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CONVERSION OF VERBENOLS TO VERBENONE BY YEASTS ISOLATED FROM *Dendroctonus ponderosae* (COLEOPTERA: SCOLYTIDAE)[1]

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Abstract—A variety of symbionts associated with bark beetles are capable of producing compounds that are used as pheromones by their hosts. We report that two yeasts associated with *Dendroctonus ponderosae* Hopkins, *Hansenula capsulata* Wickerham, and *Pichia pinus* (Holst) Phaff, are capable of converting *cis*- and *trans*-verbenol efficiently into verbenone. *trans*-Verbenol, which is produced by female *D. ponderosae*, acts as an aggregation pheromone for this scolytid, while verbenone, which other studies have indicated that microbe-reduced *D. ponderosae* are incapable of producing, acts as an antiaggregation pheromone. *D. ponderosae* appears to rely primarily on microbial symbionts for terminating aggregation and mass attack on individual host trees.

Key Words—*Dendroctonus ponderosae*, Coleoptera, Scolytidae, *Hansenula capsulata*, *Pichia pinus*, pheromones, *trans*-verbenol, verbenone.

INTRODUCTION

The hypothesis that microorganisms are involved in the production or modification of bark beetle pheromones has been debated for over half a century. Person (1931) hypothesized that the western pine beetle, *Dendroctonus brevicomis* Leconte, introduced a yeast into the inner bark of host trees that produced

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fermentation products responsible for secondary attraction. Callaham and Shifrine (1960) reported that yeast-inoculated phloem was attractive to various species of *Dendroctonus*.

More recently it has been confirmed that a variety of symbionts associated with bark beetles and other coleoptera are capable of producing compounds that are used as pheromones by their hosts. Bacteria isolated from female grass grub beetles, *Costelytra zealandica* (White), produced an unidentified compound that attracted male *C. zealandica* under field conditions (Hoyt et al., 1971). A *Serratia* species isolated from the bark beetle, *Phloeosinus armathus*, converted sabinene into terpinene-4-ol and α-terpineol, both of which were attractive to male and female *P. armathus* (Chararas et al., 1980). A strain of *Bacillus cereus* Frankland and Frankland, which was isolated from the guts of *Ips paraconfusus* Lanier, converted α-pinene to the host's pheromone *cis*-verbenol (Brand et al., 1975). Brand et al. (1976) demonstrated that fungi from the mycangia of *Dendroctonus frontalis* Zimmerman were capable of oxidizing *trans*-verbenol to the antiaggregation pheromone verbenone. The yeasts *Hansenula holstii* Wickerham and *Pichia pinus* (Holst) Phaff, also isolated from *D. frontalis*, produced metabolites that enhanced attraction of these beetles to a 1:1:12 mixture of frontalin–*trans*-verbenol–turpentine (Brand et al., 1977). French et al. (1984) reported that flying *Scolytus multistriatus* (Marsham) were attracted to agar cultures of *Bacillus subtilis* (Ehrenberg) Cohn, *Bacillus pumilis* Meyer and Gottheil, and *Enterobacter cloacae* (Jordan) Hormaeche and Edwards, isolated from elm trees. Several species of yeasts isolated from *Ips typographus* (L.) were capable of interconverting *trans*- and *cis*-verbenol and verbenone (Leufvén et al., 1984), of which the latter two function as pheromones for the host insects. These same yeasts were capable of producing a variety of oxygenated monoterpenes when grown in a phloem medium (Leufvén et al., 1988). Other more general examples of fungi and bacteria that are capable of oxidizing α-pinene and other monoterpenes are found in Bhattacharyya et al. (1960), Prema and Bhattacharyya (1962), Shukla et al. (1968), Fonken and Johnson (1972), and Keislich (1976).

