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Biocontrol of eyespot disease on two winter wheat cultivars by an anecic earthworm (*Lumbricus terrestris*)

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ABSTRACT

Eyespot is a major fungal disease of winter wheat, mostly affecting the base of the stem. The development of biological control approaches, using organisms such as earthworms, represents a potential alternative strategy for eyespot control. In a greenhouse experiment, we analyzed the response of two wheat cultivars (Soissons and Aubusson) to the presence of the pathogenic fungus *Oculimacula yallundae* and the anecic earthworm *Lumbricus terrestris*, alone and in combination. We assessed necrosis frequency, necrosis severity, wheat biomass, resource allocation and soil mineral concentrations. Disease incidence was lower in the presence of earthworms: the frequency of necrosis was 44% lower for Soissons and 70% lower for Aubusson. Necrosis severity was also lower for both cultivars (50% lower for Soissons and 80% lower for Aubusson) in the presence of earthworms. Earthworms had no detectable effect on the shoot and root biomasses of plants exposed to the fungus, but they modified resource allocation between plant organs and nutrient translocation within the plant. Our results suggest that earthworms are a potentially effective biocontrol agent for eyespot, and we discuss the possible underlying mechanisms.

1. Introduction

Over the last 20 years, studies of belowground–aboveground interactions (Hooper et al., 2000) have greatly modified our vision of soil function and plant growth. The underlying mechanisms are increasingly understood, but the potential of this complex feedback system has not yet been fully exploited in agriculture. The sustainability of agriculture could be improved by effective management of belowground–aboveground feedback in the field (Hooper et al., 2000; Wardle et al., 2004; Bardgett et al., 2005). This might allow the replacement of chemical inputs with ecological processes, in the framework of agro-ecology (Altieri, 1989) or ecological engineering (Barot et al., 2012). In this respect, the biocontrol of crop diseases is a key opportunity.

Eyespot, a fungal disease caused by *Oculimacula yallundae* (s yn. *Tapesia yallundae*), previously known as *Pseudocercosporella herpotrichoïdes*), remains problematic even in conventional agriculture and may decrease yield by up to 40% (Meyer et al., 2011). *O. yallundae* is an ascomycete that causes necrosis at the stem base, thereby impairing the uptake of nutrients and increasing the risk of lodging at the end of the crop cycle. The mycelium survives on crop residues, and plants are contaminated by spores transported in the water film present on the soil surface after rain (Matusinsky et al., 2009) or via conidia disseminated by wind and rain splash. The main control methods, in addition to chemical fungicides and the use of resistant cultivars, are the limitation of disease incidence through the use of a diversified crop rotation, the burial of crop residues and cropping practices favoring the rapid infiltration of water into the soil (Colbach et al., 1999).

Biological processes in the soil may also help to control pathogenic fungi, as shown in several studies. For example, litter ingestion by *Lumbricus terrestris* decreases attacks by *Venturia inaequalis*, an ascomycete responsible for apple scab (Hirst and Stedman, 1962). Wolfarth et al. (2011) showed that the presence of *L. terrestris* decreased the incidence of fungal disease due to *Fusarium culmorum* on winter wheat crops. This effect was attributed to the burial of infected residues by the worm. The presence of earthworms (genus *Apporectodea*) has been shown to decrease attack rates for two soil-borne fungal diseases: *Rhizoctonia* bare patch, caused by *Rhizoctonia solani* (Stephens et al., 1993;

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Stephens and Davoren, 1997) and take-all, caused by *Gaeumannomyces graminis* var. *tritici* (Stephens et al., 1994). This control of fungal pathogens by earthworms may reflect the important contribution of microorganisms to the diet of earthworms, which prefer fungi to bacteria (Shan et al., 2013). Moreover several studies (e.g., Bonkowski et al., 2000) have suggested that earthworms may feed selectively on fungi, with pathogenic fungi preferred over non-pathogenic fungi.

We hypothesized that similar mechanisms might underlie the biological control of eyespot, caused by *O. yallundae*, by the earthworm *L. terrestris*. Indeed, the burial of crop residues and the rapid infiltration of water into soil are two processes stimulated by anecic earthworms (Bouché and Al-Addan, 1997). Moreover, many studies have shown that earthworms have a positive effect on plant growth (Brown et al., 1999; Scheu, 2003; Van Groenigen et al., 2014), which might improve plant resistance to disease. We hypothesized that these effects might also result in a lower incidence of eyespot in the presence of earthworms. The mechanisms underlying this control of parasite incidence in the presence of earthworms may involve indirect effects due to improvements in the nutritional status of the plant in the presence of earthworms (Whalen and Parmelee, 2000) or direct effects on plant defense mechanisms and the induction of induced systemic resistance (Puga-Freitas et al., 2012b; Puga-Freitas and Blouin, 2015).

We set up a greenhouse experiment, carried out in microcosms, in which two wheat cultivars were inoculated with eyespot in the presence or absence of *L. terrestris* earthworms. The frequency and severity of stem necrosis were recorded. We also monitored plant growth and development by analyzing (i) the morphology of plant above- and below-ground organs, (ii) resource allocation between the various organs and (iii) tissue N content. We also assessed soil C, N and P availability for the different treatments.

