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HUMORAL DEFENSE RESPONSE OF EARTHWORM *Lumbricus terrestris* AGAINST HERBICIDE; NOMINEE

Abd-Allah, Salwa M.

Central Lab of Agricultural Pesticides (CAPL), Agricultural Research Center, Ministry of Agricultural, Alexandria, Egypt.

ABSTRACT

Earthworms are common test organisms in terrestrial ecotoxicology. Continuous use of chemicals leads to loss of soil fertility and soil organisms. The susceptibility of the earthworm *Lumbricus terrestris* for exposure to the commercial herbicide (Nominee) over 14 days was explored and resulting toxicological endpoints were recorded. Furthermore, earthworms were exposed to sub – lethal doses; 10.666 mg kg^{-1} and 34.554 mg $kg^{-1}$of nominee for 7 days based on the EC_{50} value, and a control was maintained. Analysis of protein content in earthworm plasma disclosed that the whole protein content decreased in a dose/time dependent manner. Assessment of protein quality via electrophoresis of plasma proteins and analysis of amino acid profile exhibited marked changes in peptide position, number and consequently function particularly after 24 hr of exposure to lower dose meanwhile, both levels of treatment resulted in a marked suppression of protein synthesis. SDS-PAGE showed appearance of small number of peptides in case of exposure to lower dose which may be attributed to hyper-synthesis of sulfur-containing proteins with low molecular weights (<10 KDa). Exposure to nominee at tested concentrations led to a marked effect on amino acid composition and the pattern revealed the dominance of hydrophobic, acidic and basic amino acids which may reflect the nature and structure of such proteins. Isozyme analysis of earthworm protease as an essential component of humoral response, showed marked variations in isozyme number and activity between control and exposed earthworms. The inducing effect of proteases due to nominee treatment may be considered as a stress protein acts as a protective agent of earthworm cells against environmental stressors.

INTRODUCTION

Earthworms are important model soil macro-invertebrates used to assess the general impact of pesticide pollution in soil (Menzie *et al*., 1992). Earthworms feed, cast and burrow in soil and are exposed to soil contaminants via their intestine or skin (Morgan and Morgan 1999). The widely used OECD-Earthworm-Toxicity-Test comprises both an acute (International Standard ISO 11268-1, 1993) and a reproduction tests

(International Standard ISO 11268-2, 1998). Endpoints in earthworm eco-toxicology scheduled in guidelines are mortality and reproduction rates. However, not only the direct influence of pollutants on population parameters but also changes in the biological and immunological functions can have an important impact on soil ecosystems.

Several pesticides have a detrimental effect on earthworms and toxicity varies widely among types of pesticides (insecticides, fungicides, herbicides, nematicides, fumigants, and vermicides). Historically, many studies have evaluated the toxic effects of pesticides on earthworm populations (Thompson, 1971; Randall *et al*., 1972; Benz and Altwegg, 1975; Stenerson, 1979; Inglesfield, 1984; Lee, 1985; Roberts and Dorough, 1985; Potter, 1990; Aly, 2005; Yasmin and D'Souza, 2007; Zhou *et al*., 2008; Pereira *et al*., 2009 and Xu *et al*., 2011). It was realized that fumigants, carbamates and vermicides are the most extremely toxic pesticides to earthworms and most other soil organisms. Herbicides, at the other extreme, pose relatively little threat of earthworm toxicity however, earthworms were found to directly influence the persistence of herbicides in soil by metabolizing a parent compound in their gut, by transporting herbicides to depth and increasing the soil bound (non extractable herbicides) fraction in soil or by absorbing herbicide residues in their tissues (Gobi and Gunasekaran, 2010). Literature have rare information that discussing the toxic impacts of nominee on earthworm; *Lumbricus terrestris*.

The effect of a pesticide on earthworms may be immediate or acute resulting in the death of the worm. Conversely, this impact may also be sub-lethal or chronic whereby a reduction in reproductive capabilities may occur, or a decreased functioning in life-supporting behaviors such as food-getting may be realized. Enzymes, such as oxidoreductases (e.g., superoxide dismutase, catalase), glutathione-S-transferase, acetylchloinesterase in earthworm are regarded as fast and prognostic indices of individual reaction to the environmental stress (Saint-Denis *et al*., 1998&1999; Booth *et al*., 2000 and Loureiro *et al*., 2005).

Earthworm defense system against different environmental stressors comprises two essential components; their innate immune system and protective proteases. The earthworm immune system consists of two major components, humoral and cellular (Bilej 1993a, 1993b; Cooper and Roch 1993). Cellular defense mainly involves the activity of free coelomocytes, macrophages and leukocytes. The coelomic fluid of earthworms contains cells and many molecular components involved in innate immunity of these species. Lysozyme and multifunctional proteins released by leukocytes and chloragogen cells are part of the humoral systems (Roch 1979; Kauschke and Mohring 1987; Bilej *et al*., 1995). Further humoral components phenoloxidase, proteases and other enzymes are mainly involved in the elimination of foreign material (Kauschke, 2007). Earthworm proteases are multicomponent and different species of earthworms have different resultant protease isozymes with different molecular masses (Mihara *et al*., 1983; Lu *et al*., 1988 and Zhou *et al*., 1988). Thus, one isozyme may have multiple names. Proteases were located in circulating earthworm leucocytes (Roch *et al*., 1991; Leipner *et al*., 1993). Serine protease activity has been reported

earlier in *Lumbricus terrestris* (Leipner *et al*., 1993; Kauschke *et al*., 1997). According to observations, serine proteases are usually stored inside the cells and released upon cell activation via environmental stressors (RedOrbit, 2004) reflecting the role of proteases in cytotoxic cascade and immune competence. Many metabolic cascades are regulated by the protease/anti-protease equilibrium. In *Lumbricus rubellus*, six fibrinolytic enzymes were isolated with different molecular masses and having more asparagine and aspartic acid residues (Mihara *et al*., 1983&1991; Nakajima *et al*., 1993& 2005).

