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Effect of Monoterpenes on Growth of Cellular Slime Mold, *Dictyostelium discoideum* Ax-2

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We examined 12 important monoterpenes found on the forest floor under *Pinus thunbergii*, and monitored their effect on the growth of a slime mold, *Dictyostelium discoideum* Ax-2. Four concentrations were tested for each compound (3.3, 0.33, 0.033, and 0.0033 μL/mL). Relative growth rates were determined by comparing the cell counts of treated organisms with those from the controls. At a concentration of 3.3 μL/mL, (1S)-(-)-α-pinene, (−)-menthone, (−)-camphene, (S)-(+)-carvone, and (1R)-(−)-fenchone strongly inhibited the development of this slime mold. In contrast, (+)-sabinene, (R)-(+)-limonene, and myrcene showed no inhibitory effects, even at the highest concentration tested. By comparing individual growth rates with the control during the incubation period, we could classify these monoterpenes into three groups: I., compounds that were able to inhibit Ax-2 growth at all concentrations; II., compounds that showed a strong inhibitory effect at treatments between 3.3 and 0.033 μL/mL, and mild anti-microbial activity at the lowest concentration; and III., compounds that inhibited growth at higher concentrations (3.3 and 0.33 μL/mL), but enhanced it at lower levels (0.033 and 0.0033 μL/mL). Based on these results, we suggest that the inhibitory and enhancing effects of selected monoterpenes depend upon the concentration of the individual compound.

Keywords: cell count, cellular slime mold, *Dictyostelium discoideum* Ax-2, growth enhancement, inhibition effect, monoterpenes

Plants produce secondary metabolites that are released into the environment (Rice, 1984). When these compounds reach the humus layer, they influence the growth of soil microorganisms either through stimulation (Nes and Skjelkvale, 1982; Schmidt et al., 2000; Souto et al., 2000a) or inhibition (Deans and Ritchie, 1987; Janssen et al., 1987; Silvropoulou et al., 1995; Souto et al., 2000b). Different varieties of microorganisms will show a range of responses, depending on the type and concentration of secondary metabolite present. The pine forest floor has several kinds of secondary metabolites, especially phenolic compounds and terpenes (Feliciano and Lopez, 1991). Studies have been conducted to determine the relationships among phenolic compounds (Kubo et al., 1992; Mishra and Dubey, 1994; Muller-Riebau et al., 1995; Adam et al., 1998; Karamanoli et al., 2000), terpenes (Silvropoulou et al., 1997; Karamanoli et al., 2000; Tellez et al., 2000), and soil microorganisms, including fungi and bacteria. In addition, essential oils and their derivatives, which are composed of monoterpenes and sesquiterpenes, are aromatic and volatile compounds that can act against a wide variety of microorganisms (Helander et al., 1998). For example, Karapinar and Aktug (1987), Oosterhaven et al. (1996), and Smid et al. (1996) have used pure forms of small terpenes to demonstrate the anti-microbial activity of certain essential oils.

Dictyostelium discoideum, or cellular slime mold (CSM), lives on the forest floor. Its life cycle is divided into two phases: 1) feeding, in which the single-celled myxamoebae multiply by binary fission, using soil bacteria as food; and 2) differentiation, during which these hitherto solitary myxamoebae associate on a solid surface to form multi-cellular and macroscopic aggregates. Over time, the component cells from this phase become either the stalk or the spore cells of a mature fruiting body (Olive, 1975; Loomis, 1982; Raper, 1984; Spudich, 1987). Watts and Ashworth (1970) isolated a *Dictyostelium* strain that would grow on a simpler axenic medium; earlier studies of endocytosis were almost entirely restricted to establishing axenic cultures of Strain Ax-2, which then became the most widely used for research. These culturing techniques enlarged the scope of investigations possible with such a genus because they eliminated the need for a food organism and facilitated biochemical and genetic studies (Watts and Ashworth, 1970; Olive, 1975).

As the next worthwhile step in the study of slime molds found on the forest floor, it is important to determine whether their monoterpenes possess any anti-microbial properties. Therefore, the objective of our study was to investigate the effects of selected monoterpenes on the growth of *D. discoideum* Ax-2 over its lifespan.

