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The Effects of *Pseudotsuga menziesii* Monoterpenoids on Nitrification

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*Pseudotsuga menziesii*의 Monoterpenoid가 질화작용에 미치는 효과

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ABSTRACT

Nitrification potential bioassay and terpenoid analyses were performed to determine the roles of terpenoid as an inhibitor of nitrification in the Douglas fir (*Pseudotsuga menziesii*) forests. The effect of terpenoids in the forest floor was also tested by adding 10μg/ml of four terpenoids(α-pinene, β-pinene, γ-terpinene, and terpinolene) to mineral soils.

The amount of terpenoids in the litter was higher than that in the soil and varied over time, but the amount of terpenoids in the soils was relatively constant. The correlation between the amount of terpenoids in the litter and ammonium oxidation was in inverse proportion to that in the mineral layers (r^2=0.678). Inhibition of ammonium oxidation by terpenoids in the litter was always higher than in the mineral layer, but nitrite oxidation was different from the ammonium oxidation. The fact that there was greater nitrate production from ammonium in the mineral layer than in the forest floor layer seems to be due to the less amounts of terpenoids in the mineral layer.

The result of the experiment in which four terpenoids were added to the mineral layer suggests that, after some lag time, the four terpenoids were effective in inhibiting ammonium oxidation. However, nitrite oxidation did not appear to be affected by the four terpenoids. Accordingly, all of our results suggested that terpenoids in Douglas fir forests apparently would act as a part of the inhibitors of nitrification.

Key words: Ammonium oxidation, Inhibition, Monoterpenoids, Nitrification, Nitrite oxidation, *Pseudotsuga menziesii*

INTRODUCTION

Rates of nitrification have been recorded as being low or nonexistent in coniferous forests (Kilham 1990). Lodhi and Killingbeck (1980) reported that the relatively low numbers of *Nitrosomonas* and *Nitrobacter* found in the soil due to low N concentrations may account for the low nitrate : ammonium ratio. They suggested that microbial oxidation for production of NO_3-N was severely inhibited by plant secondary products in soil. Several allelochemical components in the litter of some conifer species inhibit nitrifiers (Olson and Reiners 1983, Turner and Franz 1985, White 1986). Since coniferous monoterpenes have been hypothesized to inhibit nitrification (White 1986, 1988, 1990, 1991), four of the five major redwood monoterpenoids have been demonstrated to inhibit ammonium oxidation by *Nitrosomonas europaea* in pure culture (Courtney *et al.* 1991), and the correlation of monoterpenoid total yield of the forest floor and mineral soil nitrification potential rates was investigated in Douglas fir forests.

Little is known about the concentration, flux, and effects of monoterpenoid on soils and soil organisms (White 1991). Monoterpenoids inhibit electron transport and uncouple oxidative phosphorylation of microbial membrans (Knobloch *et al.* 1986) and have been shown to inhibit or stimulate fungal growth (Espinosa-Garcia and Langenheim 1991). Fifty percent of the dry weight total yield of redwood monoterpenoids remain in senescing needles (Hall and Langenheim 1986).

The nitrification potential bioassay measures the maximum potential ammonium and nitrite oxidation in 24 hours in soils that have been optimized for nitrification with respect to all ammonium, pH and temperature. The rates may then be used as an index for the size of the nitrifying population in each sample. Using the nitrification potential bioassay a synthetic mixture of the four most concentrated Douglas fir monoterpenoids (both in fresh needles and the forest floor) were added to the bioassay to determine the relative effects on ammonium oxidation and nitrite oxidation.

The purpose of our research is to determine the possible roles of monoterpenoids in inhibition of nitrification during the process of decomposition of leaf litter from Douglas fir.

MATERIALS AND METHODS

Study site

The area of investigation was located in reserve on the University of California, Santa Cruz Campus, which lies on the southeastern end of Ben Lomond Mountain, a major ridge in the Santa Cruz Mountains of central California. The sample sites were located in the upper campus at 1,000m elevation in a Pacific coast mixed evergreen forest. The forest at the site contained predominantly madrone, California bay laurel, tan oak and Douglas fir (*Pseudotsuga menziesii*), and was considered to be a successional stage of a redwood com-

munity. Soil samples were collected under Douglas fir trees and analysed for total yield in addition to the nitrification potential. The soils were classified as either Watsonville or Felton Lompico series cotina (fine-loamy mixed mesic Ultic Agrixerolls) (Soil Conservation Society).