The current study aims to (1) assess the toxicity of nominee herbicide upon earthworms, (2) evaluate some toxic effects upon exposure to sub-lethal doses and (3) introduce a comprehensive image about humoral defense mechanisms in earthworm due to exposure.

MATERIALS AND METHODS

Earthworms

Adults of earthworms [*Lumbricus terrestris* (*Oligochaeta, Lumbricidae*)] were obtained through a commercial biological supply and maintained in a moistened peat moss at 20 °C without light for 14 days prior to experimentation. Soil fertility test was firstly determined to identify fertility needs and can help to maintain healthy turf.

Nominee exposure

After acclimation, worms were exposed to nominee (2, 6- bis[(4,6-dimethoxy-2-pyrimidinyl)oxy]benzoic acid, CAS No.: 125401-92-5), 2% SL (supplied via KZ Company for Chemicals& Pesticides). Precisely, different concentrations (50-150 ppm) in deionized water were mixed with appropriate amount of artificial soil (450 g; 10% peat, 20% kaolin, 70% sand) in plastic cups. Five worms (300-600 mg) were distributed in each cup and lids were adjusted loosely onto cups and then incubated for 7 days according to official procedures of acute toxicity test (OECD, 1984). Controls were identically prepared except that earthworms were not exposed to nominee.

Treatment regimen

Earthworms were subjected to sub-lethal concentrations (1/3 and 1/10 EC_{50}). Different time intervals post-exposure (0, 1, 3 and 7 days) were tested for exploring toxicity due to contact and potential ingestion. Three cups for each time containing 5 worms per cup were prepared. Prior to cell harvesting, earthworms were placed on wet cotton wool, allowing defecation, to avoid contamination during cell harvesting. Plasma cells representing each time were pooled in eppendorf tubes containing a film of phenylthiourea to prevent blood oxidation, then kept in deep freeze until subsequent analyses.

Protein pattern distribution

Protein concentration in pooled plasma cells was estimated in aliquots of diluted samples (1:200) according to Lowery *et al*. (1951). Plasma proteins were separated by native-PAGE (Hames and Rickwood,1981) and SDS-PAGE on 10% polyacrylamide gels, topped by 4% stacking gel

according to Lammeli (1970) using the Mini-Protean 3 (Bio-Rad). Gel electrophoretic bands were analyzed for protein intensities using Total Lab software program, version X.

Amino-acid composition

Plasma samples (50mg protein/ ml) were subjected to acid hydrolysis (according to Ozolos, 1990) by addition of 6N-HCl and 2-Mercaptoethanol followed by heating at 110 C for 18 Hr. Hydrolyzates were dried and redissolved in citrate buffer (pH 2.2), filtered and injected into amino acid analyzer (A Beckman- Coulter Porton LF 3000G). Amount of mg a.a/100 ml hydrolyzate was estimated.

Protease activity

Separating native-PAGE (10%) containing gelatin (1%) as a substrate (Heussen and Dowdle, 1980) was used for analyzing proteases present in plasma samples (5 µL). Proteolytic activity was expressed as a zone of clearance in a dark blue background (Cheesman, 1963**).**

Statistical analysis

The 7 days EC_{50} and respective 95% confidence limits were analyzed by Probit analysis (Finney, 1971). Data of protein content were analyzed by using SPSS Student's t-test and presented as mean ±SD. Values of $p< 0.05$ were considered as statistically significant. Gel electrophoretic bands were analyzed for protein intensities using Total Lab software program.

RESULTS AND DISCUSSION

Short-term toxicity assessment: The susceptibility measurement of nominee-contaminated soil on earthworm over 14 days was performed and toxicity endpoints were represented in Table (1).The higher slope of regression line (>1) suggested a greater homogeneity among the tested worms in terms of their responses to the tested herbicide. No mortality rates were detected in the control which means that the results obtained by the toxicity test were reliable. EC_{50} value of nominee on *Lumbricus terrestris* earthworm under our lab conditions recorded a value of 106.661 mg Kg-1 although it was reported earlier that worm NOEL (14 d) for *Eisenia foetida* was > 1000mg Kg-1 soil (Chem-OnLine, 2012)

Table (1): Toxicological endpoints of nominee 2% SL on earthworm after 14 days of exposure.

Organism	EC values (mg/Kg soil)	95% Confidence limits	Slope	Regression equation	Variance
Lumbricus terrestris	EC_5= 43.629	(36.778- 51.739)	4.237	Y=4.237X-8.592	0.169
	EC_{50}=106.661	(99.784-114.015)			
	EC_{95}= 260.756	(214.715-316.792)			

Protein-pattern distribution: Analysis of protein content in plasma of earthworm exposed to nominee-treated soil; at different doses and different time intervals; disclosed that the whole content of protein is decreasing in a time/ dose dependent manner. Data were expressed as percent of control as

shown in Table (2). Protein content was significantly declined after 7 days of exposure to both tested doses.

Table (2): Protein content (mg/ml) in earthworm plasma after different treatment regimen of nominee. Data are expressed as mean± SD.

Treatment	Time interval (Days)	Protein content (mg/ mL)	% of Control
1/10 EC_{50} value	0- time	35.134± 0.315	120
	1	24.082± 0.497	82
	3	20.783± 0.578	71
	7	17.814± 0.210	61
1/3 EC_{50} value	0- time	26.804± 0.731	92
	1	35.381± 0.512	121
	3	20.206± 0.357	69
	7	9.319± 0.298	32

Protein quality describes characteristics of a protein in relation to its ability to achieve defined metabolic actions. Gel Electrophoresis of earthworm plasma proteins disclosed huge structural proteins (3^{rd} structure) which are stable by nature and are suggested to play central roles in many biological systems including humoral immune system against different environmental stressors. Native-PAGE patterns of control earthworm proteins showed appearance of 8 peptide bands differed in their migration positions (Fig. 1A & Table 3); By treatment with 1/3 EC_{50} (35.555 mg Kg-1), two peptide bands (indicated by small arrows) were disappeared from the gel at zero time of exposure (i.e. prompt response at the beginning of exposure). The same pattern was noticed after1, 3 and 7 days of exposure. After 24 hr of exposure, 3 other minor faster peptides were appeared (I, J, K).

