APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Sept. 1993, p. 2945–2950
0099-2240/93/092945-06$02.00/0

Vol. 59, No. 9

Metabolism of Linoleic Acid or Mevalonate and 6-Pentyl-α-Pyrone Biosynthesis by *Trichoderma* Species

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Received 1 February 1993/Accepted 2 July 1993

The understanding of the biosynthetic pathway of 6-pentyl-α-pyrone in *Trichoderma* species was achieved by using labelled linoleic acid or mevalonate as a tracer. Incubation of growing cultures of *Trichoderma harzianum* and *T. viride* with [U-^{14}C]linoleic acid or [5-^{14}C]sodium mevalonate revealed that both fungal strains were able to incorporate these labelled compounds (50 and 15%, respectively). Most intracellular radioactivity was found in the neutral lipid fraction. At the initial time of incubation, the radioactivity from [^{14}C]linoleic acid was incorporated into 6-pentyl-α-pyrone more rapidly than that from [^{14}C]mevalonate. No radioactivity incorporation was detected in 6-pentyl-α-pyrone when fungal cultures were incubated with [1-^{14}C]linoleic acid. These results suggested that β-oxidation of linoleic acid was a probable main step in the biosynthetic pathway of 6-pentyl-α-pyrone in *Trichoderma* species.

Lactones are generally very pleasant, potent, flavor materials which are widely distributed in nature (9). Relatively old publications indicate that some microorganisms biosynthesize lactones (18, 20). Although many microbial processes able to produce interesting flavors have been described (7), the number of industrial applications is limited. The reason for this is the low yield in most cases. The microbial flavors are often present only in low concentrations in the fermentation broths, resulting in high costs for downstream processing. This is compensated by the fact that the market price of natural aromas is 10 to 100 times higher than that of synthetic aromas. 6-Pentyl-α-pyrone (6PP), an unsaturated δ-lactone with a strong odor of coconut, was first detected in *Trichoderma viride* cultures (4). This compound is in great demand in the food industry principally as a flavor enhancer in soft drinks or yogurts.

Various studies have been carried out to improve the microbial production of this lactone (16, 25), but to our knowledge no metabolic studies have been reported. Most of the research has been focused on the metabolic pathways of saturated lactones. These molecules are generally formed by successive β-oxidations of saturated and unsaturated hydroxy acids or their lipid precursors up to the formation of a hydroxylated carbon at the C-4 or C-5 position. Thus, γ- and δ-lactones may be formed by esterification (lactonization) of the C-4 (γ) or C-5 (δ) hydroxyl group with the terminal carboxylic group from the same molecule (12, 13). Lactones may also be produced from various keto acids after reduction to their corresponding hydroxy acids (22). An alternative biosynthetic pathway involves glutamic acid metabolism (23).

With a view to reaching a better knowledge of the physiological mechanisms involved in 6PP biosynthesis by *Trichoderma* spp., we have recently studied lipid accumulation by two *Trichoderma* strains able to biosynthesize 6PP (17). This work was done to evaluate the lipid origin of 6PP in *Trichoderma* species by studying the metabolism of exogenous linoleic acid or sodium mevalonate.

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MATERIALS AND METHODS

Materials. [U-^{14}C]linoleic acid (1 Ci/mmol) was purchased from NEN Research Products (Les Ulis, France), [1-^{14}C]linoleic acid (54.6 mCi/mmol) was from Amersham (Les Ulis, France), and [5-^{14}C]sodium mevalonate (50.49 mCi/mmol) was from Dositek (Orsay, France). Unlabelled linoleic acid was obtained from Aldrich (Strasbourg, France).

Fungal strains and inoculum preparation. *T. viride* 26, isolated from soil in (our laboratory), and *T. harzianum* (IMI 206040) were used for the investigation. The strains were kept on potato dextrose agar medium. Preculture medium consisted of malt extract (20 g/liter) and glucose (10 g/liter). It was inoculated with 5-day-old mycelium (1-cm^2 disk from conservation medium) and incubated in a 150-ml Erlenmeyer flask at 27°C for 72 h on a rotary shaker at 100 rpm. The mycelium was recovered after 20 min of centrifugation at 8,000 × *g*. The mycelium suspension (10 g/liter) was homogenized in a VirTis grinder (The VirTis Co., Inc., Gardiner, N.Y.) to obtain mycelial fragments 50 to 200 μm in length.

Culture conditions. A medium for lipid accumulation (C/N = 60) was used in this study (17). The basal medium composition was as follows (in grams per liter): glucose, 30; $(NH_4)_2SO_4$, 0.94; KH_2PO_4, 7; Na_2HPO_4, 2; $MgSO_4 \cdot 7H_2O$, 1.5; $CaCl_2 \cdot 6H_2O$, 0.008; $ZnSO_4 \cdot 7H_2O$, 0.0001. Cultures were carried out in 10 ml of medium in 50-ml Erlenmeyer flasks. The media were inoculated with 0.2 ml of the previously described mycelial suspension and incubated on a rotary shaker (110 rpm) at 27°C. After 48 h of incubation, the mycelium biomass was aseptically harvested, washed, and resuspended in fresh basal medium containing unlabelled linoleic acid (10 g/liter) but no glucose. [U-^{14}C]linoleic acid (0.45 μCi) or [5-^{14}C]sodium mevalonate (2.27 μCi) was previously homogenized with the unlabelled linoleic acid in a small volume of ethanol before addition to the basal medium. Mycelial growth was monitored by dry weight determination. After incubation, the mycelium was harvested, washed, and dried at 105°C to constant weight. Experiments were run in triplicate.

Lipid class separation. The mycelium was broken up by ultrasonic treatment, and total lipids were recovered as described previously (17). Bond Elut NH_2 aminopropyl columns (Prolabo, Paris, France) were used with two 2-ml

portions of chloroform under vacuum (10 kPa) for lipid fractionation. Five to 10 mg of total lipids was applied to the column under vacuum and eluted by consecutive passage of 4 ml of each of the following mixtures: chloroform-propanol (2:1), acetic acid (2%, vol/vol) in diethyl ether, and methanol. The fractions obtained in order of elution were neutral lipids (NL), free fatty acids (FFA), and phospholipids (PL) (10). An aliquot of each fraction was put into scintillation vials, and fractions were counted in 4 ml of counting solution in a Packard Tri-Carb counter (model 4000; United Technologies Packard, Downers Grove, Ill.).

Aroma extraction and analysis. After incubation, the aroma compounds were extracted from the remaining culture medium by distillation-extraction in a Lickens-Nickerson apparatus modified by Godefroot et al. (6). Samples (10 ml) were distilled simultaneously with methylene chloride (20 ml) at 115 and 70°C, respectively, for 1 h. The organic phase was 10-fold concentrated under nitrogen flux and quantified by gas chromatography. Internal standard, γ-undecanoic acid lactone (0.2 mg in methylene chloride), was added to samples before extraction. Radioactivity was measured as described above with 100 μl of concentrate.

Chromatographic analyses were carried out on a Spirawax column (internal diameter, 0.32 mm, and film thickness, 0.25 μm; Spiral, Dijon, France) using a Packard chromatograph, model 627A (Chrompack, Middelburg, The Netherlands), equipped with a flame ionization detector. The oven rise temperature was set from 40 to 230°C, with an initial rate of 10°C/min for 14 min followed by a rate of 3°C/min. Injector and detector temperatures were 200 and 300°C, respectively. Nitrogen was used as a gas vector. Mass spectrometry (Ribermag-R-10-10C; Nermag, Rueil-Malmaison, France) was used in tandem with gas chromatography. The substances were determined by electron ionization (70 eV).

