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The Effect of Monoterpenoids on Growth of a Cellular Slime Mold, *Polysphondylium pallidum*

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A cellular slime mold, *Polysphondylium pallidum* was isolated from the forest floor of Mountain Muhak. The effect of 11 selected monoterpenoids on the growth of *P. pallidum* was studied. We tested four different concentrations (1, 0.1, 0.01, and 0.001 μg/μl) for each compound by using a disk volatilization technique. Each compound was treated after germination of spores of *P. pallidum*. The growth of *P. pallidum* was inhibited by the treatment of the monoterpenoids at all concentrations tested. The microscopic analysis further supported these results. Most of the inhibitory effects of the compounds were represented by changes in the shapes of the fruiting bodies, such as very short sorophores, smaller sized sori and sori without spores. Especially, the monotepenoids changed the shape of whorls of side branches. These results suggested that selected monoterpenoids inhibit the growth of *P. pallidum*.

Keywords: cellular slime molds, fruiting bodies, inhibitory effect, monoterpenoid, *Polysphondylium pallidum*

Monoterpenoids produced by plants are released into the environment (Rice, 1984). When these compounds reach the humus layer, they have an influence on soil microorganisms. The ecological roles of monoterpenoids are well known in combination with their allelopathic effects (Yun and choi, 2002) and antimicrobial activity (Silvropoulou et al., 1997; Vokou and Liotiri, 1999; Schmidt et al., 2000). In comparison, the process of decomposition of terpenoids has been poorly studied (Kainulainen and Holopainen, 2002). Recently, it has been suggested that degradation of monoterpenoids in the needle litter of Scots pine is a slow process and these compounds might have effects on decomposer organisms for several years after needle abscission (Coûteaux et al., 1998). On the other hand, other microorganisms might stimulate the degradation of monoterpenoids. Harder and Probian (1995) described microbial degradation of monoterpenes under anaerobic conditions. They suggested that anaerobic bacteria use the monoterpenoids as sole carbon and energy sources. However, the degradation of monoterpenoids depends on the kinds of microorganisms, compounds and their concentrations. It has been proposed that monoterpenoids play a role in the control of microbial processes in environment where they are abundant, such as pine forest soils.

Genus *Polysphondylium*, belonging to cellular slime molds (CSMs) is a common inhabitant of most litter-rich soils (Cavender, 1972; Hagiwara, 1989). They feed on bacteria, which decompose organic materials of dead plants, especially fallen leaves and dead wood. Therefore, CSMs have a very important role in the ecosystem of forest soil (Feest and Madelin, 1988). Furthermore, as *Polysphondylium* lives in the topsoil, it should be worthwhile to evaluate the activity of monoterpenes present in pine forest floor on the growth of *Polysphondylium*. However, to our knowledge, although the effect of monoterpenoids on the growth of soil bacteria and fungi has been extensively studied, the effect of monoterpenoids on the growth of CSMs has been poorly understood. In this study, we investigated the effect of selected monoterpenoids on the growth of *P. pallidum* during its lifespan.

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MATERIALS AND METHODS

Isolation of CSM from Soil of Forest Floor

Soil samples containing CSM were collected from the humus and fermentation layers of five sites of the forest floors in Mt. Muhak, Masan, and processed in a way identical to that reported by Cavender and Raper (1965).

The isolation of *P. pallidum* was performed as follows; 1) Soil sample was placed in a 300 ml flask containing 50 ml of sterile distilled water, and the mixture was agitated using a shaker operating at 120 rpm for one hour. The sample mixture was filtered using cotton

gauze to remove the suspension, and the liquid material was transferred to hay infusion isolation media (5 g/L rice straw, 20 g/L agar) and spread out evenly using a sterile L-shaped glass rod. 2) The spore mass were transferred on 2% agar cultivation media (Hagiwara, 1989) from the isolation media, and then a few drops of *E. coli* were put on the spore mass. These plates were incubated at 20°C. Two replicates per sample were prepared using both methods.

