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Relative Toxicity of two Selected Fungicides on Acid Phosphatase and Alkaline Phosphatase activity of Epigeic Earthworm Eisenia fetida (Oligochaeta)

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Abstract
Eisenia fetida was exposed to two selected fungicides, carbendazim and captan. Previously determined LC_{50} value of the two fungicides were 5.38mg/kg soil and10.41mg/kg soil respectively. The specimens were exposed to sub lethal doses of both the fungicides, i.e. 25% of LC_{50} (T2) value and 50% of LC_{50} (T3) value, along with the control set (T1). The acute toxicity test i.e. 96 hours mortality test showed the carbendazim as more toxic over captan. The enzyme activity measured on the 28th day. The acid phosphatase activity inhibition was observed in both the fungicides in both the doses but in case of alkaline phosphatase activity in both the fungicides in both the doses was increased. The inhibition in the level of acid phosphatase may occur to encounter the stress situation and the changes in the alkaline phosphatase activity may be indicative of an adaptive rise.

Keywords Eisenia, Carbendazim, Captan, Sub lethal, Phosphatase.

Introduction
Dependence on agro-chemicals for enhancing productivity is a great concern. There are 60,000varieties of chemicals in use with several thousand being added annually [1]. Besides seeds, nutrients, water etc, use of pesticides including fungicides is indispensable. Alarming population growth throughout the globe necessitates more food and cash crops production results rapid growth of pesticide market [2]. In spite of their benefits, increasing trend of fungicide application has deleterious effect on human environment and agro-ecosystem. Regular use of fungicides can potentially pose a risk to the environment, particularly if residues persist in the soil or migrate off-site and enter waterways (e.g. due to spray drift, runoff) [3, 4, 5, 6]. If this occurs it could lead to adverse impacts to the health of terrestrial and aquatic ecosystems. For instance, concerns have been raised over the long term use of copper-based fungicides, which can result in an accumulation of copper in the soil [6, 7]. This in turn can have adverse effects on soil organisms (e.g. earthworms, microorganisms) and potentially pose a risk to the long-term fertility of the soil [6, 7]. Extensive use of insecticides in agricultural field produces several deleterious effects on soil ecosystems. Insecticides produce inhibitory effect on the macrofaunal, mesofaunal and microfaunal population of the soil and disturb the equilibrium of soil organisms. Since earthworms constitute about 92% of the invertebrate biomass of the soil, researchers around the world have used earthworms as model organisms for soil toxicity testing. The inception, testing and standardization of the acute earthworm toxicity test by OECD (1984) [8] and EPA (1996) [9] have been the catalysts for the emergence of earthworms as one of the key organisms in environmental toxicologyIn the present study, two fungicides carbendazim and captan were used for acute toxicity test and also used for the determination of the toxic effects of the sub-lethal doses on the Acid Phosphatase and Alkaline Phosphatase activity, of the epigeic earthworm *Eisenia fetida* and the experiments were done following the Walter and Schutt, 1974 [10].

2. Materials and Method
2.1 Period of study:
Total period of study was of 28 days

Table 1: The fungicides used in the study with their respective RADs.

Chemical Name	Trade Name	RAD*(mg/kg)	Source of Procurement
Carbendazim	BAVISTIN	0.96	Rallis, TATA Enterprise (Local Dealer, Midnapore, West Bengal)
Captan	CAPTAF	4.80	BASF, Germany (Local Dealer,Midnapore, West Bengal)

2.3 Specimen used

Age synchronized clitellete *Eisenia fetida* each weighing about 150-250 mg was used for the test.

2.4 Biology

Total length, diameter and number of segments of the body of *Eisenia fetida* ranges from 35 to 130mm (generally >70mm), 3 to 5mm and 80 to120 segments respectively. The life cycle of *Eisenia fetida* has been studied by several researchers including cocoon production [11], effect of temperature on the reproduction [12], incubation time and hatching rate [13]. The life span of *Eisenia fetida* is reported to range from 4 to 5 years [14]. The worms become clitellate and began to produce cocoons by 4-6 weeks and after about 27 weeks the rate of cocoon production declines [11]. Cocoons of *E. fetida* are even smaller than a grain of rice, shaped like lemon and yellow-coloured. The incubation period of the cocoon is about 23 days. The cocoons gradually change its colour from golden yellow to deep red; much like maroon as 4 to 6 embryonic red wiggler worms develop inside. Cocoons hatch at a temperature of 68° to 77° F (20° to 25° C). The juveniles emerge from the cocoons at about 3-4 weeks. Juveniles are about $^1/_2$ inch in length and do not have any genital markings or the clitellum. Once they hatch they readily become organic waste eating machines. About 40-60 days are required for the juveniles to develop into an adult. It develops the genital markings and clitellum. The clitellum contains their reproductive organ and can only be seen when *E. fetida* is ready to reproduce and the clitellums are orange in colour [14]. *Eisenia fetida* can be easily bred in the laboratory using variety of organic medium and has a short generation time. Therefore, the species is very appropriate for toxicity studies [8, 15].