Analyses of Douglas fir essential oil

Fresh leaves were collected from 5 trees from the mid branches on opposite sides of each tree to determine total yield and relative concentration of leaf essential oils by organic extraction and analysis by gas chromatography (Perkin-Elmer Sigma 300). 3g subsamples of the needles were ground with dry ice and extracted immediately after collection with 50ml nano-grade n-pentane. The resulting extract was concentrated by evaporation with a gentle stream of N gas. 5μl of the resulting extract was injected to a split injector (split ratio 20:1) Perkin-Elmer Sigma 300 GC using a 25m DB 5 column with an inside diameter of 0.2mm and a flame ionization detector (FID). The injector temperature, detector temperature, and flow rate were 220℃, 320℃, and 1.8ml /min, respectively. The initial oven temperature was 37℃ for five minutes, and increased to 180℃ at a rate of 5℃ per minute, then by 20℃ per minute until 320℃. Monoterpenoids were identified by peak enrichment of known standards. Concentrations were determined by converting peak area to mass via a calibration curve.

Total yield of monoterpenoids in the forest floor and mineral soil

Two horizons were analysed for total monoterpenoid content and nitrification potential. Five or six samples from 25 × 25cm^2 quadrats each of the forest floor and the mineral soil to a depth of 0~15cm (A_1 horizon) were collected at approximately one month intervals from June to November, 1991. All samples were transported to the laboratory, weighed and dried immediately. Subsamples of the forest floor (3g) and mineral soil (5g) from all the sites were analysed for total monoterpenoid yield by organic extraction with nano grade n-pentane. 1μl of the extract was injected splitless into a GC and analysed as described above.

Nitrification potential bioassay

Three gram of fresh mineral soil samples and 5g or 7g of fresh forest floor samples were incubated in shaking 250ml flasks with 100ml culture solution (1.4 ml /L of 0.2M KH_2PO_4, 3.6ml /L of 0.2M K_2HPO_4, and 10ml /L of 50mM $(NH_4)_2SO_4$ adjusted to pH 7.2) at room temperature for 24 hours. 10ml subsamples of the slurry were taken from each flask at 1, 4, 8, 16, and 24 hours and centrifuged. The supernatant was partitioned and frozen until anlysis for nitrite and nitrate concentration.

Nitrite concentration was measured colorimetrically by the reaction of 4ml supernatant with 0.1ml of 0.1% sulfanilamide HCl and 0.1ml of 0.1% naphthylenediamine at 540nm. Nitrate concentration was determined by the reaction of 1.0ml of supernatant with 1.0ml

of 5% salicylic acid, followed by 10ml of 4M NaOH after cooling. The samples were allowed to cool to room temperature and the absorbance measured at 410nm.

The effect of added monoterpenoids on nitrification potential

10μg /ml of four most concentrated Douglas fir monoterpenoids found in the forest floor were added to mineral soil collected in October and November. β-pinene (5μg /ml), α-pinene (2μg /ml), terpinolene (2μg /ml), and γ-terpinene (1μg /ml) were mixed to simulate the relative composition of monoterpenoids which were similar to those occurring naturally in the Douglas fir forest floor. Although the percentage of γ-terpinene was lower than terpinene-4-ol, citronellol, and bornyl acetate, it was recognized as important because γ-terpinene occurred in all the sites, but in smaller amounts. The synthetic essential oil mixture was added to the soil bioassays in the beginning or 8hrs after treatment and the rates of ammonium and nitrate oxidation were measured as described above.

All statistical analyses were performed with the SAS programs (Statistical Analysis System, Version 5). Analysis of variance (ANOVA) followed by factor analysis was used to analyze for the effect of monoterpenoids on each nitrification rate. The t-test was performed for the difference of terpenoid in the forest floor and the mineral layer.

The regression and correlation coefficient were calculated for the relation between the amount of terpenoid in forest floor and the production concentration of nitrite and nitrate in each layer.

RESULTS AND DISCUSSION

Approximately 16 monoterpenoids and other compounds were identified in the Douglas fir needles, however many were present only in small or trace amounts (Fig. 1). The total yield ranged from 0.503 ~ 1.46mg /g dry weight leaf tissue and percent composition of the Douglas fir essential oil is presented in Fig. 2. β-pinene, terpinolene, α-pinene, γ-pinene, terpinene-4-ol, α-terpinene, bornyl acetate, and sabinene were found as the major constituents in variable concentrations, which is typical in the coastal-intermediate chemotype described by Von Rudloff (1975).

