Applied Soil Ecology 121 (2017) 143–151

Contents lists available at ScienceDirect

Applied Soil Ecology

journal homepage: www.elsevier.com/locate/apsoil

Survival of chicken ascarid eggs exposed to different soil types and fungi

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ARTICLE INFO

Keywords:
Fungi
Pochonia
Soil type
Ascaridia
Heterakis
Nematodes

ABSTRACT

The eggs of intestinal ascarid parasites (*Ascaridia galli* and *Heterakis* spp.) of chickens can survive long-term in soil and this makes contaminated yards and pastures infective to chickens for years. The fungi *Pochonia chlamydosporia* Biotype 10 and *Metarhizium brunneum* KVL04-57 can kill ascarid eggs in agar assays but their efficacy against these eggs in soil is unknown. We therefore initially tested the ovicidal effect of the two fungi in laboratory soil assays. Unembryonated eggs were added to sterilised and non-sterilised soil with or without fungi, and egg recovery was examined before and after incubation (22 °C, 30 days). Egg recovery was substantially reduced by *P. chlamydosporia* and *M. brunneum* in sterilised soil. However, in non-sterilised soil only *M. brunneum* slightly reduced egg counts. Notably, egg recovery was reduced markedly in non-sterilised soil though no fungi were applied. To test if this is a general characteristic of natural soil, eggs were incubated in similar assays comprising 7 different soils (sterilised and non-sterilised). After incubation, egg recoveries were reduced substantially in all non-sterilised soils whereas there were only minor reductions in sterilised soils. We next isolated and examined if particular egg-degrading fungi occurred in the non-sterilised soil that had exhibited the most extreme ovicidal activity (99%). The fungal isolates belonged to two genera (*Metarhizium* and *Acremonium*) with reported egg-degrading properties but when tested *in vitro* in agar assay, none of the three tested isolates of native fungi degraded > 34% eggs, suggesting that these fungi may not individually have caused the reduction in egg survival in the non-sterilised soil. In conclusion, a range of natural soils showed inherent properties related to biotic factors to degrade nematode eggs. The biocontrol efficacy of both *P. chlamydosporia* Biotype 10 and *M. brunneum* KVL04-57 was very good in sterilised soil but currently limited in non-sterilised soil, potentially due to competition with natural soil biota in fungal establishment. Further research may determine if efficacy may be increased by changing the formulation.

1. Introduction

Ascaridia galli (intestinal roundworm) and *Heterakis* spp. (caecal roundworm) are economically important ascarid parasites of chickens worldwide. Heavy infections with the ascarids can cause health problems (Ikeme, 1971; Schwarz et al., 2011a,b), behavioural problems (Gauly et al., 2007) and decreased productivity in chickens (Phiri et al., 2007; Skallerup et al., 2005). *A. galli* may also cause aesthetic problems to consumers as worms may occasionally migrate from the host's intestine into the oviduct via the cloaca and accidentally be trapped inside a chicken egg (Bigland and Graesser, 1961; Fioretti et al., 2005; Reid et al., 1973). These ascarids have a simple life-cycle (Ackert, 1931; Clapham, 1933). Unembryonated eggs excreted with chicken faeces develop to infectivity in the litter and soil environment if temperature and humidity are suitable. Birds acquire the infections by ingesting infective eggs and worms develop to sexual maturity in the small intestine (*A. galli*) or caeca (*Heterakis* spp.). Both nematode taxa are highly prevalent in outdoor based systems (free-range and organic) for laying hens (Kaufmann and Gauly, 2009; Permin et al., 1999; Thapa et al., 2015a).

