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SHORT COMMUNICATION

Influence of diterpenes (colophony and abietic acid) and a triterpene (beta-sitosterol) on net N mineralization, net nitrification, soil respiration, and microbial biomass in birch soil

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Abstract The aim of this study was to examine the effect of common diterpenes (colophony, abietic acid) and triterpene (beta-sitosterol) on carbon (C) and nitrogen (N) transformations in soil under birch (*Betula pendula* L.). Samples were taken from the organic layer at two study sites, Kivalo (N-poor soil) and Kerimäki (N-rich soil), and incubated with the above-mentioned terpenes in laboratory conditions. Carbon dioxide evolution (C mineralization), net N mineralization, nitrification, and N and C in microbial biomass were measured. All these terpenes increased C mineralization, but decreased net N mineralization. The potential to decrease net N mineralization depended on amount of terpenes, with a stronger effect at a higher amount. Net nitrification in Kerimäki soil (N-rich soil) decreased but was not completely inhibited by terpenes. Effect of terpenes on soil microbial biomass C and N was not so clear, but they tended to increase both. Our study suggests that higher terpenes can act as a carbon source for soil microbial communities.

Keywords C mineralization · Forest soil · Microbial biomass C and N · Net N mineralization · Nitrification

Introduction

Terpenes, also called terpenoids or isoterpenoids, represent the largest group of plant secondary compounds (Obst 1998). The concentration of terpenes in plant leaves usually ranges from 1% to 2% of the dry weight (Langenheim 1994). They are also produced by microbes, including soil microbes (Stahl and Parkin 1996), certain marine organisms (Jansen and De Groot 2004), and insects (Laurent et al. 2003). Qualitative and quantitative production of terpenes depends on species and on environmental conditions (e.g., Munne-Bosch et al. 2000). Coniferous trees, such as pine and spruce, contain sesquiterpenes, diterpenes and triterpenes and also monoterpenes; but birch does not contain diterpenes, producing mainly triterpenes and sesquiterpenes (Obst 1998). The concentrations of the sum of sesquiterpenes, diterpenes, and triterpenes were about 1.0 (silver birch) to about 2.5 (Norway spruce and Scots pine) grams per kilogram dry mass (d.m.) of leaf and needle material (Kanerva et al. 2008), providing important input of terpenes to the soil. Ground vegetation can provide an additional source of terpenes (Kanerva et al. 2008). In litter layer of a tree species experiment in Northern Finland, Kanerva et al. (2008) found diterpenes up to 15 and triterpenes up to 2 g kg^{-1} of organic matter (o.m.), and in the humus layer up to 4 and 0.8 g kg^{-1} of o.m., respectively. Beta-sitosterol was dominating triterpene in all soils (Kanerva et al. 2008).

Monoterpenes may influence nitrogen (N) cycling in forest soil, e.g., by inhibiting net N mineralization (White 1994; Smolander et al. 2006), net nitrification (White 1994; Paavolainen et al. 1998), and by decreasing carbon (C) and N contents in microbial biomass (Smolander et al. 2006). Canal oleoresin emitted from coniferous plants after injury, and consisting of volatile monoterpenes and higher terpenes, showed similar effects on N and C mineralization and nitrification (Uusitalo et al. 2008). The influence of higher terpenes, like diterpenes and triterpenes on soil

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processes is poorly known. These terpenes can have antibacterial and antifungal effects (Aderiye et al. 1989; Smania et al. 2003; Popova et al. 2009).

As discussed above there is evidence that monoterpenes may regulate N cycling in forest soils but there is no information how higher terpenes act in C and N cycling; are they good carbon sources, or relatively recalcitrant compounds, or are they showing inhibitory effects. The aim of this study was to evaluate the influence of diterpenes (abietic acid and colophony—a mixture of diterpenes) and a triterpene (beta-sitosterol) on C mineralization, net N mineralization and nitrification, and microbial biomass of two different silver birch soils. We used the birch organic layer because it contains very small amounts of diterpenes, and beta-sitosterol is common in all forest soils. Experimental design included incubations of soil exposed to these terpenes in controlled laboratory conditions. Our assumption was that di- and triterpenes affect microbial processes related to C and N cycling.