Furthermore, protein patterns showed a similar trend after exposure to lower dose (10.666 mg Kg-1) where marked changes in peptide number and positions particularly after 24 hr of exposure (as indicated by small arrows). Additionally, other changes were recorded in plasma samples collected after 3 and 7 days of exposure. Based on the above results, it can be concluded that both levels of treatments resulted in a marked suppression of protein synthesis particularly low molecular weight polypeptides.

It is important to mention here that preliminary results from invertebrates suggest that high specific growth rates may be achieved by relatively low rates of protein turnover (Houlihan, 1991), which can be divided into 3 processes; protein synthesis, protein growth and protein degradation. At any particular time (either under environmental stress or not), protein growth (as a percentage of the total protein mass) is the net balance between protein synthesis and protein degradation.

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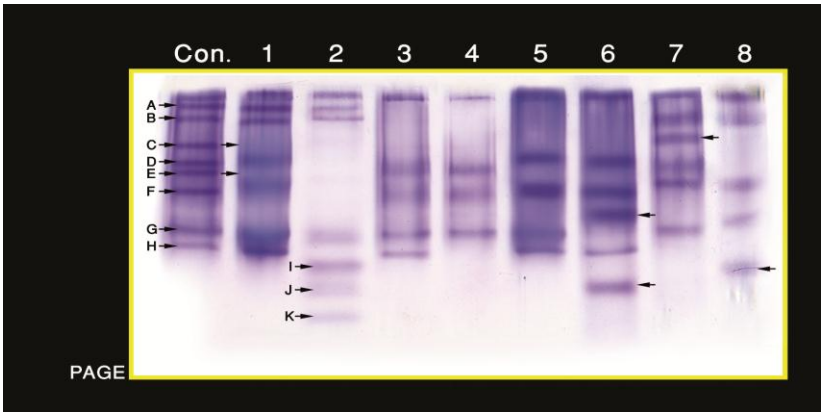

Fig (1a): PAGE- electrophoretic pattern (T%= 10%) of plasma proteins of earthworms exposed to nominee. Lane con represents control, Lanes 1-4 represent plasma proteins at o-time,1,3, 7 days of exposure to 1/3 EC_{50} of nominee. Lanes 5-8 represent plasma proteins after o-time,1,3, 7 days of exposure to 1/10 EC_{50} of nominee.

Additionally faint bands observed in SDS-PAGE (Fig. 1B) supported such evidence and highlighted the possibility of gene suppression and consequently low protein synthesis. On the other hand, appearance of small number of peptides in case of treatment with lower dose compared with those appeared in case of treatment with higher dose may be attributed to hyper-synthesis of sulfur-containing proteins (may be due to gene mutation) which may be fragmented into very small peptides (<10 KDa) due to SDS treatment or disappeared into the gel (10%).

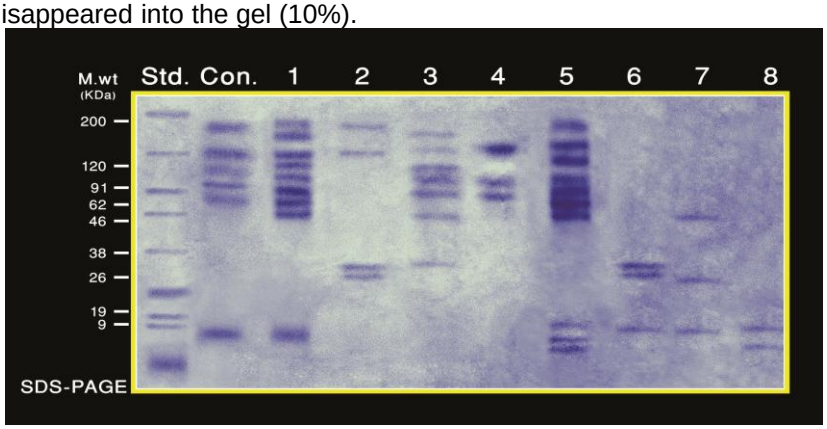

Fig (1b): SDS-PAGE pattern of plasma proteins of earthworms exposed to nominee. Lane con represents control, Lanes 1-4 represent plasma proteins at o-time,1,3, 7 days of exposure to 1/3 EC_{50} of nominee. Lanes 5-8 represent plasma proteins after o-time, 1, 3, 7 days of exposure to 1/10 EC_{50} of nominee.

Our data are matching with the recent finding that certain types of proteins can be secreted upon exposure to different pollutants where stress protein response (Hsp70) was detected in *Eisenia fetida*, *Aporrectodea caliginosa* and *Lumbricus terrestris* when exposed to imidacloprid (Dittbrenner *et al*., 2012) however, hsp70 was not a good indicator of imidacloprid toxicity given the low induction for the selected test species.

Amino-acid profile

Amino acids are the building blocks of proteins and the amino acid pattern of the whole animal is mainly determined by the pattern of body proteins (Mente *et al*., 2002). Assessment of protein quality by amino acid scoring is reflecting a complex adaptive response to varying intakes of protein and amino acids. Table (4) shows amino acids composition of plasma samples of earthworms exposed to nominee compared with control samples. Data exhibited that in both control and examined samples, hydrophobic, acidic and basic amino acids constitute the major composition. On the other hand, sulphur- containing amino acids constitute the lowest ratio particularly for methionine. For control, hydrophilic amino acids comprise 11.27% versus 28.96% for hydrophobic amino acids. Basic amino acids represented 21.14% versus 27.34% for acidic amino acids while aromatic ones represented 10.47% of total amino acids.

