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Multiple effects of secondary metabolites on amino acid cycling in white clover rhizosphere

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ABSTRACT

Secondary metabolites secreted by microbes and plants act as mediators in plant-microbe interactions including nutrient uptake. However, until now very little is known about their role in nutrient assimilation, particularly amino acids, which are important compounds due to their high N content. Here we show that the addition of flavonoid secondary metabolites, derived from clover, to soil changed the bacterial diversity, enhanced the flux of asparagine, and increased the pools of glutamine/glutamate in the soil. This indicates that flavonoids are functionally important qualitative and quantitative components of clover root exudates. Furthermore, the addition of microbial secondary metabolites negatively affected clover uptake of asparagine and plant performance, which demonstrates that microbial competition for nutrients may have multiple physiological targets in the plant. Finally, the detection of intact asparagine in clover roots confirms that amino acid uptake is significant to the plant in agricultural soil. In conclusion, amino-acid flow in the clover rhizosphere can be modified by the effects of clover-derived flavonoids on the bacterial community structure, which affects the flux and pools of amino acids; microbial secondary metabolites, which reduce clover uptake of asparagine; and direct recapture of amino acids by clover.

1. Introduction

White clover (*Trifolium repens*) releases fixed nitrogen (N) to soil in the form of amino acids, with glycine and serine being the most abundant (Fustec et al., 2010; Lesuffleur and Cliquet, 2010). However, amino acid losses are counteracted by re-uptake. Based on the detection of L-asparagine-$^{13}C_4$,$^{15}N_2$, we have recently reported clover uptake rates of 0.4 and 0.04 μmol g^{-1} DW h^{-1} in a sterile hydroponic solution at field-relevant concentrations, in both the presence and absence of inorganic N (Czaban et al., 2016a). In contrast to sterile conditions, microbes effectively compete for nutrients in soil (Bardgett et al., 2003). Experiments in soil systems are thus needed to adequately address the significance of amino acid uptake by clover.

As a good source of C and N, amino acids can be rapidly taken up by microbes directly or after extracellular mineralization (Ma et al., 2009). A half-life of 55 min for Asn in soil was recently reported (Czaban et al., 2016b). Thus, microbial competition limits the "opportunity" of the plant to assimilate amino acids from soil. Microbial release of secondary metabolites, which modify root affinity towards those compounds, may increase microbial competitiveness towards amino acids (Ma et al., 2009). Alteration in amino acid transport in legume roots in response to ubiquitous metabolites produced by *Pseudomonas fluorescens,* was reported by Phillips et al. (2004). They treated hydroponically grown alfalfa with phenazine (PHE) and 2,4-diacetylphloroglucinol (DAPG) and observed a 98% reduction in ^{15}N-alanine uptake. Moreover, Naseby et al. (1999) reported that inoculation of pea (*Pisum sativum*) with *P. fluorescens* producing the same compounds resulted in a considerably increased fraction of organic acids in the pea rhizosphere and a smaller shoot/root ratio. These studies highlight the effect of DAPG and PHE on amino acid cycling and root performance in legumes, but we lack knowledge of their impact on clover, which is important in the sense of net amino acid outflow from the roots due to N_2 fixation. We hypothesize that DAPG/PHE reduce clover uptake of amino acids, which also increases their accumulation in soil.

Similarly, clover can affect soil nutrient cycling by e.g., root exudation of secondary metabolites—flavonoids. Due to their structural diversity, they possess multiple activities, which help the plant withstand the challenges of nutrient deficiencies. This includes among other