Materials and methods

Study sites and sampling description

The two study sites were located in Kivalo (66°20'N, 26°40'E, northern Finland) and Kerimäki (61°51'N, 29°22'E, southeastern Finland). The Kivalo soil was less N-rich (C to N ratio, 30) than Kerimäki soil (C to N ratio, 19.5) and had originally been a homogenous Norway spruce stand. Now it includes an 80-year-old stand of silver birch (*Betula pendula* Roth.) in three study plots (25×25 m). The soil was a Podzol with mor humus. Characteristics of the study site have previously been described (Smolander and Kitunen 2002; Uusitalo et al. 2008). Kerimäki study site, originally a Norway spruce stand too, was clear-cut in 1993 and planted with silver birch in 1994. Samples for this study were taken from previously N-fertilized plots (N fertilization totaling 860 kg N ha^{-1} during the 30 years before clear-cutting). The soil type was a Podzol and humus type was mor. A more detailed description of this study site can be found in Smolander et al. (2000) and Uusitalo et al. (2008).

In Kivalo, representative samples (20 cores; diameter, 5.8 cm) of the humus layer (Ofh) were taken from all the birch plots in September 2008 and in Kerimäki in May 2009. These were then combined to give one composite sample per site. Samples were sieved (4 mm mesh). The Kivalo soil was frozen until the laboratory experiments began and preincubated in +4°C for 3 days prior to use. The Kerimäki soil was used fresh. Soil water holding capacity (WHC), dry matter, and organic matter contents and pH were measured as described by Priha and Smolander (1999). Organic matter content was 81% and 30% for Kivalo and Kerimäki soil, respectively. According to the previous study (Uusitalo et al. 2008), total C content was 600 and 53 kg^{-1} o.m. in Kivalo and Kerimäki, respectively. Corresponding values for total N content were 20 and 27 g kg^{-1} o.m., respectively (Uusitalo et al. 2008). Terpene concentrations of both soils were measured as described by Kanerva et al. (2008) with gas chromatography–mass spectrometry (HP6890 gas chromatograph equipped with an HP 5973 MS-detector, having DB-5MS column, 30 m long; I.D., 0.25 mm). Split injection system was used and temperature program started from 100°C, and separation of terpenes was obtained during increase from 180°C to 280°C (5°C per minute). Abietic acid (heptadecanoic acid as internal standard) was used as a reference compound to all resin acids and response factor of cholesterol (cholesterol as internal standard) was used for triterpene analysis. Dried soil samples were dissolved in 250 μl pyridine and 250 μl of *N*,*O*-Bis (trimethylsilyl) trifluoroacetamide. Samples were incubated at 90°C (for 1 h) and filtered. For Kivalo soil the concentration of total resin acids was 0.26 g kg^{-1} o.m., abietic acid 0.11 g kg^{-1} o.m., and beta-sitosterol 0.24 g kg^{-1} o.m. The corresponding values for Kerimäki soil were 0.85, 0.33, 0.12 g kg^{-1} of organic matter, respectively.

Laboratory experiments

We used commercial abietic acid, beta-sitosterol, and colophony (Sigma); colophony 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%) (GC-MS analysis).

The effects of colophony, abietic acid, and beta-sitosterol on C and N transformations in soil were studied in experiments in which soil samples were incubated at constant moisture (60% WHC) and constant temperature (+14°C). For Kerimäki, the incubation period was 6 weeks (incubations with colophony, abietic acid, and beta-sitosterol); for Kivalo the incubation lasted 4 weeks (incubations with colophony and beta-sitosterol). Soil samples (corresponding to 20 ml as d.m. Kerimäki soil 4.6 g, Kivalo soil 2.3 g) were placed in 125-ml glass bottles. Terpenes (0.01 or 0.05 g) were mixed with silica gel in the proportion 1:3 (*w*/*w*) and added to the soil. Silica gel alone was shown not to affect the measured parameters (Kanerva et al. 2006). Addition of only silica gel was used in control. To determine the initial values, some of the bottles with only soil and silica gel were stored at −20°C. Glass bottles were covered with gas-tight septa and were aerated every second day. All analyses were made using three replicate bottles for each treatment. The CO_2 evolution was measured by sampling the headspace and analyzing the amount of CO_2 by a gas chromatograph (Hewlett Packard HP 6890 series, GC System) according to

Smolander et al. (1994); the bottles for CO_2 measurements were aerated with pressurized air (for 30 s) 24 h before CO_2 was measured.