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MATERIALS AND METHODS

Culture of *D. discoideum*

The cellular slime mold, *D. discoideum* Ax-2, was obtained from the Department of Botany at Kyoto University. Although wild-type cells grow only via phagocytosis of bacteria, the strain Ax-2 can grow on liquid nutrients in the absence of bacteria (i.e., under axenic conditions; Spudich, 1987). Sussman and Sussman (1967) have studied the serial passage of this slime mold on a complex axenic medium in which the myxamoebae can grow. Therefore we used the axenic media described by Watt and Ashworth (1970), which comprised, per liter, 14.3 g bacteriological peptone, 7.15 g yeast extract, 30.8 g D-glucose, 1.28 g $Na_2HPO_4 \cdot 12H_2O$, and 0.49 g KH_2PO_4 (final pH 6.7). Our cultures were grown on a shaking incubator at 25°C.

Determination of Cell Growth Rates in Response to Monoterpene Treatments

Compositions of the monoterpenes (Table 1) that were extracted from the fallen leaves of *Pinus thunbergii* were previously identified by Kang and Kim (1997). The 12 monoterpenes selected for this study were purchased from Aldrich Chemical, Inc. and Fluka Chemical Co., and included the following: myrcene, (R)-(+)-limonene, (-)-menthone, (S)-(+)-carvone, (1R)-(-)-fenchone, (1S)-(-)-α-pinene, (-)-camphene, (1S)-(-)-verbenone, (+)-β-pinene, (+)-sabinene, geranyl acetate, and (-)-bornyl acetate. Stock solutions (3.3 μL/mL) of all compounds were prepared in liquid culture, and each compound was diluted in an axenic solution to make up four separate concentrations -- 3.3, 0.33, 0.033, and 0.0033 μL/mL. The control was a concentration of zero for each compound. The *D. discoideum* Ax-2 cells were then inoculated to a final concentration of about 10^5 to 10^6 cells/mL in the four concentrations of each compound. We used the axenic solution dilution technique to quantitatively evaluate the effects of individual monoterpenes on slime mold development. The ability to either inhibit or enhance growth by each compound was determined by comparing the cell counts of treated *D. discoideum* Ax-2 cells with those of the controls. Cells were counted twice daily with a blood cell counter for 3 to 4 d. The growth rates, based on logarithmic cell counts at each concentration, were estimated by regression equations; a particular compound was considered inhibitory if the rate for treated cells was lower than for the particular control. All experiments were replicated four times, and a *t*-test was performed on the data to identify treatment effects for each compound and its control.

Table 1. Composition of major monoterpenes found in fallen needles of *P. thunbergii* (from Kang and Kim, 1997). Compounds appearing in bold type were used in this study.

Monoterpene	Composition (%)
Tricyclene	11.24
(1S)-(-)-α-pinene	8.38
(-)-camphene	33.91
(+)-sabinene	2.46
(+)-β-pinene	37.66
Myrcene	1.09
N.I	1.94
(1R)-(+)-limonene	0.00
(1R)-(-)-fenchone	0.07
N.I	0.37
(-)-menthone	1.31
(1S)-(-)-verbenone	0.11
(1S)-(+)-carvone	0.07
(-)-bornyl acetate	0.17
geranyl acetate	1.21

RESULTS AND DISCUSSION

To assess the anti-microbial properties of our monoterpenes, we estimated the relative cell growth rates for *D. discoideum* 2 d after inoculation and found that, at a concentration of 3.3 μL/mL, (IS)-(-)-α-pinene, (-)-menthone, (-)-camphene, (s)-(+)-carvone, and (1R)-(-)-fenchone all inhibited slime mold development (Table 2). In addition, the inhibitory effect of menthone was strong even at a concentration of 0.33 μL/mL. Growth of *D. discoideum* Ax-2 was remarkably different in its response to sabinene, myrcene and (R)-(+)-limonene, with no inhibition being seen, even at the highest level. The remaining compounds showed variable results, depending on their concentrations.