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their effect on the composition and activity of beneficial soil microorganisms. For example, flavonoids induce nodulation in *Rhizobium leguminosarum* bv. *trifolii*, which initiate N_2 fixation (Cesco et al., 2012) and stimulate root colonization by arbuscular mycorrhizal fungi, which help the plant to supply P (Savana da Silva et al., 2016). Therefore, the release of flavonoids into clover rhizosphere is one of the mechanisms to increase nutrient uptake by the plant. Other studies have reported effects of flavonoids on bacteria and nutrient cycling including stimulation of *Nitrosomonas* activity involved in nitrification (Venkata et al., 2015) and reduction of bacterial activity engaged in denitrification (Bardon et al., 2016). Of the processes investigated so far, no information exists on the role of flavonoids in amino acid cycling in the clover rhizosphere (Czaban et al., 2016). Can they contribute to clover amino acid uptake by manipulating specific microorganisms, and thereby mineralization/immobilization processes, to enhance amino acid availability in soil? Such property would for example be important for clover grown in organic and low-input agriculture, where most of N is accumulated in organic form. Therefore, exudation of flavonoids could be regarded as a tool to modify the microbial status and ameliorate "opportunity" of the plant to assimilate amino acids from soil. We predict that the release of flavonoids by clover affects amino acid cycling through their impact on soil bacteria and thus changes amino acid availability for plant uptake.

The objectives of this study were to elucidate the temporal effects of a) clover plants and the addition of the common clover flavonoids—formononetin and kaempferol (Carlsen et al. (2012) on the bacterial community structure and thereby changes in amino acid quantities in soil, and b) the addition of microbial secondary metabolites—DAPG and PHE—on clover performance and the subsequent cycling of amino acids in soil. Labeled L-asparagine-$^{13}C_4{}^{15}N_2$ ($^{13}C_4{}^{15}N_2$-Asn) was selected for the present study because it is the most abundant amino acid in clover root extracts and the primary N transport molecule in various legumes (Paynel and Cliquet, 2003; Lea et al., 2007; Lesuffleur et al., 2007; Varin et al., 2010).

2. Materials and methods

2.1. Soil characteristics

The soil used in the study (sandy loam) was collected in April 2014 from the A horizon (5–15 cm) at the CENTS long-term field experiment on reduced vs. normal tillage located in Foulum, Denmark (56°30′ N, 9°35′ E) and was stored at 4 °C. The full soil properties and the homogenization process are described in the Supplementary data. The main soil properties were the following: water holding capacity (WHC), 34.4%; pH 6.1, bulk density, 1.25 g cm^{-3}; total C, 18 g kg^{-1}; total N, 1.6 g kg^{-1}; and organic matter, 3.5% (Chatskikh and Olesen, 2007; Hansen et al., 2011). Homogenized natural soil was sieved (3 mm) and mixed, and the water content was determined (100 °C, 24 h). A portion of the soil was sealed in plastic bags (1.7 kg) and radiated with an electron beam (20 kGy) to reduce the microbial abundance and activity.

2.2. Experimental design

Two sub-experiments were conducted; one on the effect of clover and flavonoids using natural soil (NS), natural soil with the addition of flavonoids (NS + F), and natural soil with clover (NS + C), and second one on the effect microbial metabolites on clover growth using radiated soil with clover (RS + C) and radiated soil with clover and microbial metabolites (RS + C + Mm).

2.2.1. Clover and soil establishment

Seeds of white clover (*Trifolium repens* L., cv. Rivendel) were germinated in pots (PlantStart Rootrainers, Bjarne's frø og planter, Denmark) containing homogenized soil (160 g) in a greenhouse (day/night temperature of 18/8 °C; 12 h photoperiod of 70 μmol m^{-2} s^{-1} light intensity). For the treatments with radiated soil, seeds were first surface-sterilized by shaking in 5% sodium hypochlorite (10 min) and 70% ethanol (10 min) (Pedersen et al., 2011), and then germinated. Seedlings were irrigated manually using Milli-Q water (Merck Millipore, Denmark); sterilized seeds were watered with autoclaved Milli-Q-water (121 °C, 60 min). 40-d old plants were removed from the pots, and transferred to nylon mesh bags (18 × 3.5 × 2 cm) with one plant per bag. Extra natural or radiated soil was added to each bag (1.2 g cm^{-3}). Four bags with plants were placed next to each other in a PCV rhizobox (37 × 20 × 2 cm, Zakład Produkcyjno-Usługowy Ryszard Saługa, ASSR, Dębowa Góra, Poland) (Paszt and Zurawicz, 2005) (Fig. S1). In total, there were 6 rhizoboxes with natural soil and clover (hereafter referred to as NS + C) and 12 rhizoboxes with radiated soil and clover (hereafter referred to as RS + C). Due to poor germination, the 2 rhizoboxes with radiated soil contained 3, rather than 4, bags. All rhizoboxes were kept at a 60° angle and placed in the greenhouse conditions under the same conditions as during germination. The soil water content in the bags and rhizoboxes (50% of WHC) was maintained by monitoring the total weight of the rhizoboxes and adding unsterilized (NS + C) or autoclaved (RS + C) Milli-Q water.

