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Control of Plant Bacterial Pathogens by Earthworm *Lampito mauritii* (Kinberg) Exudates

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To examine the influence of different preparations from the body of earthworm *Lampito mauritii* on selected soil borne bacterial pathogens. The experiments were based on the principle of agar diffusion, minimum inhibitory concentration and rate of germination. This experiment involved the recording of inhibition zones formed by various extracts amount of turbidity and suppression of rate of germination by different extracts. Colonic fluid, body wall and gut extracts were found to be inhibitory to *Xanthomonas campestris, Ralstonia solanacearum*, *Erwinia cartovora*. The mixed extract when tested on this pathogen has shown a strong clear inhibition zone. The study has proved that earthworm extracts can be effectively used for suppression of soil borne pathogens and that it can be evolve as potential biopesticide.

Key words: Plant pathogen, Earthworm, Antibacterial activity.

Annelids are capable of defending themselves from pathogens and of recognizing degenerated self tissue. The reactions require specialized immune mechanisms that are affected by proteins and cellular reactions. Vermicomposting is a process that relies on earthworms and microorganisms to help stabilize active organic materials and convert them to a valuable soil amendment and source of plant nutrients. Earthworm casts are covered with mucus from their intestinal tract; this layer provides a readily available carbon source for soil microbes and leads to a flush of microbial activity in fresh casts. Use of vermicompost as an organic amendment to agricultural lands is practiced by many Indian farmers and they opine that the application of vermicompost brings down the incidence of many soil borne diseases in crops. The earthworm secretion and microbes harbored in the vermicompost act as plant growth stimulators. The suppression of disease caused by soil borne pathogens on application of vermicompost has been reported (Somshekara *et al* 2001). Though many researchers have reported the suppression of diseases on application of vermicompost tea there has been no work carried out on using earthworm and its secretions directly as antimicrobial agents. The aim of the study was to find out the effect of aqueous earthworm extracts (body wall extract, gut extract and coelomic fluid) of the earthworm on *Lampito mauritii* selected soil-borne plant pathogens.

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MATERIAL AND METHODS

Collection of *Lampito mauritii*

Adult (sexually mature) *Lampito mauritii* were obtained from Annamalai University, Annamalai Nagar. The earthworms were

maintained at 15ºC in the moist soil and used for experiments. This experiment was used mature earthworms about 8cm long. Each experiment earthworms were separated into groups of 3 worms and housed in glass jars with sterile damp paper towel bedding. The bedding was kept damp with bottled spring water and food was provided once a week.

Coelomic fluid extract

To extract the coelomic cells the worms had to be prepared. This was achieved by taking the three worms out of the jar and placing those 5ml of PBS in a petridish. The worms were shocked with a 9 volt battery for 1-2 seconds. After 5minutes the exuded coelomic cells were collected and centrifuge in micro-centrifuge tubes. The micro-centrifuge tubes were decanted and the coelomic cell pellets were pooled into one centrifuge tube and diluted with 0.5ml of PBS. The cells were identifying using a Nikon phase contrast microscope.

Gut and body wall extracts

The filter paper fed earthworms were narcotized after collection of coelomic fluid by swabbing with 70% alcohol and then dissected to separate body wall and gut. Gut and body wall were macerated separately and homogenized using sterile glass homogenizer, a pinch of sterile sand and sterile distilled water to get 10% glass homogenizer, a pinch of sterile sand and sterile distilled water to 10% aqueous homogenate. The homogenates were centrifuged at 5000rpm separately and supernatant was transferred into sterile test tubes. About 6ml of gut extract and 12ml of body wall extract were collected from 10 earthworms.

Processing of extracts

The extracts collected were filtered using membrane filter technique. The filtered extracts were stored in the refrigerator at 4ºC until use. The frozen extracts were thawed slowly at room temperature and then used for experiments. Fresh extracts and stored extracts were used to test the effect of storage on the pathogens.

Bacterial Inoculation

The bacterial pathogens *Xanthomonas campestris*, *Ralstonia solanacearum* and *Erwina carotovora* was grown in nutrient broth (NB) overnight. The bacteria were washed in Phosphate buffered saline (PBS) and then suspended in PBS to 10^6cfu/ml. The worms were anesthetized in 5ml of 5% ethanol and PBS for 5-10 minutes (Affar, 1988), and injected in between the 4th and 6th segment behind the clitellum. Next an amount of 0.05ml of bacteria was inoculated into the experimental group of anesthetized worms was inoculated with 0.05ml of PBS. The experimental worms were separated into five jars, with each glass jar containing three bacterially inoculated worms. The control jars, with each glass jar containing three bacterially inoculated worms. The control group was then separated into five jars, with each control jar containing three PBS inoculated worms. The same experiment was done in *Xanthomonas campestris*, *Ralstonia solanacearum* and *Erwina carotovora*.

Maintenance of stock cultures

The pure cultures were stored in the refrigerator and were sub cultured often so as to maintain purity and viability of cells. Bacteria were sub cultured once in 3-4 days by streak plate method.

Preparation of culture suspension

Uniform and even distribution of culture could not be achieved by taking the culture directly from the plate. Hence culture suspensions were prepared. Only fresh culture suspensions were used for the study.

Bacterial culture suspension

Nutrient broth was used for the preparation of bacterial culture suspension. Using a sterile loop, the bacterial cells were transferred from the stock culture plate into a small quantity of nutrient broth taken in a sterile test tube. It was used for studies only after 18 hours of incubation at room temperature.

