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Coelomic Fluid of Earthworm, Eudrilus Eugeniae, Inhibits the Growth of Fungal Hyphae, in Vitro

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Abstract*: Knowledge on interactions between earthworms and microbes at molecular level has been obscure. Mechanism by which earthworms defend themselves against different microorganisms in the soil is largely unknown. Coelomic fluid from earthworm species has been shown to have antimicrobial activity. In this study we report that coelomic fluid isolated from Eudrilus eugeniae has antifungal activity against four different phytopathogens such as Rhizoctonia solani, Fusarium oxysporum, Aspergillus flavus and Verticillium dahlia. Incubation of coelomic fluid with fungal hyphae of all these four strains for 12 hours revealed that the growth of hyphae was greatly inhibited. Light microscopy studies confirmed that the hyphae growth was greatly reduced by incubation of coelomic fluid. Analysis of the fluid in the microscope showed the presence of different types of coelomocytes in the mucous fluid. Other molecules and compounds in the coelomic fluid might also have played role in antifungal activity.

***Keywords*: Earthworm, Eudrilus eugeniae, coelomocytes, plant fungal pathogens, antifungal.**

I. INTRODUCTION

Fungi are one of the major pathogens of most of the eukaryotic organisms. Plant pathogens such as *Rhizoctonia solani, Fusarium oxysporum, Aspergillus flavus and Verticillium dahliae* can be diagnosed by different methods [1]. *Rhizoctonia solani* is one of the significant pathogens in the genus of *Rhizoctonia* due to its wide range of hosts and the nature of virulence. It infects many families of plants such as *Poaceae, Fabaceae, Solanaceae, Amarathaceae, Brassicaceae, Rubiaceae, Malvaceae, Asteraceae* etc. Resistant gene for this pathogen has not yet been reported in the plants. Application of synthetic fungicides to control the fungal infection led to development of resistance towards the pathogen in rice [2]. Foliar spray of silver nano particles over rice leaves has been shown to reduce the lesion size of fungus compared to control plants [3].

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It causes sheath blight disease in rice plants all over the world. This fungus causes approximately 20 to 50 % yield loss in rice depends on its severity. *Fusarium oxysporum* includes wide diversity of strains causing diseases such as wilts or rots in many crop and ornamental plants [4]. *Aspergillus flavus* infect wide range of crop plants and produces toxic substances such as aflaotoxins. Current advances in biotechnology viz. RNAi technology, genetic engineering and plant breeding offered methods to reduce these toxic substances in crops [5]. *Verticillium dahliae* is another notorious fungal pathogen causes wilt disease in plants throughout the world. Genome analysis of this fungal pathogen revealed the presence of extensive bacterial DNA [6].

Earthworms are beneficial in both agriculture and pharmaceutical fields [7, 8]. They play significant role in population of soil microorganisms in crop fields. Plant pathogenic fungi such as *R. solani* appeared to be one of the food sources of earthworms [9]. Fungal species isolated from the gut region of earthworms in crop fields indicated that it is passed through the earthworm naturally through soil or other plant litter [10].

The spores of *Fusarium loteritium* were found to be not viable after it had been passed through earthworm gut regions [11]. Besides, midgut fluid of earthworms has been shown to inhibit the growth of few fungal species compared to hindgut fluid of same worms [12]. These data indicate that the microbial community in the soil is considerably influenced by the population of earthworms in the field. Extracts prepared from earthworm powders or pastes were also shown to have antifungal activity [13, 14]. Besides, antimicrobial peptides were identified from two different species of earthworms [15, 16]. The molecular basis for antifungal activity from earthworms was not revealed until few years ago. Coelomocytes of earthworms have been shown to inhibit the growth of phytopathogenic fungus *Fusairum oxysporum* [17]. Interestingly, the protein-carbohydrate fraction obtained from coelomic fluid has been shown to inhibit the growth of human fungal pathogen *Candida albicans* [18].