Used Monoterpenoids and Their Dilution

The following 11 monoterpenes were tested for their effects on the growth of, *P. pallidum*: myrcene, (R) –(+) limonene, (–)-menthone, (S)-(+)carvone, (1R)-(–)fenchone, α-pinene, (–)camphene, (1S)-(–)verbenone, β-pinene, geranyl acetate and bornyl acetate. The monoterpenoids were purchased from Aldrich Chemical (USA). and Fluka Chemical (JAPAN). Each monoterpeneoid was tested individually at four different concentrations (1, 0.1, 0.01, and 0.001 μg/μl), which were then applied to the filter papers (Millipore hawp 04700, 5 mm in diameter). The commericial monoterpenoids were diluted serially using axenic liquid media (per liter, 14.3 g bacteriological peptone, 7.15 g yeast extract, 30.8 g D-glucose, 1.28 g $Na_2HP0_4 \cdot 12H_20$, and 0.49 g KH_2P0_4, pH 6.7) as described by Watts and Ashworth (1970), because the axenic solution alone does not inhibit or enhance the growth of CSM amoebae. The culture solution with no monoterpenoids was used as a control.

The Effect of Monoterpenoids on *P. pallidium*

The effect of volatile oils on microorganisms has been tested with a disk diffusion technique and was measured by the clear zone of microorganisms (Vokou and Margaris 1984; Thangadurai and Anitha, 2002). Since *P. pallidum* migrates after they are aggregated, a clear zone was not formed. Based on this result, we devised a new method, the disk volatilization technique we use in this study. This new method uses volatilization of selected monoterpenes after germination of *P. pallidum* spores. The surface of 2% agar medium plate was inoculated with *E. coli,* as a feeding source, and *P. pallidum*, and each compound was inoculated on filter paper disks (Millipore hawp04700) containing 1 μl of diluted substance. The plate was incubated at 22°C for 3 days.

The growth area of *P. pallidum* was measured by planimeter at 24 h intervals. We estimated the growth rate as a logarithmic growth area for each concentration by using the regression equation. The developmental stage (fruiting body; sorophore and sorus) of *P. pallidum* affected by each monoterpenoid was observed under an anatomic microscope (x 50). All of the photographs were shown in the same absolute scale and magnification to compare relative sizes between observations. All data was obtained from four independent experiments. Data was analyzed by using ANOVA. An LSD test was performed to evaluate treatment effects for each concentration using Excel program.

RESULTS

The effect of monoterpenoids on growth rate of *P. pallidum* is shown in Fig. 1. All of the compounds at all concentrations (except menthone at 0.001 μg/μl) had very strong inhibitory effects on cell growth in this

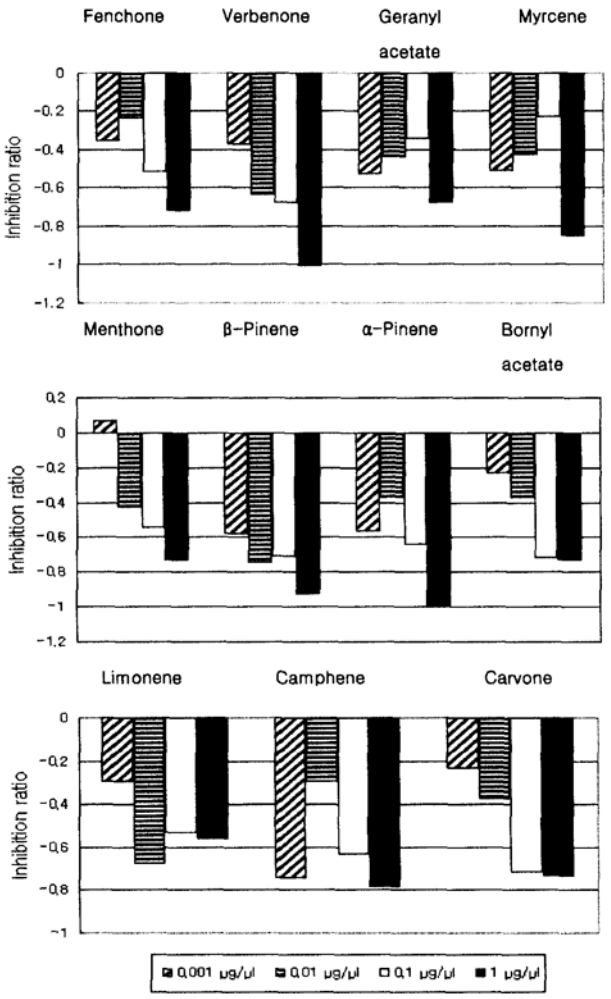

Figure 1. Effect of monoterpenoids on growth of *P. pallidum*. The control value is zero.

