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Linalyl Acetate Is Metabolized by *Pseudomonas incognita* with the Acetoxy Group Intact

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Metabolism of linalyl acetate by *Pseudomonas incognita* isolated by enrichment culture on the acyclic monoterpene alcohol linalool was studied. Biodegradation of linalyl acetate by this strain resulted in the formation of linalool, linalool-8-carboxylic acid, oleuropeic acid, and Δ^5-4-acetoxy-4-methyl hexenoic acid. Cells adapted to linalyl acetate metabolized linalyl acetate-8-aldehyde to linalool-8-carboxylic acid, linalyl acetate-8-carboxylic acid, Δ^5-4-acetoxy-4-methyl hexenoic acid, and geraniol-8-carboxylic acid. Resting cell suspensions previously grown with linalyl acetate oxidized linalyl acetate-8-aldehyde to linalyl acetate-8-carboxylic acid, Δ^5-4-acetoxy-4-methyl hexenoic acid, and pyruvic acid. The crude cell-free extract (10,000 *g* of supernatant), obtained from the sonicate of linalyl acetate-grown cells, was shown to contain enzyme systems responsible for the formation of linalyl acetate-8-carboxylic acid and linalool-8-carboxylic acid from linalyl acetate. The same supernatant contained NAD-linked alcohol and aldehyde dehydrogenases involved in the formation of linalyl acetate-8-aldehyde and linalyl acetate-8-carboxylic acid, respectively. On the basis of various metabolites isolated from the culture medium, resting cell experiments, growth and manometric studies carried out with the isolated metabolites as well as related synthetic analogs, and the preliminary enzymatic studies performed with the cell-free extract, a probable pathway for the microbial degradation of linalyl acetate with the acetoxy group intact is suggested.

Acyclic monoterpene alcohols are widespread in nature, and many of the essential oil-producing higher plants store significant levels of these compounds in the form of acetates or glycosides. Soil microorganisms, through a process of natural selection, acquire the ability to degrade these compounds of natural or plant origin. However, few reports have appeared in the literature regarding the microbial degradation of these compounds as compared with steroids and aromatic compounds. The first detailed work on the biotransformation of acyclic monoterpenes has been reported by Seubert et al. in *Pseudomonas citronellolis* (19, 20). These reports have demonstrated that the bacterial degradation of citronellol, geraniol, and farnesol is initiated by the oxidation of the primary alcohol group to carboxylate. Further degradation involves carboxylation of the β-methyl group and subsequent elimination of the latter as acetic acid. Later Cantwell et al. established further details of the pathways of degradation of citronellol and geraniol and also screened several representative *Pseudomonas* species for their ability to utilize other acyclic isoprenoids (6).

In contrast to the above findings, higher plants and mammalian systems metabolize geraniol and nerol by a totally different pathway (1, 9, 13). Both these living systems specifically carry out the oxidation of the C—8 methyl (ω-methyl) group of geraniol and nerol. This ω-hydroxylation reaction has been conclusively shown to be mediated by a cytochrome P-450 system (9, 13). We were interested in finding a microbial system that could mimic the mode of metabolism of acyclic monoterpene alcohols observed in higher plants and mammals. A search for such a microbial system led to the isolation of a soil pseudomonad, identified as *Pseudomonas incognita*, capable of utilizing monoterpene alcohol, linalool, as the sole source of carbon and energy (12). This versatile organism has also been shown to utilize geraniol, nerol, and citronellol as growth substrates (12, 18). Ramadevi et al. have studied the metabolism of geraniol and nerol by this organism and have proposed three different pathways for their degradation (18). One of them resembles the pathway proposed by Seubert et al. (19, 20). The other pathways proposed either involve the oxidative attack of the 2—3 double bond or the oxygenation of the C—10 methyl group. However, the relative importance of these pathways in the metabolism has not been fully assessed.

We reported earlier the probable pathways for the degradation of linalool by *P. incognita*, based on the identification of various metabolites as well as growth and oxygen uptake studies (12). It is interesting to note that linalool is metabolized by different pathways which have not been observed in the case of geraniol and nerol. One of the routes for the degradation is initiated by the oxidation of the C—8 methyl group of linalool. The other pathway, which appears to be a minor one, comprises the prototropic cyclization of linalool to α-terpineol and its further metabolism. The intriguing aspect of linalool metabolism by *P. incognita* is that the organism initiates the major energy-yielding pathway by the C—8 methyl oxygenation, whereas it fails to carry out similar oxidations with geraniol and nerol. The C—8 methyl oxidation pathway has also been implicated by Murakami et al. for the degradation of linalool by an unidentified *Pseudomonas* species (15). The earlier studies have clearly demonstrated that 8-hydroxy linalool is further oxidized to linalool-8-carboxylic acid (12, 18). However, the mode of further metabolism and the mechanism involved in the cleavage of the C—C bond during the degradation of linalool-8-carboxylic acid are not known.

We have noticed that *P. incognita* accepts linalyl acetate better than linalool as the sole source of carbon. This particular observation prompted us to undertake the present investigation to determine whether the organism initiates the degradation by hydrolyzing the acetate to free alcohol or whether it has the ability to metabolize the molecule keeping the acetate moiety intact. Besides, our objective was also to obtain insight into the mode of further metabolism of linalool-8-carboxylic acid. Since linalyl acetate has an asymmetric center, we were interested in ascertaining whether the organism can affect the asymmetric hydrolysis of the molecule. In fact it has been reported earlier that certain microorganisms are known to carry out the asymmetric hydrolysis of the acetates of the racemic acyclic terpene alcohols (16).

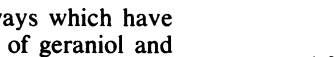
In the present paper, we report a new pathway for the degradation of linalyl acetate, the probable mode of further metabolism of linalyl acetate 8-carboxylic acid, and the nature of hydrolysis of linalyl acetate.

MATERIALS AND METHODS

Chemicals. Linalyl acetate and linalool were generous gifts from V. Paul, Hindustan Lever Ltd., Bombay. They were freshly distilled before use, and the purity was above 99.5%. All chemicals used for the preparation of the media were of chemically pure grade.

Linalyl acetate-8-aldehyde and 8-hydroxy linalyl acetate were synthesized by oxidizing linalyl acetate with selenium dioxide in refluxing ethanol for 3 to 4 h as previously reported (14).

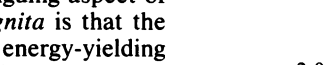
Linalyl acetate-8-aldehyde had the following nuclear magnetic resonance (NMR) ($CDCl_3$) δ: 1.5 (3H, s, C—10 Hs), 1.6 (3H, s, C—9 Hs),



1.94 (3H, s, —O—C—CH_3),

2.2 (4H, m, C—4 and C—5 Hs), 5.0 (2H, octet, C—1 Hs), 5.8 (1H, *dd*, C—2 H), 6.25 (1H, t, C—6 H), 9.4 (1H, s, aldehyde H).

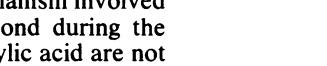
8 Hydroxy linalyl acetate had the following NMR ($CDCl_3$) δ: 1.5 (3H, s, C—10 Hs), 1.6 (3H, s, C—9 Hs),



2.0 (7H, m, —O—C—CH_3

and C—4, C—5 Hs), 2.45 (1H, s, disappears on addition of D_2O), 3.85 (2H, s, C—8 Hs), 5.0 (3H, octet, C—1 and C—6 Hs), 5.8 (1H, *dd*, C—2 H).

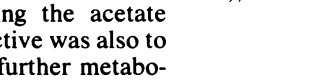
The reaction of racemic linalyl acetate with equimolar quantity of monoperphthalic acid in dry ether at 0°C yielded 6,7-epoxy linalyl acetate in quantitative yield (17). 6,7-Epoxy linalyl acetate had the following NMR ($CDCl_3$) δ: 1.23, 1.26 (6H, 2s, C—8 and C—9 Hs), 1.47 (3H, s, C—10 Hs),



2.0 (3H, s, —O—C—CH_3),

2.6 (1H, t, C—6 H), 5.0 (2H, octet, C—1 Hs), 5.8 (1H, *dd*, C—2 H).

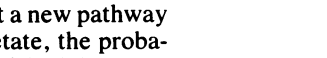
6,7-Dihydroxy linalyl acetate was prepared by treating 6,7-epoxy linalyl acetate with 1% aqueous H_2SO_4 at O°C for 4 h as reported earlier (17). 6,7-Dihydroxy linalyl acetate had the following NMR ($CDCl_3$) δ: 1.1, 1.15 (6H, 2s, C—8 and C—9 Hs), 1.5 (3H, s, C—10 Hs),



2.0 (3H, s, —O—C—CH_3),

2.6 (2H, broad s, disappears on addition of D_2O), 3.2 (1H, m, C—6 H), 5.0 (2H, octet, C—1 Hs), 5.8 (1H, *dd*, C—2 H).

Δ^5-4-Acetoxy-4-methyl hexenal was synthesized by oxidizing the diol (6,7-dihydroxy linalyl acetate) with sodium metaperiodate in pyridine (4). Δ^5-4-Acetoxy-4-methyl hexenal had the following NMR ($CDCl_3$) δ: 1.53 (3H, s, C—4 methyl Hs),



1.95 (3H, s, —O—C—CH_3),

2.2 (4H, m, C—2 and C—3 Hs), 5.0 (2H, octet, C—6 Hs), 5.8 (1H, *dd*, C—5 H), 9.45 (1H, ill-defined triplet, aldehyde proton).

Chromatography. Separation and purification of the metabolites were accomplished by preparative thin-layer chromatography (TLC) using hexane-ethyl acetate as the solvent system (80:20 [vol/vol], system I; 70:30 [vol/vol], system II). The purity of the metabolites was visualized either by exposing the TLC plates to iodine vapors or by spraying them with 1% vanillin in concentrated H_2SO_4. TLC of 2,4-dinitrophenylhydrazones (DNPHs) of oxo acids were performed with

hexane-ethyl acetate-acetic acid (39:21:1 [vol/vol], system III). Neutral DNPHs were analyzed by TLC with benzene as the solvent system (system IV). Ascending unidimensional paper chromatography of DNPHs of oxo acids was carried out with Whatman no. 1 sheets and *n*-butanol–ethanol–0.5 N sodium hydroxide (7:1:2 [vol/vol], system V) as the developing solvent.

High-performance liquid chromatography (HPLC) was carried out on a Water Associates ALC/GPC 244 series instrument with a fixed-wavelength (254 nm) UV analytical flow cell detector (model 440). The analysis was performed on a μ-Porasil normal phase column with chloroform-methanol (95:5) as the solvent system. Solvents were pumped isocratically at a flow rate of 0.8 ml/min, and the eluents were monitored with a UV detector.

Microbiological methods. (i) Growth of the organism. The *P. incognita* used in this study was propagated on nutrient agar slants (11). The organism was grown with linalyl acetate as the sole carbon source in a mineral salts medium as described earlier (12). The growth was monitored by measuring the absorbance at 660 nm (A_{660}). The organism was maintained regularly in liquid medium containing 0.3 to 0.4% linalyl acetate. Whenever starter culture was required to carry out incubations with different substrates, a sample (5 ml) from the maintenance culture was transferred to 100 ml of a sterilized liquid mineral salts medium containing 0.3% linalyl acetate and incubated on a rotary shaker (220 rpm) at 28 to 29°C for 24 h. In the present studies 24-h cultures were used as the inoculum.

(ii) Growth studies. Organisms grown with linalyl acetate for 24 h were harvested, washed well with sterile mineral salts medium, and suspended in the same medium to give a final A_{660} of 1.2. This was used as the inoculum for the growth study. Inoculum (2 ml) was added aseptically to a 500-ml Erlenmeyer flask containing 100 ml of sterile mineral salts medium and 0.2 ml of the substrate used for the growth study. Incubation was carried out on a rotary shaker (220 rpm) at 28 to 29°C. Growth was followed by measuring the A_{660} with a Bausch & Lomb colorimeter.

(iii) Manometry. Manometric experiments were performed with a Gilson differential respirometer (model G 14) at 30°C. The 24-h cells adapted to linalyl acetate were harvested, washed, and suspended in phosphate buffer (0.05 M, pH 7.2) to give a final A_{660} of 1.2.

(iv) Incubation conditions. Samples (100 ml) of sterile salt medium were taken in 500-ml Erlenmeyer flasks to which 0.3% linalyl acetate and 4% 24-h cells (A_{660}, 1.3) adapted to linalyl acetate were added. The flasks were incubated at 28 to 29°C on a rotary shaker (220 rpm) for 36 h.

Incubation conditions for the metabolism of linalyl acetate-8-aldehyde were essentially same as described above for the metabolism of linalyl acetate, except that 0.2 ml of the substrate (linalyl acetate-8-aldehyde) was used for every 100 ml of the mineral salt medium and incubated for 48 h at 28 to 29°C on a rotary shaker.

Extraction and isolation of metabolites. At the end of the incubation period, the contents from all the flasks were pooled, acidified to pH 3 to 4 with 2 N HCl, and then extracted with distilled ether. The ether extract was washed with water, concentrated, and separated into acidic and neutral fractions by treating with 5% sodium bicarbonate solution. The bicarbonate extract was acidified with 2 N HCl, and the liberated acidic metabolites were reextracted with ether and dried over anhydrous sodium sulfate. The acidic fraction was methylated with diazomethane.

Transformation of linalyl acetate-8-aldehyde by resting cells. Bacteria grown on linalyl acetate were harvested in the late exponential growth phase, and the cells were washed once with potassium phosphate buffer (0.025 M, pH 7.2). The cell paste was then suspended in the same buffer to give a final A_{660} of 1.5. Washed cell suspensions (200 ml) were incubated at 30°C with 400 mg of linalyl acetate-8-aldehyde for 4 h on a rotary shaker. Control experiments were run without the substrate. After the incubation period, the contents of the flask were acidified with 2 N HCl and extracted three times with distilled ether. The ether extract was separated into acidic and neutral components as described above.

Another set of experiments was performed as described above with 150 mg of linalyl acetate-8-aldehyde- and linalyl acetate-grown resting cells (100 ml; A_{660}, 1.5). At the end of the incubation period, the reaction was arrested by acidification with 2 N HCl, and the denatured protein was removed by centrifugation. The supernate was treated with 15 ml of DNPH reagent (0.25% solution of the reagent in 6 N HCl) and allowed to stand for 4 h at room temperature. The precipitated hydrazones were extracted three times with 15 ml of ethyl acetate and separated into neutral and acidic hydrazones by extraction with bicarbonate. The acidic derivatives were recovered from the bicarbonate extract after acidification and extraction with ethyl acetate. The identical procedure was followed while working up the control experiments.

Resting cell experiment with 6,7-epoxy linalyl acetate. In another set of experiments, 50 ml of cell suspension prepared as described above was incubated with 50 mg of 6,7-epoxy linalyl acetate at 28 to 29°C for 4 h. A cell blank and a substrate control were also run side by side. After the incubation period the cells were centrifuged and washed with saline. The supernatant and the saline wash were pooled, extracted with ether to take out all neutral metabolites, and then acidified with 2 N HCl. It was extracted with ether to isolate the acidic metabolites. This modification in the extraction procedure was adapted to avoid the nonenzymatic epoxide opening in the acidic medium. The ether extracts from all of the extractions were pooled and were separated into acidic and neutral components. The cell blank was extracted in the same manner. The substrate blank was extracted with ether without acidification.

Preparation of cell-free extract. The cells were grown for 18 to 20 h with 5% inoculum and 0.15% linalyl acetate and harvested by centrifugation (5,000 × *g*, 20 min) at 0 to 4°C. The cells were then washed twice with ice-cold Tris-hydrochloride buffer (50 mM, pH 7.4) and suspended in the same buffer (2 ml/g [wet weight] of cells). The cell suspension was sonicated in a Branson B-30 sonifier with cooling for four 30-s intervals at maximum output. The sonicate was centrifuged at 10,000 × *g* for 30 min. The supernatant was diluted with Tris buffer (50 mM, pH 7.4) to a final protein concentration of 10 to 11 mg/ml. Protein determinations were conducted by the method of Lowry et al. (10).

Conversion of linalyl acetate to linalyl acetate-8-carboxylic acid by cell-free preparations. The cell-free

extract (110 mg of protein) prepared in 50 mM Tris-hydrochloride buffer (10 ml, pH 7.4) was incubated in the presence of 25 μmol of linalyl acetate in 10 μl of acetone and 37 μmol of NADH in a total volume of 10.2 ml. The reaction mixture was incubated aerobically for 3 h at 30°C on a rotary shaker. The boiled control was run simultaneously. The above assay was repeated with NADPH as the cofactor. At the end of the incubation period, the reaction was terminated with 2 N HCl and extracted with distilled ether. The ether extract was then separated into acidic and neutral metabolites as described above. The acidic metabolites were methylated with diazomethane and analyzed by TLC (system I) and HPLC.

Alcohol and aldehyde dehydrogenases. The presence of 8-hydroxy linalyl acetate and linalyl acetate-8-aldehyde dehydrogenases was determined by incubating the cell-free extract (5 ml, 55 mg of protein) with 20 μmol of 8-hydroxy linalyl acetate or linalyl acetate-8-aldehyde in 10 μl of acetone and 25 μmol of NAD^+ in separate experiments. The reaction mixture was incubated at 30°C for 1 h on a rotary shaker. A boiled control was run for each experiment. The products formed during the reaction were analyzed by TLC and HPLC as described above.

RESULTS

Identification of the organism. Based on the various morphological, cultural (short rods, gram negative, polar flagellation), and biochemical characteristics, the organism has been identified as belonging to the genus *Pseudomonas* as per *Bergey's Manual of Determinative Bacteriology* (5); the organism possesses properties very similar to those of *P. incognita*. It was therefore identified as a strain of *P. incognita*. This organism does not produce any fluorescent pigments when cultured on nutrient agar; however, in medium B of King et al. (8); it excretes fluorescent yellow green pigments. Hence the organism may be classified under fluorescent pseudomonads.

Metabolites of linalyl acetate. Fifty flasks were inoculated with 4 ml each of a 24-h culture (A_{660}, 1.3), and 0.3% linalyl acetate was added. Three such batches (150 flasks) were run. The flasks were incubated for 36 h at 28 to 29°C on a rotary shaker (220 rpm). At the end of the incubation period, the contents were pooled and processed as described above.

From 150 flasks, 2.94 g of neutral metabolites (excluding unmetabolized linalyl acetate) and 750 mg of acidic metabolites were obtained.

Acidic metabolites. Examination of the methyl esters of the acidic fraction by TLC with solvent system I revealed the presence of at least three major acids (R_f, 0.52, 0.35 and 0.26) and five minor acids. Two major acids (R_f, 0.52 and 0.35) were separated and purified by repeated preparative TLC with solvent system I.

One of the major acid methyl esters (R_f, 0.52) had the following spectral characteristics. Infrared (IR) spectrum (liquid film) ν_{max}: 1,740, 1,435, 1,245, 990, 925 cm^{-1}. NMR ($CDCl_3$) δ: 1.53 (3H, s, C—4 methyl Hs),

1.95 (3H, s, —O—C—*CH*$_3$),

2.2 (4H, m, C—2 and C—3 *Hs*),

3.6 (3H, s, —C—O—*CH*$_3$),

5.0 (2H, octet, C—6 Hs), 5.8 (1H, *dd*, C—5 H). Based on the above data the compound was identified as the methyl ester of Δ^5-4-acetoxy-4-methyl hexenoic acid.

The IR spectrum of another major acid methyl ester (R_f, 0.35) showed bands for a tertiary hydroxyl group (ν_{max}: 3,500, 1,280 cm^{-1}), an α,β-unsaturated carboxyl group (1,700 cm^{-1}), and a terminal methylene (1,640, 1,430, 990, 910 cm^{-1}). NMR ($CDCl_3$) δ: 1.2 (3H, s, C—10 Hs), 1.4 to 1.65 (2H, m, C—4 Hs), 1.8 (3H, s, C—9 Hs), 2.0 to 2.4 (3H, m, C—5 Hs and one hydroxyl proton, disappears on adding D_2O),

3.66 (3H, s, —C—O—*CH*$_3$),

5.0 (2H, octet, C—1 Hs), 5.8 (1H, *dd*, C—2 H), 6.67 (1H, t, C—6 H). This metabolite was characterized as the methyl ester of linalool-8-carboxylic acid. This was further confirmed by comparing the IR and NMR spectra with an earlier report (12) on the same compound.

A major acid methyl ester corresponding to an R_f of 0.26 was purified by preparative TLC with solvent system II. IR spectrum (liquid film) ν_{max}: 3,400, 1,680, 1,640, 1,250 cm^{-1}. NMR ($CDCl_3$) δ: 1.2 (6H, s, C—9 and C—10 Hs), 1.8 to 2.4 (8H, m, C—3, C—4, C—5, C—6 Hs and one hydroxyl proton, disappears on adding D_2O),

3.66 (3H, s, —C—O—*CH*$_3$)

and 6.9 (1H, t, C—2 H). This compound was identified as the methyl ester of oleuropeic acid. The spectral characteristics fully agreed with the earlier report on this compound (21).

Other acidic metabolites could not be isolated and identified due to paucity of the material.

Neutral metabolites. The neutral fraction contained mainly linalool with some polar air oxidized products which were not isolated and identified further.

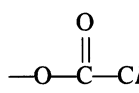
Metabolites of linalyl acetate-8-aldehyde. Fifteen flasks each containing 100 ml of sterile salt medium were inoculated with 4 ml of 24-h linalyl

acetate-adapted culture (A_{660}, 1.3), and 0.2 ml of substrate (linalyl acetate-8-aldehyde) was added. The flasks were incubated at 28 to 29°C for 48 h on a rotary shaker (220 rpm). At the end of the incubation period, the contents were pooled and analyzed as described above.

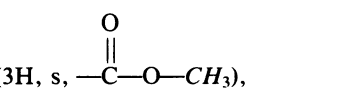
Acidic metabolites. The total amount of acidic metabolites was 550 mg. The methylated acidic fraction showed the presence of three major (R_f, 0.36, 0.57, and 0.25) and two minor (R_f, 0.52 and 0.87) acid methyl esters in solvent system I. The methyl esters were purified on a silica gel column with hexane-ethyl acetate as the eluent. The least polar acid methyl ester (R_f, 0.87) was eluted with hexane. However, this compound could not be identified due to paucity of the material. Acid methyl esters corresponding to R_f of 0.57 and 0.52 eluted with 5% ethyl acetate in hexane and were further purified by preparative TLC with 15% ethyl acetate in hexane as the developing solvent. Acid methyl esters corresponding to R_f of 0.36 and 0.25 were eluted with 15 and 30% ethyl acetate in hexane, respectively.

The IR and NMR spectra of acid methyl esters with R_f of 0.36 and 0.52 corresponded with the methyl esters of linalool-8-carboxylic acid and Δ^5-4-acetoxy-4-methyl hexenoic acid, respectively.