In this study we aimed to find out whether the coelomocytes of earthworm *Eudrilus eugeniae* can inhibit the hyphae growth of four different plant pathogenic fungal strains. Our results of *in vitro* experiments suggest that coelomic fluid with coelomocytes of earthworms inhibit the growth of fungal hyphae from four different strains.

II. MATERIALS AND METHODS

A. Fungal culture and L15 Media

All four different fungal strains were obatined from Microbial Type Culture Collection (MTCC) and Gene Bank, India, viz. *Rhizoctonia solani* (MTCC-4634), *Fusarium oxysporum* (MTCC-284), *Aspergillus flavus* (MTCC-873) and *Verticillium dahliae* (MTCC-9998). They were inoculated on PDA broth (Himedia-M403) with gentamycin (30 µg/mL) and grown at 28°C for 48 hours in shaker. The culture was centrifuged at 3000 rpm for 5 mins to remove aggregates. Supernatant was transferred and the OD was measured at A600 and adjusted to 0.1 and used in all the experiments. L15 (leibovitz's-Himedia-AT011) medium was prepared as per the instructions of supplier and filtered with sterile 0.2 µm membranes. Sterilized glycerol was added to the medium at a final concentration of 10%.

B. Extrusion of coelomocytes

Healthy adult earthworms weighing 1.0 to 1.5 gm were used for coelomic fluid collection. Ten worms were washed with distilled water to remove the soil and other solid particles and dried in tissue paper for few minutes. Later earthworms were kept in a glass Petri plate and 1X L15 medium (1ml/worm) with glycerol was added and kept in icebox for 10 mins. The collected coelomic fluid was centrifuged at 1000 rpm for 30 seconds to remove solid particles and transferred to a new tube centrifuged again at 3000 rpm for 2 mins to bring down the cells to bottom of the tubes. Concentrated cells were suspended in 250 µl of 1X L15 medium. We equalized the number of coelomocytes in each treatment by using one common stock of coelomic fluid. This stock was further validated by checking the cells in the light microscope.

C. Microscopy

Concentrated coelomocytes were placed on the glass slide and observed at 10X or 40 X magnification using light microscope. Coelomocytes were stained with safranin 0.5%W/V for 1 minute and subsequently washed with distilled water 3 times. Few drops of ethanol were used to remove excess stain if there was any and observed under light microscope. Following antifungal assay 20 µl from reaction was taken to observe the growth of fungal hyphae in light microscope and pictures were taken using the software, ScopeImage 9.0.

D. Antifungal assay

Concentrated coelomocytes in coelomic fluid with 1X L15 medium were kept in one master tube and used for different treatments to minimize the variation. Total reaction volume for each treatment was 100 µl and it was carried out in 0.5 ml eppendorf tubes. Four different reactions were set as described in the Table I. Incubation was carried out at 28°C for 12 hours. Following incubation, 20 µl from each reaction tube was placed over the PDA agar plates containing gentamycin. Gentamycin was used to inhibit the bacterial growth if there was any contamination. Fungal growth was monitored after 12 or 24 hours.

Table I. Reaction mixture in antifungal assay

Treatments	Sterile water (µl)	1X L15 Medium (µl)	Fungal Culture (µl)	Coelomocytes in CF- L15 medium (µl)
Positive control	-	80	20	-
Negative control	20	80	-	-
Coelomocytes in CF	20	-	-	80
R. solani with coelomocytes	-	-	20	80

III. RESULTS AND DISCUSSION

Coelomic fluid in earthworms is secreted through dorsal pores in their skin. This fluid contains many enzymes viz., proteases, lysozymes, metalloenzymes, fibrinolytic enzymes and polysaccharides, antimicrobial proteins, nutrients etc. [18]. Bioactive compounds in this fluid have been shown to have antimicrobial, anti-inflammatory, antioxidant and antitumor activities [19], [20]. It also possesses different forms of cells called coelomocytes [21].

Fungal cultures of *R. solani* were prepared using liquid medium. In order to normalize the quantity of fungal hyphae, different aliquots of same volume were spotted on PDA agar and the growth was monitored. The fungal hyphae growth in PDA agar from different aliquots of same volume yielded similar growth as shown (Fig. 1). It suggested that this could be followed to use similar number of fungal hyphae in all treatments of an experiment.