The total yield of Douglas fir monoterpenoids in the forest floor horizon ranged between 0.053 ~ 0.2649mg /g dry weight. The composition of the essential oils in the forest floor was similar to that found in the fresh needles (Fig. 2). Fig. 3 shows that the total amount of monoterpenoids in the forest floor were significantly higher than in the mineral soil horizon($t=4.184$, $p<0.0005$). Monoterpenoids in the forest floor varied greatly over time ($t=10.39$, $p<0.0001$), but there was no qualitative difference between the samples. All showed very similar proportions of terpenoids except for terpinolene. There was much smaller total yield of monoterpenoids in the mineral layer and this was relatively constant (Fig. 3).

The highest total yield of monoterpenoids in the forest floor was found on Novemer 19

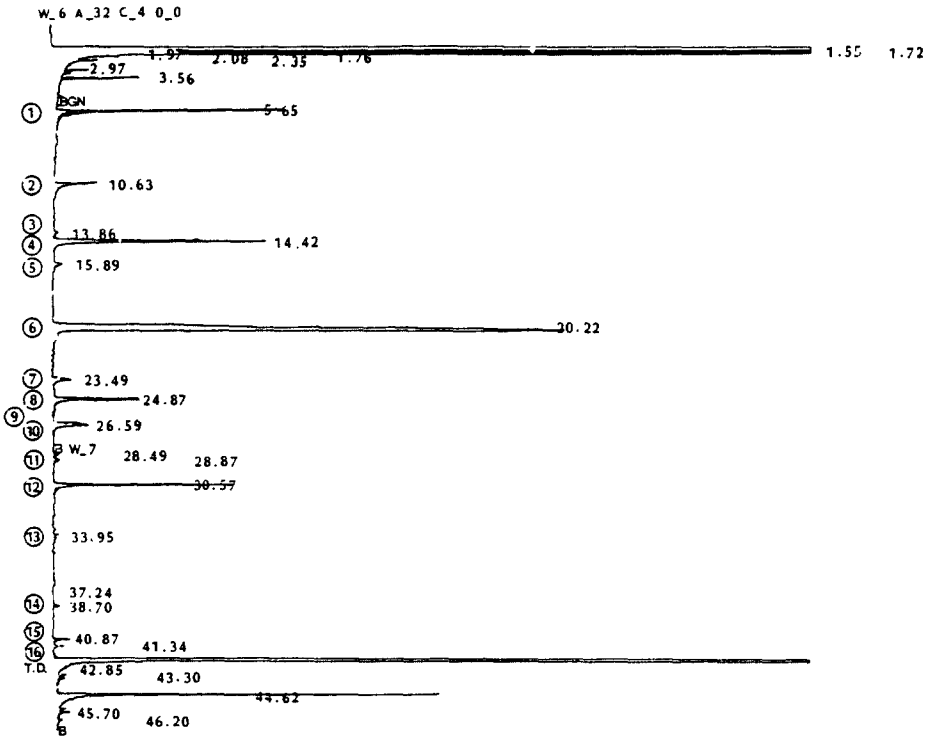

Fig. 1. Gas chromatographic assessment of the extraction from Douglas fir fresh leaf. ① Santene ② Tricyclene ③ α-Thujene ④ α-Pinene ⑤ amphen ⑥ β-Pinene ⑦ Sabinene ⑧ α-Terpinene ⑨ Limonene ⑩ Ocimene ⑪ γ-Terpinene ⑫ Terpinolene ⑬ Terpinen-4-ol ⑭ Citronellol ⑮ Bornyl acetate ⑯ Gernyl acetate T. D: Tetra Decane

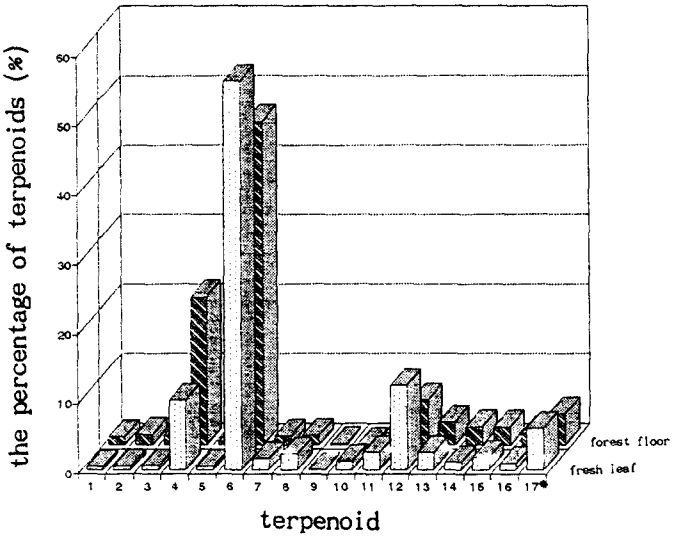

Fig. 2. The average percentage of terpenoids in fresh leaf and litter layer. Numbers are the same as in Fig. 1. 17* : total of sesquiterpenoids, oxygenated terpenoids and other minor compounds.