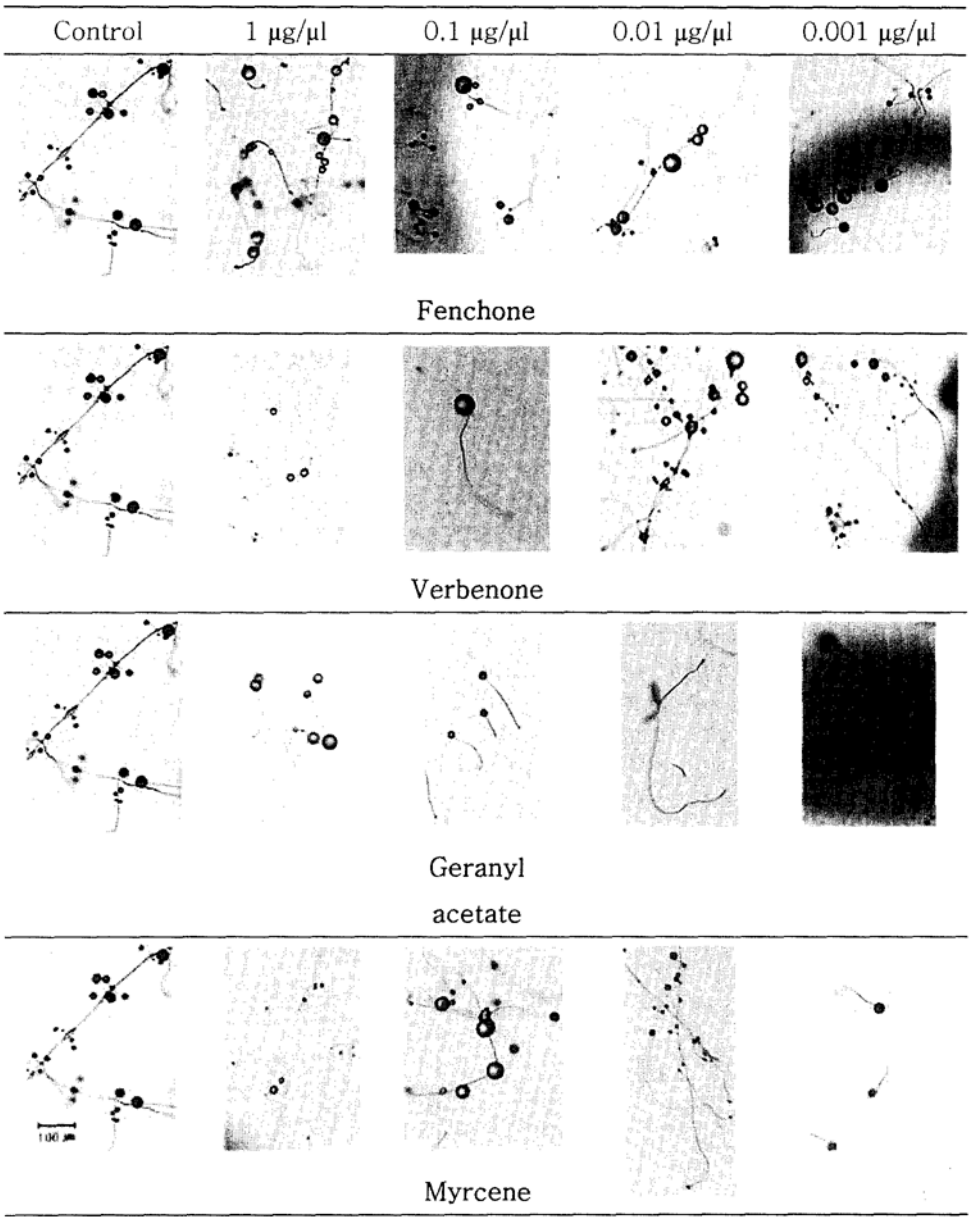

Figure 2. Effect of (1R)-(−)-fenchone, (1S)-(−)-verbenone, geranyl acetate, and myrcene on *P. pallidum*. The control photos in this figure represent perfect fruiting bodies with sori, whorls of side branches, and sorophores. The photos are represented inthe same scale with control.

species, resulting in a growth rate much lower than the control. The inhibitory effects of monoterpenoids on growth of *P. pallidum* were increased by the concentration-dependent manner in most compounds. To see if the treatment of monoterpenoids changes cellular morphology of *P. pallidum*, microscopic analysis was performed. Microscopic analysis revealed that the cells treated with monoterpenoids were less healthy than the control (Fig. 2, 3 and 4). We could see very short sorophores, as well as smaller-sized sorus, and no spores in sori compared to the control. Especially, shapes of the whorls of side branches changed and/or disappeared by the treatment of monoterpenoids.

