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Cooperation of earthworm and arbuscular mycorrhizae enhanced plant N uptake by balancing absorption and supply of ammonia

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ABSTRACT

Earthworms and arbuscular mycorrhizal fungi (AMF) interact to regulate plant nitrogen (N) supply, but the mechanisms through which they affect plant N uptake are unclear. We hypothesized that earthworms, plants and the associated AMF exhibit different preferences for different forms of inorganic N (NH_4^+ and NO_3^-), which could regulate the effect of earthworms and AMF interaction on plant N acquisition. We outlined three independent but complementary experiments to test this hypothesis in the context of exotic earthworm, *Pontoscolex corethrurus*. The earthworm is dominating the plantation forests in subtropical and tropical regions of China, which have their understory dominated by the fern *Dicranopteris dichotoma*. By employing an excised root ^{15}N incubation experiment and a field *in situ* ^{15}N experiment, we found that the fern prefers to use NH_4^+ rather than NO_3^-. Then we did a 2 × 2 factorial microcosm experiment using AMF (*Rhizophagus intraradices*) and earthworms (*P. corethrurus*). The exotic earthworm increased soil NH_4^+ concentration but did not affect soil NO_3^- concentration, while the AMF decreased soil NH_4^+ concentration but had no effect on soil NO_3^- concentration. The increase in soil NH_4^+ induced by the earthworms was efficiently utilized by the AMF, and significantly increased the total N uptake by the fern. In contrast, the AMF alone increased the N concentration of leaves and coarse roots, but not the total plant N uptake, primarily due to the lower levels of available NH_4^+ compared with the earthworm treatments. The uninoculated fern did not benefit from the earthworm-induced increase in soil NH_4^+, suggesting that the root of the fern cannot access the 'NH_4^+ hotspots' created by the earthworms. Our work suggests that successful cooperation of earthworms and AMF on plant N uptake depends on the correct match in N-form.

1. Introduction

Linkages between above- and below-ground biota are a cornerstone of modern ecology; they are critical in regulating ecosystem functioning (Wardle et al., 2004). Soil organisms affect nutrient supply for plants not only by decomposition but also through parasitism and mutualism. Thus, nutrient supply is best interpreted in the context of interactions between soil organisms (Lavelle et al., 2006; Wall and Bardgett, 2012).

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Earthworms and arbuscular mycorrhizal fungi (AMF) each trigger influences on plant nutrients, stimulating decomposition and root uptake, respectively (Hodge and Storer, 2015; Lavelle, 1988). How earthworms and AMF interact with each other, however, to affect plant nutrient uptake, has not been represented in perspectives on the linkages between above- and below-ground biota (Fitter and Garbaye, 1994; Paudel et al., 2016).

Soil nitrogen (N) is the main limiting nutrient for plant growth in most ecosystems (Chapin et al., 2011). Most soil N is present in organic forms; however, plants take up N mainly in inorganic forms (NH_4^+ and NO_3^-) (Vitousek and Howarth, 1991). Earthworms can enhance organic N mineralization by feeding, digestion and casting activities, which often lead to increased soil inorganic N (Araujo et al., 2004; Blair et al., 1997). This increase in N availability has been recognized as, perhaps, the most important effect of earthworms on plant growth (Brown et al., 2004; Barot et al., 2007), but it is still unclear whether plant N-uptake benefits from the increased N availability induced by earthworms (Blouin et al., 2006; Cao et al., 2016; Domínguez et al., 2004; Lubbers et al., 2011). Earthworms may have different effects on soil NH_4^+ and NO_3^-, and subsequently on N acquisition of plants (Domínguez et al., 2004; Hale et al., 2008; Lubbers et al., 2011). Such results may be because plant species prefer different inorganic N forms in different soil conditions. In general, plants that are adapted to low pH and reducing soil conditions tend to take up NH_4^+, whereas plants that adapted to higher pH and more aerobic soils prefer NO_3^- (Maathuis, 2009; Masclaux-Daubresse et al., 2010). Therefore, a focus on soil inorganic N forms can improve predictions of the interactions between earthworm activities and plant N acquisition.

Another important determinant of plant N uptake is AMF, which form symbioses with more than two thirds of terrestrial plants (Hodge and Storer, 2015). However, it is unclear how their function responds to earthworms. AMF take up soil N and transport it to host plants (Jin et al., 2012; Thirkell et al., 2016). Most AMF species predominantly utilize NH_4^+, having low NO_3^- assimilation rates (Cheng et al., 2012; Hodge and Storer, 2015; Perez-Tienda et al., 2012; Tanaka and Yano, 2005).

The cumulative influences of earthworms and AMF on plant N acquisition are complex. Earthworms may reduce AMF abundance by eating fungal hyphae or destroying them as they move through the soil. But earthworms may enhance AMF abundance by dispersing spores (Paudel et al., 2016). Earthworms may affect AMF functioning by enhancing N availability and altering the forms in which N occurs. Previous work has emphasized the amount, over the form, of N that may be present and available for AM fungal uptake (Li et al., 2013; Cao et al., 2016). Here, we propose that the effects of earthworms, AMF, and their interaction with AMF on plant N-uptake are strongly driven by inorganic N forms.