Net N mineralization and net nitrification were determined after extraction with 40 ml 1 M KCl as described by Smolander et al. (1995); net N mineralization was estimated as the accumulation of NH_4^+–N and (NO_2+NO_3^-)–N during incubation. Ammonium N and nitrite and nitrate N were measured with a flow injection analyzer (FIA Star 5020, Tecator). Nitrogen and C in the microbial biomass were determined using the fumigation–extraction method, as described previously (Kanerva and Smolander 2007). Briefly, soil samples were fumigated for 24 h at 28°C with ethanol-free chloroform vapor. C and N flushes from the microbial biomass were calculated by subtracting K_2SO_4-extractable organic C and N in unfumigated control samples from those in fumigated samples. C and N flushes were converted to microbial biomass with the formulas of Martikainen and Palojärvi (1990).

The original pH was 4.2 and 5.0 for Kivalo soil and Kerimäki soil, respectively. In the control treatment, the pH increased to 4.6 (Kivalo soil) and decreased to 4.4 (Kerimäki soil) during incubation. All terpene treatments changed the soil pH slightly—at the end of the experiment the pH was 0.1–0.2 unit lower in Kivalo soil treated with terpenes compared with the control; in Kerimäki soil the pH was 0.01–0.3 unit higher in soil treated with terpenes compared with the control. All reagents were purchased from Sigma

Statistical analysis

Differences in the measured values between treatments were compared with analysis of variance (ANOVA), followed by Tukey's test using a significance level of $P<0.05$. When needed, the results were transformed to fulfill the assumptions of the analysis of variance. All statistical analyses were made using "Statistica" software (StatSoft Inc.).

Results and discussion

Diterpenes (colophony and abietic acid) and triterpene (beta-sitosterol) increased CO_2 evolution in both soils (Fig. 1). Addition of C was on the same level (0.01 g beta-sitosterol contains 0.0084 g C, 0.01 g of colophony or abietic acid contains 0.0079 g C) in all treatments. Higher amounts of terpenes increased CO_2 evolution more than the lower amount, colophony more than beta-sitosterol or abietic acid, and the effect lasted for a longer time in the more N-rich (Kerimäki) soil than in Kivalo soil. Higher results for diterpenes can be explained by comparison of the