In the European Union member states, free-range and organic systems require that hens have outdoor access to promote natural behaviour (Anonymous, 2008, 1999). Unfortunately, ascarid eggs may remain viable and infective outdoors for at least 2–4 years (Farr, 1961; Thapa et al., 2017) and there are currently no effective methods to sanitize pastures once they are contaminated. Control of the infections in outdoor systems therefore relies entirely on flock treatment with a limited number of synthetic anthelmintics (AH) but reinfections are

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http://dx.doi.org/10.1016/j.apsoil.2017.10.001
Received 24 May 2017; Received in revised form 21 September 2017; Accepted 2 October 2017
Available online 12 October 2017
0929-1393/

inevitable if birds have continuous access to contaminated premises, e.g. pastures and yards. Overuse of AH may increase the risk of anthelmintic resistance as seen for other parasitic nematodes (Sutherland and Leathwick, 2011). This together with increased consumers' demand for products with minimal synthetic chemical input emphasizes the need of alternative approaches to parasite control.

One approach could be biological control using fungi that degrade the parasite eggs in the environment before they can infect their host (Braga and De Araújo, 2014). *Pochonia chlamydosporia* (syn. *Verticillium chlamydosporium*) is one of the most studied species of ovicidal fungi and has been developed as a biological control agent against many plant-parasitic root-knot and cyst nematodes (Kerry and Hirsch, 2011; Sellitto et al., 2016; Stirling and Smith, 1998; Tobin et al., 2008; Verdejo-Lucas et al., 2003). The fungus occurs naturally in soil as a saprophyte and upon contact with nematode eggs, the fungus switches to a parasitic phase and colonizes the eggs (Kerry and Hirsch, 2011) by hyphal penetration (Escudero and Lopez-Llorca, 2012; Lopez-Llorca et al., 2002; Segers et al., 1996) followed by enzymatic degradation of egg shell protein and chitin components (Kunert, 1992; Segers et al., 1996; Zhang et al., 2009). Recent results show that one isolate of *P. chlamydosporia* and one isolate of a closely related insect-pathogenic fungus *M. brunneum* can degrade a large number of chicken ascarid eggs within four weeks on agar using an *in vitro* assay (Cheta, 2015; Thapa et al., 2015b) but it is not known if these fungi could be equally effective under more natural conditions in soil assays.

The overall aim of this study was therefore to investigate the survival of chicken ascarid eggs in a series of laboratory based soil assays with or without inoculum of two isolates of *P. chlamydosporia* and *M. brunneum* using sterilised and non-sterilised natural soil in parallel. This allowed for evaluation of the biocontrol potential of the microfungi against ascarid eggs within the soil matrix with and without competition from the soil biota.

2. Materials and methods

In the present study, we initially tested the ovicidal effect of the microfungi *P. chlamydosporia* and *Metarhizium brunneum* in sterilised and non-sterilised soils along with a non-ovicidal fungus *Trichoderma harzianum* as control for egg detection despite fungal growth (Experiment (Exp.) 1). Results from Exp. 1 surprisingly indicated a significant inherent ability of the un-inoculated non-sterilised soil to reduce ascarid egg survival. Consequently, we wanted to evaluate if this was a general trait of natural soils. We therefore collected soils, exhibiting different characteristics and microbial activity, from a well-described long-term (> 10 years) field experiment to compare the relative ovicidal effect in sterilised and non-sterilised soil (Exp. 2). Lastly, we obtained fungal isolates from samples of the initial non-sterilised natural soil to investigate the occurrence of nematophagous fungal species. The obtained fungal isolates were identified by DNA sequencing and selected isolates were inoculated on agar supplemented with ascarid eggs to compare their effect with that of *P. chlamydosporia* (Exp. 3).

2.1. Ovicidal effect of exogenous microfungi (Exp. 1)

2.1.1. Chicken ascarid eggs

Ascarid (*A. galli* and *Heterakis* spp.) eggs were isolated from fresh chicken faeces collected from a Danish organic layer farm (Thapa et al., 2017). Viability (i.e. ability to develop a fully formed embryo) of the egg batch (94%) was determined by incubating a subsample of eggs in 0.1 N H_2SO_4 (25 °C, 15 days).

2.1.2. Soil

A loamy fine sandy soil (FF) was collected in December 2014 from a Danish fallow-field (55°15′ 0.4″N, 11°51′29.3″E) that had not been fertilised since 1997. A total of 10 kg soil was collected from 0 to 15 cm depth from six areas. After sieving (3 mm) and mixing thoroughly, a portion of the soil was sterilised by autoclaving (120 °C, 30 min) inside polypropylene bags (Labsolute®, 150 g soil per bag).