Alongside the rhizobox preparations, 48 pots (5 × 5 × 5 cm) were filled with natural soil (1.11 g cm^{-3}), and the water content was adjusted to 50% WHC (hereafter referred to as NS). Pots were kept under the same conditions as the rhizoboxes. The plants in the rhizoboxes and the soils in the pots were kept for an additional 2 weeks, and labeling experiments were conducted at three times: 2, 3 and 4 weeks after transferring the clover to the rhizoboxes.

2.2.2. Soil supplementation with secondary metabolites

One day before the first addition of $^{13}C_4{}^{15}N_2$-Asn, 24 pots with natural soil (NS) and 6 rhizoboxes with clover in radiated soil (RS + C) were supplemented with the secondary metabolites. Pots with natural soils received 2 mL of each clover flavonoid (formononetin and kaempferol) in water solution (6.93 μg mL^{-1}), introduced at 3 cm depth by raising the pipette while injecting to give a final concentration of 0.1 μg g^{-1} soil DW (hereafter referred to **as NS + F).** The concentrations were based on the concentrations reported in the clover rhizosphere (Carlsen et al. (2012). Similarly, bags in the rhizoboxes with radiated soil received 2 mL of each microbial compound (DAPG and PHE) in a 2% ethanol solution (330 μg mL^{-1}), introduced at 5 cm depth by raising the pipette while injecting to give a final concentration of 4.36 μg g^{-1} soil DW (hereafter referred to as RS + C + Mm). Injections were made at the same angle as the inclination angle of the rhizoboxes of 60°. The DAPG and PHE concentrations were based on Phillips et al. (2004). Secondary metabolites were always supplemented one day before the addition of $^{13}C_4{}^{15}N_2$-Asn, resulting in the soils from the 2nd, 3rd and 4th week sampling being supplied with secondary metabolites 1, 2, and 3 times, respectively.

2.2.3. Addition and uptake of $^{13}C_4{}^{15}N_2$ Asn

Two weeks after the clover plants were transferred to rhizoboxes and the pots were placed in the greenhouse, $^{13}C_4{}^{15}N_2$-Asn (98 atom% ^{13}C, 98 atom% ^{15}N) was added to three rhizoboxes (NS + C, RS + C, and RS + C + Mm, each containing 4 plants) and to 8 pots (4 from NS, 4 from NS + F). Volumes of 3.14 and 2.88 mL of $^{13}C_4{}^{15}N_2$-Asn aquatic solution (2000 μM) were introduced at 5 cm (bag) and 3 cm (pot) depth by raising the pipette while injecting to give a final concentration of 0.04 μmol g^{-1} soil DW. A similar set of samples was prepared with unlabeled Asn (≥98%) as a control for the natural abundance of the labeled compounds. Injections of labeled and unlabeled Asn were added at the same angle of 60° as the inclination angle of the rhizoboxes. After 60 min, rhizoboxes were opened and bags with plants were removed and cut. Roots and soil were separated, and the soil was collected in a plastic bag. Roots and shoots were thoroughly washed in 0.5 mM $CaCl_2$, dried with a paper towel, and frozen in liquid N. Soils from the pots were separately collected in plastic bags. Each rhizobox and pot soil