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# Effect of seed treatment with coelomic fluid secreted by *Perionyx excavatus* on corn seedling and control of *Aspergillus flavus*

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## Abstract

**Importance of the work**: *Aspergillus flavus* contamination could lower corn seed quality and aflatoxin production. Depending on the concentration, certain chemicals used for fungal control are harmful to the environment; consequently, natural products are increasingly used as alternatives. Coelomic fluid secreted by the blue worm, *Perionyx excavatus*, has been found to affect plant growth and to inhibit growth of *Aspergillus* species.

**Objectives**: This work investigated the appropriate coelomic fluid concentration to promote corn seedling and inhibit the growth of *A. flavus*.

**Materials & Methods:** Corn seeds were treated with 0%, 1%, 2.5%, 5%, 10%, 15%, 20% or 30% coelomic fluid solution.

**Results**: The seeds treated with coelomic fluid solutions had higher shoot lengths ($p < 0.01$) than the control but there were no significant differences between the concentrations. Mean (± SD) shoot lengths of seeds treated with 0% and 1% coelomic fluid solution were 19.07 ± 0.89 cm and 23.14 ± 1.32 cm, respectively. In the second experiment, corn seeds were contaminated with *A. flavus* and subsequently soaked in 100% coelomic fluid or fungicide solution and compared with non-contaminated seeds and non-treated seeds. The results indicated that the coelomic fluid had the greatest inhibition on the growth of *A. flavus*. The level of fungal infection of coelomic fluid solution treatment was 0%, while the mean (± SD) infection levels for the fungicide treatment and non-treated seed were 1.67 ± 1.92% and 27.5 ± 11.34%, respectively.

**Main finding**: This study revealed that coelomic fluid secreted by *P. excavatus* significantly enhanced seedling growth and inhibited the growth of *A. flavus*.

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## Introduction

*Aspergillus flavus* is a soil-borne pathogen that produces a carcinogenic aflatoxin during its growth in the field, both before and after crop harvest, and during storage (Klich, 2007). Aflatoxin contamination in food and feed, such as in corn, can lead to food rejection and feed refusal (Carvajal-Moreno, 2021). On the other hand, the fungal infection has impacted seed germination and seedling growth in many species (Christensen, 1978; Krishnamurthy et al., 2008; Safdar et al., 2013; Ng'ang'a et al., 2016). Risk factors affecting the mycotoxin production include the climatic conditions and pest infestation; thus, control of aflatoxin contamination can be achieved through manipulating these biotic and abiotic stresses (Fountain et al., 2014; Mahuku et al., 2019). Fungicides are commonly used in seed treatment to ensure the seeds are fungal free (Munkvold, 2009). However, in recent years, the environmental effects of chemicals used in agriculture have been of concern, with the utilization of natural products proposed as a simple alternative toward productive farming and a healthier environment (Bonome et al., 2020). Epigeic earthworms (living at the soil surface) have been recognized for their excellent contribution to increasing soil fertility by decomposing matter using their body secretion and castings into vermicomposts (Sharma et al., 2005; Hatti et al., 2010; Domínguez, 2018; Singh, 2018). During the vermicomposting process, organic matters are converted into various inorganic plant nutrients such as N, P, K and Ca that could be used for plant growth (Nath et al., 2009). Vermiwash (the substances collected from washing vermicomposts and coleomic fluid collected from earthworms) has been reported to contain nutrients, plant growth regulators and antimicrobial effects (Krishnamoorthy and Vajranabhaiah, 1986; Sundaravadivelan et al., 2011; Nadana et al., 2020). Hatti et al. (2010) reported that foliar spraying vermiwash of the blue earthworm, *Perionyx excavatus*, containing high levels of macro and micronutrients every alternate day for 20 d increased the growth of *Vigna mungo*, *V. radiata* and *Sesamum indicum*. Suthar (2010) treated *Cyamopsis tetragonoloba* and *Trigonella foenum-graecum* seeds by adding vermiwash on blotting papers and found that the seed germinations of both species increased from 55% in the control (distilled water) to more than 85% in 50% vermiwash. That author also reported that the total protein, total soluble sugars and starch in the seedling tissues significantly increased in the 100% vermiwash foliar spray treatment. Nadana et al. (2019) found that the coelomic fluid of *Eudrilus eugeniae* increased the seedling growth of *V. radiata* to 128% when coelomic fluid (1:10 dilution) was applied to the seeds twice daily for 7 d. Byzov et al. (2007) examined the action of the gut fluids from three earthworm species (*Aporrectodea caliginosa*, *Lumbricus terrestris* and *Eisenia fetida*) on soil bacteria (25 species) and fungi (30 species) and found that the gut fluids from those earthworms inhibited the spore germination and reduced the fungal and bacterial growth rates. Plavšin et al. (2017) reported that extracts of coelomic fluid of *Dendrobaena veneta* and *E. fetida* at concentrations of 4,500 coelomocytes/mL reduced the growth of *Fusarium oxysporum* up to 50%. Antimicrobial activities of dried earthworm powder have also been reported by Punu et al. (2016) who demonstrated that the powder derived from *P. excavatus* at a concentration of 1:5 w/v was more effective against Gram-negative aerobic bacteria (*Pseudomonas aeruginosa* ATCC27583, *Escherichia coli* ATCC25922 and *Staphylococcus aureus* ATCC25923) and fungi (*Candida albicans* ATCC24055, *Aspergillus flavus* and *A. niger*) than at concentrations of 1:1 and 1:3.

