Journal of Chemical Ecology, Vol. 14, No. 1, 1988

OXYGENATED MONOTERPENES PRODUCED BY YEASTS, ISOLATED FROM *Ips typographus* (COLEOPTERA: SCOLYTIDAE) AND GROWN IN PHLOEM MEDIUM[1]

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(Received October 27, 1986; accepted February 3, 1987)

Abstract—When yeasts associated with *Ips typographus* beetles were grown in an aqueous phloem medium for two days, the main oxygenated monoterpenes produced were α-terpineol and borneol. Terpinene-4-ol, myrtenol, and *trans*-pinocarveol were also found but in lesser amounts. Of the six strains used in this study, *Hansenula capsulata* and *Candida nitratophila* produced the largest amounts of oxygenated monoterpenes. Addition of α-pinene to the phloem medium generally reduced the amounts of oxygenated monoterpenes, probably because this substance is toxic to all tested yeast species. Our *Candida diddensii* strain seemed to be particularly sensitive to α-pinene. None of the yeast strains produced *cis*-verbenol, *trans*-verbenol, or verbenone from the medium or from added α-pinene.

Key Words—Microbial transformations, microorganisms, yeasts, bark beetle, Coleoptera, Scolytidae, *Ips typographus*, oxygenated monoterpenes, α-terpineol, borneol.

INTRODUCTION

Yeasts are commonly associated with bark beetles (Shifrine and Phaff, 1956; Callaham and Shifrine, 1960; Whitney, 1971), but it is uncertain what role they play in the attack sequence of tree-killing bark beetles (Bridges et al., 1984). It

[1]This study was made within the project "Odour Signals for Control of Pest Insects."

0098-0331/88/0100-0353$06.00/0

is generally supposed that beetles inoculate a tree with different fungi in order to overcome the tree's resistance to attack (Whitney, 1982). One line of defense that the trees have against a bark beetle–fungus attack is a resin containing toxic terpenoids; the beetles have adapted to this by detoxifying oxygenation of some of the compounds (Hughes, 1973). In view of the general occurrence of mixed function oxidases (White et al., 1980), it is reasonable to believe that fungi and bacteria possess a similar detoxification system. A *Bacillus cereus* strain isolated from the North American bark beetle *Ips paraconfusus*, could convert α-pinene to *trans*-verbenol, *cis*-verbenol, and probably myrtenol (Brand et al., 1975). The verbenols are among those substances known as attractants for many bark beetle species, but in view of other findings (Byers and Wood, 1981; Conn et al., 1984), it is still uncertain how microorganisms are involved in the production of pheromones.

In our work with the spruce bark beetles, *Ips typographus* (L.), and its associated yeasts, we found that some of the yeasts could convert the beetles' aggregative pheromone component *cis*-verbenol to verbenone (Leufvén et al., 1984), a substance suspected to have antiaggregative properties (Bakke, 1981). The oxygenated monoterpenes found by Birgersson et al. (1984) in guts of *I. typographus* beetles attacking a Norway spruce (*Picea abies*, Karst.), were both qualitatively and proportionally different from those found in the gallery walls surrounding the beetles (Leufvén and Birgersson, 1987). There is a striking similarity between the shape of the curves depicting the varying amounts of certain oxygenated monoterpenes found in phloem surrounding *I. typographus* beetles from different attack phases (Leufvén and Birgersson, 1987) and the curve showing the total number of colony-forming units (produced on Sabouraud medium) originating from beetles in different attack phases (Leufvén and Nehls, 1986).

In the present study, we have investigated the ability of *I. typographus* associated yeasts to produce, from a water extract of spruce phloem, some of the oxygenated monoterpenes found in phloem from the walls of bark beetle galleries. To see which oxygenated monoterpenes, if any, the isolated yeast strains could produce from the pheromone precursor α-pinene under our *in vitro* conditions, we added this substance to some of the incubations.

METHODS AND MATERIALS

The yeast strains used in this study were *Candida diddensii* (CCUG 11142), *Candida nitratophila* (CCUG 11139), *Cryptococcus albidus* var. *diffluens* (CCUG 11141), *Cryptococcus laurentii* var. *magnus* (CCUG 11125, now reclassified as *Cryptococcus heveanensis*), *Hansenula capsulata* (CCUG 11140),

Hansenula holstii (CCUG 11128) and *Pichia pinus* (CCUG 13846). All of these strains were isolated from natural Swedish populations of *Ips typographus* bark beetles. The strains, which are deposited with the Culture Collection University of Göteborg (CCUG), have also been used in previous studies (Leufvén et al., 1984; Leufvén and Nehls, 1986).

