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Monoterpenes and higher terpenes may inhibit enzyme activities in boreal forest soil

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ABSTRACT

Plant secondary compounds, including terpenes, potentially play an important role in controlling the decomposition process in boreal forest soil. However, the role of terpenes is not well understood, and their direct influence on enzyme activity is not well-known. The aim of this study was to examine the possible effects of common monoterpenes and higher terpenes on the activity of enzymes crucial in C, N, P, S cycling, i.e. β-glucosidase, chitinase, protease, acid phosphatase and arylsulfatase. Monoterpenes (α-pinene, carene, myrcene), diterpenes (abietic acid and colophony), and triterpene (β-sitosterol) were used. Studies were done in two environments, *in vitro* (studies without soil) and *in vivo* (studies with soil). Soil experiments were conducted using humus layers of two different birch stands, the first N-poor with high organic matter content and the second N-rich with a lower organic matter content. In general, all the terpenes studied showed inhibitory potential against enzymes in *in vitro* studies. In the soil incubation studies, both of the measured enzymes, chitinase and β-glucosidase, showed some decrease in activity when exposed to different terpenes. Our study suggests that terpenes modify the enzyme machinery in boreal forest soil.

1. Introduction

Decomposition process in boreal forest soil seems to be tightly controlled by numerous factors, including plant secondary compounds, among which terpenes are the largest group (Obst, 1998; Smolander et al., 2012). Chemically, terpenes are hydrocarbons derived from isoprene units. In leaves terpenes may even represent up to 20% of the dry mass (Langenheim, 1994). Qualitative and quantitative differences in the production of terpenes are plant species-specific, but they are also modulated by environmental conditions, like season and biotic and abiotic stress (Klepzig et al., 1995; Rivoal et al., 2010; Smolander et al., 2012). The knowledge about concentration and role of terpenes in soil is scarce. Some of the few results available showed presence of higher terpenes and monoterpenes in boreal forest soil (Smolander et al., 2012, 2013). The most common soil monoterpenes are α- and β-pinene, Δ-3-carene, camphene, myrcene and limonene (White, 1991; Smolander et al., 2006; Asensio et al., 2008).

Terpenes may potentially influence the decomposition process in soil. Certain monoterpenes inhibit nitrification, net N mineralization, and decrease microbial biomass C and N (White, 1991, 1994; Paavolainen et al., 1998; Smolander et al., 2006; Uusitalo et al., 2008). It has been shown that monoterpenes inhibit methanotrophic bacteria and methane oxidation (Amaral and Knowles, 1998; Maurer et al., 2008) and trigger off substantial changes in the composition and functioning of the soil's microbial communities (Asensio et al., 2012). The influence of higher terpenes is far less known. It has been shown that they modify bacterial and fungal growth in pure cultures (Aderiye et al., 1989; Smania et al., 2003; Popova et al., 2009) and in boreal forest soil (Adamczyk et al., 2013). Moreover, the addition of higher terpenes to boreal forest soil partially inhibited nitrification (Adamczyk et al., 2011, 2013).

There is little information on the direct effect of terpenes on activity of enzymes, key factors for every biological process in soil. Some of a few results available indicate that there is an inhibitory effect of terpenes on enzymes, but most of these studies were conducted in the field of medicine: Ganatra and Suchak (2012) showed the inhibitory effect of terpene-based phytochemicals on cyclin-dependent kinase 2. Moreover, Bustanji et al. (2010) showed an inhibition of pancreatic lipase by terpenes from *Ginkgo biloba*.

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With regard to forest soil it has been shown that monoterpenes inhibit a primary enzyme in the nitrification process, ammonia monooxygenase (White, 1988, 1990; Ward et al., 1997).

The aim of this paper was to study the response of enzymes involved in C, N, P and S mineralization to certain common monoterpenes (α-pinene, carene, myrcene) and higher terpenes (abietic acid, colophony, β-sitosterol). We hypothesize that monoterpenes and higher terpenes directly inhibit enzymes (*in vitro* studies); the same effect appears in soil conditions. We studied the activities of a) β-glucosidase, involved in the degradation of cellulose to glucose, b) chitinase (β-glucosaminidase) degrading chitin to amino sugars (Ekenler and Tabatabai, 2002), c) protease releasing amino acids/peptides from peptides/proteins (Nduwimana et al., 1995), d) acid phosphatase, which produces plant available phosphates and e) arylsulfatase producing plant available sulfates (Acosta-Martinez et al., 2007). To ensure that we recognize the possible effect of terpenes on enzymes, we used two different soils, different concentrations of terpenes and different exposure times. Studies were conducted in two environments, *in vitro* (studies without soil) and *in vivo* (studies in soil); studies in soil included only enzymes involved in C and N cycling as P and S are not limiting nutrients in soils used (Finer et al., 2005). To assess how the addition of terpenes affected overall soil microbial activity, we measured the CO_2 production. We used birch soil as it contains less terpenes than spruce or pine soil (Kanerva et al., 2008), and therefore the effects of added terpenes may be more noticeable. Birch soil was taken from two different forest sites: one was N-poor with high organic matter content and the other was N-rich with a lower organic matter content. We used two forest sites to observe possible site-specific differences.

2. Materials and methods

At first we will describe compounds (terpenes and enzymes) and soils used in the study (Section 2.1.), later the experimental design. In studies without soil (*in vivo*) we investigated direct response of enzymes to terpenes (Section 2.2.), we measured amount of used monoterpenes (Section 2.2.3.) and precipitation of enzymes by higher terpenes (Section 2.2.4.). In studies *in vivo* we investigated the response of enzymes and CO_2 production to terpenes (Section 2.3.).