Among the epigeic earthworms, *P. excavatus* is commonly found over large areas of tropical Asia (Gates, 1972 as cited in Edwards et al., 1998). Compared to *P. sansibaricus, P. excavatus* grows and reproduces relatively better which renders the latter species more suitable for large-scale vermiculture operations (Nadana et al., 2019).

Although the use of *P. excavatus* as a biofertilizer in the form of vermicompost and vermiwash has been commonly reported, the effects of coelomic fluid as a corn seed treatment on germination, seedling growth and control of seed fungi have not been investigated. Based on the literature review, the current study hypothesized that optimizing the concentration of coelomic fluid could effectively enhance corn seedlings and prevent mold, especially *A. flavus*, growth. Therefore, various concentrations of coelomic fluid secreted by *P. excavatus* were tested for corn seed quality and the control of *A. flavus*.

## Materials and Methods

### *Culturing of earthworms*

Earthworms (*P. excavatus*) derived from two commercial farms in Nakhon Pathom, Thailand, were raised for 60 d to the adult stage in plastic buckets filled with bedding material made of 3:1 mixture of cow manure and coconut coir (Muthukumaravel et al., 2008). The bedding was moistened and turned every 2 wk to ventilate. Then, the earthworms were fed with compost food scraps twice weekly.

### *Obtaining secretion from earthworms*

The coelomic fluid extraction was conducted separately in two experiments to study the effect of coelomic fluid on seed germination and seedling growth in the first experiment and to control *A. flavus* in the second experiment. In the first experiment, 20 healthy adult earthworms of equal weight were used for each extract. The earthworms were removed from the bedding and washed with distilled water, kept on tissue paper to absorb surface body moisture and then transferred into a 15 mL tube. The earthworms were stress-induced to obtain coelomic fluid by covering a tube with a cotton containing three mL of ether for 90 s. Then, the earthworms were removed and returned to the bedding. Six mL of distilled water was added to wash the secretion in the tube. The obtained light yellow-colored coelomic fluid, a mixture of the washing water and the secrete, was collected. A pool of eight coelomic fluid extracts was kept at -20°C until used. The coelomic fluid extraction of the second experiment was performed for five extracts following the same steps as in the first experiment but 50 healthy adult earthworms were used for each extract in a 45 mL tube and 5 mL of the washing water was added to wash the secretion. To concentrate the coelomic fluid extracts in the second experiment, a pool of the five extracts was freeze-dried and each dissolved with 20 mL of distilled water before using.