Based on these results, we then categorized individual monoterpenes into one of three classes: Group I compounds, which inhibited growth of Ax-2 cells at all concentrations, and included (IS)-(-)-α-pinene, (-)-menthone, (+)-sabinene, and (R)-(+)-limonene (Fig. 1a). However, it should be noted that, even though the values for the last two compounds were both negative, their anti-microbial activity did not differ significantly ($p > 0.1$) from that of the control. Group II compounds were able to inhibit growth at concentrations of 3.3 to 0.033 μL/mL, but showed only mild activity against Ax-2 at a level of 0.0033 μL/mL. This group comprised (-)-camphene, (-)-bornyl acetate, and geranyl acetate (Fig. 1b). Finally, Group III compounds inhibited growth at high concentrations (3.3 to 0.33 μL/mL), but had an

Table 2. Monoterpene activities in *D. discoideum* Ax-2. The value for growth ratio was obtained by comparing the number of cells from inoculated tissue versus the control after 2 d. Experiments were replicated four times.

Compound	Concentration	Growth ratio
(IS)-(–)-α-pinene	0.0033 µL/mL	0.90
	0.033 µL/mL	0.87
	0.33 µL/mL	0.53
	3.3 µL/mL	0.00
(–)-menthone	0.0033 µL/mL	1.05
	0.033 µL/mL	0.31
	0.33 µL/mL	0.00
	3.3 µL/mL	0.00
(+) sabinene	0.0033 µL/mL	0.69
	0.033 µL/mL	1.03
	0.33 µL/mL	0.86
(–)-camphene	0.0033 µL/mL	1.30
	0.033 µL/mL	0.60
	0.33 µL/mL	0.02
	3.3 µL/mL	0.00
(–)-bornyl acetate	0.0033 µL/mL	1.54
	0.033 µL/mL	0.53
	0.33 µL/mL	0.16
	3.3 µL/mL	0.09
geranyl acetate	0.0033 µL/mL	1.75
	0.033 µL/mL	0.92
	0.33 µL/mL	0.16
	3.3 µL/mL	0.10
(S)-(+)-carvone	0.0033 µL/mL	1.58
	0.033 µL/mL	1.79
	0.33 µL/mL	0.01
	3.3 µL/mL	0.00
(+)-β-pinene	0.0033 µL/mL	1.33
	0.033 µL/mL	2.21
	0.33 µL/mL	0.01
	3.3 µL/mL	0.02
(1R)-(–)-fenchone	0.0033 µL/mL	1.13
	0.033 µL/mL	1.13
	0.33 µL/mL	0.04
	3.3 µL/mL	0.00
(1S)-(–)-verbenone	0.0033 µL/mL	2.05
	0.033 µL/mL	1.30
	0.33 µL/mL	0.14
	3.3 µL/mL	0.24
Myrcene	0.0033 µL/mL	1.43
	0.033 µL/mL	1.31
	0.33 µL/mL	1.20
(R)-(+)-limonene	0.0033 µL/mL	0.91
	0.033 µL/mL	1.03
	0.33 µL/mL	1.03

enhancing effect at lower concentrations (0.033 to 0.0033 µL/mL). They included (+)-β-pinene, (S)-(+)-carvone, (1R)-(-)-fenchone, myrcene, and (1S)-(-)-verbenone (Fig. 1c). These results were consistent with those