When earthworms were exposed to nominee, different patterns of amino acid distribution were recorded. No significant increase in acidic amino acids after exposure however, after 1 day of exposure to lower dose, a significant increase was recorded (aspartic& glutamic were increased). Contrarily, basic amino acids decreased slightly after exposure. A pronounced decrease was noticed after 1 day of exposure to high dose (15.81% with particular decline in histidine) and to lower dose (11.64% with particular decline in lysine). Exposure has led to a significant increase in SH-amino acids percent after 1, 3 and 7 days. The effect of high dose was more remarkable particularly after 3 days of exposure (2.14% with particular surge in methionine).

An irregular trend was recorded in pattern of hydrophilic amino acids but a significant increase was recorded after 1 day of exposure to both doses (15.29, 16.41%; respectively with particular increase in serine) as compared with control. Hydrophobic amino acids decreased significantly after 1 day of treatment with low dose (22.23% with particular increase in glycine accompanied with decrease in content of alanine, proline, valine, leucine& isoleucine). As related to aromatic amino acids, exposure to lower dose for 1 day caused significant decrease (5.60% with particular decline in phenyalanine& tyrosine) compared with the higher dose.

Table (4): Amino-acid composition in plasma samples of earthworm (*Lumbricus terrestris*) exposed to nominee.

Amino acid (a.a.)	Control	Percentage/ time interval							
		Exposure to 1/3 EC_{50}				Exposure to 1/10 EC_{50}			
		0-time	1 day	3 days	7 days	0-time	1 day	3 days	7 days
Acidic a.a	**27.34**	**28.32**	**25.65**	**27.83**	**27.66**	**28.66**	**42.33**	**26.81**	**26.58**
Aspartic	14.59	14.71	12.50	14.89	16.11	13.93	21.59	14.30	13.55
Glutamic	12.75	13.61	13.15	12.94	11.55	14.73	20.74	12.51	13.03
Basic a.a.	**21.14**	**20.07**	**15.81**	**20.58**	**20.21**	**22.45**	**11.64**	**20.78**	**15.90**
Lysine	6.78	7.15	5.57	6.54	4.86	9.59	2.96	8.64	5.30
Arginine	7.29	5.43	5.46	7.31	8.36	7.23	4.87	6.63	5.73
Histidine	7.07	7.49	4.78	6.73	6.99	5.63	3.81	5.51	4.87
SH-containing a.a	**0.89**	**1.03**	**1.83**	**2.14**	**1.67**	**0.84**	**1.79**	**1.34**	**1.39**
Cystiene	0.74	0.89	1.29	0.91	0.61	0.73	0.63	1.04	1.04
Methionine	0.15	0.14	0.54	1.23	1.06	0.11	1.16	0.30	0.35
Hydrophilic a.a.	**11.27**	**8.66**	**15.29**	**9.00**	**10.94**	**7.54**	**16.41**	**12.95**	**12.60**
Serine	7.07	5.64	9.69	5.57	7.45	4.93	10.48	7.89	7.21
Theronine	4.20	3.02	5.60	3.43	3.49	2.61	5.93	5.06	5.39
Hydrophobic a a.	**28.96**	**30.71**	**29.28**	**29.72**	**28.88**	**28.84**	**22.23**	**28.15**	**31.18**
Glycine	4.86	4.67	5.68	4.59	3.80	4.07	8.89	5.14	5.99
Alanine	6.71	7.56	7.61	7.06	5.47	7.12	3.28	7.59	7.38
Proline	1.25	1.17	0.93	1.17	0.46	1.01	1.69	1.19	1.22
Valine	3.98	4.60	5.68	3.95	5.47	4.48	2.65	4.77	5.82
Leucine	8.62	9.07	6.25	9.84	10.03	8.23	4.13	6.85	7.47
Isoleucine	3.54	3.64	3.13	3.11	3.65	3.93	1.59	2.61	3.30
Aromatic a.a.	**10.47**	**11.27**	**11.74**	**10.88**	**10.33**	**11.71**	**5.60**	**10.05**	**12.25**
Phenyl alanine	6.34	7.42	5.71	7.06	6.99	7.85	3.70	6.40	5.13
Tyrosine	4.13	3.85	6.03	3.82	3.34	3.86	1.90	3.65	7.12

According to the above results, it can be concluded that exposure of earthworms to nominee at both tested concentration levels, led to marked effect on amino acid composition pattern. Effect of exposure to lower dose was more pronounced than the higher one. Meanwhile, the amino acid pattern of earthworm plasma proteins revealed the dominance of acidic and hydrophobic amino acids which may reflect the nature and structure of such proteins and their roles in defense mechanisms in earthworm.

Comparatively, our study identified17 amino acid in earthworm plasma which is matching with other study that reported 9 essential amino acids namely, lysine, histidine, arginine, threonine, valine, methionine, isoleucine, leucine and phenylalanine and 8 non-essential amino acids namely, aspartic acid, serine, glutamic acid, proline, glycine, alanine,cystine and tyrosine, analyzed in 4 earthworm species from Nigeria (Dedeke *et al*., 2010). In another study, it was found that in *L. rubellus*, the highest essential amino acid of earthworm was dominated by histidine (0.63% of dry matter basis) (Istiqomah *et al*., 2009). No comparable studies dealing with the effect of pesticides on amino acid profile in earthworm were found in the literature.

Proteolytic activity

Protease isozyme forms in earthworm samples using native-PAGE are shown in Fig (2). Enzyme patterns showed marked variations in protease isozymes number and activity between control and samples of exposed earthworms. No activity of proteases was recorded at all in control samples

either at 0-time or after 7 days, while a marked protease activity was exhibited after exposure to nominee at both tested concentrations. Protease activity varied considerably between high and lower doses as well as among different time intervals of each dose. After treatment with high dose, it was noticed that at zero-time, three isozyme forms were found, and their number was increased to seven isozymes after 24 hrs (as indicated by small arrows). After 3 and seven days of exposure, only one isozyme form was found (spreaded band with no sharp end). Comparatively, after exposure to lower dose, only one isozyme form was recorded at zero-time, which increased to seven isozymes followed by significant decrease to one isozyme form at day 3 but the enzyme activity increased again after 7 days of treatment (many diffused bands appeared).

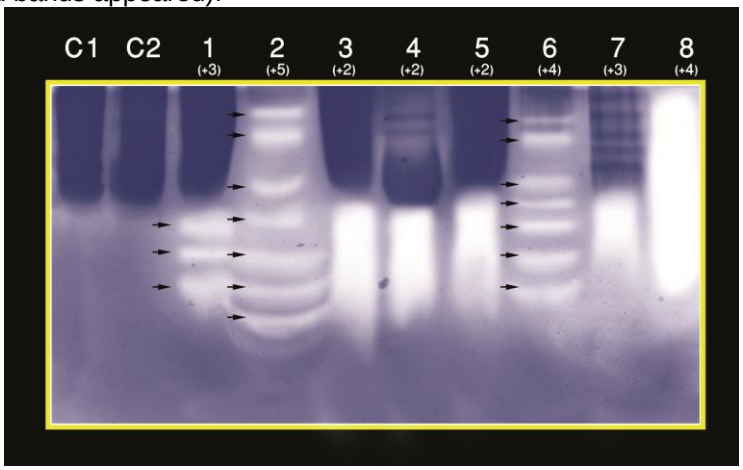

Fig (2): Proteases *isozyme* pattern based on native-PAGE (10%) of earthworm plasma samples taken after nominee exposure. Clearance zones indicate protease activity. Lanes C1, C2 represent control samples at 0-time and after 7 days of experiment initiation; lanes 1-4 represent enzyme activity after exposure to 1/3 EC_{50} at 0-time, 1d, 3d and 7d;respectively; lanes 5-8 represent enzyme activity after exposure to 1/10 EC_{50} at 0-time, 1d, 3d and 7d;respectively.

Based on the above results, it can be concluded that there were slight differences in the effect of low and high dose of nominee. Both levels of treatment caused a pronounced effect on protease activity particularly after 24 hr of exposure. The inducing effect of proteases due to nominee treatment may be considered as a stress protein acts as a protective agent of earthworm cells. Such enzyme was found to comprise many isoforms and the number of these isoforms was affected by the extent of exposure.

Comparative studies showed that intracellular protease activity was increased after Arochlor and carbaryl exposures and the stimulating effect was being evident with the lowest dose of carbaryl (0.1 pg/ cm). Also, protease activity tended to increase with 2, 4 D and T2 toxin exposures, but the changes were statistically insignificant (RedOrbit, 2004). Additionally, it was revealed that

wounding as well as the injection of foreign material into the coelomic cavity increases coelomocyte numbers and the activity of some easily measurable humoral immune factors like proteases, agglutinins and lysins in several earthworm species (*Allolobophora chlorotica, Apporectodea caliginosa, Dendrodrillus rubidus, Eisenia fetida, Lumbricus rubellus, L. terrestris*), Moreover, newly synthesized proteins were identified within the protein pattern of coelomic fluid after challenging, suggesting their involvement in immune reactions (Kauschke, 2007). Differences in protease activity become obvious especially by comparing enzyme patterns of untreated and stimulated coelomic fluid samples separated by electrophoresis. An enhanced lytic activity was evident for some species after stimulating the immune response. Other literatures strengthened our findings where humoral and cellular immunodefense responses of the earthworms, *Eisenia fetida andrei, Eisenia hortensis, and Lumbricus terrestris,* have been affected after exposure to the PCB Arochlor 1254. Responses mediated by in vitro assays for lysozyme, hemolysis, and proteases, were increased and nonspecific cellular functions, including phagocytosis and those related to wound healing, decreased dramatically in all earthworms (Ville *et al*., 1995).

Taking all these facts it can be summarized that disturbance of the homeostasis in earthworm results in an altered immune response. Some of the measured proteins might be considered as suitable bio-markers in monitoring of environmental pollutants. Protease pattern and activity may assume to be promising candidates in this regard and are easy to analyze.

Conclusion

The current data exhibited that earthworm is a quite sensitive biomarker for soil contamination with nominee. Exposure of earthworms to sublethal doses for 7 days led to alteration of protein turnover as measured by suppression of protein synthesis, hypersynthesis of low M.wt of polypeptides (< 10 KDa) accompanied with altered amino acid profile. Additionally, protease enzyme as an important constituent of earthworm humoral immune response was found to comprise many isoforms and the number of these isoforms was affected by extent of exposure. An inducing effect on protease upon exposure was recorded after exposure to both doses. Such data may illustrate; to some extent, the affection of humoral mechanisms of earthworm immune system due to nominee exposure. Also, it is worthy to mention that there is a potential of earthworms to transfer pesticides to vertebrate predators and a secondary poisoning may occur. The potential of contamination of food chain (soil - earthworm - bird/mammal) should be emphasized through testing appropriate risk models.

REFERENCES

Aly, N.M. (2005). Earthworm as a biomarker for pesticide toxicity. Alex. Sci. Exch. J., 26: 229-335.

Benz, G. and Altwegg, A. (1975). Safety of *Bacillus thuringiensis* for earthworms. J. Inverteb. Pathol., 26: 125-126.

Bilej, M. (1993a). Humoral defense mechanisms. In: Velvicka, V.; Sima, P.; Cooper, E. L.; Bilej, M. and Roch, P. (eds.) Immunology of Annelids. CRC Press, Boca Raton, pp. 245-262.

