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Stable isotope labelling of earthworms can help deciphering belowground–aboveground interactions involving earthworms, mycorrhizal fungi, plants and aphids

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ABSTRACT

Functional relationships between belowground detritivores and/or symbionts and aboveground primary producers and their herbivores are not well studied. In a factorial greenhouse experiment we studied interactions between earthworms (addition/no addition of *Lumbricus terrestris*; Clitellata: Lumbricidae) and arbuscular-mycorrhizal fungi (AMF; with/without inoculation of *Glomus mosseae*; Glomerales: Glomeraceae) on the leguminous herb *Trifolium repens* (Fabales: Fabaceae) and associated plant aphids (*Aphis gossypii*, *A. craccivora*; Hemiptera: Aphidoidea). In order to be able to trace organismic interactions, earthworms were dual-labelled with stable isotopes (^{15}N-ammonium nitrate and ^{13}C-glucose). We specifically wanted to investigate whether (i) isotopic signals can be traced from the labelled earthworms via surface castings, plant roots and leaves to plant aphids and (ii) these compartments differ in their incorporation of stable isotopes. Our results show that the tested organismic compartments differed significantly in their ^{15}N isotope enrichments measured seven days after the introduction of earthworms. ^{15}N isotope incorporation was highest in casts followed by earthworm tissue, roots and leaves, with lowest ^{15}N signature in aphids. The ^{13}C signal in roots, leaves and aphids was similar across all treatments and is for this reason not recommendable for tracing short-term interactions over multitrophic levels. AMF symbiosis affected stable isotope incorporation differently in different subsystems: the ^{15}N isotope signature was higher below ground (in roots) but lower above ground (leaves and aphids) in AMF-inoculated mesocosms compared to AMF-free mesocosms (significant subsystem × AMF interaction). Aphid infestation was unaffected by AMF and/or earthworms. Generally, these results demonstrate that plants utilize nutrients excreted by earthworms and incorporate these nutrients into their roots, leaf tissue and phloem sap from where aphids suck. Hence, these results show that earthworms and plant aphids are functionally interlinked. Further, ^{15}N-labelling earthworms may represent a promising tool to investigate nutrient uptake by plants and consequences for belowground-aboveground multitrophic interactions.

Introduction

In the last decade it has increasingly been recognized that a combined aboveground–belowground approach is necessary to understand the functioning of terrestrial ecosystems (Wardle et al., 2004; van der Putten et al., 2009; Bardgett and Wardle, 2010; Eisenhauer, 2012). Plants thereby play an essential role as they interlink above- and belowground subsystems. Factors above the soil surface can directly or indirectly influence the plant itself, but can also affect soil processes and soil organisms that can feed back to plants (Bardgett and Wardle, 2003; Porazinska et al., 2003; Schröter et al., 2004; Wardle et al., 2004; van der Putten et al., 2009). Several studies investigating the functional diversity and multitrophic interactions in terrestrial ecosystems have shown that aboveground–belowground interactions can have consequences at the ecosystem level (Scheu, 2001; Wardle et al., 2004; Megías and Müller, 2010; Eisenhauer and Schädler, 2011; Zaller et al., 2011b; Eisenhauer, 2012; Arnone et al., 2013). However, so far only a few studies have focussed on the effects of belowground detritivores and symbionts on aboveground herbivory (e.g. Poveda et al., 2003;

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Megías and Müller, 2010; Wurst, 2010; Wurst and Rillig, 2011; Trouvé, 2013).

Earthworms make up the majority of the soil faunal biomass in temperate grasslands (Lee, 1985; Curry, 1994) and are considered as ecosystem engineers (e.g. Jones et al., 1994). They alter the quality of resource inputs either directly, through the return of up to 45 ton ha^{-1} a^{-1} of nutrient rich casts (Bohlen et al., 1997), or indirectly by altering soil processes through bioturbation, acceleration of decomposition of organic materials and an increase in microbial activity and nutrient mineralization, which results in increased plant nutrient uptake and plant growth (Brussaard, 1999; Bonkowski et al., 2001). Despite the plethora of information on the effects of earthworms on soil structure, nutrient availability and plant growth (Bohlen et al., 1997; Scheu, 2003), little is known about their effects on aboveground herbivores (Wurst, 2010). A few studies report earthworm-stimulated herbivory (Scheu et al., 1999; Wurst and Jones, 2003; Poveda et al., 2005), but it appears that no effects or negative effects of earthworms on aboveground herbivores are more often found (Bonkowski et al., 2001; Wurst et al., 2003; Wurst et al., 2004; Trouvé, 2013; Zaller et al., 2013a).

Arbuscular mycorrhizal fungi (AMF) form a widespread mutualism between the plant roots of over 80% of all families of land plants (Smith and Read, 2008). In most environmental conditions, these fungi are beneficial to their host plants, by providing access to limiting soil nutrients, increasing photosynthetic rates and resistance to drought, insect herbivores and fungal pathogens (reviewed in van der Heijden and Sanders, 2002; Smith and Read, 2008). Studies on the effects of AMF on herbivores show either positive or no effects of AMF on phloem feeding insects (Koricheva et al., 2009; Reidinger et al., 2012).