All incubations were made in aqeuous phloem and bark medium prepared in the following way. Phloem and bark of Norway spruce were cut in centimeter-size pieces and extracted for 4 hr in tap water at room temperature; approximately 250 g phloem and bark were used per liter of water. After removal of the solid material by filtration through coarse paper, the medium was frozen in 0.5-liter bottles and stored until used. After thawing and autoclaving, the phloem medium had a pH just below 6.

Cotton-wool-stoppered, 250-ml Erlenmeyer flasks with 50 ml of phloem medium were used for all incubations. After being heavily inoculated with yeasts from Sabouraud's solid medium, the culture flasks were kept at room temperature and continually agitated for two days. At the end of the incubation period, two 4-ml aliquots were transfered to screw-capped centrifugation tubes. To facilitate extraction, yeast cells were removed by centrifugation; in a preliminary experiment, only very small amounts of extractable oxygenated monoterpenes were found in the yeast pellet. Each of the two supernatants were extracted three times with a total of 2.5 ml of a mixture of 10% diethyl ether in pentane. The extracts of the two supernatants were combined, and 2.5 μg of heptyl acetate in hexane were added as a standard.

In some experiments, racemic α-pinene (EGA-Chemie) dissolved in ethanol was added during the incubation. At the time of inoculation, 5 mg of α-pinene was added, followed by another 10 mg after one day of incubation. The total volume of added ethanol was 300 μl. Prior to its use, the α-pinene was passed through a short, heat-activated, silica column in order to remove oxygenated monoterpenes. Camphene and β-pinene, 0.56% and 1.22%, respectively, were present as impurities in the α-pinene used. Phloem medium without inoculated yeasts was used as a control in all incubation experiments.

The ether–pentane extracts were concentrated by evaporation and analyzed on a Finnigan 4021 GC-MS system equipped with a 25-m fused silica column, ID 0.15 mm, coated at the Department of Chemical Ecology with a 0.30-μm-thick film of Superox FA (RSL). The initial temperature in the gas chromatograph was 50°C for 4 min, followed by an increase of 8°C/min up to 200°C and then kept isothermal. Compounds were quantified by measuring the area of prominent and/or typical mass spectral fragments and comparing the calculated total peak area with that of added heptyl acetate (cf. Leufvén and Birgersson, 1987). The mass fragments used for each compound are shown in Figures 1 to 3.

RESULTS

The major oxygenated monoterpenes found in the phloem medium after the two-day incubation period were α-terpineol, borneol, terpinene-4-ol, *trans*-pinocarveol, myrtenol, and an unidentified alcohol. The amounts of these major components, per 50 ml of medium, are presented in Figures 1 and 2. The bars represent the means from three different incubations, except the controls in which five (no α-pinene added) or six (α-pinene added) incubations were carried out.

Under the in vitro conditions used in this study, our *Candida nitratophila* and *Hansenula capsulata* strains produced the largest amounts of oxygenated monoterpenes. *Candida diddensii*, *Hansenula holstii*, and *Pichia pinus* also produced relatively large amounts of certain of these monoterpenes, whereas *Cryptococcus albidus* var. *diffluens* and *Cryptococcus laurentii* var. *magnus* usually only produced minor amounts.

The main product after incubation in the phloem medium was α-terpineol (Figure 1), especially when the *C. nitratophila* strain was used. However, if α-pinene was not added, the amounts produced by this strain, per 50 ml medium, varied greatly: i.e., from 60 to 250 μg. Large amounts of α-terpineol were also produced by *H. capsulata* but quantities varied largely for this strain as well.

The compound found in the second largest amounts was borneol (Figure 1), the largest amounts of which were produced by *H. capsulata* followed by *C. nitratophila* and *C. diddensii*. The amounts of borneol produced by the different yeast strains was partly paralleled, but on a smaller scale, by the amounts of *trans*-pinocarveol produced (Figure 2). Terpinene-4-ol (Figure 2) was found as a major oxygenated monoterpene, especially after incubations with *H. capsulata*, but was also present in relatively large amounts in control incubations without yeasts. Myrtenol and an unidentified monoterpene alcohol (having 79 and 93, respectively, as characteristic mass spectral fragments) were produced in incubations with *H. capsulata*, in amounts of 5–10 μg/50 ml of medium. The unidentified terpene was also produced in incubations with *C. nitratophila* (Figure 2).