Fig. 2 shows various sizes of fruiting bodies of *P. pallidum* affected by (1R)-(−)-fenchone, (*1S*)-verbenone, geranyl acetate, and myrcene. In this figure, the sorus treated with fenchone at all concentrations and verbenone at 0.01 and 0.1 μg/μl seemed to be a little larger than control. These shapes of sorus were represented that sorus contains water and there were no spores.

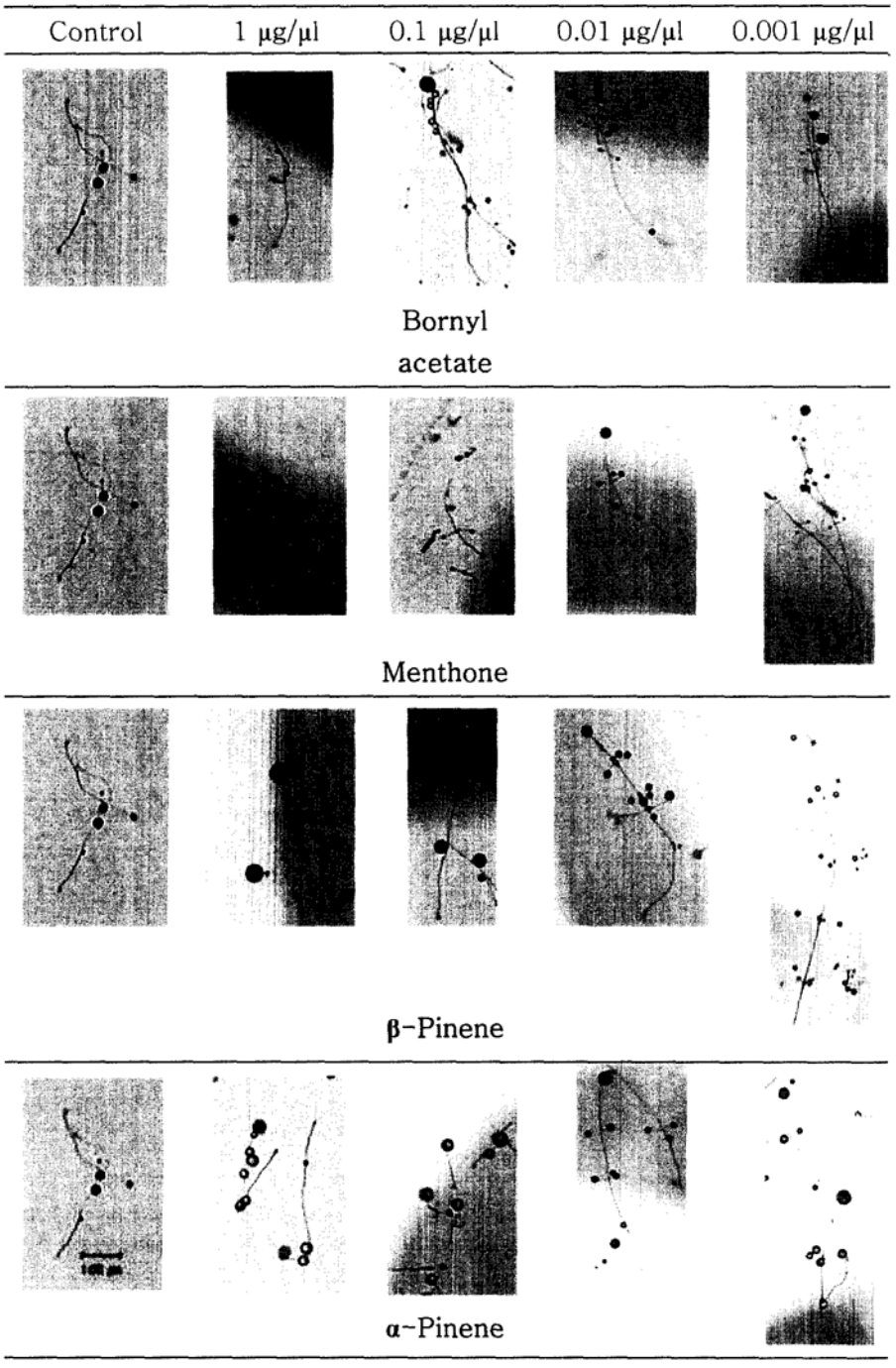

Figure 3. Effect of bornyl acetate, (–)-menthone, (+)-β-pinene, and (1S)-(–)-a-pinene on *P. pallidum*. The control photos in this figure show young fruiting bodies. The photos are represented in the same scale with control.