Detection and purification of ^{14}C-labelled 6PP after distillation. An aliquot of each aroma concentrate obtained as described above was subjected to thin-layer chromatography on silica gel plates to purify the 6PP molecule. The chromatogram was developed with a solvent system composed of hexane-diethyl ether-acetic acid (80:20:1, vol/vol/vol). The 6PP was located on the plate by comparison with the retention front of synthetic 6PP. Radioactive 6PP was detected by autoradiography. The spot areas were scraped off and extracted with methanol (100 μl) before radioactivity was counted as described above.

RESULTS

Incorporation of [U-^{14}C]linoleic acid in fungal cells. After 48 h of incubation on lipid accumulation medium with glucose, fungi were transferred to a basal medium containing linoleic acid. Incorporation of [U-^{14}C]linoleic acid by *T. harzianum* and *T. viride* was monitored for 48 h, when more than 50% of the labelled compound was detected in the cells. Labelled fatty acid was principally incorporated into NL in both strains (Fig. 1) and was weakly incorporated into PL. For *T. harzianum* cultures, maximal incorporation of labelled fatty acid was observed at 24 h. At this time, the radioactivity in NL, FFA, PL, and the aqueous phase represented 61, 24, 13, and 2%, respectively, of the total radioactivity associated with the cells.

Under the same conditions, the maximal incorporation of [U-^{14}C]linoleic acid in *T. viride* cells was observed at 48 h (Fig. 1). The intracellular distributions of labelled linoleic acid in NL, FFA, PL, and the aqueous phase were 46, 37, 13, and 4%, respectively.

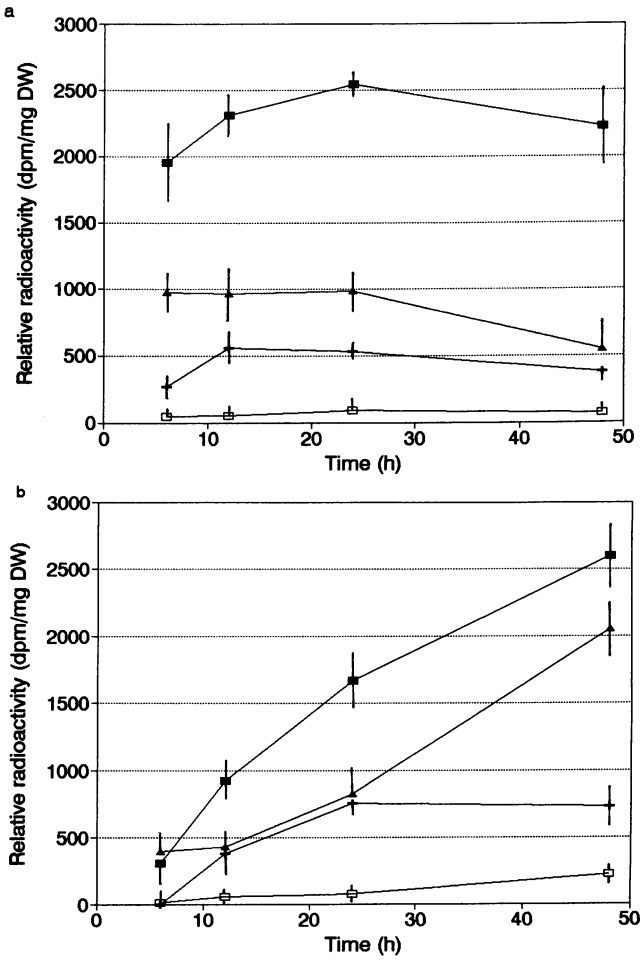

FIG. 1. Distribution of radioactivity after incorporation of [U-^{14}C]linoleic acid into lipid classes of *T. harzianum* (a) and *T. viride* (b). ■, NL; ▲, FFA; +, PL; □, aqueous phase. [U-^{14}C]linoleic acid (0.45 μCi) was added to fungal cultures after 48 h of incubation in lipid accumulation medium. Results represents the average of three replicates ($P < 0.05$).