The coelomic fluid collected from adult earthworms by cold stress was spun down to remove the solid and crusts as reported earlier [7]. Coelomic fluid having the cells, coelomocytes, was used to set *in vitro* antifungal assay as described in the methods section (Table I). Following 12 hours of incubation, aliquots from *in vitro* assay were analysed in light microscope. It showed that fungal hyphae were grown well in the control samples where there was no coelomic fluid (Fig. 2A). However, growth of hyphae was greatly reduced when it was incubated with coelomic fluid (Fig. 2B).

Coelomic fluid containing coelomocytes of earthworms *Dendrobaena veneta* and *Eisenia fetida* has been shown to inhibit the growth of plant parasitic fungus *Fusarium oxysporum* after 48 or 72 hours [17].However, in their experiments, coelomocytes were incubated over the solidified agar medium in a small size Petri plate that had the fungal agar block in the middle. In contrast, we incubated the *R. solani* hyphae with coelomic fluid in L15 liquid medium to support the growth of both coelomocytes and fungal cells. In L15 liquid medium coelomocytes might act better compared to solid medium. Interestingly after 12 hours of incubation time, inhibition of fungal hyphae growth was observed.

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Fig. 1. Normalization of R. solani culture volume. Different aliquots of same volume from single mother culture in PDA agar plate is shown.

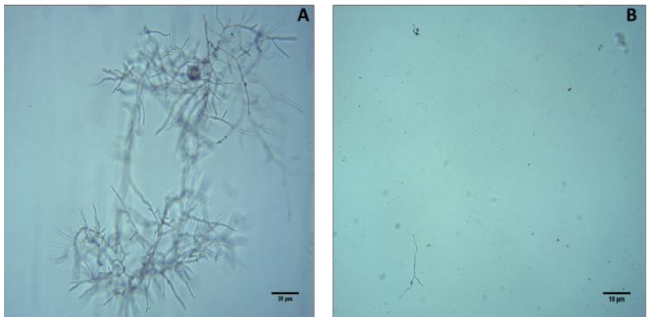

Fig. 2. Inhibition of R. solani hyphae growth by coelomic fluid. Panel A represents hyphae growth in control and B shows the growth in coelomic fluid incubated samples. Photographs were taken using light microscope with 10X magnification. Scale bar represents 1 µm.

Coelomic fluid containing coelomocytes of earthworms *Dendrobaena veneta* and *Eisenia fetida* has been shown to inhibit the growth of plant parasitic fungus *Fusarium oxysporum* after 48 or 72 hours [17].However, in their experiments, coelomocytes were incubated over the solidified agar medium in a small size Petri plate that had the fungal agar block in the middle. In contrast, we incubated the *R. solani* hyphae with coelomic fluid in L15 liquid medium to support the growth of both coelomocytes and fungal cells. In L15 liquid medium coelomocytes might act better compared to solid medium. Interestingly after 12 hours of incubation time, inhibition of fungal hyphae growth was observed.

To confirm further, after 12 hours of incubation, aliquots were taken from different treatments and spotted in PDA agar plates and allowed to grow for another 12 hours. Fungal growth was better in positive controls (Fig. 3A) where there was no coelomic fluid. Coelomic fluid was tested exclusively in the PDA agar to exclude the possibility of fungal contamination. The symptoms for any fungal growth were not observed from coelomic fluid (Fig. 3B). In contrast, fungal growth was greatly inhibited from three different replicates containing coelomic fluid (Fig. 3C). Plain L15 medium did not have any growth of fungal hyphae (Fig. 3D). These results strongly suggest that molecules in coelomic fluid and coelomocytes might act individually or synergistically to inhibit the growth of fungus. Other than coelomocytes cells, coelomic fluid has many molecules such as proteins, enzymes, carbohydrates and small molecules and they would have contributed in antifungal activity. It has been shown recently that the protein-carbohydrate fraction from coelomic fluid of earthworm (*Dendrobaena veneta*) inhibit the growth of *Candida albicans* [18]. It clearly shows that other than coelomocytes, components in the coelomic fluid also have significance in antifungal activity. Besides, the rate of fungal inhibition was directly proportional to the number of coelomocytes available in a reaction [22]. These data suggest that the significance of both coelomocytes and other components in the fluid in their antifungal activity.