### *Optimization of coelomic fluid concentration to increase germination rate and seedling growth*

The new hybrid field corn seeds of variety KSX5720 used in the experiment were from the National Corn and Sorghum Research Center, Nakhon Ratchasima, Thailand. A completely randomized design (CRD) with four replications was used for the experiment. Approximately 200 seeds were soaked in 16 mL of the 0%, 1%, 2.5%, 5%, 10%, 15%, 20% or 30% (volume per volume, v/v) coelomic fluid for 24 hr at room temperature. In each replication, the 50 soaked seeds were planted in moist sand and kept at 30°C for 7 d, with four replications for each treatment. The control treatment was unsoaked seeds. Seedlings having the first emergence of the coleoptile above the sand were counted at 1500 hours daily for 7 d. The percentage germination was calculated using Equation 1 (International Seed Testing Association (ISTA), 2020);

$$\text{Germination (\%)} = \frac{\text{Total number of normal seedings in a particular treatment}}{\text{Total number of seed treated in a particular treatment}} \times 100 \quad (1)$$

The mean germination time was calculated using the formula described by Matthews and Khajeh Hosseini (2006) as $\frac{\sum nt}{\sum n}$, where n is the number of seeds newly germinated at time t; t is the number of days from planting and Σn is the final number of germinated seeds.

All normal seedlings were cut at sand level to measure the shoot length and dried at 70°C for 48 hr to measure the shoot dry weight.

### *Evaluation of A. flavus and aflatoxin inhibiting ability of coelomic fluid of earthworm*

A CRD experiment with four replications was conducted. There were four corn seed treatments: 1) without *A. flavus* inoculation and without coelomic fluid (negative control; $AF^-$, $CF^-$); 2) with *A. flavus* inoculation and without coelomic fluid ($AF^+$, $CF^-$); 3) with *A. flavus* inoculation and with fungicide (positive control; $AF^+$, $F^+$); and 4) with *A. flavus* inoculation and with 100% coelomic fluid ($AF^+$, $CF^+$). The *A. flavus* was derived from the Thailand Institute of Scientific and Technological Research. Preparation of *A. flavus* for seed inoculation was conducted following the method of Sinha et al. (1993). Briefly, *A. flavus* was cultured on potato dextrose agar medium and incubated to full growth at room temperature for about 7 d; then, the spores were collected to prepare the spore suspension at $1 \times 10^6$ spores/mL. To inoculate the *A. flavus*, the corn seeds were soaked with 10% Clorox for five minutes, rinsed with sterilized distilled water and put into a 250 mL flask containing 20 mL of the spores suspended in 0.1% (v/v) Tween 80 (for better spore separation) with shaking at 1.5 revolutions per second for 4.5 hr. The treatments were conducted by soaking the 80 seeds in 20 mL of distilled water or fungicide (metalaxyl) at the rate of 22 mL per 45 kg of seed or 20 mL of 100% coelomic fluid for 24 hr at room temperature. The fungal growth was evaluated using the 5-day blotter method modified from ISTA (2020) and Niaz and Dawar (2009). Briefly, for each treatment, corn seeds were placed on three layers of moistened blotter paper, with 10 seeds per Petri dish and two Petri dishes per replication, and four replications per treatment. The dishes were incubated at 28°C in alternating cycles of 12 hr light and 12 hr darkness for 5 d. Seeds with fungal spores were counted as infected seeds and determined for aflatoxin production using an Aflatoxin ELISA Test kit (Chinaphuti, 2006). The percentage frequency (PF) of fungal occurrence on seed was calculated using Equation 2:

$$\text{PF(\%)} = \frac{\text{Number of infected seeds per plate}}{\text{Total number of seeds per plate}} \times 100 \quad (2)$$

### *Statistical analysis*

Analysis of variance for the CRD experiment with four replications and Duncan's multiple range test ($p < 0.01$) for mean comparisons were performed using STAR 2.0.1 (International Rice Research Institute, 2014). Results were presented as the mean ± SD.

### *Ethics statements*

Experimental procedures related to animals were approved by the KASETSART UNIVERSITY Institutional Animal Care and Use Committee (Approval no. ACKU64-AGK-029) and conducted in accordance with relevant guidelines.