Incorporation of [5-^{14}C]sodium mevalonate in fungal cells. Only 15% of the total radioactivity from [5-^{14}C]sodium mevalonate added to cultures was recovered in fungal cells after 72 h of incubation (Fig. 2). In *T. harzianum* cultures, the assimilation of radioactivity from labelled sodium mevalonate into NL varied from 49% (6 h) to 89% (72 h) of total intracellular sodium mevalonate. The [5-^{14}C]sodium mevalonate content in the aqueous phase varied from 49 to 16%. Weak amounts of radioactivity were found in FFA and PL (<5% for both fractions). The kinetics of [5-^{14}C]sodium mevalonate incorporation in *T. viride* cells also showed a maximum at 72 h (Fig. 2). The radioactivity detected in NL increased from 28% (6 h) to 80% (72 h) of the total, while in the aqueous phase the radioactivity decreased from 69 to 14%. The radioactivity contained in FFA and PL fractions represented <6% of the total radioactivity incorporated into the cell.

Incorporation of labelled compounds in 6PP. A mass spectrum of 6PP obtained from a fungal culture, after its purification by thin-layer chromatography, is shown in Fig. 3. Incorporation ratio kinetics of radioactivity from [U-^{14}C]li-

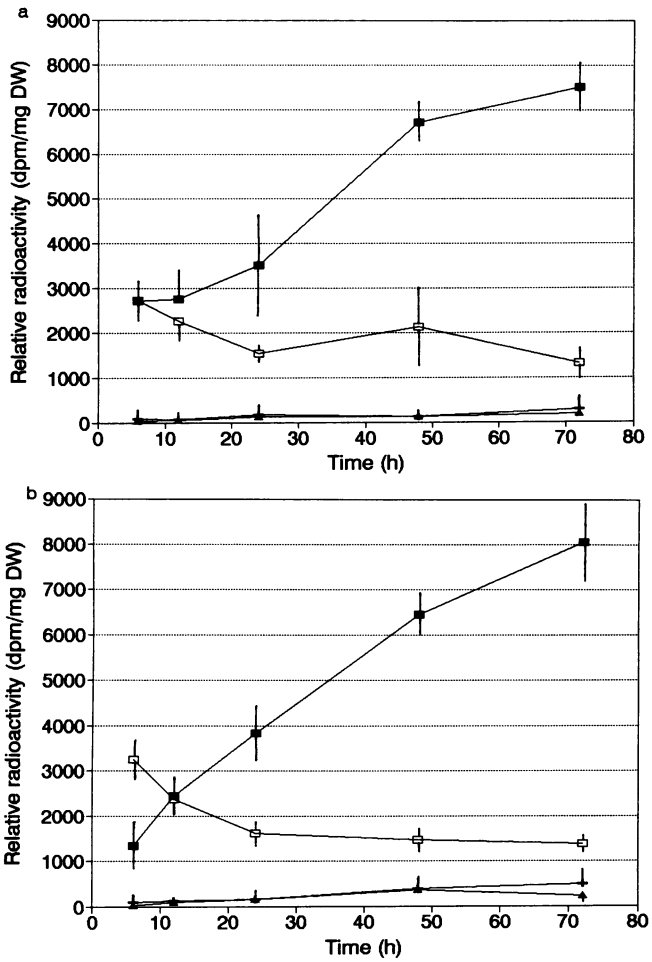

FIG. 2. Distribution of [5-^{14}C]sodium mevalonate incorporated into lipid classes of *T. harzianum* (a) and *T. viride* (b). ■, NL; ▲, FFA; +, PL; □, aqueous phase. [5-^{14}C]sodium mevalonate (2.27 μCi) was added to fungal cultures after 48 h of incubation in lipid accumulation medium. Results represent the average of three replicates ($P < 0.05$).