Midgut fluid of three different earthworm species was shown to inhibit the growth of bacteria and delayed the germination of spores of few fungal species [12]. Midgut fluid might be containing coelomocytes and other components of coelomic fluid. Interestingly, heated midgut fluid at 98°C for 10 minutes did also show inhibition of bacterial growth but relatively less compared to unheated fluid. Coelomocytes and many enzymes would not be biologically active after the heat treatment. The reduction in the inhibition could be the result of inactive coelomocytes and proteins present in the fluid. Altogether the data suggest that both coelomocytes and other components in the coelomic fluid play role in inhibition of microbial growth.
Following 12 hrs of incubation in PDA agar plates, the growth of hyphae of *R. solani* was examined in light microscope. The growth level of fungal hyphae was enormous in the controls as shown (Fig. 4A). In contrast, the hyphae growth was greatly reduced in samples where it was incubated with coelomic fluid containing coelomocytes (Fig. 4B). The structure of fungal hyphae was different in appearance after incubation with coelomic fluid. It suggests that the components in the coelomic fluid greatly influence the growth and physiology of fungal hyphae. Phagocytes, type of cells in coelomocytes, from Eisenia *fetida were* shown to phagocytose few bacterial strains [23]. Phagocytes were reported to phagocytose clearly different cells such as bacteria or red blood cells rather than other types of cells in coelomocytes [24]. Preincubation of yeast cells with the coelomic fluid considerably increased the rate of phagocytosis [25].

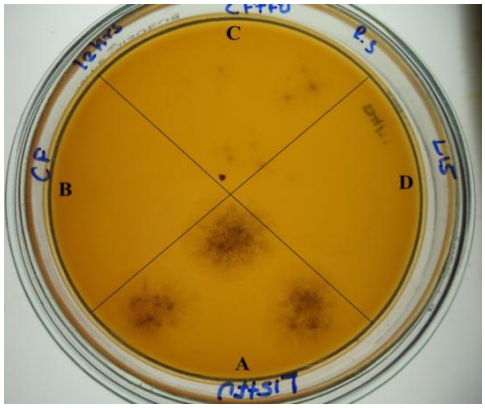

Fig. 3. Inhibition of fungal growth after incubation with coelomic fluid. Three samples of 4 different conditions were tested in PDA agar plate. A: condition of fungus with L15 medium, B: coelomic fluid in L15 medium, C: fungus with coelomic fluid and D: L15 medium. Photograph was taken after 12 hours of incubation.

Coelomic fluid of *Eudrilus eugeniae* was analyzed further to identify different types of coelomocytes cells. Light microscopy studies showed the presence of different types of cells in coelomic fluid (Fig. 5 A-D). It was observed that some cells were much bigger than others as shown in Fig. 5A and B. Other types of cells were grouped or clumped together in nature as shown in Fig. 5C and D. Coelomic fluid had different types of cells as reported earlier [26], [27]. These coelomocytes might be playing significant role in the inhibiting the growth of fungal hypahe. Coelomic fluid is secreted from its skin during adverse conditions. It suggests that it might play a role in defense mechanism of earthworms. To identify if there was similar inhibition in growth from other phytopathogenic fungal strains, *Fusarium oxysporum*, *Aspergillus flavus* and *Verticillium dhaliae* were examined similarly like *R. solani*. In all the three strains the growth of fungal hyphae in control treatments was very dense. In contrast, the growth of fungal hyphae was very poor when they were incubated along with coelomic fluid of *E. eugeniae* (Table II). It suggests that coelomic fluid has components to disturb the growth or lysis of foreign cells. Lytic ability of coelomic fluid of earthworm in species of *Eisenia fetida* and *Lumbricus terrestris* has already been shown [28]. Interestingly, coelomic fluid from earthworm, *Eisenia fetida* has been shown to induce apoptosis in HeLa cells [29]. Besides, analysis of coelomic fluid from earthworm species of *Eisenia* revealed the presence of species-specific aromatic metabolites and its exact biological signicance is not known [30]. Earthworm secretions and ingestions appear to influence the fungal population in its skin. Our data support that coelomic fluid from *E. eugeniae* inhibits the growth of four different phytopathogenic fungal strains. Hence, the protein-carbohydrate fraction has recently been shown to inhibit the growth of fungus [18], it is clear that both coelomocytes and other bioactive components in the coelomic fluid act together to defend them in the hostile environment. Molecular interactions between earthworms and soil pathogens should be studied in detail to understand better and design environment-friendly methods to control the pathogens of crop plants.

