European Journal of Soil Biology 78 (2017) 1–6

Contents lists available at ScienceDirect

European Journal of Soil Biology

journal homepage: http://www.elsevier.com/locate/ejsobi

Original article

Inhibitory effect of earthworm coelomic fluid on growth of the plant parasitic fungus *Fusarium oxysporum*

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ARTICLE INFO

Article history:
Received 23 June 2016
Received in revised form
9 November 2016
Accepted 22 November 2016
Available online 8 December 2016

Handling Editor: S. Schrader

Keywords:
Earthworms
Coelomic fluid extract
Fungi
Growth inhibition
Earthworm-fungi interactions

ABSTRACT

Interactions between soil biota communities play an important role as intrinsic factors of suppression and degradation of different phytopathogenic soil fungi. There is significant evidence that earthworms can directly affect fungal populations by feeding or by dispersing fungal propagules. Possible effects of indirect contact by secretion of mucus and coelomic fluid were not assessed previously. In the present laboratory study the effects of earthworms' coelomic fluid extract on the growth of *Fusarium oxysporum*, a phytopathogenic fungi species important in agricultural production, were assessed. The results showed that extracts of coelomic fluid of both tested earthworm species (*Dendrobaena veneta* and *Eisenia fetida*) have negative effects on fungal growth. After 48 h, a significant growth reduction was observed in groups treated with extracts containing 2250, 4000 and 4500 coelomocytes/mL. Growth reduction was even more pronounced 72 h after the treatment. The obtained results indicate the possibility that earthworms can affect soil fungi not only by ingestion, but also by contact interaction. This study proved that earthworm coelomic fluid extract shows antifungal activity in *in vitro* testing. For better understanding of the exact mechanism, studies with soil as a substrate are required, as well as in depth investigation of contact interactions between earthworms and fungi.

1. Introduction

Fusarium is a widespread genus of fungi including more than 1000 species. Even though most of them are saprophytic, there are some species that are phytopathogenic [22,52]. On plants, *Fusarium* causes a wide range of diseases such as cortical, root and fruit rots, head blights, leaf spots and vascular wilt diseases [33,40]. *Fusarium* infection can seriously affect food quality and consequently lead to great economic losses. Once *Fusarium* infects plants they become able to produce mycotoxins which may induce toxic effects endangering the health of animals or even humans [49]. One of the economically most important *Fusarium* species is *Fusarium oxysporum* Schlecht. emend. Snyder and Hansen since it is pathogenic to a variety of hosts including the most important cultivated crops: wheat, corn and rice. More specifically, it is a species complex distributed worldwide, which consists of both pathogenic and nonpathogenic strains [20,26,36]. Currently, the control of fungal phytopathogenic species is based mostly on the use of the resistant crop cultivars and synthetic agrochemicals. The current ways of control, mostly by using conventional chemical fungicides to cope with pathogenic fungal infections, are becoming less effective due to generated resistance [36]. Therefore, there is a growing need for development of new methods for phytopathogenic fungi control.

One of the most attractive alternatives to the use of synthetic agrochemicals is the biological control of phytopathogenic fungi [34,46]. Due to the important role of different members of soil fauna and their interactions with fungi, it has been suggested that soil organisms have a great potential to be used as alternative way of crop and soil protection [19]. Earthworms are among most widely investigated soil fauna members because of their indisputable importance as ecosystem engineers [47]. They play a major role as primary and secondary decomposers, in altering soil structure and water movement, and are important for plant pathogens surviving reduction [15,32,41,53]. The existence of interactions between earthworms and fungi in the soil is already well known. It has been shown that earthworms may affect populations of soil fungi, although the exact mechanisms remain controversial [8,10]. There are significant evidences that earthworms can directly affect fungal populations by feeding or by dispersing fungal

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http://dx.doi.org/10.1016/j.ejsobi.2016.11.004
1164-5563/

propagules, and that fungi are among the most preferable food sources [2,14,38]. Although it is well known that earthworms ingest a large amount of fungal propagules with other soil particles, little information exists about the exact impact of gut fluid on their viability [10]. Beside direct impact, some authors point out the possibility of indirect effects on fungi by alteration of the physical and chemical state of environment and producing unfavorable life conditions which is extremely important in terms of phytopathogenic fungi survival [51,52].

In context of potential negative impacts on fungal growth, indirect contact by secretion of mucus and coelomic fluid should also be considered. Namely, upon irritation by different physical and chemical factors, earthworms can discard mucus and coelomic fluid through the dorsal pores in the body wall [21,44]. Coelomic fluid contains coelomocytes – earthworms' immune cells which play a significant role in defence reactions and have strong phagocytic properties [6,27]. Also, coelomic fluid itself possesses a large variety of biological effects such as antimicrobial, proteolytic, hemolytic and many other [7,13,18]. Considering all the above mentioned, earthworms coelomic fluid may potentially have a significant impact on the fungal growth.