Introduced earthworms now occur in most terrestrial biogeographic regions (Hendrix et al., 2008). Previous studies suggests the effects of exotic earthworms on aboveground biota are mediated by AMF (Lawrence et al., 2003; Nuzzo et al., 2009), so AMF-mediated effects of exotic earthworms on ecosystems might be common (Paudel et al., 2016).

Earthworm invasion is common in disturbed plantations and secondary forests in southern China (Du et al., 2008; Gao et al., 2010; Zhang et al., 2010). These forests commonly have an understory that is dominated by a fern (*Dicranopteris dichotoma*) which grows rapidly when light intensity is high. *D. dichotoma* often forms a dense mat-like understory layer. This influences the soil microclimate (Zhao et al., 2013, 2012), nutrient cycling (Liu et al., 2012; Wu et al., 2011), biodiversity (Zhao et al., 2013, 2012), erosion and nutrient leaching (Zheng et al., 2008), and even overstory tree growth (Wan et al., 2014). In our study site, the pantropical peregrine earthworm *P. corethrurus* (González et al., 2006) accounts for 95% of the earthworm biomass, while *D. dichotoma* accounts for 40% of the understory plant biomass; they most likely co-exist (unpublished data).

In the present experiment, we focused on the interactive effects of the exotic earthworm *P. corethrurus* and the AMF *Rhizophagus intraradices* on *D. dichotoma* N uptake. We outlined three independent but complementary experiments. First, we asked which form of soil inorganic N (NH_4^+ or NO_3^-) the fern prefers; this involved two approaches; a lab experiment that measured uptake of ^{15}N-substrates by excised root segments, and a field ^{15}N labeling experiment. Second, we asked which form of soil inorganic N the earthworms tend to stimulate and the AMF tend to absorb. We approached this by determining soil NH_4^+ and NO_3^- in a ^{15}N-microcosm experiment, in which earthworm and AMF abundance were manipulated as factors. Third, we asked whether N-uptake by the fern was influenced by the differential effects of earthworms and AMF on the balance of NH_4^+ vs. NO_3^- in the microcosm experiment.

2. Materials and methods

2.1. Field site

The field site is located in a plantation mono-cultured with *Schima superba* Gardn. et Champ, at the Heshan National Field Research Station of Forest Ecosystem (112°54′E, 22°41′N), Chinese Academy of Science (CAS), Guangdong Province, China. The climate is subtropical monsoon with distinct wet (from April to September) and dry (from October to March) seasons. The mean annual precipitation is 1580.4 mm and the mean annual temperature is 21.9 °C from 1985 to 2014 (Gao et al., 2017). The soil is an Acrisol (FAO, 2006), the sand, silty and clay content are 48.5%, 13% and 38.5%, respectively. The soil C and, N contents are 56.1 and, 4.3 g kg^{-1} dw soil, respectively. The soil pH was 3.7. The understory vegetation is dominated by *D. dichotoma*; other common understory plants include *Miscanthus sinensis* and *Rhodomyrtus tomentosa*.

2.2. Excised root segment experiment

An excised root technique was adopted to assay the physiological uptake capacity of *D. dichotoma* roots for NH_4^+ and NO_3^- (Rothstein et al., 2000). In April 2017, three clusters of *D. dichotoma* were collected and brought to the laboratory within hours. Fine roots (< 1 mm) of each cluster were removed and washed under 0.5 mM CaCl to maintain membrane integrity. Fifteen 0.1 g fresh weight subsamples of fine roots from each cluster were used to assay for ^{15}N uptake rate. Three kinds of labeling markers were used, $^{15}NH_4NO_3$ (10 atom% ^{15}N) with nitrification inhibitor dicyandiamide (DCD), $^{15}NH_4NO_3$ (10 atom% ^{15}N) and $NH_4{}^{15}NO_3$ (10 atom% ^{15}N). Each form of N label was tested at five concentrations (10, 50, 100, 250, and 500 μM). For all $^{15}NH_4NO_3$ + DCD solutions, the concentration of DCD was 120 μM. DCD was used to inhibit the conversion of NH_4^+ to NO_3^- (McGeough et al., 2016); therefore, the uptake rate for NH_4^+ estimated by the treatment $^{15}NH_4NO_3$ + DCD may be more accurate than the results from the treatment without DCD. However, DCD may be toxic for some plants (Reeves and Touchton, 1986), so the treatment $^{15}NH_4NO_3$ without DCD was kept. All uptake solutions contained 0.5 mM CaCl and 1% sucrose. Each subsample was incubated in 100 ml labeling solution for 30 min. After incubation, roots were washed three times (with about 100 ml each time) with 5 mM KCl and 0.5 mM CaCl to remove any ^{15}N labeling marker absorbed by the root surface. The 45 labeled root samples and 3 unlabeled root samples (one per cluster) were oven dried at 60 °C for 3 days, then ball milled and sieved to 100 mesh before analyzing them for total N content and atom% ^{15}N of the root samples by an elemental analyzer (Vario EL Cube, Elementar Analysensysteme GmbH, Germany) interfaced to a IRMS (Iso-prime 100 IRMS, Isoprime Co., UK).

Atom% excess ^{15}N (APE) was calculated as the atom% ^{15}N difference between labeled samples and unlabeled samples. The ^{15}N uptake rate by roots was estimated by ^{15}N excess in unit mass of root per unit time (μg ^{15}N excess g^{-1} dw root h^{-1}). This is calculated as: