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Some Effects of Douglas Fir Terpenes on Certain Microorganisms

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The Douglas fir terpene α-pinene was shown to inhibit the growth of a variety of bacteria and a yeast. Other terpenes of the Douglas fir, including limonene, camphene, and isobornyl acetate, were also inhibitory to *Bacillus thuringiensis*. All terpenes were inhibitory at concentrations normally present in the fir needle diet of Douglas fir tussock moth larvae. The presence of such terpenes in the diet of these insects was found to strongly influence the infectivity of *B. thuringiensis* spores for the Douglas fir tussock moth larvae. The terpene α-pinene destroyed the cellular integrity and modified mitochondrial activity in certain microorganisms.

Several studies have provided some insight into the effects of terpene on the growth of bacteria (7, 9, 12). The terpenes or terpene mixtures in the latter tests were employed at levels greatly exceeding those found under natural conditions. However, the specific action of terpenes has not yet been treated.

The larvae of the Douglas fir tussock moth (DFTM), *Orgyia pseudotsugata*, feed on the Douglas fir and other materials rich in terpene content (15). The digestive system of these larvae is relatively free of common microflora (R. E. Andrews and K. D. Spence, unpublished data). This is by no means the case with many other insects (3, 5). For example, common microbes found in the midgut of *Scotia segetum* (turnip moth) larvae include *Streptococcus faecalis*, *Escherichia coli*, *Citrobacter freundii*, *Klebsiella pneumoniae*, *Proteus* spp., *Erwinia* spp., and *Pseudomonas* spp. (3).

To investigate the action of terpenes, several different microorganisms were employed, and the terpenes were employed at levels comparable to those found in the normal diet of the DFTM larvae. These studies report the relationship between terpenes and the growth rate, cellular integrity, mitochondrial activity, and infectivity of the selected microbial organisms.

MATERIALS AND METHODS

Chemicals and media. α-Pinene, limonene, and camphene were obtained from Eastman Chemical Co., isobornyl acetate was obtained from Aldrich Chemical Co., and cyclohexane came from Baker Chemical Co. Brain heart infusion broth and nutrient broth were obtained from Difco. Medium B, a minimal medium for growth of *Saccharomyces cerevisiae*, was prepared as described by Spence et al. (13). Medium G for growth of *Bacillus thuringiensis* was prepared as formulated by Nakata (10). Yeast complete medium was prepared as specified by Ferro and Spence (4).

Inhibition studies. *Bacillus* species were grown in brain heart infusion broth or medium G. *S. cerevisiae* was grown in yeast complete medium, and all other bacteria were grown in nutrient broth. All microorganisms were laboratory strains. Cells were preadapted to the medium, removed from midlog-phase cultures, and inoculated into new, terpene-containing medium at an initial concentration of 1 Klett unit. Incubation was carried out at 30°C with vigorous shaking in a water bath shaker, and growth was followed on a Klett-Summerson colorimeter with a green filter. All of the terpenes have a low solubility in water. The individual effects of the solubilized and nonsolubilized terpenes (which existed as microdroplets in the growth media) are not known. However, it was assumed that their combined effect approximated the action of terpenes in the insect natural diet. The exponential growth phase data were fitted to a line, and the slope was used as the growth rate. The percent inhibition of growth rate was obtained by the equation ([1 − slope with terpene]/slope without terpene) × 100.

Terpene content of fir needles. To determine the terpene content of Douglas fir needles, the needles were collected, frozen in liquid nitrogen, ground, and then placed in CS_2 (5 ml/g of needle). The suspensions were then refluxed for 30 min, followed by filtration on Whatman no. 41 paper. Pooled filtrates from three successive refluxes were concentrated by distilling away the CS_2. The extracts were fractionated and identified by using a Hewlett-Packard series 5700 gas chromatograph coupled to a Hewlett-Packard 5900 mass spectrograph with a 5930 data system.