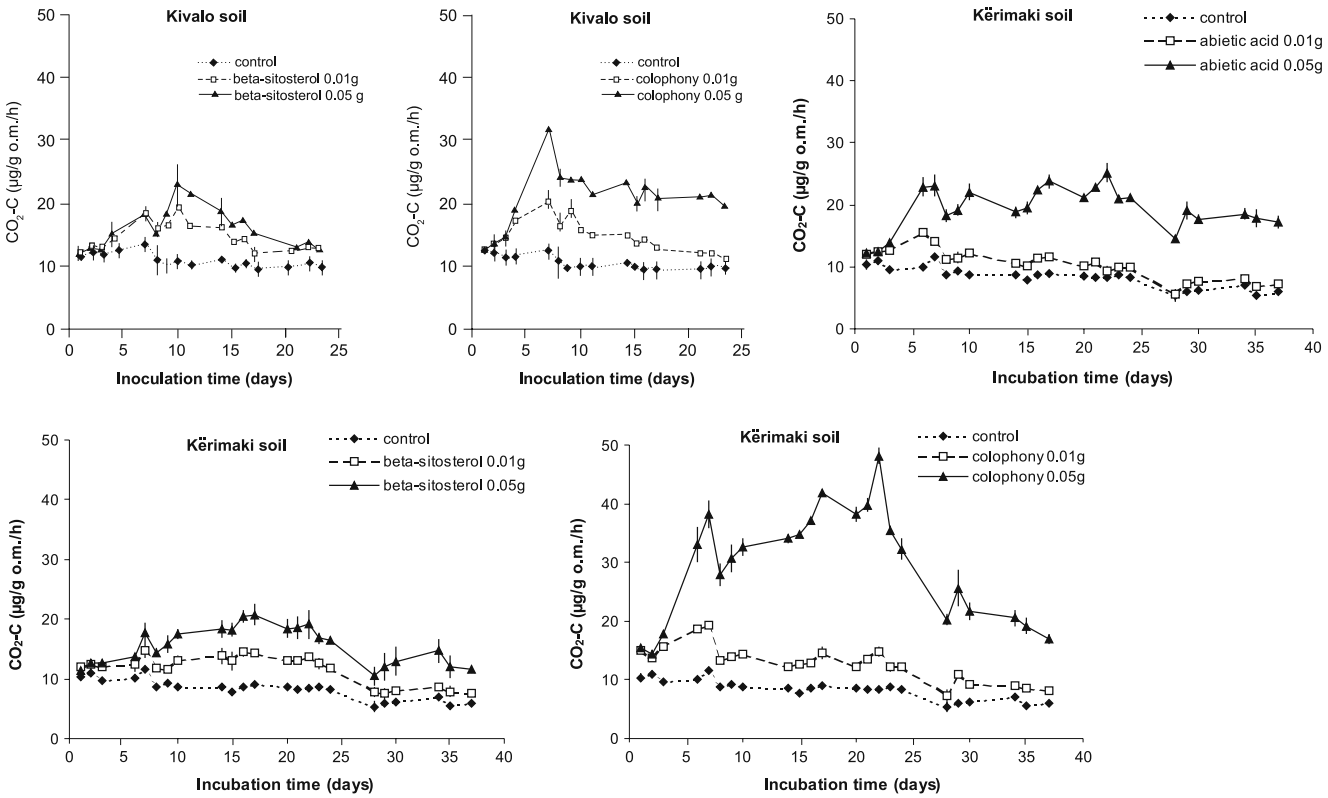

Fig. 1 CO_2–C evolution in Kivalo soil and Kerimäki soil after adding terpenes. Mean and SD for three replicates