We could see very short sorophores, a smaller sized sorus and sorus without spores, and abnormal shape of sorus by geranyl acetate and myrcene even at the lowest concentration. Especially, whorls of side branches were disappeared and/or changed. We recognized that these shapes were affected by all monoterpenoids we tested, and corresponded with the growth rate results mentioned above. Fig. 3 clearly shows the effect of bornyl acetate, (–)-menthone, (+)-β-pinene, and (1S)-a-pinene on *P. pallidum* fruiting bodies. We could see that the sizes of the fruiting bodies at all concentrations of all compounds (except for menthone at 0.001 μg/μl) were

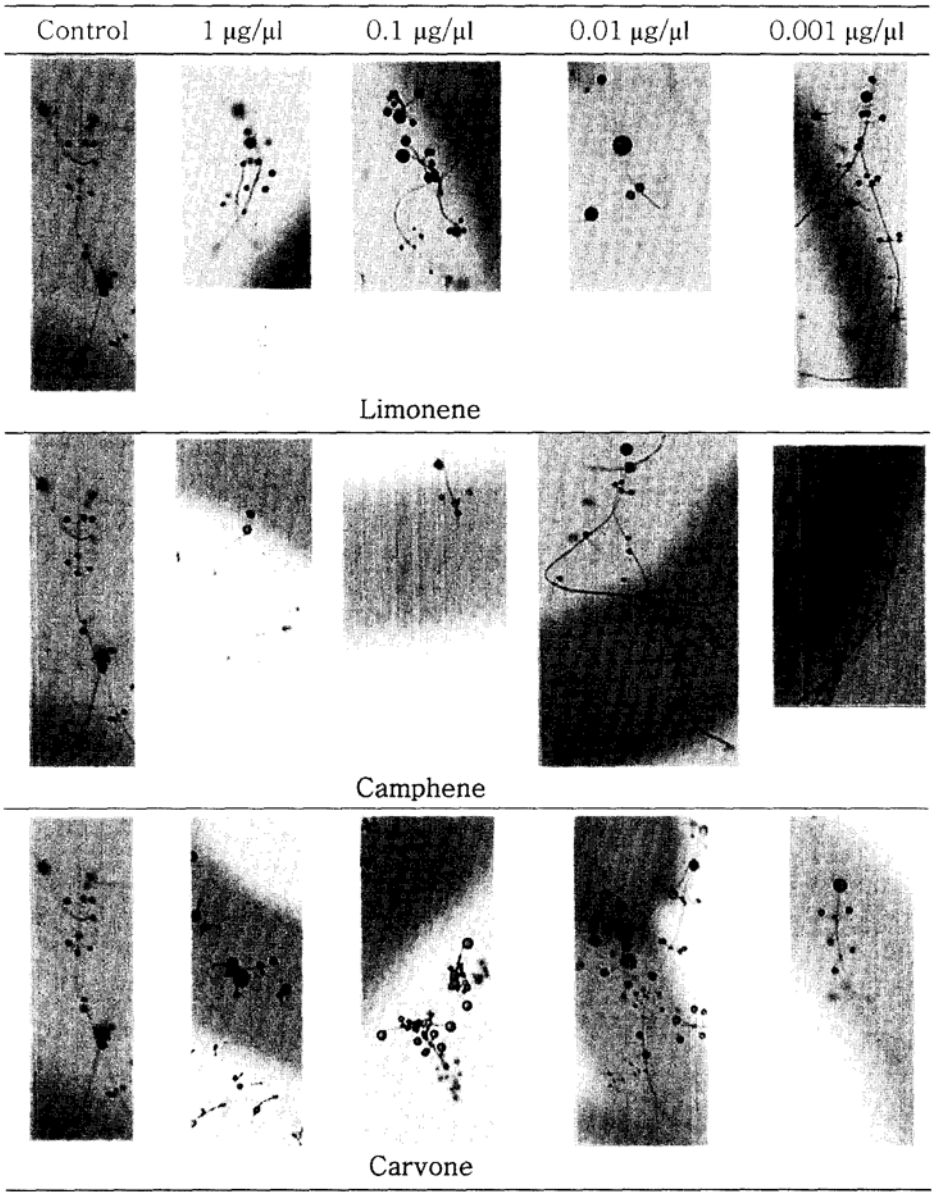

Figure 4. Effect of (R)-(–)-limonene, (–)-camphene, and (S)-(+)-carvone on *P. pallidum*. The control photos in this figure show the perfect shape of fruiting bodies with sori, sorophore, and whorls of side branches. The photos are represented in the same scale with control.

prominently reduced with empty sorus, shorter sorophores compared with control. Although size and shape of the sorus treated with menthone at 0.001 μg/μl concentrations were similar to those of control, there was no significant difference in growth rate compared with the control. We also found that the shape of the sorus was somewhat elongated by menthone at 0.1 μg/μl, unlike the control. The whorls of side branches also were obserbed in some compounds, but the shape of those was different from the control. (*R*)-(–) limonene, (–)-camphene, and (*S*)-(+)carvone also inhibited growth of *P. pallidum* at all concentrations (Fig. 4). Shape of the fruiting body treated with limonene at 0.001 μg/μl concentration was similar to the control, but their growth rate was remarkably lower than the control. Effect of camphene on the fruiting body was represented by very shorter sorophores at 0.1 and 1 μg/μl concentrations, and changed shape of whorls of side branches at 0.01 and 0.001 μg/μl concentrations. Carvone severely inhibited the fruiting body even at the lowest concentration. The fruiting bodies had short sorophores, no spores in sori, and no or changed whorls of side branches.

DISCUSSION