Individual monoterpenes or extracts of resin have been found to be toxic to microorganisms associated with bark beetles, such as several *Ceratocystis* species (Cobb et al., 1968; DeGroot, 1972), *Trichosporium symbioticum* Wright (Raffa et al., 1985), and fungal symbionts of *Dendroctonus ponderosae* Hopkins (Shrimpton and Whitney, 1968), as well as being toxic to *Dendroctonus* species (Smith, 1965; Reid and Gates, 1970; Coyne and Lott, 1976; Raffa and Berryman, 1983b). Therefore, it is of adaptive advantage for the microorganisms to be capable of allylic oxidations of monoterpenes to detoxify these compounds and secondarily to use them as energy sources.

Our objective was to determine whether microorganisms associated with

the mountain pine beetle, *D. ponderosae*, were capable of the production or interconversion of pheromones of their host. We report the results of a study conducted on two yeasts associated with *D. ponderosae*, *Hansenula capsulata* Wickerham and *Pichia pinus*.

METHODS AND MATERIALS

Microorganisms. Cultures of two species of yeasts that are frequently associated with *D. ponderosae* were obtained from H.S. Whitney (Pacific Forestry Centre, 506 West Burnside Road, Victoria, B.C. V8Z 1M5, Canada), and were maintained on Sabouraud dextrose agar (SDA) (Difco Laboratories, Detroit, Michigan). These two species were identified by L.J. Wickerham (Northern Utilization Research and Development Division, U.S. Department of Agriculture, Peoria, Illinois; now retired) as *Pichia pinus* and *Hansenula capsulata*.

Yeast Conversion Experiments. Conversion experiments were conducted using a procedure similar to that employed by Leufvén et al. (1984). α-Pinene, *trans*-verbenol, *cis*-verbenol, and verbenone were dissolved individually at 3 mg/ml in 95% ethanol; 250 μl of one of the ethanolic solutions was added to 250 ml Erlenmeyer flasks containing 50 ml of Sabouraud dextrose broth that had just been inoculated by aseptically transferring a small amount of cells from a fresh SDA culture of one of the yeasts. After 24 hr at 21–23°C with slight shaking, another 250 μl of the ethanolic solution was added, yielding a final ethanol concentration in the medium of 1% (v/v). All the flasks that contained broth that had been inoculated with one of the yeasts appeared cloudy after 24 hr, indicating growth of the yeasts. After a further 24 hr of incubation, 5 ml of the medium was extracted three times with 1 ml each of distilled pentane. Each treatment for each yeast was replicated three times, as were control treatments in which a sterile inoculating loop was dipped into the medium.

The *trans*-verbenol used in this study, which was 75% (−)-25% (+) and was contaminated with approximately 12% *cis*-verbenol and 0.8% verbenone, was obtained from Phero Tech Inc. (Vancouver, B.C.). Racemic *cis*-verbenol, which was contaminated with approximately 14% *trans*-verbenol, was obtained from Borregaard, A.S. (Sarpsborg, Norway), and racemic α-pinene (>99% pure) was obtained from Aldrich Chemical Co. (Milwaukee, Wisconsin). Racemic verbenone (97% pure, as determined by gas chromatography) was obtained from Indukern (Barcelona, Spain).

Gas Chromatographic Analyses. Pentane extracts of yeast cultures were analyzed on a Hewlett Packard 5880A gas chromatograph equipped with a capillary inlet system and a flame ionization detector. A glass capillary column (30 m × 0.66 mm ID) coated with SP-1000 (Supelco, Inc., Bellefonte, Pennsyl-

vania) was used with the following temperature program: 120°C for 2 min, then increased by 4°C/min to 180°C. The injection port temperature was 260°C, the flame ionization detector temperature was 275°C, and helium was used as the carrier gas.

