concentrations of Cr for all the samples were lower than the WHO/FAO safe limit of 1.30mg/kg in vegetables (CODEX STAN 193-1995).

Conclusion: The use of Lambda-cyhalothrin even under GAP appears to be associated with the danger of the deposition of this pesticide residue above the MRL. Results from the present study clearly positions *A. niger* as a very effective bioremediation agent capable of effectively reducing Lambda-cyhalothrin residue in *L. sativa* to levels below the MRL. Also, the use of Lambda-cyhalothrin on *L. sativa* under GAP does not pose any risk nor threat of the deposition of heavy metals above the WHO/FAO recommended safe limit on vegetables.

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