The major acid methyl ester corresponding to an R_f of 0.57 had the following spectral characteristics. IR spectrum (liquid film) ν_{max}: 1,710, 1,640, 1,430, 1,240, 1,000, 920 cm^{-1}. NMR ($CDCl_3$) δ: 1.53 (3H, s, C—10 Hs), 1.8 (3H, s, C—9 Hs), 2.0 (7H, a prominent singlet with a diffused multiplet at the base),

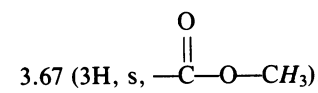


C—4 and C—5 Hs),



5.1 (2H, octet, C—1 Hs), 6.0 (1H, *dd*, C—2 H) and 6.8 (1H, t, C—6 H). From the spectral data the compound was identified as the methyl ester of linalyl acetate-8-carboxylic acid.

The IR spectrum of the polar major acid methyl ester (R_f, 0.25) showed intense absorption at a ν_{max} of 3,500 cm^{-1}, indicating the presence of a hydroxyl function and 1,700 cm^{-1} characteristic of an α,β-unsaturated carboxyl group. NMR ($CDCl_3$) δ: 1.6 (3H, s, C—10 Hs), 1.83 (3H, s, C—9 Hs), 2.0 (1H, s, disappears on addition of D_2O, one hydroxyl proton), 2.2 (4H, m, C—4 and C—5 Hs),



4.07 (2H, d, C—1 Hs), 5.4 (1H, t, C—2 H) and 6.7 (1H, t, C—6 H). Based on the above data the structure for this compound was assigned to be the methyl ester of geraniol-8-carboxylic acid.

Resting cell experiments carried out with linalyl acetate-8-aldehyde as described above provided 150 mg of the acidic fraction. Examination of the methyl esters by TLC in solvent system I indicated the presence of one major and one minor acid. The major acid methyl ester corresponded with the methyl ester of linalyl acetate-8-carboxylic acid, whereas the minor one corresponded with Δ^5-4-acetoxy-4-methyl-hexenoic acid methyl ester. This was further confirmed by spectral analysis.

With the resting cell experiment, carried out as described above with linalyl acetate-8-aldehyde with DNPH as a trapping reagent, it was possible to show the formation of one acidic and three neutral phenylhydrazones. The acidic DNPH that had the same mobility as DNPH of pyruvic acid in both TLC (system III; R_f, 0.24) and paper chromatography (system V; R_f, 0.35) was purified by preparative TLC (system III) and recrystallized from ethanol (decomposed near mp 207°C; previously reported to decompose at 208 to 210°C [7]). The IR spectrum (KBr pellet) of the purified acidic DNPH was superimposable with that of DNPH of authentic pyruvic acid. Analysis of the neutral DNPH fraction by TLC (system IV) showed the presence of three minor compounds with R_f of 0.75, 0.62, and 0.22. However, they could not be isolated in amounts sufficient for further identification.

Resting cell experiments carried out with linalyl acetate 6,7-epoxide did not yield Δ^5-4-acetoxy-4-methyl hexenoic acid or its analogs. The oxidation of the C-8 methyl of compound 14 was not observed. On the other hand, it was noticed that most of the substrate added (~90%) could be reisolated, indicating its resistance for further metabolism. However, small amounts of air-oxidized compounds were formed which could not be isolated and identified.

Optical rotations were measured for unmetabolized linalyl acetate, linalool, methyl esters of linalyl acetate-8-carboxylic acid, and Δ^5-4-acetoxy-4-methyl hexenoic acid. None of them was found to be optically active.

Growth and manometric studies. To obtain corroborative evidence for the probable pathways of degradation of linalyl acetate, growth and manometric studies were carried out with metabolites isolated from the culture media as well as synthetic probable intermediates. Linalyl

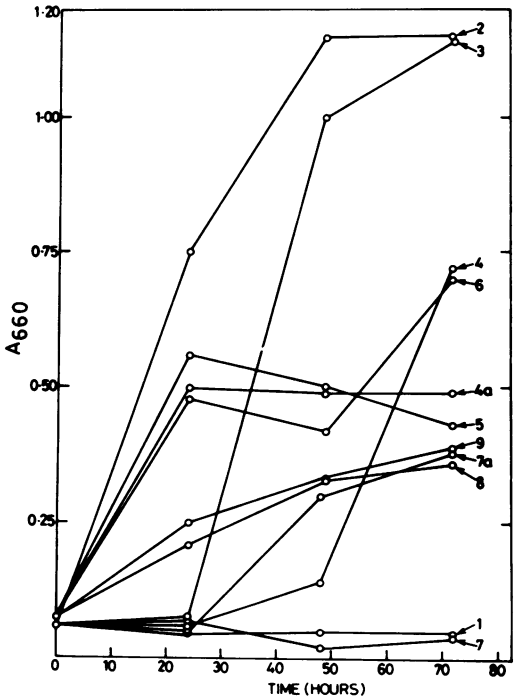


FIG. 1. Growth of *P. incognita* on the following compounds: 1, Control; 2, linalyl acetate; 3, linalool; 4, 8-hydroxy linalool (200 mg); 4a, 8-hydroxy linalool (50 mg); 5, 8-hydroxy linalyl acetate; 6, linalyl acetate-8-aldehyde; 7, α-terpineol (200 mg); 7a, α-terpineol (50 mg); 8, 6,7-epoxy linalyl acetate; 9, 6,7-dihydroxy linalyl acetate.

acetate-grown cells simultaneously adapted to 8-hydroxy linalyl acetate and linalyl acetate-8-aldehyde. Linalool, 8-hydroxy linalool, and α-terpineol required a lag period possibly due to the surface toxic effects exhibited by these alcohols in liquid culture experiments. However, the bacterium grew much better when these substrates were supplied at lower levels (Fig. 1). Such an effect has been reported earlier in *Pseudomonas citronellolis* when exposed to citronellol, geraniol, and other alcohols (6). Both linalyl acetate 6,7-epoxide and the diol (6,7-dihydroxy linalyl acetate) were very poorly accepted by the linalyl acetate-adapted cells (Fig. 1).