2.5 Instruments Used

Instruments Used	Company and Model No.
Electronic Balance	Mettler Toledo (New Classic MS)
Environmental TestChamber	IIC-INSTIND
Homogenizer	Remi Electrotechnik Ltd (Type RQP-127/A).
Centrifuge	Remi Cooling Centrifuge (C-24BL).
Spectrophotometer	Systronics (UV-VIS Spectrophotometer 117)

3. Experimental Procedures:

The acute toxicity test of the two fungicides was performed for a period of 96 hours. Studies were performed with age synchronized specimens (150-250 mg). Experiments were conducted in small inert polythene boxes (16 X 12 X 1 cm; total area, 192 cm^2) containing soil, collected from grasslands, as the test medium. Soil samples were sun dried, grinded and sieved to get a particle size of 0.25 mm before filling in the experimental boxes. The moisture content of the soil was measured by Infrared Torsion balance moisture meter [16]. Finally the experimental boxes were kept in an Environmental Chamber at a constant temperature of 28±0.5 °C and 60-65% relative humidity. The physiochemical parameters of the soil media, viz, pH and Organic carbon Content were measured (Table 2) and the temperature and moisture content were kept constant.

Table 2: Physiochemical parameters of the natural soil used as medium in both the acute toxicity test and Enzyme activity estimation.

Natural soil parameters	Values
P^H	6.90
Organic Carbon Content	1.18%
Moisture	61.2%

3.1 Acute Toxicity Test:

Different levels of the carbendazim based on their recommended agricultural doses (RAD) (viz RAD, 1/2XRAD, 2X-RAD and 3X-RAD) were administered into the test boxes with a micropipette [17]. The amount of a fungicide required was determined from the total area of the experimental box and was converted into mg per kg soil taking into consideration the total amount of soil (200 g) contained in one box. The experiment was setup with three replicates for each level of the fungicide and control. The boxes were then left undisturbed for about 30 min for uniform spreading of the fungicides in the soil medium. Five numbers of age synchronized specimens of *Eisenia fetida* were then transferred into the boxes. Observations were made every 24 h. Those individuals, who showed no apparent sign of life, even when poked with a needle, were considered dead and were removed. The total mortality obtained after 96 h of exposure were subjected to probit analysis by EPA probit analysis program, version 1.5 [18] to determine LC_{50} value (Table 3) and 95% confidence limit of each insecticide. The entire experiment was repeated three times [19].

Table 3: LC_{50} values of the two fungicides used in the Acute Toxicity

Chemical Name	Trade Name	LC_{50} Values
Carbendazim	Bavistin	5.38 mg/kg
Captan	Captaf	10.41 mg/kg

3.2 Chronic toxicity Test:

Bioassays were made with age synchronized specimens in the same small inert polythene boxes as described above. Dry (500 g) finely ground soil(0.25 mm particle size) were laid in the experimental boxes and 25g dried and grounded cow dung as food was added [8]. Soil moisture of the test soil was maintained at 60-70 %. Two sub-lethal doses (T2 and T3) of each fungicide were applied along with control (T1), 25% (T2) and 50% (T3) of the LC_{50} value of the respective fungicides.

Ten number age synchronized adult specimens were introduced in each experimental box. Before introduction, the worms were rinsed with water, blotted dry on a filter paper. Five replicates for each dose were maintained and control boxes for each dose were maintained simultaneously. Finally the experimental boxes were kept in an Environmental Chamber at a constant temperature of 28 ± 0.5ºC and moisture of 65%. Finely ground cow manure (5 g dry weight) moistened to 50% (w/w) was added each week to provide food for the growing worms. Additional food was given when all the food added was consumed. Moisture loss from the test soil was checked by weighing the test containers at weekly intervals and replenished if needed [15].

Result

The 96 hrs acute toxicity tests showed that Carbendazim with an LC_{50} value of 5.38 mg/kg soil was more toxic than Captan, LC_{50} value 10.41 mg/kg soil. The LC_{50} value of carbendazim is about five times higher than its RAD and in case of captan it is about two times higher than its RAD.

The acid phosphatase activity was decreased in both the sub lethal doses i.e.T2 (25% of LC_{50} value) and T3 (50% of LC_{50} value) in both the fungicides application as compared to the control set i.e. T1. The inhibition of the activity of the acid phosphatase in T3 was most severe in case of captan than in carbendazim. In T2 dose the acid phosphatase activity was also decreased largely in case of captan while in carbendazim the activity inhibition was more in T3 than in T2.In both the sub lethal doses i.e. T2 and T3 the alkaline phosphatase activity were increased in both the fungicides, in respect of the control set i.e. T1. The enzyme activity elevation in T2 of captan was most severe. In T3 of captan also increased markedly but not as in T2.Whereas in carbendazim enzyme activity elevation was not so remarkable.