## Results

### *Effects of coelomic fluid on germination rate and seedling growth*

The experimental results are shown in Table 1. There was no significant difference in the germination percentage (98.50 ± 1.00% to 100 ± 0.00%) and mean germination time (2.02 ± 0.10 d to 2.36 ± 0.42 d). Significant differences were observed in the shoot dry weight and shoot length of seedlings from the corn seeds treated with the coelomic fluid at all concentrations, with both dry weight and shoot length being higher than for non-treated seeds (control) and water-soaked seeds. The shoot seedling dry weight and shoot length of the control (53.08 ± 2.20 mg/plant and 19.07 ± 0.89 cm, respectively) were lower than those of the water treatment (60.07 ± 3.00 mg/plant and 21.08 ± 1.51 cm, respectively) while those of the coelomic fluid treatments ranged from 62.32 ± 3.00 mg/plant to 65.55 ± 0.82 mg/plant and from 23.08 ± 0.90 cm to 23.56 ± 1.33 cm, respectively.

Nevertheless, there were no significant differences in the shoot dry weight and shoot length of seedlings among the concentrations of coelomic fluid used in this study.

### *Ability of secretion of earthworms on growth inhibition of A. flavus and aflatoxin production*

The average level of fungal infection was highest in the seeds with *A. flavus* inoculation and without coelomic fluid ($AF^+$, $CF^-$) at 27.5 ± 11.34% followed by the *A. flavus* and with fungicide treated seeds ($AF^+$, $F^+$) at 1.67±1.92%, while there was no fungal infection in the seeds without *A. flavus* inoculation and without coelomic fluid ($AF^-$, $CF^-$) and the seeds with *A. flavus* inoculation and with 100% coelomic fluid ($AF^+$, $CF^+$), as shown in Table 2 and Fig. 1. However, aflatoxin was not found in any treatment, even in the *A. flavus*-inoculated seeds without fungicide or coelomic fluid (Table 2).

**Table 1** Germination rate, mean germination time, shoot seedling dry weight and shoot length of corn seeds treated with coelomic fluid (mean ± SD from 4 replications)

| Treatment | Germination (%) | Mean gemination Time (d) | Shoot seedling dry weight (mg/plant) | Shoot length (cm)** |
|---|---|---|---|---|
| Control | 100.00±0.00$^a$ | 2.32±0.43$^a$ | 53.08±2.20$^c$ | 19.07±0.89$^c$ |
| Water | 98.50±1.00$^a$ | 2.03±0.05$^a$ | 60.07±3.00$^b$ | 21.08±1.51$^b$ |
| Coelomic fluid 1% | 100.00±0.00$^a$ | 2.35±0.40$^a$ | 63.73±1.60$^{ab}$ | 23.14±1.32$^a$ |
| Coelomic fluid 2.5% | 98.50±1.91$^a$ | 2.09±0.08$^a$ | 62.32±2.02$^{ab}$ | 23.41±0.70$^a$ |
| Coelomic fluid 5% | 100.00±0.00$^a$ | 2.08±0.06$^a$ | 63.47±3.98$^{ab}$ | 25.13±0.57$^a$ |
| Coelomic fluid 10% | 100.00±0.00$^a$ | 2.36±0.42$^a$ | 62.49±2.22$^{ab}$ | 23.08±0.90$^a$ |
| Coelomic fluid 15% | 99.50±1.00$^a$ | 2.06±0.03$^a$ | 62.96±0.15$^{ab}$ | 23.56±1.33$^a$ |
| Coelomic fluid 20% | 99.00±1.15$^a$ | 2.34±0.47$^a$ | 65.25±0.78$^a$ | 23.43±0.66$^a$ |
| Coelomic fluid 25% | 99.50±1.00$^a$ | 2.02±0.10$^a$ | 65.28±1.56$^a$ | 23.18±0.75$^a$ |
| Coelomic fluid 30% | 100.00±0.00$^a$ | 2.06±0.04$^a$ | 65.55±0.82$^a$ | 23.55±0.74$^a$ |

Mean±SD values in the same column superscripted with different lowercase letters are significantly ($p < 0.01$) different.