The ability of *P. incognita* whole cells to oxidize proposed linalyl acetate pathway intermediates and synthetic analogs was determined by measuring oxygen uptake rates with induced cells. These studies (Table 1) indicated that linalyl acetate-8-aldehyde, 8-hydroxy linalyl acetate, and Δ^5-4-acetoxy-4-methyl hexenal (metabolites of pathway B, Fig. 2) showed good oxygen uptake in contrast to linalyl acetate 6,7-epoxide and linalyl acetate 6,7-diol. However, the rates of oxygen uptake observed with the

TABLE 1. Oxygen uptake by *P. incognita* whole cells[a]

Substrates	QO_2 (μl/mg dry wt/h)
Linalyl acetate	110.0
Linalool	102.9
8-Hydroxy linalool	98.7
8-Hydroxy linalyl acetate	59.1
Linalyl acetate-8-aldehyde	72.8
Δ^5-4-acetoxy-4-methyl hexenal	42.0
6,7-Dihydroxy linalyl acetate	17.9
6,7-Epoxy linalyl acetate	15.8
α-Terpineol	29.4
Oleuropyl alcohol	40.0

[a] Each flask contained 1.5 ml of freshly washed 24-h linalyl acetate-grown cells (A_{660}, 1.4; 2.2 mg [dry wt]), 1.0 ml of phosphate buffer (0.05 M, pH 7.0), 10 to 15 μmol of the substrate (in the sidearm), and 0.2 ml of 5 N KOH (in the central well). The total volume was made to 3.0 ml with glass-distilled water. Temperature was 30°C. The endogenous oxygen uptake rate was substracted from the total observed rate to give the values listed.

metabolites of pathway A (Fig. 2) were certainly higher than the rates noticed for the metabolites of pathway B (Fig. 2), indicating the order of preference by which the bacteria carry out the biodegradation. These studies clearly indicated that the pathway involving the degradation of linalyl acetate with the acetoxy group intact (pathway B) seems to be more prominent than the one involved in the prototropic cyclization of linalool to α-terpineol and its further metabolism. However, further work must be done to determine the nature of induction and the rate of synthesis of the enzymes involved in the pathway of degradation. It is apparent that the linalyl acetate pathway enzymes are not constitutive, since little activity was observed with any of the substrates with glucose-grown cells (data not shown).

Incubation of the cell-free extract with linalyl acetate in the presence of NADH yielded metabolites which were separated into acidic and neutral compounds as described above. The TLC profile (system I) of the methyl esters revealed the presence of two compounds having R_f values identical with that of authentic methyl esters of linalyl acetate-8-carboxylic acid and linalool-8-carboxylic acid. The presence of these two compounds was further confirmed by subjecting the methyl esters of enzymatically formed acids to HPLC analysis (Fig. 3), where the peaks corresponding to these two methyl esters (retention times, 3.7 and 4.1 min) were enhanced when mixed with authentic methyl esters of linalyl acetate-8-carboxylic acid and linalool-8-carboxylic acid. When NADH was replaced with NADPH, the levels of both these