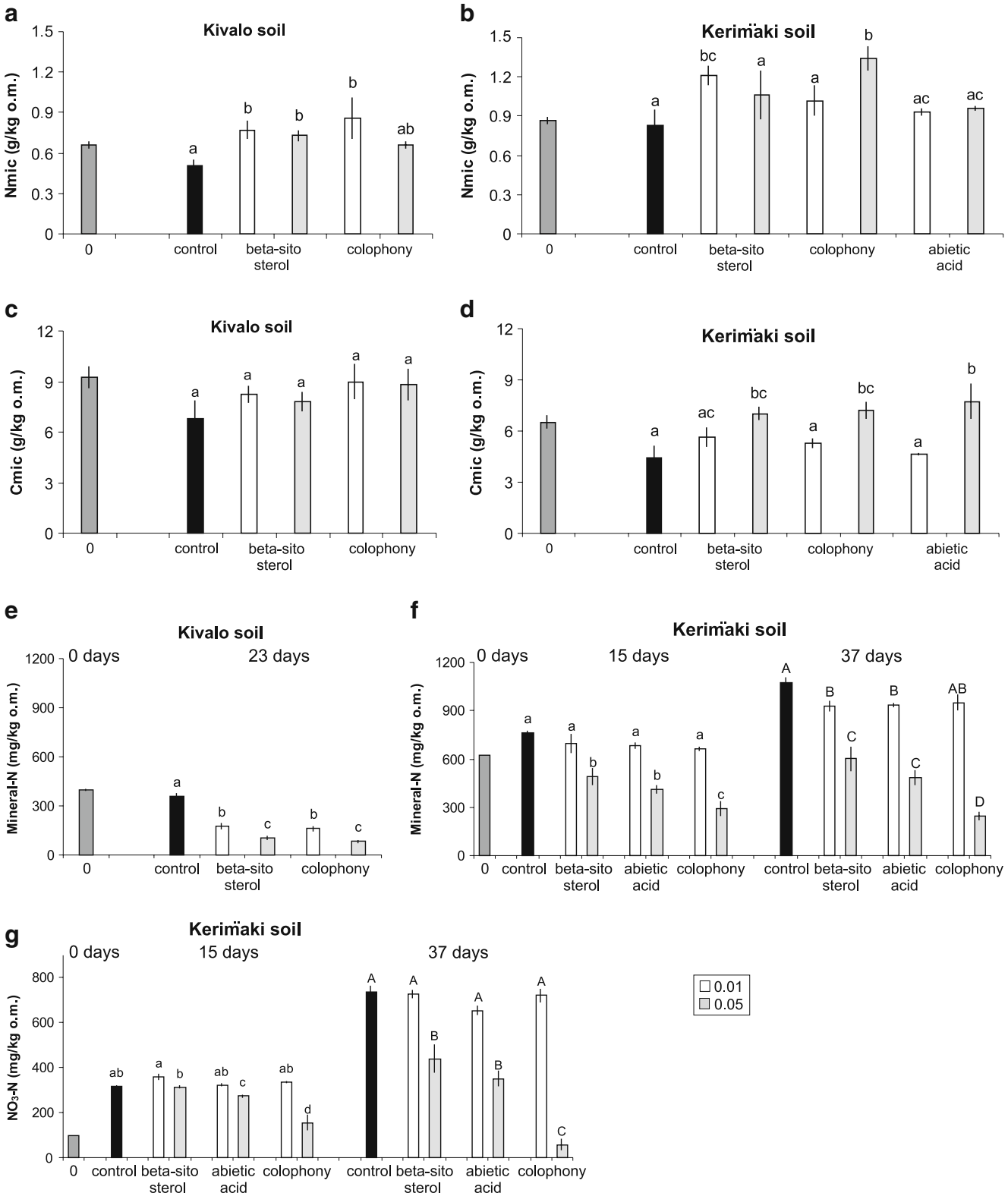

Fig. 2 Effect of terpenes on **a**–**d** microbial biomass C and N, and **e**-**f** net N mineralization in Kivalo soil and Kerimäki soil, and **g** net nitrification in Kerimäki soil. Statistical analysis was performed separately for two soils and for two different incubation periods for Kerimäki soil. Statistically significant differences ($P<0.05$) between means are marked with different letters (for longer incubation period, with capitals). Mean and SD for three replicates

chemical structure of these compounds in light of their digestibility; degradation of beta-sitosterol, especially its side chain is a formidable task (Mahato et al. 1981). On the other hand, degradation of the diterpene, abietic acid does not seem to be such challenge because it was even proven that this diterpene can act as the sole carbon source for *Alcaligenes* isolated from the soil (Cross and Myers 1968).

Microbial biomass C showed also a tendency to increase after these di- and triterpene treatments in Kivalo soil; in Kerimäki soil this increase was statistically significant for higher amounts of compounds (Fig. 2). Also, microbial biomass N showed a tendency to increase in these treatments. Increase in both C mineralization and microbial biomass indicates that these terpenes were used as a C source for soil microbes.

All treatments decreased net N mineralization in both soils, in Kivalo this decrease was statistically significant even after use of lower amount of terpenes, in Kerimäki soil only higher amount of terpenes gave statistically significant difference as compared to control (Fig. 2). As these higher terpenes seemed to act as a source of C for microbes, the decrease in net N mineralization in our study may be caused, at least partially, by microbial N immobilization.

The stimulation of C mineralization and inhibition of net N mineralization by volatile monoterpenes was observed earlier; however, some monoterpenes decreased soil microbial biomass (Smolander et al. 2006). Different behavior of microbial biomass and CO_2 evolution (and thus C mineralization) probably depended on preferences of some soil microbial species for monoterpenes, with others being negatively affected (Amaral and Knowles 1998; Smolander et al. 2006). Surviving soil microorganisms can use debris produced by soil microorganisms for which terpenes are not toxic. The same can occur for higher terpenes. Future research should determine the effects of higher terpenes on different microbial populations in soil.

In Kivalo soil, net nitrification was negligible in all treatments, with ammonification contributing to the results for net N mineralization. On the contrary, in the more N-rich Kerimäki soil, nitrification was high but decreased with higher amount of all terpene additions; but values were still relatively high even after the highest addition (0.05 g) (Fig. 2). The inhibition was strongest with colophony, which decreased net nitrification to half of that in the control already at first measuring point (after 15 days), while with beta-sitosterol and abietic acid this decrease took more time and was detected at second measuring point (after 37 days). Ammonium was always found in soil samples at the end of all incubations, so the amount of ammonium was not restricting nitrification process. The decreased net nitrification can result from either decreased mineralization of N (decrease in substrate NH_4^+–N) or from direct inhibition. Monoterpenes are known to completely inhibit net nitrification (White 1994; Paavolainen et al. 1998; Uusitalo et al. 2008). This "weaker" influence of these diterpenes and triterpene than monoterpenes could be related to the different effects of various terpenes on nitrification; monoterpenes inhibit ammonia oxidizers in forest soils (i.e., Ward et al. 1997), while the effect of higher terpenes on ammonia oxidizers is unknown and needs future research.

Our study was made in controlled laboratory conditions. However, we used terpene amounts which correspond to soil concentrations; additions were 0.01 or 0.05 g which correspond to about 5 and 30 g kg^{-1} o.m., respectively. Diterpene concentration of the humus layer of the pine stand at the Kivalo study site was up to 4 g kg^{-1} of o.m. and that of triterpenes was 1 g kg^{-1} of o.m., and values of the litter layer were much higher (15 g kg^{-1} of o.m. and 2 g kg^{-1} of o.m., respectively). Concentrations of resin acids and beta-sitosterol in the soils were below 1 g kg^{-1} of organic matter, since we used birch soils. Thus, we can assume that the effects of diterpenes and triterpene observed in our study can also occur in natural conditions, at least in coniferous soils.

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