**Table 2** Level of *Aspergillus flavus* infection (mean±SD from 4 replications) on non-inoculated and inoculated corn seeds with and without treatment of the coelomic fluid and treated with fungicide

| Seed treatment | Level of fungal infection (%) | Production of aflatoxin |
|---|---|---|
| Without *A. flavus* inoculation and without coelomic fluid | $0^{b}$ | ND |
| With *A. flavus* inoculation and without coelomic fluid | $27.50\pm11.34^{a}$ | ND |
| With *A. flavus* inoculation and with fungicide | $1.67\pm1.92^{b}$ | ND |
| With *A. flavus* inoculation and with coelomic fluid | $0^{b}$ | ND |

Mean±SD values in the same column superscripted with different lowercase letters are significantly ($p < 0.01$) different. ND = not detected

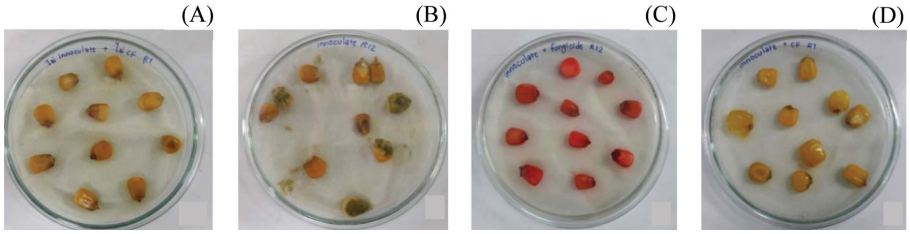


**Fig. 1** Fungal growth on seeds: (A) without *Aspergillus flavus* inoculation and without coelomic fluid; (B) with *A. flavus* inoculation and without coelomic fluid; (C) with *A. flavus* inoculation and with fungicide; (D) with *A. flavus* inoculation and with coelomic fluid

## Discussion

The results indicated that the coelomic fluid from *P. excavatus* increased the seedling growth but had no effect on the germination and the mean germination time. These results differed from Suthar (2010) who reported higher germination of *Cyamopsis tetragonoloba* and *Trigonella foenum-graecum* seeds germinated on blotting papers wetted with 50% vermiwash. However, the seed germination of the two species reported in Suthar (2010) were as low as 55% in the distilled water treatment (control), while in the current study using the water-soaked hybrid corn seed, seed germination was 100%. This indicated that future experiment should be conducted using seeds with a low germination percentage to allow for investigation of the effect of the *P. excavatus* coelomic fluid.

Similar to germination percentage, the coelomic fluid treatments did not affect the mean germination time. Mean germination time is an indicator of seed vigor that correlates with water uptake at the beginning of seed germination processes, as the uptake of water accelerates various metabolic processes resulting in active growth of the embryonic axis (Delouche, 2016). Seeds with higher vigor have a lower mean germination time than lower vigor seeds. In the current study, non-treated seeds and seeds treated with distilled water and the coelomic fluid did not differ significantly in mean germination time. The effects of the coelomic fluid on mean germination time may not be detected in high vigor seeds.

The current findings indicated a positive effect of the coelomic fluid of *P. excavatus* by incresing the shoot length and seedling dry weight of corn seedlings, even in seeds treated with the minimum concentration of coelomic fluid (1%). This suggested the existence of plant growth regulators in the coelomic fluid of *P. excavatus*. Indole acetic acid (IAA)-like substances have been found in earthworm extracts (Nielson, 1965; El Harti et al., 2001; Brown et.al, 2004). IAA (an auxin) plays a role in controlling tissue differentiation in shoots and roots (Reed, 2001; Tanimoto, 2005). Recently, Nadana et al. (2019) identified a volatile compound called heneicosane, a plant growth regulator and an antimicrobial agent in coelomic fluid extracted from *E. eugeniae*, and reported that the coelomic fluid (1:10 v/v) increased the growth of *V. radiata* seedlings. The plant growth-promoting properties of humic acid have been reported by Canellas et al. (2002) who investigated the effects of humic acids isolated from cattle manure earthworm compost on corn seedlings and found that the humic acids stimulated the plasma membrane $H^{+}$-ATPase activity leading to accelerated germination and elongation of seedlings. It was likely that the current trial earthworms produced humic substances that affected the corn seedling growth; however, more verification on the key factors stimulating growth is needed to confirm this hypothesis. Quantitative analysis of the key active compounds would allow investigation of the minimum effective concentration.