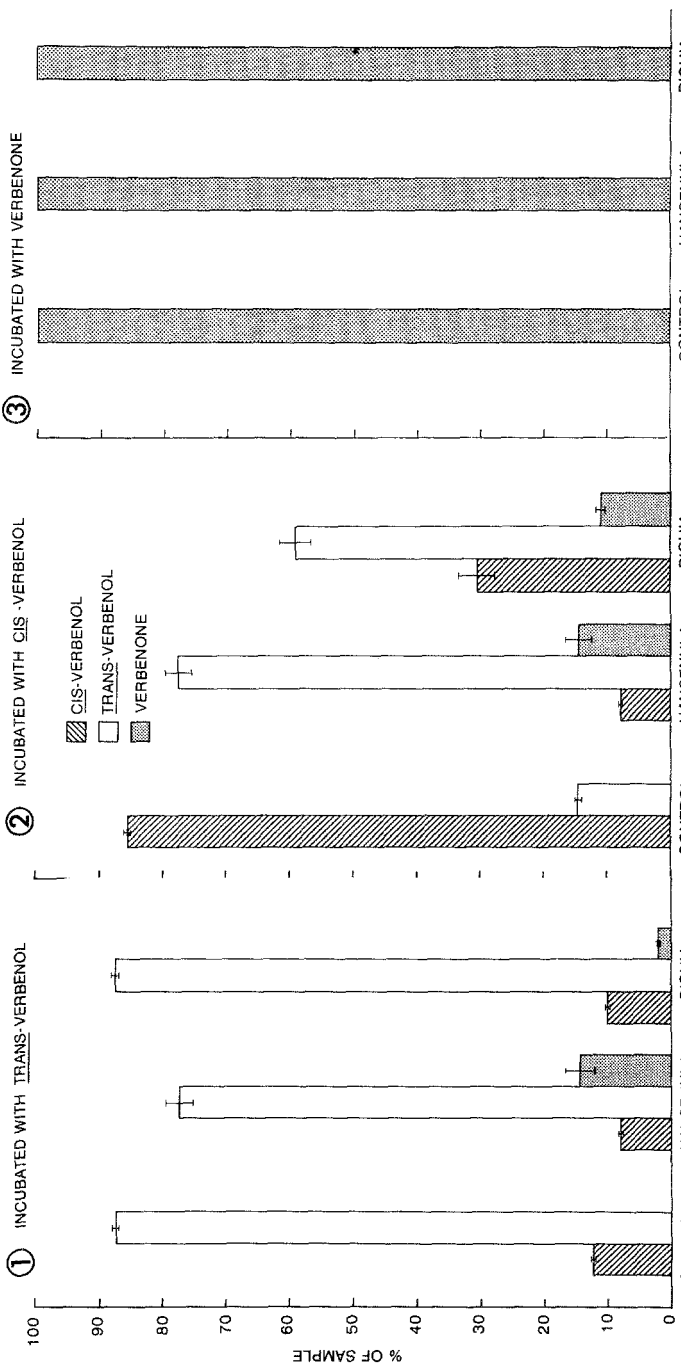
Each day that samples were analyzed by gas chromatography, a standard sample made up of α-pinene, *cis*- and *trans*-verbenol, and verbenone was also analyzed to aid in the identification of unknown compounds in the samples by comparison of retention times. In addition, these compounds were added periodically to an extract and that mixture was analyzed to ensure correct identification by cochromatography with unknown compounds in the extract. Selected extracts were analyzed using gas chromatography–mass spectroscopy to ensure proper identification of compounds. Amounts of detected compounds in yeast extracts were evaluated by comparing the relative proportions of the gas chromatographic peak areas (Leufvén et al., 1984). The sum of the gas chromatographic peak areas of *trans*- and *cis*-verbenol and verbenone was approximately the same after all incubations, indicating that the compounds were primarily interconverted, and not metabolized into other compounds.

RESULTS

Incubation of either yeast with *trans*-verbenol resulted in accumulations of verbenone, while the *trans*-verbenol control flasks, which were incubated without yeasts, contained no more verbenone than the 0.8% in the starting material (Figure 1). Incubation of *H. capsulata* with *trans*-verbenol caused an accumulation of 14.5 ± 2.1% verbenone, while incubation with *P. pinus* caused an accumulation of 2.2 ± 0.1% verbenone (Figure 1). For *H. capsulata* the decline in the relative amount of *trans*-verbenol, compared to that in the control flasks, appeared to indicate that the verbenone resulted from conversion of *trans*- as opposed to *cis*-verbenol.