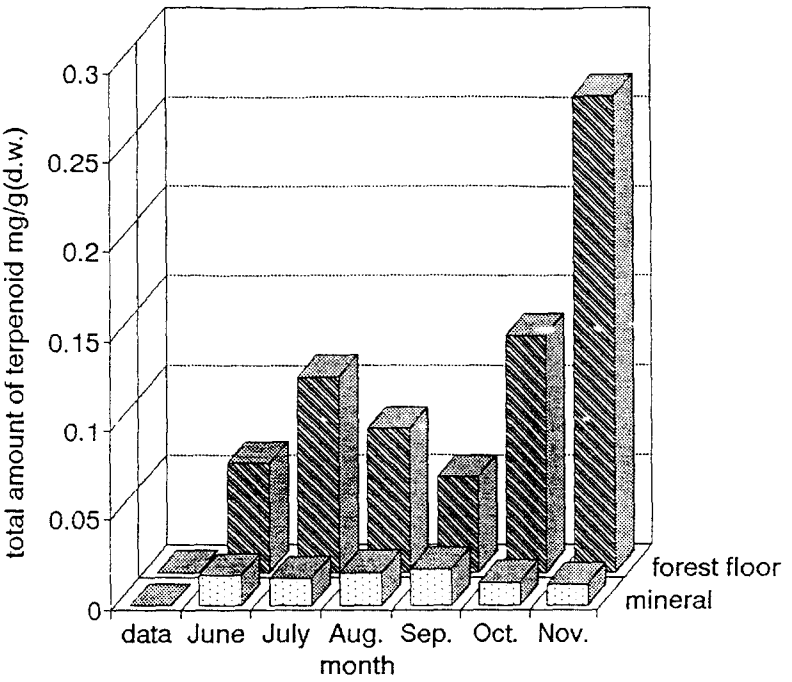

Fig. 3. The amount of monoterpenoids in forest floor and mineral against time.

which may be due to the addition of terpenoids from newly fallen needles. Although the total yield of γ-terpinene was less than terpinene-4-ol, it occurred more frequently than terpinene-4-ol in the forest floor. Therefore γ-terpinene may be more important monoterpenoid with respect to microbial effects than terpinene-4-ol in the Douglas fir forest floor.

There was a significant difference in the rates of ammonium oxidation and nitrite oxidation between the forest floor and the mineral layer during incubation (Table 1). The rate of ammonium oxidation was higher in the mineral soil horizon than the forest floor at all sampling times. The regression analysis (Fig. 4a, b) showed that the rate in ammonium oxidation in the mineral layer (7.202, r^2=0.98) was significantly higher than in the forest floor (2.201, r^2=0.915) in the August sample.

All rates of ammonium oxidation from the nitrification potential bioassay were similar to each other except for samples collected and incubated October 28 and November 19. The rates of nitrite oxidation, however, did not decrease over time. All rates of nitrite oxidation in the forest floor were higher than the mineral soil (Table 1). Higher rates may be due to higher initial substrate concentrations in the forest floor from input of newly fallen litter.

There was an inverse relationship between the total yield of monoterpenoids in the forest floor and ammonium oxidation rates (−22.042, r^2=0.678) in the mineral soil. White (1991) found a strong correlation between monoterpenoid total yield in the litter layer and nitrification rates in the mineral soil. We also found that after addition of the four major

Table 1. The differences in the rates of ammonium oxidation and nitrite oxidation in mineral and litter layers over time.