Discussion

The activity of acid phosphatase decreased in both the doses of all the fungicides tested, but the inhibition was less in carbendazim. Work of [20, 21] shows the increase in activity of acid phosphatase in earthworms exposed to LC_{25} and lower doses of the insecticides. More works on different organisms other than earthworms with insecticides shows the increasing activity of this enzyme. Acid phosphatase is a lysosomal enzyme that hydrolyses the ester linkages of phosphate esters and helps in autolysis of cells after it's death. Phosphatase enzymes are thus related to metabolism of glycogen, phosphoprotein and nucleotides. Decreased level of acid phosphatase at higher doses of insecticides has been related to inhibition of ATPase activity in the gut of insects which may affect active ion transport due to toxic effects of the insecticides on the membrane permeability, especially on the gut epithelium [22]. Thus in our study in *E.fetida* higher dose treatment may have altered the membrane physiology for which the acid phosphatase activity have been affected. In our present study the acid phosphatase activity decreases significantly in both the doses namely T2 and T3 in respect of the control i.e. T1 in both the fungicides. But there was no significant changes have been occurred in T2 in respect of T3 and vice versa. As the increase in lactic acid dehydrogenase level caused by the insecticide exposure induce tissue necrosis in insects while increase in acid phosphatase level induce lysosomal activities in cells. This leads to biochemical stress in insects [23]. Therefore a decline in the level of above enzyme also may occure to encounter the stress situation.The alkaline phosphatase activity elevation observed in both the fungicides application in the T2 and T3 doses in relation with the T1. The elevation in case of captan was significant but not in carbendazim. The elevation in T2 is more significant than in T3.

The changes in the alkaline phosphatase activity may be indicative of an adaptive rise in enzyme activity to the persistent stress [24]. It is established that insecticide exposure causes reduction in soluble protein and there is a relationship between growth rate, protein content, transaminases and phosphatases and thus an increase in phosphatase may result. However elevation of alkaline phosphatase has been reported to be related to resistance of the organism towards the insecticides and the level of pathological and physiological damage causes to the particular organism [25, 26].

The particular work on the influence of the fungicides carbendazim and captan on the acid phosphatase and alkaline phosphatase activity of *E. fetida* has not been reported so far.

Conclusion

From the above study it can be concluded that carbendazim shows less toxicity upon the earthworm than captan. In this regard carbendazim can be treated as an ecologically safe fungicide whereas due to high responsive result captan is not a so much safe fungicide in the agro ecological view. Last of all, it can be concluded that these enzyme parameters can be used as potential biomarker to detect pesticide pollution in agro ecosystem.

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Figures

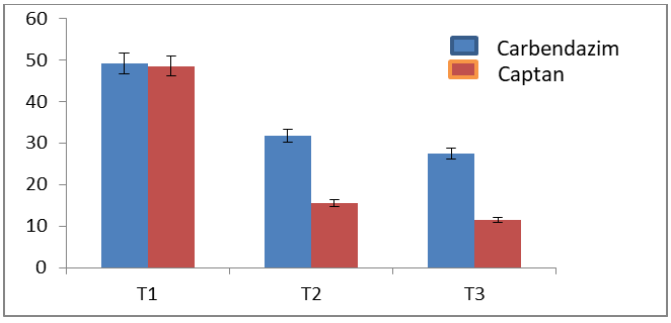

Fig.1: Acid Phosphatase activity inhibition comparison of the two fungicides**.**

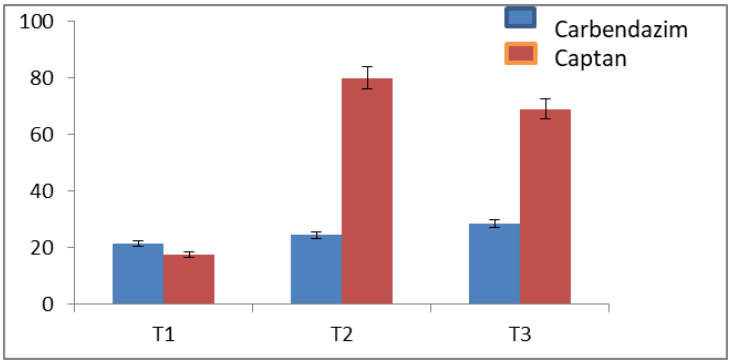

Fig.2: Increased Alkaline Phosphatase activity comparison of the two fungicides

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