Incubation of either yeast with *cis*-verbenol resulted in accumulations of *trans*-verbenol and verbenone, while the *cis*-verbenol control flasks, which were incubated without yeasts, contained both verbenols as well as verbenone at the original levels (Figure 2). Neither of the yeasts produced *trans*- or *cis*-verbenol from verbenone (Figure 3).

Extracts of α-pinene incubations did not contain detectable levels of α-pinene or terpene alcohols, even when the medium was extracted immediately following the addition of α-pinene. Thus the α-pinene was apparently bound very rapidly to an unidentified constituent of the medium, or it reacted to form a product that we did not detect through gas chromatographic analysis.



FIGS. 1–3. Mean percent content (±SE) of *trans*- and *cis*-verbenol and verbenone in cultures of *H. capsulata* or *P. pinus* incubated with *trans*-verbenol (Figure 1), *cis*-verbenol (Figure 2), or verbenone (Figure 3) for 48 hr. $N = 3$ for each treatment in each experiment.

DISCUSSION

The aggregation of *D. ponderosae* on host trees is due partially to the beetle-produced terpene alcohol *trans*-verbenol, in combination with host tree monoterpenes (Pitman et al., 1968), although low concentrations of the male-produced, multifunctional pheromones *exo*-brevicomin and frontalin may also be involved (Rudinsky et al., 1974a; McKnight, 1979; Conn et al., 1983; Borden et al., 1983, 1987; Chatelain and Schenk, 1984). Once a certain attack density is reached, the attack switches to adjacent trees (McCambridge, 1967; Geiszler and Gara, 1978; Geiszler et al., 1980), preventing the increased competition within hosts and reduced brood survival that occur at overly high attack densities (Reid, 1963). This switching appears to be due to a number of factors, including a decrease in resin flow (Renwick and Vité, 1970) and *trans*-verbenol production (Renwick and Vité, 1969; Borden et al., 1987) and the production of antiaggregation pheromones (Rudinsky et al., 1974a). Although several compounds produced by *D. ponderosae* have proven to be inhibitory to these beetles in laboratory and field tests (Ryker and Libbey, 1982; Ryker and Rudinsky, 1982; Libbey et al., 1985; Hunt and Borden, 1988), leading to speculation that they may act as antiaggregation pheromones, the antiaggregative activity of verbenone (Ryker and Yandell, 1983) is generally thought to be the most significant.

In lodgepole pine trees attacked by *D. ponderosae*, aggregation generally peaks on approximately the second day of attack, and then declines to zero within four to seven days of the initiation of attack (Raffa and Berryman, 1983a). The conversion of *trans*-verbenol into verbenone (Figure 1), particularly by *H. capsulata*, suggests that this termination of aggregation on trees attacked by *D. ponderosae* is the result of verbenone production by yeasts introduced into the trees by the attacking beetles. It is our hypothesis that the yeasts that are introduced into the attacked trees by *D. ponderosae* require a few days to reach a population size in the galleries large enough to enable them to convert significant quantities of *trans*-verbenol, which has been produced in large quantities by the beetles for the first few days, into verbenone. The switching of the attack to adjacent trees (McCambridge, 1967; Geiszler and Gara, 1978; Geiszler et al., 1980) thus would be due largely to the production of verbenone by yeasts (Figures 1 and 2).

The hypothesis that fungi introduced into attacked trees by bark beetles are responsible for the termination of aggregation has been proposed for other species. Brand et al. (1976) hypothesized that fungi introduced into the galleries of *D. frontalis* may convert *trans*-verbenol, which is involved in the aggregation of this bark beetle (Renwick and Vité, 1969, Payne et al., 1978), into verbenone, which can function as an antiaggregation pheromone (Rudinsky, 1973; Rudinsky et al., 1974a). Also, Leufvén et al. (1984) hypothesized that yeasts

introduced into the galleries of *I. typographus* may convert the aggregation pheromone *cis*-verbenol (Bakke et al., 1977) into verbenone, an antiaggregation pheromone (Bakke, 1981).