2.1.3. Preparation of fungal inocula

P. chlamydosporia Biotype 10 (obtained from Rothamsted Research Ltd., The United Kingdom; originally isolated from *Meloidogyne incognita* eggs, Brazil) was cultured on Sabouraud Dextrose Agar (SDA; Merck KgaA, Germany) at 22 °C for 5 wk. Spores (conidia and chlamydospores) were harvested using 0.05% Triton-X and the suspension was filtered (900 µm) and collected in centrifuge tubes. The fungal suspension was washed three times by centrifugation (1831 × *g*, 3 min) and re-suspended in 0.05% Triton-X. The concentration of chlamydospores and conidia in the suspension was determined in diluted samples using a haemocytometer (Thapa et al., 2015b). Spore viability (93 and 90% for chlamydospores and conidia, respectively) was determined by incubating diluted subsamples on SDA (23 °C, 30 h). *M. brunneum* (KVL04-57; originally isolated from infected *Cydia pomonella* larva, Austria) and *T. harzianum* isolate T22 (originally produced by protoplast fusion of two strains of *T. harzianum*, T12 and T95 (Sivan and Harman, 1991)) were cultured on SDA (room temperature, 3 wk). The conidia were harvested and spore suspensions prepared as described for *P. chlamydosporia*. The conidial germination was 98 and 99% for *M. brunneum* and *T. harzianum,* respectively.

2.1.4. Experimental setup

Petri dishes (8.5 × 1.5 cm) each containing 22 g soil, 1 ml egg suspension containing 17,430 ± 625 (mean ± S.E.) eggs in sterile H_2O and 1 ml sterile H_2O (to increase soil moisture content) were prepared for both sterilised (n = 56) and non-sterilised (n = 42) soils. For both soils, dishes were randomized into three main groups (n = 14): Control, *P. chlamydosporia* and *M. brunneum*. Additionally, a secondary negative control group (n = 14) *T. harzianum,* which does not degrade chicken ascarid eggs *in vitro* (authors' personal observation) was included for the sterilised soil to assess the efficacy of our method to recover eggs after fungal colonization within the soil matrix. Each control dish received 1 ml Triton-X (0.05%) whereas dishes from the other treatments received either 1 ml *P. chlamydosporia* spore suspension containing 0.9 × 10^6 chlamydospores and 5.4 × 10^7 conidia, 1 ml *M. brunneum* spore suspension containing 5.5 × 10^7 conidia or 1 ml *T. harzianum* spore suspension with 5.5 × 10^7 conidia. Eggs, fungal spores and soil were mixed thoroughly using a sterile applicator in the dish which was sealed with Parafilm®. The moisture content of the soil-egg-H_2O-fungus/Triton-X mixture day 0 (pre-incubation) was estimated to be 23–26%. Seven dishes per treatment were randomly selected to determine the day 0 egg recovery while the remaining dishes were incubated in the dark (22 °C, 30 days). The moisture loss during incubation was measured by recording weight of dishes at the beginning and end of experiment.

2.1.5. Recovery of eggs

On days 0 and 30, thirty ml 0.5 M NaOH was added to each dish (5 °C, 16 h). After washing the soil sequentially through 212 and 20 µm sieves, the residue on the latter was divided between two 50 ml tubes and centrifuged (253 × *g*, 7 min) and the eggs isolated according to Thapa et al. (2017). For each dish, a 20% subsample was examined for the number and development stages of eggs (unembryonated (UE), prelarval (PLE), larvated (LE) or degenerated (DE)) (Thapa et al., 2017).

2.2. Survival of eggs in different sterilised and natural soils (Exp. 2)

2.2.1. Origin and preparation of soil

Soil samples (~5 kg) were collected from the pasture of an organic layer farm (CF; 55°50′18.5″N, 12°22′40.2″E), the fallow-field (FF) used in Exp. 1, an unfertilised field (U) as well as fields fertilised with source separated organic household waste compost (CH), source separated