Antibacterial potency tests

To test the influence of extracts directly by the Agar diffusion method (Filter paper disc method) and Micro titer plate method.

Filter paper disc method

Bacterial cells are grown overnight in Luria-Bertani media and inoculate into 5ml of molten 0.6g/l Luria-Bertani broth with a final concentration of 10^7 colony forming units per ml which was overlaid onto a 90mm petridish containing 10ml of 2g/l Luria-Bertani solidified agar. After the top agar harden, sterilize blotting paper 9 about 6mm in diameter), free from any antibacterial activity are impregnate with 20µl of the fractions to be tested and placed on agar dishes inoculated

with one bacterial strain. The dishes are incubating overnight at 37ºC. Control tests are performing with paper impregnate with PBS. Antimicrobial activity was determined by observing the zone of suppression of bacterial growth around the 6mm papers.

Micro titer plate method

The micro titer trays are sterilize by immersing in 2% glutaradehyde solution for overnight sterilization and the plates are rinsed twice with sterile tap water and finally with sterile distilled water and the plates were dry under UV exposure. The sterilized plates are either use immediately or packed and keep at 4 ºC.

Inoculation of the Trays

Before inoculation of the trays the bacterial suspension are adjusted to the 0.5 Mc Farland standard , is further diluted in 1:50 dilution (0.1 ml of suspension in 4.9 ml of TSB media containing phenol red indicator) From this , 50µl of inoculum was add to each well containing 50µl of dilute antibacterial agent. The final concentration will be one half that which was originally dispensed. For example, if the original concentration is 64µg/ml, then after the addition of the inoculum, the concentration will be 32 µg/ml. Box keeps it in an incubator at 35ºc for three hours. The results are observed from third hours onwards.

Antibacterial potency of Earthworm Extracts

All the three extracts have shown inhibition zones by disc diffusion method.

RESULTS AND DISCUSSION

Results From this study it was observed that, the concentration of the extract used for testing was very critical for the antimicrobial potency to be expressed. In filter paper disc method, the extract diffused slowly into the media so that the antimicrobial activity was successfully expressed and the minimum inhibitory concentration of al the bacterial pathogen is less than 256µg concentration (Table 1) .

From the study it is evident that the earthworm extracts possess antibacterial properties. Several studies have also reported the inhibition of plant pathogens by earthworm secretions. Reiten and Salter (2002) have reported strong inhibition for *X.campestris E.carotovora*

Table 1. The test results of antibacterial potency of earthworm extracts on abacterial pathogens

Pathogen	Methods	Quantity of extract used (ml)	Results for different substances tested		
			Gut Extract	Body Wall Extract	Coelomic Fluid
Xanthomonas campestris	Filter paper disc method	Crude extract	18mm	16mm	18mm
	Microtiter method	1280 to 1µg / 1ml	64 µg	128 µg	32 µg
Ralstonia solanacearum	Filter paper disc method	Crude Extract	17mm	15mm	19mm
	Microtiter method	1280 to 1µg / 1ml	128 µg	64 µg	64 µg
Erwinia carotovora	Filter paper disc method	Crude Extract	19mm	17mm	20mm
	Micro titer method	1280 to 1µg / 1ml	64µg	32µg	16µg

Table 2. Isolation of bacteria from Earthworm Extracts

S.No	Microorganisms	Selective Media	Incubation Period
1	Bacteria	Nutrient agar	37ºC 24hrs
2	Fungi	Potato dextrose agar	25º C 3-5 days
3	Actinomycetes	Starch casein agar	25º C 4-5 days
4	Azotobacter	Ashby's agar	37º C 3-5 days
5	Phosphate solubilizing microbes	Pikovskaya's medium	37º C 14 days

culture plate method using compost tea. Their studies indicate that compost tea can be used to control *X.campestris E.carotovora* both in the laboratory studies as well as in the field. Our studies have proved inhibition of *X.Campestris*, and *E.carotovora* and *R.solanacearum* by the earthworm extracts but extracts were tested on the pathogens directly and at laboratory level. The previous report showed that the clear differentiation in the structure of oligochaete coelomocytes was associated with their functions with phagocytosis (Dales and Kalac, 1992) encapsulation, coagulation of systemic fluids (Valembois *et al.*, 1988) wound healing (parry,1975), immune reaction.

The present study showed that, coelom contains milky white fluid which contains corpuscles of phagocytes, chloragogen circular nucleated cells and the mucocytes. The previous report Valembois *et al* (1985) reported that the granular amoebocytes of earthworms are capable of phagocytosis and encapsulation.

The present study concludes the coelomic fluid, which is exuded through these pores, keeps the skin moist and destroys the bacteria that may collect on the skin. Antimicrobial peptides constitute a very important component of the innate system in organisms across the evolutionary scale (Saberwal, 1994; Nicolas, 1995; Boman, 1995; Sitaram, 1999). However the coelomic fluid is found to contain the protein, that proteins are fetidin and lysenin which possesses antibiotic property. Body wall and gut extracts occupy different types of bacteria ,fungi, Actinomycetes, Azotobacter, Phosphate solubilizing microbes, so this bacteria acting against the pathogen. From the experiment conclude that earthworm extracts have direct influence on the soil borne plant pathogens.

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