None of the yeast strains used could produce *cis*-verbenol, *trans*-verbenol, and verbenone, substances which participate in regulation of bark beetle behavior, from compounds present in the phloem medium or from the added α-pinene. Verbenone was detected in amounts of up to 2 μg/50 ml medium, but there were no differences between incubations with and without yeasts (Figure 3).

Small amounts of a nonterpenoid compound, 2-phenyl-ethanol, were produced by some of the yeast strains, particularly *H. holstii*, *H. capsulata*, and *C. nitratophila* (Figure 3).

In addition to the above-mentioned compounds, minor amounts of several

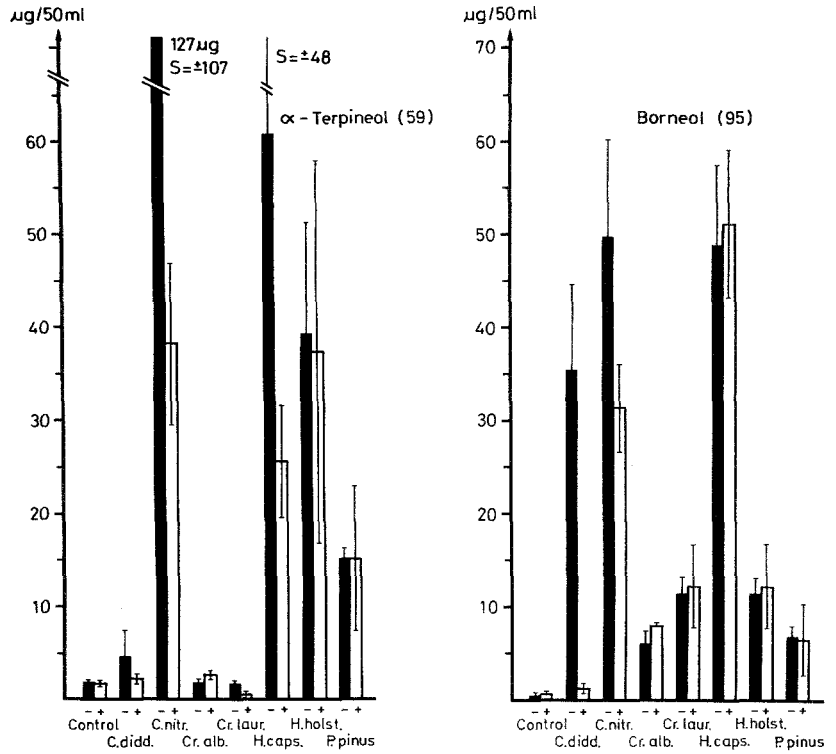

FIG. 1. Amounts of α-terpineol and borneol extracted from 50 ml of phloem medium inoculated with different strains of yeasts and incubated for two days with (solid bars) or without (open bars) added α-pinene. Thin vertical bars represent standard deviation. The mass fragment used for quantification is shown in parentheses after the compound name.

other substances were detected in the extracts from some of the inoculated incubations. Myrtenal and perilla alcohol were among those compounds, as was an unidentified substance with a mass spectrum closely resembling borneol (large *m/z* 95 fragment) but with a retention time similar to that of myrtenol. The two unidentified compounds reported from *I. typographus* gallery phloem, called monoterpene alcohol A and B (*m/z* 71 and 93) by Leufvén and Birgersson (1987), were also detected in the phloem medium after incubation with several of the yeast strains, i.e., *C. nitratophila*, *H. capsulata*, *H. holstii*, and *P. pinus*.

In general, the amounts of oxygenated monoterpenes produced in incubations to which racemic α-pinene was added (open bars in the figures) were lower than those produced in incubations without α-pinene. The most obvious examples are the production of borneol (Figure 1), *trans*-pinocarveol, myrtenol,