Effect of α-pinene on cell integrity. To test the effect of α-pinene on yeast membrane integrity, the cells of *S. cerevisiae* were initially grown for 24 h in medium B. The release of cytoplasmic materials from the cell was measured by the method described by Schlenk and Zydek-Cwick (11). Disruption of *B. thuringiensis* vegetative cells was tested by essentially the same method, except that the cells were grown in

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medium G and suspended in 0.15 N phosphate buffer before addition of the terpene. In each experiment the test solutions contained 0.27 mg of α-pinene per ml. Microorganisms in these studies were taken from the exponential phase of growth.

Mitochondrial activity. *S. cerevisiae* spheroplasts were prepared by the Glusulase method using a wild-type diploid yeast strain, MCC (14). Spheroplasts were lysed and the mitochondria were prepared according to the procedure of Astin and Haslam (1). Respiration was measured polarographically at 28°C in a Gilson oxygraph with a Clark-type electrode. The reaction volume (1.8 ml) contained the following ingredients at the concentrations shown: 0.9 M sorbitol, 1.2 mM ethylenediaminetetraacetic acid, 6 mM K_2HPO_4, 6 mM $MgCl_2$, 15 mM tris(hydroxymethyl)aminomethane, 15 mM maleate, and 0.2% bovine serum albumin (pH 6.6). Ethanol (0.5%) was the oxidizable substrate, and adenosine 5'-diphosphate was at 2.4 mg/ml; mitochondria were added to give 0.76 mg of mitochondrial protein in the reaction mixture. Oxygen consumption rates were calculated as microgram-atoms of O_2 per milligram of mitochondrial protein per minute.

Infectivity studies. Egg masses from the DFTM were collected and sent to us by David Holland, U.S. Forest Service, Albuquerque, N.M., from the Cibola National Forest in the fall of 1978. The egg masses were stored and the larvae were reared as described by Brandt et al. (2), on the artificial diet formulated by Lyon and Flake (8). This diet contains several bactericidal or bacteriostatic agents. In early fourth instar, the larvae were switched to the artificial diet with the microbial inhibitors omitted. One group of insects was fed a diet containing no α-pinene, one group was fed a diet with 0.27 mg of α-pinene per g of diet, and a third received a diet with 2.7 mg of α-pinene per g. After 2 days, each larva was placed in a rearing cup containing a portion of the same experimental diet to which an appropriate dose of *B. thuringiensis* spores had been applied. *B. thuringiensis* spores were prepared as described elsewhere (6). The larvae were examined 4 days postinfection, and the 50% lethal dose values (numbers of spores) were determined by probit analysis.

RESULTS

To determine the effect of terpenes on the growth of four *Bacillus* species, inhibition curves were performed with α-pinene. The data for *B. thuringiensis* are shown (Table 1). Other *Bacillus* species, including *B. megaterium*, *B. subtilis*, and *B. cereus*, were inhibited to essentially the same extent as *B. thurigiensis*. Maximum growth rate inhibition by α-pinene occurred at a concentration well below that found in Douglas fir needles. Since other Douglas fir terpenes could have been more or less inhibitory than α-pinene, growth rate inhibition of *B. thuringiensis* by camphene, limonene, and isobornyl acetate was also tested. All of the compounds inhibited *B. thuringiensis* at levels of the terpene below those found in the foliage of the tree (Table 1).

TABLE 1. *Inhibition of growth rate of B. thuringiensis*[a] *by various terpenes*

Supplement	Terpene concn giving 100% inhibition (mg/ml)[b]	Terpene concn in needles (mg/g)[c]
α-Pinene	0.13	0.27
Camphene	≤0.05	0.37
Limonene	≤0.05	0.09
Isobornyl acetate	≤0.04	0.37

[a] Inhibition of *B. cereus*, *B. subtilis*, and *B. megaterium* by α-pinene was essentially the same as that for *B. thuringiensis*.

[b] Each growth inhibition value is the average of five experiments.

[c] The concentration of Douglas fir terpenes in the natural diet of the insects is shown here for comparative purposes.