Table II. Growth rate of four different fungal hyphae with coelomocytes of earthworm

S.NO	Name of the Fungus	MTCC Number	Growth in Control	Growth with coelomocytes
1	*Fusarium oxysporum*	284	+ + +	+
2	*Aspergillus flavus*	873	+ + +	+
3	*Verticillium dahliae*	9998	+ + +	+
4	*Rhizoctonia solani*	4634	+ + +	+

Highly dense (+ + +) or poor (+) level of hyphae growth is shown.

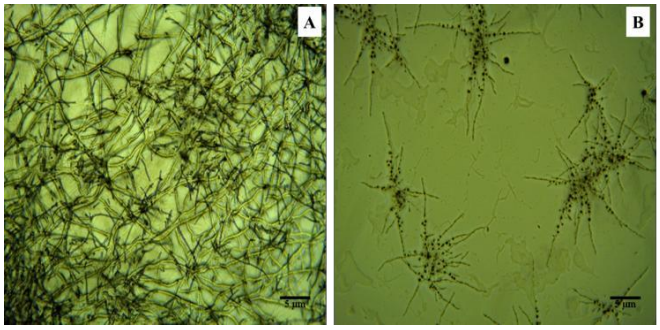

Fig. 4. Confirmation of R. solani growth inhibition by coelomic fluid with Light Microscopy. PDA agar plates showing the fungal hyphae growth in control (A) and coelomic fluid-treated fungal hyphae (B). Scale bar represents 5 µm. Photographs were taken after 12 hours of incubation in PDA agar plates, using 10X magnification lens.

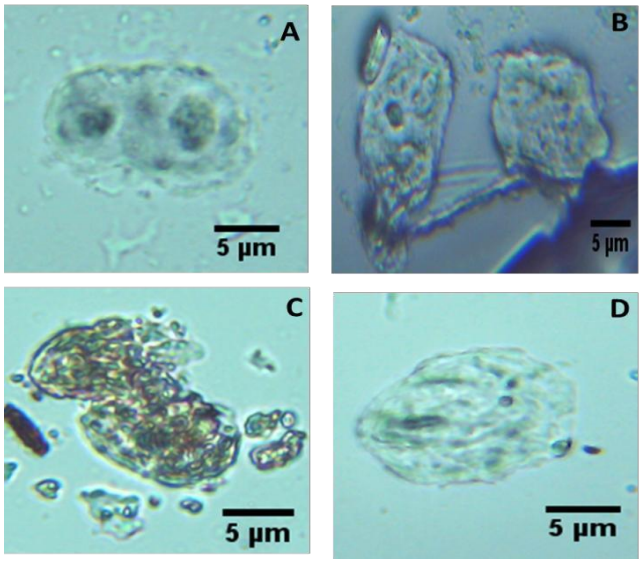

Fig. 5. Light microscopy studies on Coelomocytes. Panels A-E represents different size and shapes of coelomycete cells and panel F shows the cells stained with safranin. All images were taken using 40X objective lens. Scale bar equals 10 µm.

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