Measurements of enzyme activity were done at room temperature using a Shimadzu UV–VIS 2401 PC spectrophotometer. The results of enzymatic activity are presented as residual activity of the control (no terpene addition, 100% activity). All analyses were done using three replicates.

2.1. Terpenes, enzymes and soils used in studies

We used commercial α-pinene, carene, myrcene (monoterpenes), abietic acid (diterpene), β-sitosterol (triterpene), and colophony (a mixture of diterpenes); which consisted of abietic acid (37.7%), palustric acid (22.2%), neoabietic acid (18.4%), pimaric acid (8.4%), dehydroabietic acid (7.6%), and isopimaric acid (5.7%) (Adamczyk et al., 2011). We used following enzymes: protease (from *Aspergillus saitoi*), acid phosphatase (from wheat germ), chitinase (from *Trichoderma viride*), β-glucosidase (from *Aspergillus niger*), arylsulfatase (from *T. viride*). All the reagents were ordered from Sigma.

Soils were taken from silver birch (*Betula pendula* Roth.) study site in Kivalo (66°20′N, 26°40′E, northern Finland) and Kerimäki (61°51′N, 29°22′E, south-eastern Finland). For precise description of the sites see Smolander et al. (2000), Smolander and Kitunen (2002) and Uusitalo et al. (2008). At both sites the soil type was Podzol, and the humus type was mor. Each site has three study plots. The Kivalo soil was less N-rich than the Kerimäki soil (C-to-N ratio 30 and 19.5, respectively). Representative samples (20 cores, diameter 5.8 cm) of the humus layer (O_{fh}) were taken from each plot in September 2011, and combined to give one composite sample per Kivalo site and a second one for Kerimäki site. The dry weight (+105 °C, 16 h) was determined, and the organic matter content was measured as loss on ignition (+550 °C, 4 h). The soil pH was measured in a soil–water suspension of 15 ml of soil in 25 ml of ultrapure water. The soil organic matter (SOM) content was 67.5% in the Kivalo soil and 32% in the Kerimäki soil. Soil pH for Kivalo was 4.1 and for Kerimäki 4.5.

2.2. The response of enzyme activities to terpenes (in vitro studies)

2.2.1. Experiments with higher terpenes

Proteolytic activity was measured on the basis of the Anson method (1949). Abietic acid, colophony, and β-sitosterol (10, 20, 50 mg) were mixed with acetate buffer (0.6 ml, 0.1 M, pH 4), and, after 10 min, protease (0.2 ml, 0.1% in acetate buffer) was added. After 1 h of incubation the samples were filtrated using syringae filters (0.45 μm, PALL Corporation). Filtration was necessary, as trichloroacetic acid (TCA) added in the next step, dissolves terpenes and this can influence the results. 0.2 ml of filtrate was mixed with hemoglobin (0.2 ml, 0.5%, in acetate buffer). After 1 h TCA (0.4 ml, 10%) was added. After centrifugation (12,000*g*, 5 min), 0.2 ml of supernatant was mixed with Na_2CO_3 (0.8 ml, 6%) and Folin reagent (0.2 ml, diluted 5 times with water). After 20 min the absorbance was read at 750 nm.

A control sample (no terpenes, 100% proteolytic activity) contained 0.2 ml of 0.025% protease (the same final concentration as in the studied sample) and hemoglobin (0.2 ml 0.5%). The following steps were the same as described above. A blank sample (0% proteolytic activity) was prepared by mixing protease (0.2 ml 0.025%), TCA (0.4 ml, 10%), and, after one hour, hemoglobin (0.2 ml 0.5%) was added. The following steps were as described above.

The methods to study the activities of arylsulfatase, β-glucosidase, chitinase and acid phosphatase were very similar: differences were in the enzyme, substrate and the time of incubation (see Table 1). Terpenes (10, 20, 50 mg) were mixed with acetate buffer (0.6 ml, 0.1 M, pH 4), and after 10 min with 0.2 ml enzyme. After 1 h 50 μl of substrate was added. After incubation samples were filtrated using syringae filters (0.45 μm, PALL Corporation), and TRIS–NaOH (0.2 M, 0.4 ml; adjusted to 12 pH with 5 M NaOH) was added to 0.2 ml of filtrate. The absorbance was read at 405 nm. The control sample (no terpenes, 100% activity of enzyme) was prepared in the same way as the studied sample, but without terpene addition. The blank sample was prepared in the same way, but instead of an enzyme the same volume of water was added.

2.2.2. Experiments with monoterpenes

To study the influence of monoterpenes on enzyme activities we used similar methods to those described above but with the necessary modifications (see Table 1 and text below). A monoterpene (1 ml of α-pinene, myrcene or carene) or 1 ml of water (control) was poured into glass bottles (125 ml) which were then covered with gas-tight septa. The bottles were warmed up to 40 °C for 30 min (to increase evaporation). From each bottle 10 ml of head space was taken using a syringe with a needle and injected into the buffer (acetate buffer 0.5 ml 0.1 M, pH 4) containing an enzyme (for concentrations see Table 1). For measuring enzyme activities, 100 μl of this liquid (for proteolytic activity 200 μl) was transferred to another test-tube.

For proteolytic activity measurements, to 200 μl of liquid containing buffer, enzyme and monoterpene 100 μl ml of hemoglobin was added. After 1 h TCA (200 μl, 10%) was added. After