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Response of *Pheretima Posthuma* and Some Soil Fertility Enhancing Bacteria to the Copper Stress

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Abstract: Physiological or biological stress is an organism's response to a stressor such as an environmental condition or a stimulus. Stress is a body's method of reacting to a challenge. Toxic metals are now everywhere, and affect everyone on planet earth. They have become a major cause of illness, aging and even genetic defects. Copper is essential for the function of most living organisms. But increased concentrations of copper in soils are harmful to soil organisms. Most microorganisms are known to have specific genes for resistance to stress due to heavy metals. Some of the bacteria are resistant to heavy metals and some have attained adaptation towards the toxic effects of the metals. It is also found that soils containing significant copper residues are stressful for earthworms and have been observed to have few earthworms. From the present study also, it was seen that, copper is a stressful factor for soil bacteria and earthworms and both the organisms are affected from the experimental concentrations of Copper.

Keywords: Stress, Copper Toxicity, Bacteria, Earthworm, Karimganj, Heavy Metal, Soil

1. Introduction

The body's way to respond to stress is by sympathetic nervous system activation which results in the fight-or-flight response. But as the body cannot keep this state for long periods of time, the parasympathetic system returns the body's physiological conditions to normal (homeostasis). Metal toxicity or metal poisoning is the toxic effect of certain metals in certain forms and doses on life. Some metals are toxic when they form poisonous soluble compounds and all heavy metals are particularly not toxic also, and some are even essential such as iron and some, such as bismuth, have a low toxicity.

However, some forms of the required minerals can be highly toxic. Examples are some forms of copper, iron, manganese, hexavalent chromium, selenium and others. Too much of even the most needed minerals can also become toxic. Toxic metals replace nutrient minerals in enzyme binding sites. When this occurs, the metals inhibit, over stimulate or otherwise alter thousands of enzymes.

Copper is not only a ubiquitous metal in the technological environment, it is also essential for the function of most living organisms. Copper ions are required as cofactors by many enzymes, such as oxidases and hydroxylases, but are highly toxic when present in excess. Inside the cells, copper may be bound by various compounds to form copper complexes [1]. Copper is an essential element and required by all organisms. However, elevated concentrations of copper in soils are toxic and stressful and may result in a range of effects including reduced biological activity and subsequent loss of fertility [2].

Effects can occur at relatively low Copper concentrations and influence a number of soil processes including microbial activity, earthworm activity and bio-turbation. In most soils, copper residues are likely to remain indefinitely, and will continue to influence the health of the soil. Soils contain an extremely diverse array of microorganisms including bacteria, fungi, yeasts; photosynthetic organisms including algae, and macro-organisms such as protozoa, nematodes, mites, springtails, spiders, insects and earthworms. The functions of this complex array of biota, often referred to as

the 'soil food web,' are diverse, and include residue decomposition, nutrient storage and release, soil structure and stability, resistance against disease and degradation or immobilization of pesticides and other pollutants.

Most microorganisms are known to have specific genes for resistance to stress due to heavy metals. Mostly, the resistance genes are found on plasmids or on chromosomes. Bacteria that are resistant to heavy metals also play an important role in biogeochemical cycling of those metal ions [3]. Thus, it is worthwhile to note that although some heavy metals are important and essential trace elements and others are stressful at high concentrations to microbes, some microbes have adapted to tolerate the presence of metals or even to use them to grow. Copper is an essential micronutrient and are used for redox-processes, to stabilize molecules through electrostatic interactions, as components of various enzymes, and for regulation of osmotic pressure [4].