Although the interactions between earthworms and fungi have received considerable attention [8,10,39], the impact of coelomocytes and coelomic fluid on the fungal growth has not been investigated so far. We hypothesized that coelomic fluid could have negative effects on fungal growth. Therefore, the aim of the present study was to investigate the effects of coelomic fluid and coelomocytes on the growth of phytopathogenic fungus. For that purpose, the effects of coelomic fluid extracts of two earthworm species on the growth of *Fusarium oxysporum*, a phytopathogenic fungi species important in terms of agricultural production, were assessed.

2. Material and methods

2.1. Earthworms

Adult individuals of the earthworm species *Eisenia fetida* (Savigny, 1826) and *Dendrobaena veneta* (Rosa 1886) (the species names conform to the Fauna Europea web site: http://www.faunaeur.org/index.php) were purchased from local supplier and acclimatized in the laboratory. Both species were chosen since they are easily commercially available and commonly used for laboratory experiments. Prior to use, earthworms were rinsed with physiological saline, weighed and placed in Petri dishes on damp filter paper. The earthworms used in this assay were all adults with well-developed clitellae. Average weight (±SD) of *E. fetida* used in this experiment was 0.22 ± 0.06 g, while in case of *D. veneta* average weight (±SD) was 1.04 ± 0.21 g.

2.2. Fungi

Fungal cultures of *Fusarium oxysporum* used in this research were provided from the culture collections of Faculty of Agriculture in Osijek, Croatia. For the purpose of this study, *F. oxysporum* isolate FT4 was used. All fungal cultures were maintained in Petri dishes on potato dextrose agar (PDA, Difco, Detroit, Michigan) and kept in a growth chamber at 22 ± 1 °C, with 12 h light/12 h dark regime.

2.3. Earthworm coelomic fluid extract preparation

Earthworms were placed in physiological saline and subjected to electroshocking in order to stimulate the extrusion of coelomic fluid and coelomocytes. Each earthworm was firstly washed and weighed. Ten earthworms of *E. fetida* species or five of *D. veneta* were individually placed in Petri dishes containing 4 mL of physiological saline. Earthworms were stimulated for 30 s by electrical voltage of 12 V by using a modified charger to expel coelomic fluid – both electrodes were leaned on earthworms and the direct electricity current was applied. Coelomic fluid was then collected using a micropipette, put in a tube and kept on ice. Total amount of coelomocytes per mL was estimated using Bürker-Türk counting chamber. In case of *D. veneta*, the initial coelomocyte fluid extract had a higher concentration, so the highest concentrations of both coelomic fluid extracts were adjusted to the average concentration got in case of *E. fetida* (4500 coelomocytes/mL) to counterbalance comparison of effects of these two species on fungal growth. Coelomic fluid extracts of both earthworm species were then diluted using physiological saline in a proper ratio to get concentrations of approximately 1000, 1500, 2250 and 4000 coelomocytes/mL in order to find out which is the lowest concentration that cause inhibitory effects on fungal growth. In addition, due to the method applied for obtaining colemic fluid extract, it is likely that the extract also contained some proportion of mucus from epidermal cells.

2.4. Growth inhibition test

The effect of coelomic fluid on growth of *F. oxysporum* was examined by preparing fresh coelomic fluid extracts of two earthworm species, *E. fetida* and *D. veneta*, separately (as described in *2.3*). The maximal concentration of coelomocytes in extract that was possible to obtain with the applied method was around 4500 coelomocytes/mL. Since preliminary tests showed that concentration of 1000 coelomocytes/mL has no adverse effect on fungal growth, the following concentrations in final growth inhibition tests were applied: 1000, 1500, 2250, 4000 and 4500 coelomocytes/mL. For the growth inhibition test, small Petri dishes (Ø 5.8 cm) were filled with 5 mL of freshly prepared PDA. After cooling, the culture medium was inoculated with fungus by placing a round (Ø 4 mm) sterile agar core of fungal culture in the middle of the Petri dish. Each Petri dish was then treated with 1 mL of five different concentrations of coelomic fluid extracts of both earthworm species separately. A control batch was also maintained using the same volume of physiological saline. For each earthworm species the growth inhibition test was conducted in 4 independent replicates with 5 Petri dishes prepared for each coelomocyte concentration and control (i.e. 20 replicates per concentration). After application, Petri dishes were kept in a growth chamber at 22 ± 1 °C, with 12 h light/12 h dark regime. The diameter of the aerial mycelium was measured 24, 48 and 72 h after application.

2.5. Data analyses

All data analyses were performed using the statistical software R version 3.0.2 [43]. Growth inhibition was estimated on the basis of the aerial mycelium diameter 24, 48 and 72 h after the treatment. The significance of the results was evaluated using one-way ANOVA followed by Tukey's multiple comparison test. The probability level for statistical significance was $p < 0.05$.

3. Results

According to the data presented in Figs. 2 and 3, no significant differences were recorded 24 h after the treatment between control group and any of the group treated with different concentrations of coelomic fluid extracts. It is therefore evident that initial growth was approximately equal in all groups (control and treatments). The first sign of growth reduction was recorded 48 h after the treatment, but not in all treated groups. Treatment with extracts