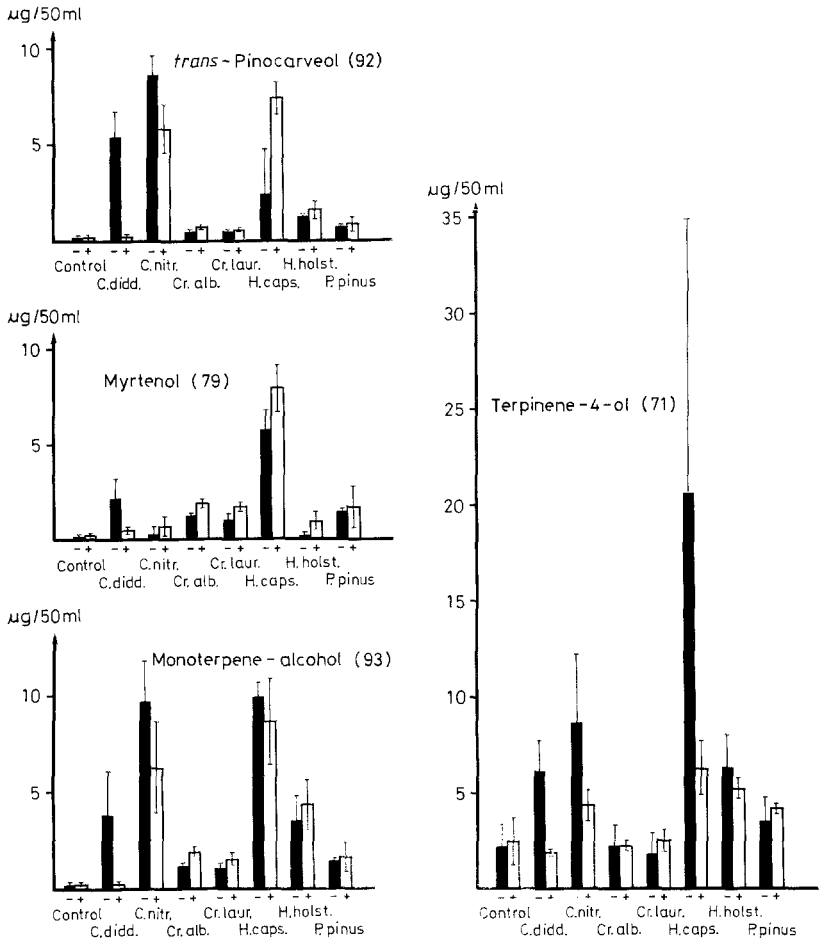

FIG. 2. Amounts of terpinene-4-ol, *trans*-pinocarveol, myrtenol, and an unidentified monoterpene alcohol extracted from 50 ml of phloem medium inoculated with different strains of yeasts and incubated for two days with (solid bars) or without (open bars) added α-pinene. Thin vertical bars represent standard deviation. The mass fragment used for quantification is shown in parentheses after the compound name.

and the unidentified monoterpene alcohol, quantified on mass spectral fragment *m/z* 93 (Figure 2), by *C. diddensii*. Contrary to this general trend, larger amounts of *trans*-pinocarveol and myrtenol were found after incubation of *H. capsulata* in phloem medium in which α-pinene was added than were found after incubation of this yeast strain in medium to which α-pinene had not been added.

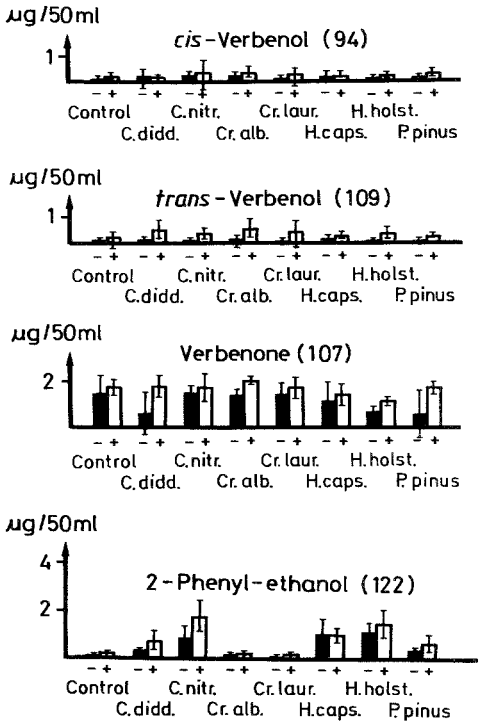

FIG. 3. Amounts of *cis*-verbenol, *trans*-verbenol, verbenone, and 2-phenylethanol extracted from 50 ml of phloem medium inoculated with different strains of yeasts and incubated for two days with (solid bars) or without (open bars) added α-pinene. Thin vertical bars represent standard deviation. The mass fragment used for quantification is shown in parentheses after the compound name.