In most terrestrial ecosystems of the temperate region, earthworms and arbuscular mycorrhizal fungi are commonly co-occurring and interacting with each other, however our understanding of these functional interactions is still very rudimentary. Generally, earthworms are thought to affect AMF populations by (i) ingesting fungal species (Dash et al., 1979; Cooke, 1983; Edwards and Fletcher, 1988; Morgan, 1988; Kristufek et al., 1992; Bonkowski et al., 2000) thus affecting the germination of ingested spores (Parle, 1963; Hoffmann and Purdy, 1964; Keogh and Christensen, 1976; Striganova, 1988) and (ii) dispersal of AMF spores (Coûteaux, 1994; Pattinson et al., 1997; Wurst et al., 2004). Even less is known about the effects of earthworms and AMF on aboveground herbivores, especially on sap-sucking herbivores such as aphids.

In both natural and agricultural ecosystems aphids are known to affect plant growth, biomass production, phenology and chemistry (Dixon, 1998). The presence of both earthworms and AMF was shown to accelerate the development of an aphid species (*Myzus persicae*), while aphids were delayed when only AMF or earthworms were present (Wurst et al., 2004). In another study aphid abundance on plants decreased in treatments with earthworms, but this was independent of the presence of AMF (Wurst and Forstreuter, 2010). Despite these important contributions, the mode of interaction between earthworms, AMF, plants and sap-feeding insects remains uncertain and mainly conceptual as traditional ecological methods provided only limited information on nutrient fluxes among organisms involved in these trophic relationships.

To better understand potential multitrophic interactions between earthworms and other organisms in terrestrial ecosystems we for the first time used ^{15}N–^{13}C dual stable isotope labelling of earthworms (Schmidt et al., 2004; Heiner et al., 2011) in experimental ecosystems comprising AMF, plants and aphids. We used ^{15}N ammonium-nitrate and ^{13}C glucose as both elements can be taken up by plant roots either directly or when incorporated in amino acids or other organic sources (Marschner, 1995). For the present study, we hypothesized that due to functional interactions the isotopic label can be detected in different parts of this model ecosystem and that AMF would increase stable isotope uptake into plants and aphids. In particular we investigated whether (i) isotopic signals would be passed on from labelled earthworms to surface castings, plants and aphids, and (ii) these compartments differ in their incorporation of stable isotopes.

These objectives were tested in a factorial experiment where we manipulated the factors earthworms (*Lumbricus terrestris*) and AMF (*Glomus mosseae*) and studied their single or interactive effects on the legume *Trifolium repens* and associated aphids (*Aphis* spp.). The chosen species are commonly co-occurring and interacting in temperate grassland ecosystems throughout Europe.

Materials and methods

Experimental setup and treatments

The experiment was carried out in the research greenhouse of the University of Natural Resources and Life Sciences, Vienna (BOKU) between March and November 2011. Using a factorial design we manipulated earthworms (two levels – addition of *L. terrestris*, +EW, without earthworms, −EW) and AMF inoculation (two levels – inoculation with *G. mosseae*, +AMF, no AMF inoculation, −AMF) to investigate interactions between earthworms, AMF, plants and aphids. We set up six replicates of each treatment totalling 24 experimental units.

Experimental units consisted of 20 L plastic planting pots (diameter: 31 cm, height: 30 cm; further called mesocosms) filled with 18 L sterilized field soil (Haplic Chernozem, silty loam; steamed at 100 °C for 15 h) and quartz sand (grain size 1.4–2.2 mm) in a ratio of 40:60 (v/v) (bulk density 1.4 g cm^{-3}, pH = 7.6, C_{org} = 22.0 g kg^{-1}, N_{tot} = 0.92 g kg^{-1}, P-CAL = 64.5 mg kg^{-1}, K-CAL = 113.6 mg kg^{-1}). We successfully used this substrate mixture in other experiments involving the same earthworm, plant and AMF taxa (e.g. Putz et al., 2011; Zaller et al., 2011c; Zaller et al., 2013b). All mesocosms were lined at the bottom with two layers of garden fleece material to prevent earthworms from escaping while still allowing water drainage.

The uppermost 10 cm soil layer (approximately 6 L) was inoculated with AM fungal propagules [*Glomus mosseae* (T.H. Nicolson & Gerdemann) Gerdemann & Trappe (La Banque Européenne des Glomales – BEG 198) (Glomeraceae)] by mixing 25 g L^{-1} of commercial AMF inoculum (Symbion, Landskroun, Czech Republic) to the substrate (treatment +AMF). AMF-free control treatments (−AMF) received the same amount of sterilized, inactive AMF inoculum (steamed at 110 °C for 2 h). In addition, each mesocosm received 100 ml microbial wash from active AMF inoculum and 300 ml microbial wash of field soil. This microbial wash corrects for possible differences in microbial communities between the different treatments (Koide and Li, 1989). It was prepared with a total of 0.5 kg AMF inoculum and 3.5 kg field soil that was wet-sieved through a cascade of sieves, where the finest sieve had a mesh size of 25 μm to receive a filtered non-mycorrhizal microbial inoculum.

Furthermore, *Trifolium repens* L. (Fabaceae) seeds from an agricultural seed supplier (Lagerhaus, Groß-Enzersdorf, Austria) were germinated in commercial steam-sterilized (105 °C for 20 h) potting soil on a greenhouse bench. Eight days after germination, 18 seedlings (approx. 2 cm high) were transplanted into each mesocosm in a consistent hexagonal pattern in equal distance of each plant individual of 5 cm (equals 240 plants m^{-2}). The greenhouse containing the mesocosms was not temperature-controlled, artificial light was only provided during the last 38 days (14 h light day^{-1} in October and November). Mean air temperature during the course of the experiment was 21.7 °C at 51.6% mean relative humidity. The mesocosms were irrigated daily by adding 500 ml of