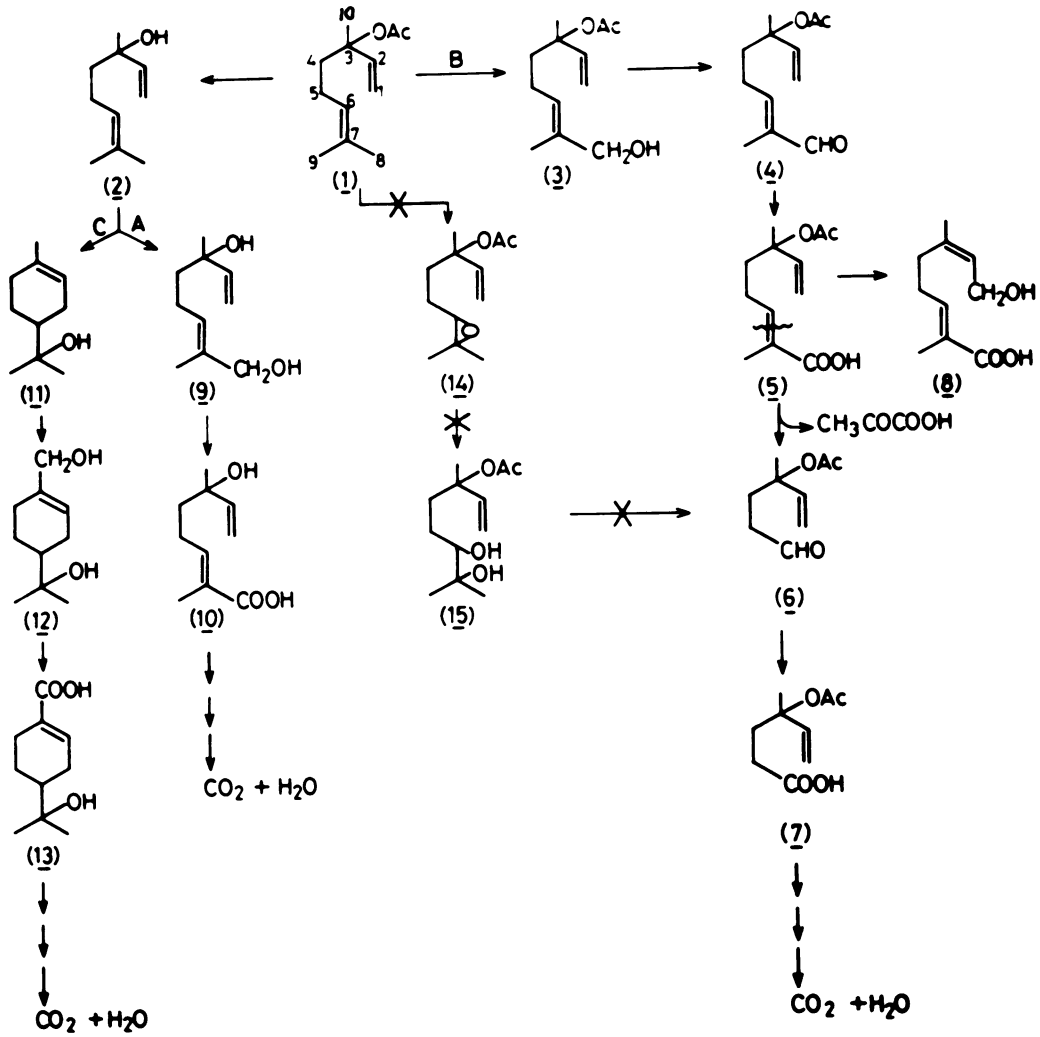


FIG. 2. Probable pathways of degradation of linalyl acetate by *P. incognita*. Compounds: 1, linalyl acetate; 2, linalool; 3, 8-hydroxy linalyl acetate; 4, linalyl acetate-8-aldehyde; 5, linalyl acetate-8-carboxylic acid; 6, Δ^5-4-acetoxy-4-methyl hexenal; 7, Δ^5-4-acetoxy-4-methyl hexenoic acid; 8, geraniol-8-carboxylic acid; 9, 8-hydroxy linalool; 10, linalool-8-carboxylic acid; 11, α-terpineol; 12, oleuropyl alcohol; 13, oleuropeic acid; 14, 6,7-epoxy linalyl acetate; 15, 6,7-dihydroxy linalyl acetate.

acids (linalyl acetate-8-carboxylic acid and linalool-8-carboxylic acid) formed were considerably lower. From the neutral fraction only unreacted linalyl acetate could be identified.

Likewise, the same two acids were shown to be formed when the crude cell-free extract was incubated either with 8-hydroxylinalyl acetate or linalyl acetate-8-aldehyde in the presence of NAD^+. The presence of linalyl acetate-8-carboxylic acid and linalool-8-carboxylic acid in the form of their corresponding methyl esters was indicated by their TLC profiles and HPLC analyses. $NADP^+$ was found to be far less efficient in carrying out this transformation.

However, when the cell-free extract was incubated with either 6,7-dihydroxylinalylacetate or 6,7-epoxylinalyl acetate in the presence or absence of NADH or NADPH, further transformations of any one of these compounds were not noticed, indicating their resistance to metabolism.

DISCUSSION

The results of the present investigation document distinctive features regarding the metabolism of linalyl acetate by *P. incognita*. The most striking part is the observation that the organism

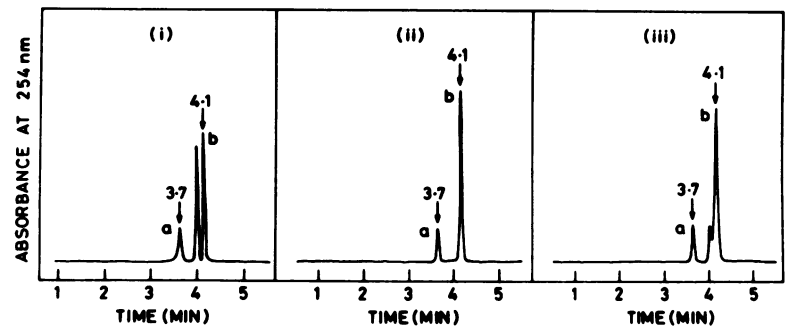


FIG. 3. HPLC analysis of the methyl esters of the acids. HPLC separation of the methyl esters of the acidic fraction obtained by incubating the cell-free extract with (i) linalyl acetate, (ii) 8-hydroxy linalyl acetate, and (iii) linalyl acetate-8-aldehyde. Details are as described in the text. Methyl esters of linalyl acetate-8-carboxylic acid (peak a) and linalool-8-carboxylic acid (peak b) had retention times (R_T) of 3.7 and 4.1 min, respectively. The control did not indicate the presence of peaks corresponding to R_T of 3.7 and 4.1 min.

has the unique ability to degrade linalyl acetate while keeping the acetate moiety intact. Cursory examination of various metabolites isolated from the culture medium indicated the existance of at least three different pathways for the biodegradation of linalyl acetate. Among them, two probable pathways (A and C, Fig. 2) involved hydrolysis of linalyl acetate to linalool and its further metabolism as previously reported (12). Hence pathways A and C (Fig. 2) will not be discussed here. The present investigation is mainly centered around the third pathway (B, Fig. 2), which appeared to be one of the two major pathways involved in the metabolism of linalyl acetate. In this mode of degradation, the acetoxy group of linalyl acetate remained intact during the metabolism as evidenced by the isolation of linalyl acetate-8-carboxylic acid and Δ^5-4-acetoxy-4-methyl hexenoic acid from the culture medium.

It appears that the first step in the degradation sequence (pathway B, Fig. 2) is the oxygenation of the C-8 methyl group resulting in the formation of 8-hydroxy linalyl acetate, which undergoes progressive oxidation to yield linalyl acetate-8-aldehyde and linalyl acetate-8-carboxylic acid. Growth and oxygen uptake studies (Fig. 1, Table 1) gave corroborative experimental evidence for the above sequence of reactions. Linalyl acetate-grown cells simultaneously adapted to oxidize 8-hydroxy linalyl acetate, linalyl acetate-8-aldehyde, and Δ^5-4-acetoxy-4-methyl hexenal. Preliminary experiments carried out with cell-free extract indicated that NAD-linked alcohol and aldehyde dehydrogenases catalyzed the conversion of 8-hydroxy linalyl acetate to its corresponding acid (linalyl acetate-8-carboxylic acid). NAD-linked dehydrogenase from *P. putida* (PL strain) have been shown to be involved in the metabolism of other terpenoids (2, 3).