Earthworms have been suggested as useful indicators of soil health [5, 6]. Soils that contain significant copper residues are stressful for earthworms and have been observed to have few earthworms [7, 8], reduced surface activity (fewer castings visible at the soil surface) and greater litter build-up [9]. At sites in a study on avocado orchards in northern NSW [10], an absence of earthworms in areas of copper contamination was accompanied by a thick layer of organic matter (ca. 10-30 cm deep) that was clearly stratified on the soil surface, with little evidence of breakdown and incorporation into the sub-surface layers. This phenomenon is not seen in other sub-tropical horticultural systems where copper fungicides are not used. In 1983, Ma & co-workers observed a strong correlation between soil-copper concentration and the level of copper in earthworm tissues. Salminen and Haimialso observed in 2001 that the enchytraeid worm *Cognettiasphagnetorum* (Oligochaeta) actively avoid copper contaminated soil.

Hence we planned our project work to screen the stress tolerance level of Copper in some soil organisms like soil bacteria and earthworms.

2. Materials and Method

2.1. Isolation of Bacteria and Earthworms

Soil samples were collected from undisturbed semi forest areas of Karimganj (Shyamaprasad Road), Assam, India in sterile plastic bags and brought to the laboratory. Earthworms were also collected from the same spot. 1g of soil was added to 100ml of sterile distilled water making a dilution of 10^{-2}. From this, 1ml was taken by pipette and added to 9ml of sterile distilled water making a dilution of 10^{-3}. Following this technique a dilution upto 10^{-5} was prepared and 1ml of the diluted soil sample from the concentration of 10^{-5} and 10^{-4} were plated in Nutrient Agar plates and incubated at 28±2°C for 24h. After 24h, the plates were observed for any

microbial growth and the microbial colonies were isolated and subcultured thrice subsequently for obtaining pure cultures. These pure cultures were used for the screening. 24h old bacterial inoculation grown in liquid culture (Nutrient broth) was used for the study. The earthworms were kept in glass beakers with soil until further use.

2.2. Screening of Copper Stress Tolerance Level

2.2.1. Bacteria

Nutrient Agar (Himedia) added with $CuSO_4$ solution at the concentration of 0.5mg/ml, 0.75mg/ml, 1mg/ml, 1.25mg/ml were prepared autoclaved and then plated in sterilized petridises and allowed to solidify. On solidification, the plates were marked into several small squares on the backside and bacterial inoculum was spotted (spot test) in individual squares and numbered according to the samples spotted. After 1/2h, the plates were sealed with parafilm and then incubated for 48hrs in inverted position at 28±2°C in an incubator. After the incubation period, the plates were checked for growth of bacteria and the data was tabulated [11].

2.2.2. Earthworm

200g of soil sample was dried in oven at 100°C for 24 h and the moisture content was found by the formula

$$MF = (I-F)/[A-(I-F)]X100 \qquad (1)$$

Where,
I=Initial wet weightof soil taken+weight of petridish (g)
F=Final dry weight of soil+weight of petridish (g)
A= Initial weight of soil taken (g)

Then, 200g of soil sample was hydrated with requisite amount of water containing $CuSO_4$ at the concentration of 0.5mg/ml, 0.75mg/ml, 1mg/ml, 1.25mg/ml in separate glass beakersupto 45% ofthe soil moisture. The amount of $CuSO_4$ solution to be added to the dried soil sample was found out by the following formulas

$$H = T-MF \qquad (2)$$

H=Hydrataion needed (%)
T=Target moisture content
MF=Initial moisture fraction of the soil sample.

$$W = (BxH/100)XC$$

W=Amount of water to be added
H=Hydration needed (from ii)
C=Conversion factor (1ml/g)

5 earthworms were then inoculated in each beaker and allowed to stay for 14 days. After 14 days, the soil samples were emptied in trays to check out the number of earthworms, study the morphological changes and weight of the surviving earthworms (Norton, 1996).

3. Results

Table 1. Responses of the bacteria against different concentration of CuSO₄.