It is unclear why α-pinene was not extractable with pentane, which is an efficient solvent for neat α-pinene. It appears that the α-pinene was bound in some way within the water phase, so it may not have been available to be metabolized by the yeasts, or extracted. Alternatively, the α-pinene may have reacted to form a product that we did not detect through gas chromatographic analysis. Additional research is required to establish the capacity of these yeasts to metabolize α-pinene.

Although under laboratory conditions many microorganisms are capable of producing or interconverting compounds used as pheromones by bark beetles (Bhattacharyya et al., 1960; Prema and Bhattacharyya, 1962; Fonken and Johnson, 1972), and some of these microorganisms are found in association with bark beetles (Brand et al., 1975, 1976, 1977; Chararas et al., 1980; Leufvén et al., 1984), there is little evidence to suggest that these conversions are of any significance to the chemical ecology of bark beetles under natural conditions. However, several pertinent observations support the hypothesis that conversion of *trans*-verbenol into verbenone by symbiotic yeasts is of genuine significance to the chemical ecology of *D. ponderosae*.

First, although certain microorganisms such as *B. cereus* (Brand et al., 1975), and *Aspergillus niger* van Tieghem (Bhattacharyya et al., 1960; Prema and Bhattacharyya, 1962) are capable of the production of verbenols, it has been established that *D. ponderosae* that are free of these readily culturable microorganisms are capable of converting α-pinene vapors into *trans*-verbenol at levels equal to or above those found in wild beetles (Hunt and Borden, 1989). In contrast, axenically reared *D. ponderosae* did not produce quantifiable levels of verbenone, while beetles with their normal complement of microorganisms, which were fed on *P. contorta*, did (Hunt and Borden, 1989), as did yeasts associated with *D. ponderosae* (Figures 1 and 2). The production of verbenone by wild *D. ponderosae* from ingested α-pinene and the inability of these wild beetles to produce verbenone from α-pinene vapors (Hunt and Borden, 1989) suggest that the conversion is done by microorganisms in their guts. Verbenone is apparently not metabolized further by *H. capsulata* or *P. pinus* (Figure 3), so it would probably build up at significant levels in attacked trees. Thus it would be of adaptive advantage for individual *D. ponderosae* to exploit verbenone as a signal of an established attack in host trees that offer limited resources. *Candida nitratophila*, the only yeast isolated by Leufvén et al. (1984) that converted *trans*-verbenol into verbenone, has also been isolated from *D. ponderosae* (Shifrine and Phaff, 1956). There is a highly persistent association between *D. ponderosae* and its associated yeasts, suggesting a mutualistic association (Whitney, 1971). *H. capsulata* and *P. pinus* were associated closely

with *D. ponderosae* during brood development in lodgepole pine, and in an extensive survey no populations were found to be free of these yeasts (Whitney, 1971). Farmer (1965) also found that these yeasts were closely associated with *D. ponderosae* in lodgepole pine. Finally, these yeasts were isolated frequently from the maxillary mycangium of *D. ponderosae* (Whitney and Farris, 1970), compelling evidence of the importance of this symbiotic relationship.

When yeasts associated with *I. typographus* were quantified, it was found that those that can convert the aggregation pheromone *cis*-verbenol into the antiaggregation pheromone verbenone (Leufvén et al., 1984) were most prevalent on the beetles and in the galleries during those phases of the attack at which verbenone is produced in much higher levels than *cis*-verbenol (Leufvén and Nehls, 1986). During early attack phases, when aggregation is occurring on the trees and the beetles are producing large quantities of *cis*-verbenol, these yeasts were present on the insects in very low numbers (Leufvén and Nehls, 1986). We hypothesize that a similar temporal relationship exists between gallery development in *D. ponderosae* and the population of yeasts therein.