We addressed the following specific questions: (1) Do earthworms decrease the frequency and severity of the disease? (2) Do they decrease the negative impact of the disease on wheat biomass? (3) Are the effects of earthworms robust enough to be observed on both cultivars?

2. Materials and methods

2.1. Experimental treatments

We used four experimental treatments for each cultivar: C: control, without earthworms or fungus; E: earthworms (no fungus); F: fungus (no earthworms); EF: earthworms and fungus.

The experiment was set up as a randomized block design with three factors (earthworms, fungus and cultivar type) and eight replicates for each of the eight treatments, for a total of 64 microcosms.

2.2. Soil, earthworms, plant material and fungal inoculum

Plastic pots (25 cm high, 9 cm in diameter) were filled with 0.8 kg of soil from the 0–30 cm surface layer of a field that had been under maize (*Zea mais*) monoculture for 10 years. This plot was chosen as the soil source because maize does not serve as a host for eyespot; the soil from this plot was, therefore, probably free of this pathogen. The soil was air-dried and sieved through a 2-mm mesh-size sieve, to eliminate earthworm cocoons. The mean characteristics of the 0-30 cm surface layer of the soil were as follows: 1.3 g cm^{-3} bulk density, pH KCl: 7.1, clay: 288 g kg^{-1}, silt: 547 g kg^{-1}, sand: 165 g kg^{-1}, organic C: 27.4 g kg^{-1}, total N: 1.26 g kg^{-1}, total $CaCO_3$: 85.6 g kg^{-1}.

Anecic earthworms (*L. terrestris*) were purchased from Le Thepault Fils (Montlhery, France). They were kept in soil containers at 4 °C for two weeks. The earthworms were then purged, cleaned and weighed. Four days after sowing, two earthworms, with a mean fresh weight of 3.43 (±0.30) g, were added to each microcosm. This density is commonly used in microcosm experiments (e.g., Laossi et al., 2010; Sizmur et al., 2011). Earthworms were overfed four times during the experiment, with 0.80 g of dehydrated alfalfa, to ensure optimal growing conditions. The same amount of alfalfa was also added, on the same dates, to the pots of treatments without earthworms. Wheat seeds, *Triticum aestivum*, from two cultivars of similar earliness, were purchased from the seed companies Florimond Deprez (cv Soissons) and Nickerson (cv Aubusson). Seeds were stored at 4 °C for 44 days. Five germinated wheat seeds were introduced into each pot. Thirty days after sowing, the number of seedlings per pot was reduced to three. Pots were placed in a greenhouse with a 16-hour photoperiod. The temperature thresholds applied were 10 °C and 9 °C for activating the cooling system during the day and night, respectively, and 16 °C and 15 °C for activating the heating system during the day and night, respectively. During the experiment, the temperature remained between 10 °C and 25 °C. Microcosms were watered regularly, to keep soil water content at about 80% of field capacity. Fertilizer (33.5% NH_4NO_3) was supplied to all the microcosms at the tillering stage (0.07 g N pot^{-1}) and at ear formation (0.21 N pot^{-1}), to provide plants with an amount of nitrogen calculated from a dose commonly provided in wheat fields (240 kg N ha^{-1}). The experiment was terminated at flowering, 119 days after sowing, on April 4th.

The eyespot inoculum was provided by GEVES (*Groupe d'Etude et de contrôle des Variétés et des Semences*, Rennes, France), the national seed evaluation agency (Angers, France), on autoclaved infested barley grains. In the treatments with fungus (F and EF), three crushed inoculated barley grains were placed on the soil surface, 8, 37 and 65 days after sowing. Water was supplied during the first five days after inoculation to favor fungus development. For the C and E treatments, we added three non infested crushed barley grains without fungus to each pot.

2.3. Measurements

At the end of the experiment, the frequency of necrosis was determined as the proportion of plants with at least one necrotic lesion. For each plant, we scored necrosis severity with a four-class visual index, based on the proportion of the stem section destroyed by the fungus: 0 = no attack; 1 = less than 1/3 of the stem section destroyed, 2 = between 1/3 and 2/3 of the stem section destroyed, 3 = more than 2/3 of the stem section destroyed. If several attacks were observed on the same plant, only the score for the most severe necrotic lesion was noted. Plant height, total number of tillers and leaf area index (LAI), determined with the LI 3100 planimeter (Li-Cor, USA), were measured for each of the three plants in each microcosm.

The main stem, secondary tillers and ears were separated, oven-dried at 80 °C for 2 days and the dry biomass was recorded. Roots were carefully washed, cut into small pieces and passed through a sieve column (Blouin et al., 2007a). Diameter classes displaying similar types of variation were grouped together, resulting in two final classes: $<$ and >400 μm in diameter.

Oven-dried roots, leaves and shoots were pooled and nitrogen concentrations were determined, according to the Dumas combustion procedure (Houba et al., 1990).

Earthworms were recovered and weighed, and cocoons were counted. In each pot, the total N content of 80 g oven-dried (80 °C for 72 h) soil samples was determined by a dry combustion procedure (NF ISO 13878). Organic C content was determined by Anne's method (Anne, 1945), by colorimetry with potassium bichromate after oxidization of the soil organic matter with sulphuric acid at 135 °C. Soil nitrate and ammonium contents were determined by