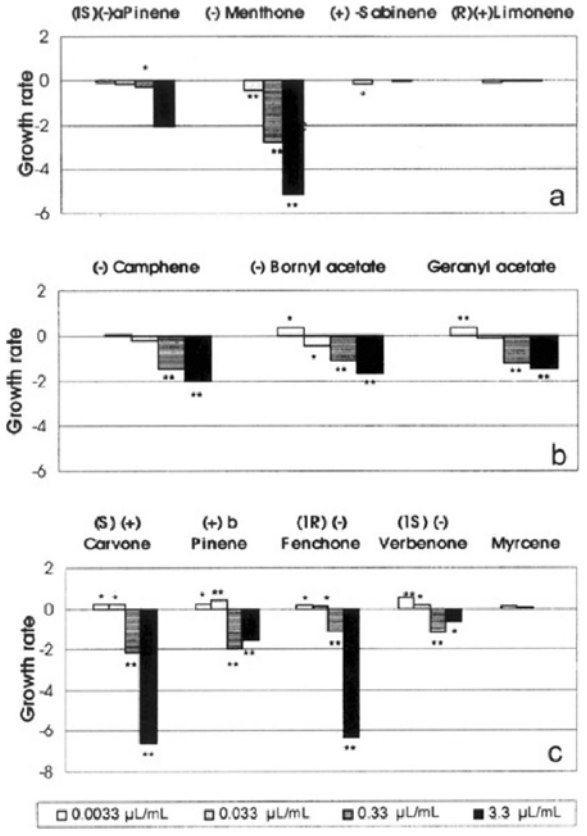

Figure 1. Effect of monoterpenes on *Dictyostelium discoideum* Ax-2, as a function of growth rates during the incubation period. Value for the control is zero. Significant difference between each compound and the control was estimated by *t*-testing at the 0.05 (*) and 0.001 (**) levels of probability.

from studies of α-pinene, β-pinene, and limonene by Gibbon and Pirt (1971), Tellez et al. (2000), and Stumpf et al. (1990).

The Ax-2 cells observed under the microscope were biodegraded after the addition of (1S)-(–)-α-pinene, (S)-(+)-carvone, (1R)-(–)-fenchone, or (-)-menthone at a concentration of 3.3 µL/mL. At the level of 0.33 µL/mL, geranyl acetate, (1R)-(–)-fenchone, (+)-β-pinene, (–)-menthone, (–)-bornyl acetate, (1S)-(–)-verbenone, (S)-(+)-carvone, and (–)-camphene all influenced the growth of Ax-2, whose cells were able to maintain their normal shape, but were incapable of further development. Because the shapes of the cells treated with monoterpenes primarily from Groups II and III appeared healthier and larger than those of the control, we had first assumed that those particular compounds had enhanced their growth.

Vokou et al. (2002) have reported that the major monoterpene fenchone, from *Lavandula stoechas*,

inhibits *Bacillus subtilis*, but has no such influence on *Escherichia coli* or other soil microorganisms. They have proposed that these organisms probably use those compounds as carbon sources. Misra et al. (1996) and Harms et al. (1999) have also suggested that certain monoterpenes can serve as sources of energy for soil bacteria. Our results support those previous studies; for example, (1R)-(-)-fenchone had no negative effect on Ax-2 cell growth at concentrations between 0.033 and 0.0033 μL/mL. Schmidt et al. (2000) attempted to estimate the biomass of a specific microbial functional group (i.e., salicylate-mineralizing microbes) in the soil beneath salicylate-producing plants. In doing so, they demonstrated that even very toxic, plant-produced phenolic compounds had positive effects on phenolic-mineralizing populations in soil. Similarly, our study showed that low concentrations of most of the tested compounds enhanced or had a mildly positive effect on the growth of *D. discoideum* Ax-2 cells.

Because the pine forest floor is comprised primarily of (+)(–)β-pinene (37%), (–)-camphene (33%), tricyclene (11%), and (IS)-(–)-α-pinene (8%), we believe that those compounds play an important role in regulating the population dynamics of microorganisms in humus. For example, in our study, low concentrations of (+)-β-pinene and (-)-camphene enhanced cell growth, which suggests that Ax-2 was benefiting from their existence. In fact, many kinds of CSMs are found in pine forests (Hong and Chang, 1990; Hwang et al., 2000), and may utilize such monoterpenes as a mineralizing source.

Nes and Skjelkvale (1982), Deans and Ritchie (1987), and Silvropoulou et al. (1995) have reported similar mixed results when studying the anti-microbial activities of some essential oils. Their research has shown that most of the tested oils inhibit bacterial growth, while others have an enhancing effect. The relationship between monoterpenes and soil microorganisms is not well understood, so it is important to continue assessing their distribution in soils and their effects on soil microorganisms. Our results suggest that the anti-microbial properties of each monoterpene vary in their effectiveness in inhibiting the growth of *D. discoideum* Ax-2 cells, and also according to the concentration tested. Therefore, further analysis is needed to better determine the negative influence of monoterpenes on CSM found on the pine forest floor.

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