The results indicated that the coelomic fluid of *P. excavatus* exhibited antifungal properties and successfully inhibited the growth of *A. flavus* on corn seeds. Other studies have identified antimicrobial peptides (such as lysenin and lumbricin, as a result of the cellular immune system trying to destroy membranes of foreign cells), in the coelomic fluid of earthworms (Bodó et al., 2019; Bilej, 2000; Lange et al., 1999; Swiderska et al., 2017) *In vitro* experiments demonstrated the negative effects of the coelomic fluid of earthworms on bacterial and fungal growth. Plavšin (2017) reported negative effects of coelomic fluid extracts of *D. veneta* and *E. fetida* on the growth of *F. oxysporum*. In addition, Punu et al. (2016) proved antibacterial properties of earthworm powder prepared from *P. excavatus* and tested against selected bacteria (*Pseudomonas aeruginosa* and *Escherichia coli*) and fungi (*A. flavus, A. niger* and *Candida albicans*).

*A. flavus* infection was observed on the non-treated control seeds and fungicide solution treatment but aflatoxin was not detected in any treatment in this experiment. There are both biotic and abiotic factors affecting aflatoxin biosynthesis. For example, aflatoxin formation is induced by simple sugars, lipid substrate, amino acid and acidic media with oxidative stress (Yu, 2012). Studies have shown that hot drought conditions promoted aflatoxin production (Cotty and Jaime-Garcia, 2007; Fountain et al., 2014). Admittedly, the optimal temperature for aflatoxin production is 28–30°C but the production is inhibited when the temperature approaches 37°C that is the optimum temperature for *A. flavus* growth (Obrian et al., 2017). Likewise, high relative humidity (RH) can reduce aflatoxin formation. For example, Guo et al. (1995) observed that the aflatoxin levels of preincubated corn seeds at 100% RH for 3 d prior to inoculation with *A. flavus* was lower than in corn seeds not preincubated. Grintzalis et al. (2014) reported that oxidative stress regulates sclerotial differentiation and aflatoxin B1 biosynthesis. Perhaps the production of aflatoxin in the current experiment might have been inhibited by the reduced oxidative stress.

Based on the preliminary experiment, the 1–30% coelomic fluid concentrations used to investigate the germination rate and the seedling growth could not control the growth of *A. flavus* but the treatment with 100% coelomic fluid was effective. Similary, Byzov et al. (2007) reported antifungal activity using 100% of 2 µL of gut fluid for 1 µL of fungal spores ($1 \times 10^5$ spore/mL) on a thin flim (1.5–2 mm) of the agar medium R2A poured on a microscope glass slide. Plavšin et al. (2017) reported reducing the growth of *F. oxysporum* using extracts of coelomic fluid of *D. veneta* and *E. fetida* with concentrations of 4,500 coelomocytes/mL. However, the minimum effective concentration (using coelomocytes/mL) of coelomic fluid secreted by *P. excavatus* for inhibiting the growth of *A. flavus* will be investigated in further study, including identifying the key plant-growth stimulating compound in the earthworm *P. excavatus*.

In conclusion, the current study indicated that the coelomic fluid of *P. excavatus* could be used as an alternative seed treatment at concentrations as low as 1% to enhance seedling growth while the concentration at 100% was able to inhibit the growth of *A. flavus*. These results indicated that treating the seeds with the coelomic fluid derived from *P. excavatus* provided improved germination and growth compared to no treatment and has potential as a cost-saving method to enhance corn growth. However, the extraction efficiency of the coelomic fluid should be improved and further research is recommended on seed treatment with the coelomic fluid in experimental fields with many microorganisms in the soil to quantify the growth and yield performance of corn.

## Conflict of Interest

The authors declare that there are no conflicts of interest.

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