Bilej, M. (1993b). Cellular defense mechanisms. In: Vetvicka, V.; Sima, P.; Cooper, E. L.; Bilej, M. and Roch, P. (eds.) Immunology of Annelids. CRC Press, Boca Raton, pp. 167-200.

Bilej, M.; Brys, L.; Beschin, A.; Lucas, R.; Vercauteren, E.; Hanusova, R. and De Baetselier, P. (1995). Identification of a cytolytic protein in the coelomic fluid of *Eisenia foetida* earthworms. Immunol. Lett., 45: 123-128.

Booth, L.; Heppelthwaite, V. and McGlinchy, A. (2000). The effect of environmental parameters on growth, cholinesterase activity and glutathione S-transferase activity in the earthworm (*Aporrectodea caliginosa*). Biomarkers, 5: 46-55.

Cheesman, G.C. (1963). Action of rennet and other proteolytic enzymes on casein in casein-agar gels. J. Dairy Res., 30: 17-22.

Chem-OnLine (2012). Nominee, http:// www.Chem-OnLine.org,

Cooper, E. L. and Roch, P. (1993). Immunological profile of annelids: Transplantation immunity. In: Vetvicka, V.; Sima, P.; Cooper, E. L.; Bilej, M. and Roch, P. (eds.) Immunology of Annelids. CRC Press, Boca Raton, pp. 201-244.

Dittbrenner, N.; Capowiez, Y.; Kohler, H. and Triebskorn, R. (2012). Stress protein response (Hsp70) and avoidance behaviour in *Eisenia fetida*, *Aporrectodea caliginosa* and *Lumbricus terrestris* when exposed to imidacloprid. J. Soils& Sediments, 12(2):198-206.

Dedeke, G.A.; Owa, S.O. and Olurin, K.B. (2010). Amino acid profile of four earthworm species from Nigeria. Agric. Biol. J. N. Am., 1(2): 97-102.

Finney, D.J. (1971). Statistical methods in biological assays. Second edition,Griffin, London.

Gobi, M. and Gunasekaran, P. (2010): Effect of butachlor herbicide on earthworm *Eisenia fetida:* Its histological perspicuity. Appl. Environ. Soil Sci., 4(1):1155-1163.

Hames, B.D. and Rickwood, D. (1981). Protein separation. In: Gel electrophoresis of proteins: A Practical approach. TRL, London, England, Publishing Co., pp. 34-37.

Heussen, C. and Dowdle, E.B. (1980). Electrophoretic analysis of plasminogen activators in polyacrylamide gels containing sodium dodecyl sulfate and copolymerized substrates. Anal. Biochem., 102: 196-202.

Houlihan, D.F. (1991). Protein turnover in ectotherms and its relationship to energetic. In: Advances in Comparative and Environmental Physiology, Vol. 7(ed. R. Gilles), pp.1-43. Berlin: Sprionger-Verlag.

Inglesfield, C. (1984). Toxicity of the pyrethroid insecticides cypermethrin and WL85871 to the earthworm, *Eisenia foetida* Savigny. Bull. Environ. Contam.Toxicol., 33: 568-570.

International Standard ISO 11268-1 (1993). Soil Quality: Effects of pollutants on earthworms (*Eisenia fetida*) – Part 1: Determination of acute toxicity using artificial soil substrate. International Organization for Standardization, Genf.

International Standard ISO 11268-2 (1998). Soil Quality: Effects of pollutants on earthworms (*Eisenia fetida*) – Part 2: Determination of effects on reproduction. International Organization for Standardization, Genf.

Istiqomah, L.; Sofyan, A.; Domayanti, E. and Julendra, H. (2009). Amino acid profile of earthworm meal (*Lumbricus rubellus*) for animal feedstuff. J. Ind. Trop. Anim. Agric., 34(4): 253-257.

Kauschke, E. (2007). Coelomic fluid proteins as basic components of innate immunity in earthworms. EarthwormDigest.Org, http : // www. wormdigest. org

Kauschke, E. and Mohring, W. (1987). Cytotoxic activity in the coelomic fluid of the annelid *Eisenia foetida*. Scan. J. Comp. Physiol., 157: 77-83.

Kauschke, E.; Pagliara, P.; Stabili, L. and Cooper, E. (1997). Characterization of proteolytic activity in coelomic fluid of *Lumbricus terrestris*. Comp. Biochem. Physiol., 116(B): 235-242.

Lammeli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. Nature, 227: 680-685.

Lowery, O.H.; Rosebrough, N.J.; Farr, A.L. and Randall, R.J. (1951). Protein measurement with the Folin phenol reagent. J. Biol. Chem., 193: 265-275.

Lee, K. E. (1985). Earthworms: Their ecology and relationships with soils and land. UsP, Australia: Academic Press.

Leipner, C.; Tuckova, L.; Rejnek, J. and Langner, S. (1993). Serine proteases in coelomic fluid of annelids *Eisenia fetida* and *Lumbricus terrestris*. Comp. Biochem. Physiol., 105: 637-641.

Loureiro,S.; Soares, A. and Nogueira, A. (2005). Terrestrial avoidance behavior tests as screening tool to assess soil contamination. Environ. Poll., 138: 121-131.

Lu, Y.; Jin, R.; Wu, Y. and Mang, X. (1988). The purification and characterization of fibinolytic enzymes from *Amynthas dancatala.* Chin. J. Bioch. Mol. Biol., 4: 166–172.

Mente, E.; Coutteau, P.; Houlihan, D.; Davidson, I. and Sorgeloos, P. (2002). Protein turnover, amino acid profile and amino acid flux in juvenile shrimp: Effects of dietary protein source. J. Exp. Biol., 205:3107-3122.

Menzie, C.A.; Burmaster, D.E.; Freshman, J.S. and Callahan, C.A. (1992). Assessment of methods for estimating ecological risk in the terrestrial component: A Case study at the Baird and McGuire superfund site in Holbrook, Massachusetts. Environ. Toxicol. Chem., 11:245-260.