It was of course possible that the terpenes exerted a simple solvent effect, extracting hydrophobic components of the cell membrane, leading to inhibition. To test this possibility, *B. thuringiensis* was exposed to the compound cyclohexane, which possesses solvent properties analogous to those of the terpenes. When exposed under conditions identical to those employed with terpenes, maximum inhibition was only 20% (Fig. 1).

To test the inhibition of other microbes by α-pinene, *E. coli* (Fig. 2a), *Serratia marcescens* (Fig. 2b), *Staphylococcus epidermidis* (Fig. 2c), and *Saccharomyces cerevisiae* (Fig. 2d) were grown in the presence of various concentrations of α-pinene. *S. epidermidis* and *S. cerevisiae* were inhibited at levels less than 0.2 mg of terpene per ml. Within the concentrations employed, *E. coli* and *S. marcescens* displayed a growth rate which was inhibited approximately 80% and 25%, respectively, as compared to the rates without α-pinene.

To test for a general membrane effect, the influence of α-pinene on the cytoplasmic membrane integrity and mitochondrial function was analyzed. It is clear (Fig. 3) that the cytoplasmic membrane was disrupted by the terpene. Both vegetative cells of *B. thuringiensis* (Fig. 3a) and *S. cerevisiae* (Fig. 3b) exhibited massive leakage of their cellular constituents when exposed to 0.27 mg of α-pinene per ml.

The effect of terpene on the mitochondrial function of *S. cerevisiae* may still be analyzed, however, since the mitochondria themselves can be isolated before exposure to the inhibitor. The data from such tests are shown in Table 2. Although the terpene α-pinene causes disruption

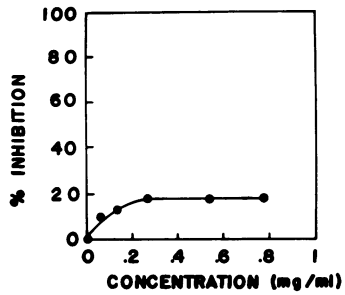

FIG. 1. *Inhibition of growth rate of B. thuringiensis by cyclohexane.*

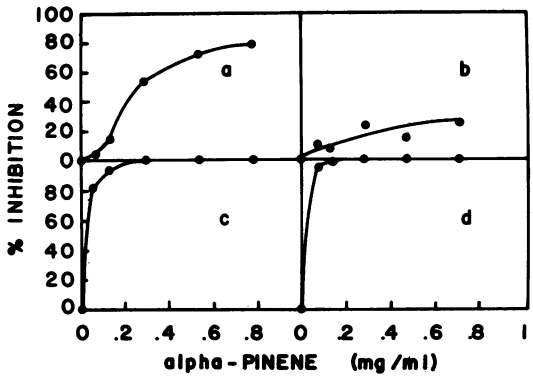

FIG. 2. *Percent inhibition of growth rate of four microbes by α-pinene. (a) E. coli, (b) S. marcescens, (c) S. epidermidis, and (d) S. cerevisiae.*

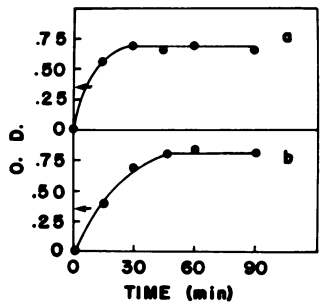

FIG. 3. *Release of 260-nm-absorbing materials from B. thuringiensis and S. cerevisiae when exposed to 0.27 mg of α-pinene per ml. (a) 1 mg (wet weight) of B. thuringiensis vegetative cells per ml in 0.15 N phosphate buffer; (b) 1 mg (wet weight) of S. cerevisiae per ml in water. Arrows indicate quantity of 260-nm-absorbing material released in the presence of 1.5 N perchloric acid in the same test solutions.*

of the cytoplasmic membrane, there is not a comparable effect on the mitochondrial membrane of yeast. The mitochondrial activity is reflected in the respiratory control value (1). The variation in the respiratory control values of 13% is considered a significant alteration in mitochondrial function (1). Although the effect is significant, this reduction is probably not sufficient to inhibit the growth of the yeast.