P. pallidum mostly showed decreased growth rate at all concentrations of all compounds. However, all compounds inhibited growth of *Dictyostellium discoideum* NC4 at 1 μg/μl, but fenchone (0.1, 0.001, and 0.001 μg/μl) and camphene (0.01 μg/μl) stimulated growth of *D. discoideum* NC4 (our unpublished data). Hwang and Kim (2002) reported that the effect of monoterpenoids on a CSM, *D. discoideum* Ax-2, which was the first study about the relationship between monoterpenoids and growth of CSM. They suggested that the inhibitory and enhancing effects of selected monoterpenoids depend on the concentration of the individual compound. There were remarkable differences in *P. pallidum* from the results of *D. discoideum* NC4 and Ax-2. It may be due to the different genus and species. The inhibition of activity and growth of some microorganisms by monoterpenoids is well known (Amaral and Knowles., 1998; Paavolainen et al., 1998; Heyen and Harder, 2000; Soliman and Badeaa, 2002). Therefore the inhibitory effects of monoterpenoids on microorganisms can be useful for medicine, food additives, repellents, and aromatherapy. In comparison, there are only a small number of studies describing the effects of monoterpenes on soil microorganisms in forest floors during decomposition (Cleveland and Yavitt, 1998; Coûteaux et al., 1998).

Hwang et al. (2000) reported that CSM occurred in the forest floor. They suggested that the occurrence of CSM might be due to forest floor composed by vegetation type. Highly volatile monoterpenoids can be initially present at relatively high concentrations in recently fallen litter (Wood et al., 1995). In addition, concentration of monoterpenes can be slower than the phenolic compounds, because monoterpenoids might be more water-insoluble and are not leached by rainwater. Therefore, monoterpenoids remain longer in pine forest floors, and have influence on soil microorganisms. Nevertheless, since the monoterpenoid fraction of forest floors could be changed rapidly by biodegradation (Misra and Pavlostathis, 1997), monoterpenoids might be the potential regulators of decomposition process in coniferous forest. Dyk et al. (1998) and White (1994) reported that some soil microorganisms could be strongly influenced by monoterpenoids. Feest reported that CSMs including *P. pallidum* occurred rare in pine forest floor. Our results suggested that one of the reasons might be the inhibitory effect of monoterpenes on the growth of *P. pallidum* as suggested by Dyk et al. (1998) and White (1994). Based on this study on the relationship between monoterpenoids and growth of *P. pallidum*, further research is needed on the decomposition process of monoterpenoids and the composition and abundance of CSMs.

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