Mihara, H.; Sumi, H. and Akazawa, K. (1983). Fibrinolytic enzyme extracted from the earthworm. Thromb. Haemost., 50: 258-267.

Mihara, H.; Sumi, H. and Yoneta, T. (1991). A novel fibrinolytic enzyme extracted from the earthworm, *Lumbricus rubellus.* Jap. J. Physiol., 41(3): 461–472.

Morgan, J.E. and Morgan, A.J. (1999). The accumulation of metals (Cd, Cu, Pb, Zn and Ca) by two ecologically contrasting earthworm species (*Lumbricus rubellus* and *Aporrectodea caliginosa*): Implications for ecotoxiclogical testing. Appl. Soil Ecol., 13: 82-86.

Nakajima, N.; Mihara, H. and Sumi, H. (1993). Characterization of potent fibrinolytic enzymes in earthworm, *Lumbricus rubellus.* Biosci. Biotech. Biochem., 57(10): 1726–1730.

Nakajima, N.; Sugimoto, M.; Tsuboi, S.; Tsuji, H. and Ishihara, K. (2005). An isozyme of earthworm serine proteases acts on hydrolysis of triacylglycerol. Biosci. Biotech. Biochem, 69(10): 2009–2011.

OECD (1984). Earthworm. Acute Toxicity Test. OECD Guideline for Testing of Chemicals. 207 Adopted: 4 April.

Ozolos, J. (1990). Amino acid analysis. In: Guide to protein purification. Murray, P.D. (ed.). Methods in Enzymology, vol. 182, Academic Press, London, pp. 587-601.

Pereira, J.L.; Antunes, S.C.; Castro, B.B.; Marques, C.R.; Goncalves, M.M.; Goncalves, F. and Pereira, R. (2009).Toxicity evaluation of three pesticides on non-target aquatic and soil organisms: Commericial formulation versus active ingredient. Ecotoxicol., 18: 455-463.

Potter, D.A. (1990). Toxicity of pesticides to earthworms (*Oligochaeta: Lumbricidae*) and effect on thatch degradation in Kentucky bluegrass turf." J. Econ. Entomol., 3: 2362-2369.

Randell, R.; Butler, J.D. and Hughes, T.D. (1972).The effect of pesticides on thatch accumulation and earthworm populations in Kentucky bluegrass turf. HortSci., 7: 64-65.

RedOrbit (2004). Earthworm immunity: A Model of immune competence. http://www.redorbit.com/earthworm.

Roberts, B.L. and Domugh, H.W. (1985). Hazards of chemicals to earthworms. Environ. Toxicol. Chem., 4:307-323.

Roch, P. (1979). Protein analysis of the earthworm coelomic fluid, I: polymeric system of the natural hemolysin of *Eisenia foetida andrei*. Develop. Comp. Immunol., 3: 599-608.

Roch, Ph.; Stabili, L. and Pagliara, P. (1991). Purification of three serine proteases from the coelomic cell of earthworm (*Eisenia fetida*). Comp. Biochem. Physiol., 98(B): 597-602.

Saint-Denis, M.; Labort, F.; Narbonne, J. and Ribera, D. (1998). Glutathione, glutathione-related enzymes, and catalase in the earthworm *Eisenia fetida andrei*. Arch. Environ. Contam. Toxicol., 35: 602-614.

Saint-Denis, M.; Narbonne, J.; Arnaud, C.; Thybaud, E. and Ribera, D. (1999). Biochemical responses of earthworm *Eisenia fetida andrei* exposed to contaminated artificial soil: Effects of benzo(a)pyrene. Soil Biol. Biochem., 31: 1837-1846.

Stenerson, J. (1979). Action of pesticides on earthworms. Part 1: The toxicity of cholinesterase-inhibiting insecticides to earthworms as evaluated by laboratory tests. Pest. Sci., 10: 66-74.

Thompson, A. R. (1971). Effects of nine insecticides on the numbers and biomass of earthworms in Pasture. Bull. Environ. Contam.Toxicol., 5: 577-586.

Ville, P.; Roch, P.; Cooper, E. L.; Masson, P. and Narbonne, J. F. (1995). PCBs increase molecular-related activities (lysozyme, antibacterial, hemolysis, proteases) but inhibit macrophage-related functions (phagocytosis, wound healing) in earthworms.
J. Invert. Pathol., 65(3): 217-224.

Xu,P.; Diao,J.;Liu,J. and Zhou, Z. (2011). Enantioselective bioaccumulation and toxic effects of metalaxyl in earthworm *Eisenia foetida*. Chemosphere, 5: 347-356.

Yasmin, S. and D'Souza, D. (2007). Effect of pesticides on the reproductive output of *Eisenia foetida.* Bull. Environ. Contam. Toxicol., 79:529-532.

Zhou, Y. C.; Zhu, H. and Chen, Y.C. (1988). The isolation and purification of earthworm fibrinolytic protease from *Eisenia fetida.* Acta Bioch. Biophy. Sin., 20: 35–42.

Zhou, S.P.; Duann, C.Q.; Wang, X.H.; Gi Michelle, W.; Yu, Z. and Fu, H. (2008). Assessing cypermethrin-contaminated soil with three different earthworm test methods. J. Environ. Sci., 20: 1381-1385.

الإستجابة الدفاعية الأولية لدودة الأرض *Lumbricus terrestris* ضد مبيد الحشائش نومينى

سلوى مصطفى عبدالله

المعمل المركزى للمبيدات- مركز البحوث الزراعية- وزارة الزراعة- الأسكندرية- مصر.