Isolate number	0.25mg/ml	0.5mg/ml	0.75mg/ml	1mg/ml	1.25mg/ml
1	+	-	-	-	-
2	+	-	-	-	-
3	+	-	-	-	-
4	+	-	-	-	-
5	-	-	-	-	-
6	+	-	-	-	-
7	+	-	-	-	-
8	-	-	-	-	-
9	+	-	-	-	-
10	-	-	-	-	-
11	+	-	-	-	-
12	-	-	-	-	-
13	+	-	-	-	-
14	+	-	-	-	-
15	-	-	-	-	-
16	+	-	-	-	-
17	+	-	-	-	-
18	+	-	-	-	-
19	+	-	-	-	-
20	+	-	-	-	-
21	-	-	-	-	-
22	+	-	-	-	-
23	-	-	-	-	-
24	-	-	-	-	-
25	-	-	-	-	-
26	-	-	-	-	-
27	+	+	+	-	-
28	+	+	+	-	-
29	+	+	+	-	-
30	+	+	+	-	-
31	+	+	+	-	-

Table 2. Responses of the earthworm against different concentration of CuSO₄.

Concentration (mg/ml)	Soil moisture (%)	Initial Number of worms	Initial weight of worms (g)	Final number of live worms	Final weight of worms (g)	Morphological changes
0.5mg/ml	45	5	25	5	24	Shrinkage, paleness
0.75mg/ml	45	5	24	5	21	Shrinkage, paleness
1.0mg/ml	45	5	27	3	20	2 earthworms were found dead. Remaining earthworms were shrunk and became thin.
01.25mg/ml	45	5	24	1	5	4 earthworms were found dead and remaining one became pale, shrank etc.

4. Discussion

Copper (Cu) is an essential nutrient for plant and microbial growth, but because only a small amount is needed, it is classified as a micronutrient. Copper promotes seed production and formation, plays an essential role in chlorophyll formation and is essential for proper enzyme activity. But due to high use of Copper-fungicides and release of toxic industrial effluents to the environment, the level of Copper is increasing and may cause severe damage to ecosystems including plants, animals, microorganisms and human health [12, 13]. Copper toxicity damage plant roots, with symptoms ranging from disruption of the root cuticle and reduced root hair proliferation, to severe deformation of root structure. Moreover excessive copper is detrimental to the growth and survivability of microbes and earthworms because it proves to be stressful and hence toxic to them and kills the life forms.

In the present work also, it was seen that maximum number of bacterial isolates could not grow at the concentration of 0.75mg/ml of Copper. At 1mg/ml concentration, only two bacterial colonies were found to grow. Almost every microbial metabolic activity can be adversely stressful by elevated heavy and toxic metal concentrations Heavy metal resistant microbes are of particular importance because if they are found to possess growth promotory activities towards plants, can be used for polluted land reclamation. In the present study two bacterial isolates were found to grow in high copper concentrations which are a new breakthrough on the studies involving metal toxicity and microorganisms. These bacterial strains will be further studied to screen their metal tolerance level using higher concentration of the salts used in the present study and

steps would be taken to identify these strains and study their other plant growth promoting activities.

In case of earthworm as well, it was seen that the mortality rate of earthworms increased gradually with the rise in copper concentration and at 1.25mg/ml of Copper, only two earthworms could survive, though they showed high variations in their morphology like shrinkage, paleness etc. High copper kills the earthworm [14]. Moreover, high copper gets accumulated in the body of the earthworms rendering the entrance of the toxic heavy metal in food chain as well [15] In the present study also, high dose of copper is stressful and killed the earthworms and made them sick. Further studies with the earthworms which could survive such a high concentration of copper, like finding out the amount of copper absorbed in the tissue, more exposure of the earthworms to further high concentration of copper etc can lead to an insight on the mechanism of adaptability of the earthworms to tolerate the metal stress or screening out some new variants which can survive in contaminated soil and act as bio-fertilizer and may prove useful to determine if the stress level of Copper concentration can pose any change in the physiology of soil bacteria and earthworms.

5. Conclusion

It can thus be concluded from the above that high dose of Copper proves to be highly stressful for the soil micro- and macro biota. The bacteria and earthworms used in the present study were killed by the levels of Copper used in the present study. Thus it is a high time for thoughtful use of copper in agricultural fields in order to reduce copper stress.

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