The dramatic effect of α-pinene on the several microorganisms in vitro suggested that the terpenes might influence the infectivity of the organisms in vivo. To determine whether α-pinene in fact influences the infectivity of *B. thuringiensis* spores in the DFTM, purified *B. thuringiensis* spores were tested in groups of larvae that were fed diets containing differing levels of α-pinene. It is clear that increasing the amount of α-pinene in the diet increases the 50% lethal dose by approximately 700-fold (Table 3).

DISCUSSION

Several terpenes normally present in the Douglas fir clearly inhibit the growth of *B. thuringiensis* at concentrations comparable to those found in the fir needle diet. This effect appears to be primarily due to the structure of the terpenes, rather than a generalized sensitivity to hydrocarbons, since exposure to similar concentrations of cyclohexane, a compound with analogous solvent properties, inhibited the growth rate to only a limited extent. Several different microorganisms were compared with respect to terpene effects. These organisms certainly show a widespread sensitivity of microbes to terpenes, and suggest as well that gram-neg-

TABLE 2. *Activity of yeast mitochondria in the presence of the terpene α-pinene*

α-Pinene concn (mg/ml)	Slope at state 4	Slope at state 3	Respiratory control value[a]
None	0.56	1.08	1.93 ± 0.2
2.06	0.56	0.94	1.68 ± 0.2

[a] Respiratory control is calculated as the ratio of rate of O_2 consumption in state 3 (+ adenosine 5'-diphosphate) to that in state 4 (adenosine 5'-diphosphate limited) (1).

TABLE 3. *Effect of α-pinene concentration on the 50% lethal dose of B. thuringiensis spores in DFTM*[a]

α-Pinene concn (mg/g of diet)	50% lethal dose (no. of spores)	95% fiducial limit[b]
None	1.7×10^5	7.9×10^4–3.3×10^5
0.27	1.1×10^7	5.2×10^6–2.1×10^7
2.7	1.2×10^8	4.7×10^7–4.8×10^8

[a] Results of one representative experiment, out of a total of three, are reported here.

[b] Limits calculated by probit analysis (number of spores).

ative and gram-positive organisms differ in their sensitivity to terpenes.

It is apparent that terpenes cause disruption of the cytoplasmic membranes of both the procaryotic *B. thuringiensis* and the eucaryotic organism *S. cerevisiae*. The damage to the membrane is obviously extreme, since the quantity of ultraviolet-absorbing materials in the filtrate was found to exceed those extracted by the rather harsh method employing 1.5 N perchloric acid (11).

The mitochondrial membrane of *S. cerevisiae* was not similarly affected, although there was a modest decrease in mitochondrial activity. The action of the terpene α-pinene seems therefore to not have a universal effect on the selected cellular membranes. The data show that the gram-negative organisms were relatively resistant to terpenes. These organisms, of course, possess an outer membrane, which may account for the increase in resistance to the terpene. The data show rather selective sensitivity of the cytoplasmic membrane to terpenes, an effect which is not a simple solvent sensitivity. The specific site of action is therefore not evident from these data.

Since the sole food source for the DFTM is the foliage of the host trees, one may tentatively assume that midgut concentrations of these terpenes would influence the type and quantity of microorganisms inhabiting the larval gut. Since the in vitro growth of bacteria was inhibited at levels of terpenes corresponding to those found in the Douglas fir needle diet, it was possible that the DFTM would be more resistant to bacterial infection on a terpene-containing diet than on a terpene-free diet. One may assume that this conclusion is correct, since there was an approximate 700-fold increase in 50% lethal dose on the terpene diets (Table 3). Although several other terpenes are both present and toxic to *B. thuringiensis*, the higher concentration of α-pinene used in the experiment was assumed to be a reasonable approximation of the effect of total fir needle terpenes on the *B. thuringiensis* infectivity for DFTM

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