DISCUSSION

The main oxygenated monoterpenes found in phloem medium after incubation with yeasts isolated from the spruce bark beetle were α-terpineol and borneol. These two compounds were also found as the major oxygenated monoterpenes in phloem surrounding spruce bark beetle galleries in an attacked Norway spruce (Leufvén and Birgersson, 1987). Terpinene-4-ol, *trans*-pinocarveol, myrtenol, *trans*-verbenol, and verbenone were also found in relatively large amounts in the phloem walls and, with the exception of *trans*-verbenol and verbenone, these compounds were also produced by some of the yeasts used in the present study. The failure of our strains to produce the verbenols and verbenone could, of course, be explained by incorrect in vitro conditions,

but we assume that the isolated yeast strains do not produce the verbenols, at least not from compounds present in, or added to, the incubation medium. The small amounts of verbenols and verbenone actually detected were probably autoxidation products from terpene hydrocarbons. This is well in accordance with the similarity in the amounts of verbenone found in all yeast incubations and controls (Figure 3). The inability of our beetle-associated yeast strains to produce verbenols and/or their previously reported ability to convert verbenol to verbenone (Leufvén et al., 1984) agrees with the findings of Conn et al. (1984), who demonstrated that axenic beetles exposed to α-pinene vapors had more verbenols in their guts than septic beetles treated in the same way. On the other hand, a bacterial strain isolated from bark beetles has been used to produce both *cis*- and *trans*-verbenol from α-pinene (Brand et al., 1975).

The origin of the oxygenated monoterpenes found in the phloem medium after incubation is still unclear. In this study, preparation of the medium by water extraction and subsequent sterilization by autoclaving probably excluded all monoterpene hydrocarbons from the incubation medium. Preliminary chromatographic investigations of the medium did not indicate the presence of any monoterpene hydrocarbons. Larger terpenoids that may have been present in the medium could have been precursors of the oxygenated monoterpenes, but this possibility has not been investigated.

Addition of α-pinene to the incubation medium usually failed to increase the amounts of oxygenated monoterpenes present after incubation. The production of *trans*-pinocarveol, and possibly myrtenol, by *H. capsulata* are exceptions. However, in most cases, the addition of α-pinene probably had a more or less toxic effect, especially in some of the incubations with *C. diddensii*. The decrease in production is not surprising, since it is known that monoterpene hydrocarbons are somewhat toxic, even to fungi associated with bark beetles (Raffa et al., 1985; DeGroot, 1972). In our experiments with the addition of α-pinene to *H. capsulata* incubations, the production of *trans*-pinocarveol, and possibly myrtenol, from α-pinene increased, while the production of α-terpineol and terpinene-4-ol from unknown sources decreased. This change in priority of production makes it very tempting to suggest that in order to detoxify α-pinene, *H. capsulata* converts it to *trans*-pinocarveol and possibly myrtenol. In view of the fact that *H. holstii* and *C. diddensii* were the yeasts isolated in the largest numbers from *I. typographus* beetles (Leufvén and Nehls, 1986), it is surprising that *C. diddensii* seems to be the yeast strain less suited to cope with the toxic properties of α-pinene. The major producers of oxygenated monoterpenes, *H. capsulata* and *C. nitratophila* were both bark beetle associates isolated in lesser numbers by Leufvén and Nehls (1986). The reason for this discrepancy concerning production ability and numbers isolated is not clear, but one has to bear in mind that conditions in the natural environment of the yeasts and during *in vitro* incubations are inevitably different and that the yeast

strains isolated by Leufvén and Nehls (1986) came from crushed beetles, not from gallery wall phloem.

The nonterpenoid compound 2-phenylethanol was not detected in gallery wall phloem (Leufvén and Birgersson, 1987), but it was found in bark beetle guts (Birgersson et al., 1984) and was found to be produced by yeasts, especially *H. holstii*, growing in synthetic Sabouraud medium (Leufvén et al., 1984), and in a phloem medium from pine (Brand et al., 1977). It is not yet evident whether or not this compound plays a part in the "attack dynamics" of the bark beetle–microorganism complex, although this has been suggested (Renwick et al., 1976).

Despite the fact that *in vitro* incubations have been used, this study shows that some of the compounds found in phloem from gallery walls of *I. typographus* beetles attacking Norway spruce can be produced by yeasts associated with the bark beetle. The actual precursors of the oxygenated monoterpenes are, however, not revealed by this study.

Acknowledgments—The staff at the Department of Clinical Bacteriology, Göteborg University, helped us with preparation of culture media and maintaining the cultures. A.-B. Wassgren prepared the GC columns and P. Ödman corrected the language. Several colleagues at our department and at other departments made valuable comments on the manuscript. This study was made as part of the project "Odour Signals for Control of Pest Insects" funded by Swedish Research Councils (NFR, FRN, SJFR, STU) and by Swedish forest industries.

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