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Research article

Soil microbial communities respond differently to three chemically defined polyphenols

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ABSTRACT

High molecular weight polyphenols (e.g. tannins) that enter the soil may affect microbial populations, by serving as substrates for microbial respiration or by selecting for certain microbes. In this study we examined how three phenolic compounds that represent some environmentally widespread tannins or their constituent functional groups were respired by soil microorganisms and how the compounds affected the abundance and diversity of soil bacteria and archaea, including ammonia oxidizers. An acidic, silt loam soil from a pine forest was incubated for two weeks with the monomeric phenol methyl gallate, the small polyphenol epigallocatechin gallate, or the large polyphenol oenothein B. Respiration of the polyphenols during the incubation was measured using the Microresp™ system. After incubation, metabolic diversity was determined by community level physiological profiling (CLPP), and genetic diversity was determined using denaturing gradient gel electrophoresis (DGGE) analysis on DNA extracted from the soil samples. Total microbial populations and ammonia-oxidizing populations were measured using real time quantitative polymerase chain reaction (qPCR). Methyl gallate was respired more efficiently than the higher molecular weight tannins but not as efficiently as glucose. Methyl gallate and epigallocatechin gallate selected for genetically or physiologically unique populations compared to glucose. None of the polyphenols supported microbial growth, and none of the polyphenols affected ammonia-oxidizing bacterial populations or ammonia-oxidizing archaea. Additional studies using both a wider range of polyphenols and a wider range of soils and environments are needed to elucidate the role of polyphenols in determining soil microbiological diversity.

1. Introduction

Soil microbial communities respond to natural processes and soil management practices [1]. For example, community structures are altered by temperature, moisture, and atmospheric CO_2 levels [2–4], while fertilizer treatments can change the diversity and abundance of ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA) in soils [5]. Multivariate analysis of soil microbial diversity in several soils with long term fertilizer treatments showed that the aromaticity of dissolved organic carbon was the most important variable correlated to bacterial community diversity [6]. Soil microbial communities can also be affected by secondary metabolites produced by plants [7]. Because of their wide distribution, high abundance and diverse bioactivities, the reactive, aromatic secondary products known as polyphenols are of particular interest as modifiers of soil microbial processes [8].

Tannins are water-soluble high molecular weight polyphenols (500–20,000 g/mol) that bind to and precipitate protein [9]. Tannins are divided into two major classes, condensed tannins (proanthocyanidins), which are polymeric compounds of flavan-3-ol units joined by carbon–carbon bonds, and hydrolyzable tannins, which are comprised of gallic acid or its derivatives esterified to a carbohydrate center [10]. Plants make complex mixtures of tannins, which may include compounds of both types, and which may vary depending on environmental, developmental, and genetic factors [11–13].

List of abbreviations: amo-A, ammonia monooxygenase gene; AOA, ammonia-oxidizing archaea; AOB, ammonia-oxidizing bacteria; CLPP, community level physiological profiling; DGGE, denaturing gradient gel electrophoresis; EGCg, epigallocatechin gallate; qPCR, real time quantitative polymerase chain reaction; R_0, starting fluorescence.

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Tannins enter soil by several different processes including rainfall through the leaf canopy, root exudation, and litter decomposition [8,14]. Estimates of the polyphenol inputs into soils range from 1 to 85 kg ha^{-1} y^{-1} for temperate prairie and forest systems [15] to 479–1710 kg ha^{-1} y^{-1}in tropical systems [14]. In soils, tannins can affect nutrient cycling by serving as substrates for respiration [16], by altering nitrogen availability [17], or by antimicrobial effects [18,19]. Variable and often contradictory effects of tannins on soils have been reported based on amendment experiments with various tannin-containing plant extracts, but this is not surprising given the molecular diversity found within this broad group of natural products.

In the last 10 years the tools for isolating and characterizing specific polyphenols from crude tannin extracts have been developed [9], and are revolutionizing our understanding of polyphenol bioactivities. Structural chemistry of polyphenols provides a logical explanation for variable microbial respiration of different "tannin" extracts. For example, extracts from sugar maple, which contain predominantly hydrolyzable tannins, are more readily respired than extracts from red oak or eastern hemlock which are mainly condensed tannins [16]. The relative ease of respiration of hydrolyzable tannins is consistent with their chemical lability compared to the much more inert condensed tannin structure [20]. Even within a specific structural classification, details of tannin structure can dictate bioactivity as illustrated by the poor respiration of predominantly *cis*-condensed tannins compared to the stereoisomeric *trans*-condensed tannins [21].