H. capsulata, *P. pinus*, and other yeasts capable of converting verbenols to verbenone (Leufvén et al., 1984), have been isolated frequently from *Dendroctonus* and *Ips* species (Callaham and Shifrine, 1960; Shifrine and Phaff, 1956). Many of these bark beetles use *cis*- or *trans*-verbenol as an aggregation pheromone and verbenone as an antiaggregation pheromone, suggesting that microbial involvement in the termination of bark beetle aggregation may be widespread. Of particular interest are insects such as *D. pseudotsugae* and *D. frontalis*, for which verbenone is a multifunctional pheromone, attractive at low concentrations and inhibitory at high concentrations (Rudinsky, 1973; Rudinsky et al., 1974a,b). For these species the low population levels of yeasts present in beetle galleries soon after the initiation of attack could contribute toward beetle aggregation by producing low levels of verbenone. In more advanced stages of attack the higher levels of verbenone produced by larger yeast populations would contribute toward terminating the attack on individual trees and directing it toward other trees.

It was our original intention to culture microorganisms from large numbers of *D. ponderosae* and to examine the abilities of the isolated microorganisms to produce and interconvert compounds that function as semiochemicals for their hosts. We now believe that such a survey would add little to what is already known about microbial involvement in bark beetle pheromone production and regulation. Considering the number of microorganisms that already have been found to be capable of producing or interconverting bark beetle pheromones in laboratory experiments, it is evident that the metabolic capacities for these conversions are common. It would be much more edifying to establish whether these metabolic capacities are relevant in nature. This question could be addressed by quantifying the production of metabolites by microorganisms

exposed to realistic levels of precursors under natural conditions and at population levels similar to those found naturally in association with bark beetles.

For the production of α-pinene-derived pheromones in *D. ponderosae*, it is now possible to refine and expand the model proposed by Borden (1984). The new model (Figure 4) proposes that most of the aggregation pheromone *trans*-verbenol formed in trees attacked by *D. ponderosae* is produced from inhaled α-pinene by the beetles' own enzymes (Conn et al., 1984; Hunt and Smirle, 1988; Hunt and Borden, 1989). The conversion of ingested α-pinene by microorganisms in the beetles' guts (Hunt and Borden, 1989), as well as autoxidation of α-pinene (Hunt et al., 1989), contribute in a minor way towards *trans*-verbenol production. *trans*-Verbenol also may be formed by microorganisms present in the beetles' galleries, although there are currently no data to support this possibility, and, therefore, this route is excluded from the model.

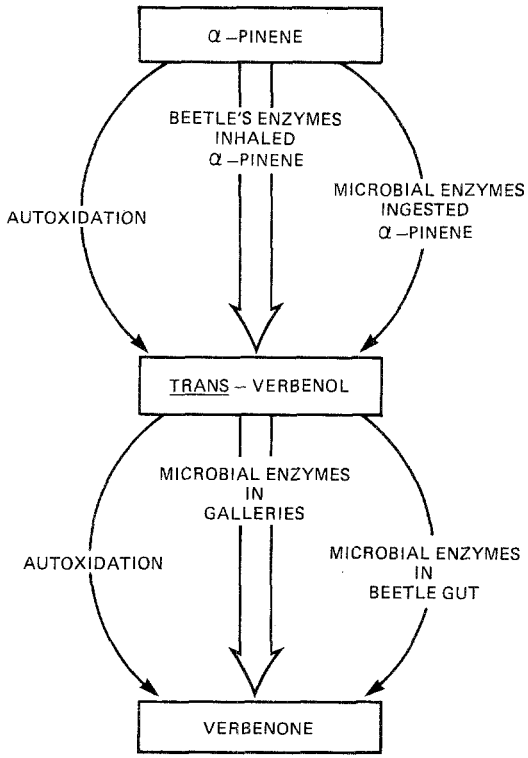


FIG. 4. Pathways for the production of α-pinene-derived pheromones in *D. ponderosae*. Wide arrows down the middle of the model denote major pathways. Narrower arrows on either side denote minor pathways.

The antiaggregation pheromone verbenone appears to be produced almost entirely by microorganisms (Hunt and Borden, 1989) (Figure 1), with the beetles' enzymes not involved. This conversion is probably performed by microorganisms in the beetles' galleries, as well as, to a lesser extent, by gut symbionts. Autoxidation of *trans*-verbenol to verbenone supplements the other routes to an unknown but minor extent (Hunt et al., 1989). There is no evidence that verbenone is metabolized further by any system.

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