Date	Ammonium oxidation		Nitrite oxidation	
	Mineral	Forest floor	Mineral	Forest floor
June	7.606	2.777	1.944	4.837a
	(0.687)	(0.441)	(0.270)	(1.223)
July	6.749[a]	1.684[b]	0.772	2.860
	(0.695)	(0.665)	(0.122)	(0.583)
Aug.	7.202[a]	2.201[a]	2.382[c]	2.537[c]
	(0.747)	(0.503)	(0.596)	(0.770)
Sep.	6.236	1.724[a]	1.098	1.890[c]
	(0.833)	(0.649)	(0.214)	(1.206)
Oct.	3.251[a]	1.688[a]	0.323[a]	0.921[a]
	(0.475)	(0.626)	(0.018)	(0.146)
Nov.	2.596	2.167	0.971[a]	2.275
	(0.424)	(0.284)	(0.395)	(0.416)

ANOVA was performed.

( ) values means standard deviation.

a,b, and c indicates numbers of rates, 5, 4, and 3, respectively.

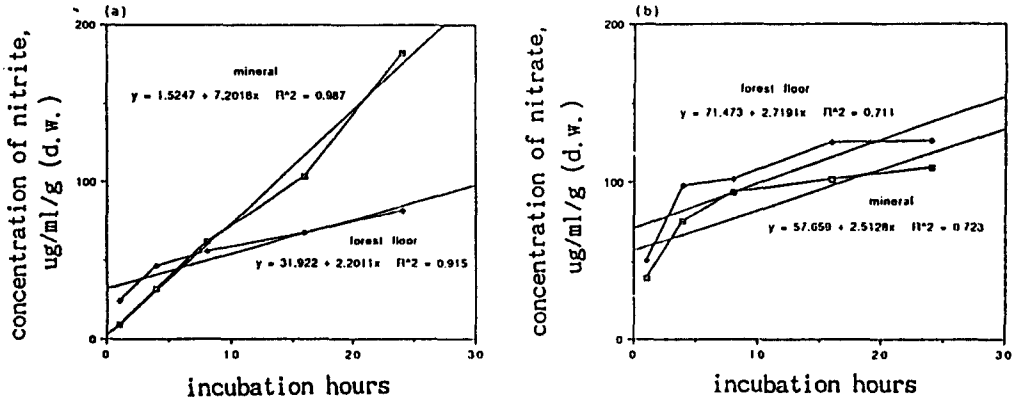

Fig. 4. The ammonium oxidation (a) and the nitrite oxidation (b) in forest floor and mineral layer in August sample.

monoterpenoids found in the forest floor, at a combined yield of 10μg /ml to the mineral layer, the rate of ammonium oxidation decreased from an average of 39μM·g^{-1}·ml^{-1}·hr^{-1} to 23μMg^{-1}·ml^{-1}·hr^{-1}(t=3, $p<0.05$). Rate of nitrite oxidation to nitrate was similar to the control and not significantly affected by the addition of the monoterpenoid mixture(13.32μ M·g^{-1}·ml^{-1}·hr^{-1}, t=1.5059, $p<0.192$) (Fig. 6a, b). This suggests that only ammonium oxidation is inhibited by the addition of monoterpenoids but nitrite oxidation is not which supports the hypothesis suggested by White(1990). He proposed that monoterpenoids bind to the ammonium monooxygenase complex (AMO) due to the similarity of their molecular structure of known nitrification inhibitors (White 1988).

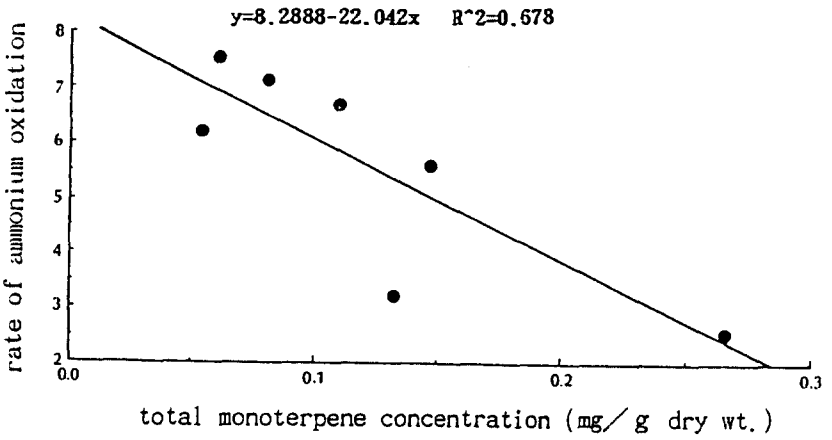

Fig. 5. The relationship between total monoterpenoid concentration and ammonium oxidation rate.