تعد دودة الأرض من الكائنات المختبرة شائعة الأستخدام فى مجال السمية البيئية البرية. ويؤدى الأستخدام المستمر للكيماويات الى فقد لخصوبة التربة ولكائناتها أيضا. وقد تم فى هذه الدراسة تقييم درجة تأثر دودة الأرض *Lumbricus terrestris* عند التعرض لمبيد الحشائش (نومينى) لمدة ١٤ يوم وقد تم تسجيل محددات السمية الناتجة. وبعد ذلك تم تعريض دودة الأرض الى جرعات تحت مميتة (١٠.٦٦٦ مجم/ كجم تربة)و (٣٥.٥٥٤ مجم/ كجم تربة) من النومينى اعتمادا على القيمة النصف مميتة وذلك لمدة ٧ أيام مع المحافظة على تواجد الكنترول للمقارنة. وقد أوضح تحليل كمية البروتين فى بلازما دودة الأرض أن المحتوى الكلى للبروتين قد أنخفض بنمط يعتمد على الجرعة ووقت التعرض. وأظهر تقييم نوعية البروتين بأستخدام الفصل الكهربائى لبروتينات البلازما و تحليل صورة الأحماض الأمينية تغييرات ملحوظة فى وضع الببتيدات وعددها وبالتبعية وظيفتها خاصة بعد مرور ٢٤ ساعة من التعرض للجرعة الصغيرة وخلال ذلك فقد أحدثت كلا الجرعتين أنخفاضا فى تصنيع البروتين. وقد أظهر تكنيك SDS-PAGE تواجد عدد صغير من الببتيدات فى حالة التعرض للجرعة الأقل مما قد يرتبط بزيادة فى تصنيع البروتينات المحتوية على الكبريت ذات الكتلة المنخفضة(< ١٠ كيلو دالتون). وقد أدي التعرض الى مبيد النومينى الى تأثير ملحوظ فى تركيب الأحماض الأمينية وأوضح نمط التأثير سيادية الأحماض الأمينية الحمضية والقلوية والغير محبة للماء مما قد يعكس طبيعة تكوين هذه البروتينات. وقد أظهر تحليل الأيزوزيم لأنزيم البروتيز (المحلل للبروتين) فى دودة الأرض (وهو مكون أساسى فى أحداث الأستجابة المناعية الأولية)- تغييرات ملحوظة فى عدد الأيزوزيم ونشاطها بين الكنترول والديدان المعرضة للمبيد. والتأثير المنشط لأنزيم البروتيز نتيجة التعرض لمركب نومينى يمكن أن يعد كبروتين ضغط أو أجهاد يعمل كعامل وقائى لخلايا دودة الأرض ضد الملوثات البيئية.

قام بتحكيم البحث

أ.د / على على عبد الهادى	**كلية الزراعة – جامعة المنصورة**
أ.د / فكرى ابراهيم الشهاوى	**كلية الزراعة – جامعة الاسكندرية**

Table (3): Protein distribution pattern in PAGE electrophoresis in earthworm plasma after exposure to nominee.

Control	Treatment/ time interval							
	High dose exposure				Low dose exposure			
	0-time	1 day	3day	7 day	0-time	1 day	3day	7 day
Protein A Bd%=7.45 Rf=0.10	**Protein A** Bd%=11.12 Rf= 0.11	**Protein A** Bd%=13.85 Rf=0.12	ND	ND	ND	ND	ND	ND
Protein B Bd%=10.04 Rf=0.15	**Protein B** Bd%=9.87 Rf= 0.16	**Protein B** Bd%=17.45 Rf=0.16	ND	ND	ND	ND	**Protein B** Bd%=20.66 Rf=0.16	**Protein B** Bd%=28.06 Rf=0.16
							Protein B2 Bd%=16.78 Rf=0.23	
Protein C Bd%=10.69 Rf=0.25	ND	ND	ND	ND	ND	ND	ND	ND
Protein D Bd%=11.81 Rf=0.32	**Protein D** Bd%=21.60 Rf=0.31	ND	ND	ND	**Protein D** Bd%=29.41 Rf=0.30	**Protein D** Bd%=23.21 Rf=0.32	**Protein D** Bd%=23.31 Rf=0.34	ND
Protein E Bd%=10.25 Rf=0.35	ND	ND	**Protein E** Bd%=35.77 Rf=0.35	**Protein E** Bd%=36.51 Rf=0.35	ND	ND	ND	ND
Protein F Bd%=20.77 Rf=0.43	**Protein F** Bd%=23.07 Rf=0.41	ND	**Protein F** Bd%=30.03 Rf=0.45	**Protein F** Bd%=32.67 Rf=0.44	**Protein F** Bd%=31.52 Rf=0.43	**Protein F** Bd%=22.14 Rf=0.43	**Protein F** Bd%=24.54 Rf=0.40	**Protein F** Bd%=31.14 Rf=0.41
						Protein F2 Bd%=28.89 Rf=0.52		
Protein G Bd%=18.02 Rf=0.58	**Protein G** Bd%=18.59 Rf=0.61	**Protein G** Bd%=21.64 Rf=0.61	**Protein G** Bd%=21.03 Rf=0.60	**Protein G** Bd%=30.82 Rf=0.59	**Protein G** Bd%=23.09 Rf=0.59	ND	**Protein G** Bd%=14.70 Rf=0.58	**Protein G** Bd%=20.42 Rf=0.53
Protein H Bd%=10.97 Rf=0.64	**Protein H** Bd%=15.76 Rf=0.66	ND	**Protein H** Bd%=13.44 Rf=0.68	ND	**Protein H** Bd%=15.98 Rf=0.65	**Protein H** Bd%=10.67 Rf=0.66	ND	ND
		Protein I Bd%=22.01 Rf=0.72						**Protein I** Bd%=20.38 Rf= 0.72
		Protein J Bd%=14.30 Rf=0.80				**Protein J** Bd%=15.09 Rf=0.80		
		Protein K Bd%=10.75 Rf=0.91						

Abbreviations: Bd%; band percentage, Rf; relative mobility or migration factor, ND; not detected