Many authors have speculated that tannins may play a critical role in controlling nitrogen cycling in soil [8,22]. Halvorson et al. [23] showed that chemical properties of the tannins such as polarity and sorption capacity [24] were the dominant factors in determining the initial effects of tannins on soluble-*N* in soils, with highly sorptive, hydrophobic tannins associated with higher *N*-retention compared to less sorptive, polar tannins. This suggests that changes in *N* solubility is at least partly due to physiochemical reactions between tannins and soil nitrogen, such as formation of soluble or insoluble tannin–protein complexes [25]. Data from many studies support the idea that tannins in soils form insoluble complexes with proteins, and that formation of these complexes limits *N* availability [20,26–29]. In addition to directly complexing protein *N*, tannin may limit nutrient availability by inhibiting enzymes including proteases, phosphatases, and glycosidases that initiate the degradation of biopolymers [30,31].

An important contributor to loss of *N* from soils is nitrification, which is the biological oxidation of ammonium to leachable nitrites and nitrates. In general, nitrification is limited when inputs of organic *N* are limited [32]. Thus it is possible that tannins in soil might select against ammonia-oxidizing bacteria and archaea by limiting available organic *N* [33–36]. There have been relatively few investigations of the ability of tannins to affect microbial community structure. In ruminants, dietary tannins enhance populations of tannin-degrading rumen microbes [37], but similar clear cut results have not been obtained in soils. In an investigation of coniferous forests in Newfoundland, addition of *Kalmia angustifolia* L. tannin to the soil system did not affect either the physiology or the diversity of the existing heterotrophic microbial communities [29]. In contrast, in an alpine ecosystem, addition of *Dryas octopetala* L. tannin to the soil selected for communities that efficiently degrade tannins [17]. Discrepancies between the studies could reflect differences between the soils, the ecosystems, or the tannins, which were condensed tannin dimers and trimers in the *K. angustifolia* study but comprised a mixture of higher molecular weight condensed tannins in the *D. octopetala* experiment [17,29].

We hypothesize that tannins will affect soil microbial diversity, especially abundance of ammonia oxidizing microbial populations. We expect that the effects will vary with tannin structure, so that different tannins have different effects on microbial populations. We tested this hypothesis by amending soil from a pine-dominated (*Pinus strobus* L.) forest with three different chemically defined polyphenols (Supplemental Fig. 1) and evaluating substrate induced respiration and soil microbial populations with molecular and physiological methods. Changes in metabolic diversity were determined by community level physiological profiling (CLPP) using a Microresp™ system [38]. The overall microbial biomass was measured by determining the abundance of the 16S rRNA gene in DNA extracted from the soil using real time quantitative polymerase chain reaction (qPCR). Diversity of the bacterial community was measured using denaturing gradient gel electrophoresis (DGGE) [39]. Using qPCR for the ammonia monooxygenase gene (*amo*-A) we also assessed relative levels of AOB and AOA [40].

2. Results and discussion

2.1. Respiration in soil amended with polyphenols

Measuring substrate-induced respiration of soil amended with tannin establishes whether a specific tannin is labile enough to serve as a substrate for microbial growth [36,41]. In this study we used the Microresp™ system to evaluate substrate-induced respiration of polyphenols. The Microresp™ system is a technique that has been used for CLPP [38,42], but there are few reports of this system being used to determine ability to respire substrates other than the limited set used in CLPP [43]. We used glucose as a substrate to determine whether the Microresp™ system measured similar kinetics of respiration and levels of respired carbon to a typical titration method using 2 g soil samples in Mason jars and base traps (data not shown) [44]. The results from both approaches were comparable, but the Microresp™ system uses less than 0.3 g soil per sample in an array set up making it well suited to study respiration of purified natural products that are difficult to produce in large quantities.

Preliminary assessment of compounds in a library of polyphenols indicated that high molecular weight proanthocyanidins are not respired by soil microbes in short term experiments. This is consistent with previous reports on poor respiration of high molecular weight condensed tannins [16,41]. In the same preliminary experiments, hydrolyzable tannins including simple gallotannins (pentagalloyl glucose) and ellagitannins (oneothein B) were respired, with the smaller and less highly cross-linked gallotannins degraded more rapidly. Low molecular weight polyphenols including methyl gallate and catechin served as good substrates. Based on this assessment, we chose to examine a series of related compounds that had different molecular weights but similar functional groups (Supplemental Fig. 1). Oenothein B (molecular weight 1568), epigallocatechin gallate (EGCg, molecular weight 458) and methyl gallate (molecular weight 184) were used in the remainder of our experiments. All three compounds are esters of gallic acid, but EGCg represents the flavonoid-derived tannins and oenothein B the hydrolyzable tannins. Aromaticity is correlated with molecular weight, with gallic acid having one, EGCg three, and oenothein B eight aromatic rings. Glucose served as a positive control and the water control samples, comprising soil with water but no exogenous substrate, provided the basal level of metabolism for all experiments.

Substrate-induced microbial respiration of glucose in the Microresp™ system was similar to that reported for many natural soils [45,46]. CO_2 was produced over the first 168 h to yield 44.4 μg CO_2–C g^{-1} soil for samples that were supplied with 1000 μg C g^{-1} soil (Fig. 1). The small polyphenol (methyl gallate) was a better substrate for respiration than the larger polyphenols