Metabolism of linalyl acetate-8-aldehyde by linalyl acetate-grown cells led to the isolation of linalyl acetate-8-carboxylic acid, Δ^5-4-acetoxy-4-methyl hexenoic acid, and geraniol-8-carboxylic acid as their methyl esters. Geraniol-8-carboxylic acid was probably formed from linalyl acetate-8-carboxylic acid upon hydrolysis followed by isomerization. A reaction very similar to this isomerization of linalyl acetate to geraniol has been reported in certain microorganisms (16). The formation of Δ^5-4-acetoxy-4-methyl hexenoic acid from linalyl acetate-8-aldehyde clearly suggests that the cleavage of the 6,7 double bond in linalyl acetate molecule occurs only after the oxidation of the C-8 methyl group. One of the possible modes for the cleavage of the 6,7 double bond is through epoxidation of the double bond and then opening of the epoxide to the corresponding diol. The resulting diol may be cleaved like the periodate oxidation of diols to form Δ^5-4-acetoxy-4-methyl hexenal, which may be dehydrogenated to the corresponding acid (Δ^5-4-acetoxy-4-methyl hexenoic acid). However, one cannot rule out the possibility of the formation of Δ^5-4-acetoxy-4-methyl hexenoic acid through β-oxidation of linalyl acetate-8-carboxylic acid in a manner similar to the pathway for the oxidation of fatty acids.

The observation that 6,7-epoxy linalyl acetate and 6,7-diol of linalyl acetate were neither growth substrates for *P. incognita* nor oxidized by induced cells indicated that these compounds are not involved in the pathway of degradation of linalyl acetate and excluded the possibility of the formation of Δ^5-4-acetoxy-4-methyl hexenoic acid from 6,7-epoxy linalyl acetate or its corresponding diol (6,7-dihydroxy linalyl acetate). The above observation was further supported by the fact that resting cell experiments carried out with induced cells indicated that 6,7-epoxy linalyl acetate was not further metabolized. Studies carried out with cell-free extract also substantiated the above findings. It is quite possible that the low oxygen uptake (Table 1)

and the slight growth (Fig. 1) noted for these compounds could be due to the presence of linalyl acetate as a trace impurity in these compounds as they were synthesized from it.

This proposed catabolic sequence was further supported by the preliminary experiments carried out with the cell-free extract to detect some of the enzymes involved in the pathway B (Fig. 2). The cell-free extract was shown to convert linalyl acetate to linalyl acetate-8-carboxylic acid and linalool-8-carboxylic acid in the presence of NADH. However, 8-hydroxy linalyl acetate and 8-hydroxylinalool, the initial products in the degradation sequence, were not detected. Even in the whole cell experiments, it was not possible to demonstrate the accumulation of these intermediates. This can be ascribed to the fact that perhaps both 8-hydroxylinalyl acetate and linalyl acetate-8-aldehyde dehydrogenases present in the cell-free extract are more active than the 8-hydroxylase system. In fact the cell-free extract was shown to contain highly active NAD-linked alcohol and aldehyde dehydrogenases responsible for the formation of linalyl acetate-8-aldehyde and linalyl acetate-8-carboxylic acid, respectively. The presence of linalyl acetate-8-hydroxylase activity in the cell-free extract can be demonstrated after the dehydrogenases are selectively removed. The above study is beyond the scope of this investigation since the present work with cell-free system is mainly intended to detect some of the enzymes involved in the metabolism of linalyl acetate.

It appears that the bacterial 8-hydroxylase prefers NADH to NADPH as the cofactor. In this respect the ω-hydroxylase system of *P. incognita* differs from the mammalian and higher plant monoterpene ω-hydroxylase systems (9, 13). Both, higher plant and mammalian monoterpene ω-hydroxylase systems have been shown to be mediated by cytochrome P-450-dependent monooxygenases (9, 13). Preliminary studies carried out on the linalyl acetate-8-hydroxylase system revealed that this enzymatic reaction seems to be mediated by a cytochrome P-450 system (unpublished observation). However, further investigations are needed to characterize this hydroxylase and to ascertain whether it is similar to the higher plant or mammalian ω-hydroxylase system.

The present studies clearly pointed out that part of the linalyl acetate was hydrolyzed to linalool during metabolism. Since linalyl acetate has an asymmetric carbon, we were interested in finding out whether the organism has the ability to carry out its asymmetric hydrolysis to optically active linalool. In fact it has been reported earlier (16) that racemic linalyl acetate is hydrolyzed to (+)-(R) linalool by *Bacillus subtilis*. However, *P. incognita* failed to bring about the enantioselective hydrolysis of racemic linalyl acetate. None of the metabolites isolated from the culture medium showed any optical activity.

Results from the growth, oxygen uptake, and metabolic studies carried out with linalyl acetate and linalyl acetate-8-aldehyde are quite compatible with the sequence of formation of different metabolites in the pathway B (Fig. 2) for the assimilation of linalyl acetate. In vitro conversion of linalyl acetate to linalyl acetate-8-carboxylic acid and linalool-8-carboxylic acid in the presence of NADH and further demonstrating the presence of NAD-linked alcohol and aldehyde dehydrogenases responsible for the formation of linalyl acetate-8-aldehyde and linalyl acetate-8-carboxylic acid, respectively, support the catabolic sequence proposed for the degradation of linalyl acetate. The evidence for the proposed cleavage of linalyl acetate-8-carboxylic acid between carbon atom 6 and 7 is obtained from the fact that linalyl acetate-8-aldehyde incubated with the resting cells yielded pyruvic acid and Δ^5-4-acetoxy-4-methyl hexenoic acid. The study points out that *P. incognita* can metabolize linalyl acetate while maintaining the acetoxy group intact, indicating great metabolic diversity in its ability to degrade organic molecules.

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