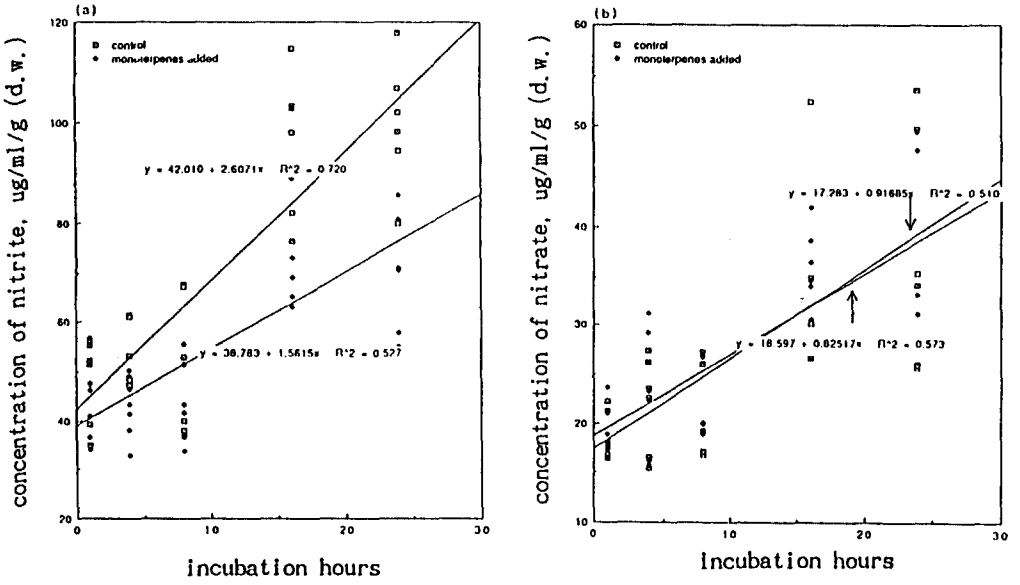

Fig. 6. The effect of monoterpenoids added to the mineral layer on ammonium oxidation (a) and nitrite oxidation (b) in November.

Sometimes nitrification has been found to be weakly correlated with pH in forest soils, but in the present study, the differences in percent nitrification did not consistently parallel the difference in pH. There may be allelochemical components in the litter of some conifer species which inhibit nitrifiers (Olson and Reimers 1983, Turner and Franz 1985, White 1986).

Accordingly, all of our results suggested that terpenoids in the Douglas fir forest floor would act as inhibitors of nitrification. The added monoterpenoids inhibited the ammonium oxidation process in the mineral soil, but did not affect nitrite oxidation in laboratory incubations, and field studies showed the same results. There is consistent evidence for the mechanism of inhibition of nitrification whether monoterpenoids inhibit the enzyme responsible for ammonium oxidation or are toxic to the microorganisms in the process.

Future studies should focus on the mechanisms by which monoterpenoids in the Douglas fir influence the growth of *Nitrobactor* species in pure culture, and simultaneously in field studies.

적 요

Pseudotsuga menziesii 임상에서 질화작용의 억제제로서의 monoterpenoids의 역할을 연구하고자 토양에서의 질화작용과 식물체잎, 낙엽 및 무기토양에서의 monoterpenoids의 함량을 분석하였다.

Pseudotsuga menziesii 잎이나 임상에서 분석된 monoterpenoids는 대략 16종 이었으며, 그 중 α-pinene, β-pinene, γ-terpinene 그리고 terpenolene이 대표적인 것들이었다. 임상에 있는 monoterpenoids의 양은 무기토양층에 비해 항상 많았으며, 계절적 변이가 있었으나 토양층은 항상 일정하였다.

질화작용 과정 중 ammonium oxidation 과정은 낙엽층이 보다 더 많은 저해를 받았으나, nitrite oxidation 과정은 두층별간 별 차이가 없었다. 또한 4가지 monoterpenoids(α-pinene, β-pinene, γ-terpinene, terpenolene)를 인위적으로 첨가한 토양에서의 질화작용에 역시 ammonium oxidation 과정은 심히 저해를 받는 반면 nitrite oxidation 과정은 저해를 받지 않는 것으로 나타났다. 이 같은 모든 결과들은 *Pseudotsuga menziesii* 임상에 있는 monoterpenoids의 영향으로 질화작용에 관여하는 미생물, 특히 *Nitrosomonas europaea*의 증식이 억제되어 ammonium oxidation 과정이 저해되었음을 시사한다.

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