TABLE 1. Incorporation of radioactivity from [U-^{14}C]linoleic acid or [5-^{14}C]sodium mevalonate into 6PP by *T. harzianum* or *T. viride*[a]

Incubation time (h)	Radioactivity of 6PP (% of total)			
	T. harzianum		*T. viride*	
	[U-^{14}C]linoleic acid	[5-^{14}C]sodium mevalonate	[U-^{14}C]linoleic acid	[5-^{14}C]sodium mevalonate
6	0.03	0.012	0.02	0.011
12	0.12	0.017	0.03	0.014
24	0.17	0.021	0.06	0.018
48	0.09	0.072	0.11	0.019

[a] [U-^{14}C]linoleic acid (0.45 μCi) or [5-^{14}C]sodium mevalonate (2.27 μCi) was added to fungal cultures aged for 48 h in lipid accumulation medium. Results represent the average of three replicates ($P < 0.05$).

noleic acid and [5-^{14}C]sodium mevalonate in 6PP are represented in Table 1. Labelled linoleic acid produced better incorporation ratios in both strains than labelled sodium mevalonate, particularly in the case of *T. viride*. However, the highest radioactivity values were obtained in *T. harzianum* cultures. No labelled 6PP was found in additional experiments when cultures were incubated with [1-^{14}C]linoleic acid (0.45 μCi).

DISCUSSION

In a previous study (17), we demonstrated that *Trichoderma* strains cultivated on basal medium with glucose (C/N = 60) were able to accumulate almost 20% of their biomass in lipid form. However, 6PP production remained low (3 mg/liter of culture medium for *T. harzianum*) or undetected (*T. viride*). 6PP production increased after 48 h of incubation on a glucose carbon source, when the fungal biomass was harvested, washed, and transferred to a similar basal medium (C/N = 60) having linoleic acid as the sole carbon source. In that case, the fungal biomass acts as it does in a bioconversion medium: little growth occurs, but 6PP production is promoted. For *T. harzianum*, 6PP production reached 50 mg/liter of culture medium after an additional 24 h of incubation.

On the basis of these observations, we then investigated the possible implication of fatty acid metabolism in 6PP production by *Trichoderma* species. The incorporation of labelled linoleic acid in fungal cultures was studied since this

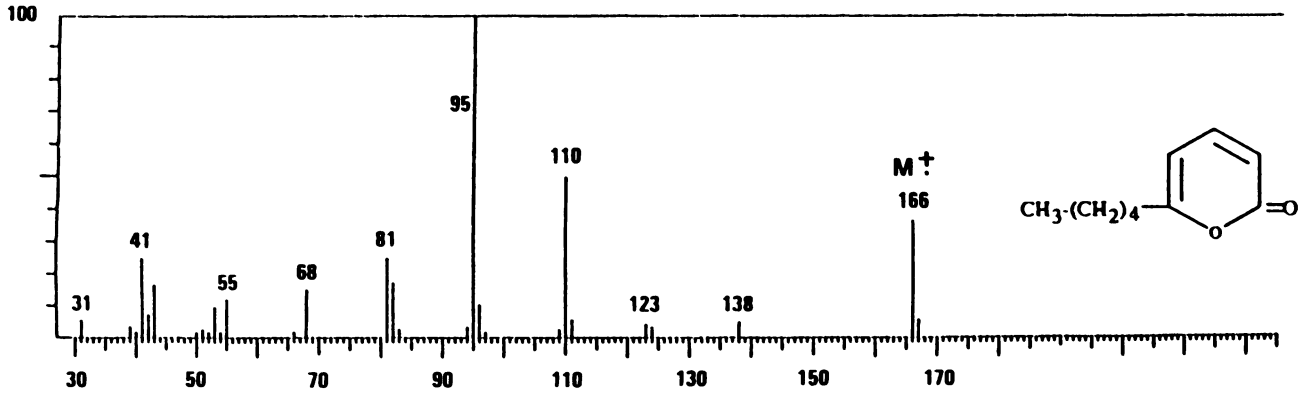

FIG. 3. Mass spectrum of 6PP from fungal culture. Sample was obtained after extraction of aroma compounds and purification by thin-layer chromatography.

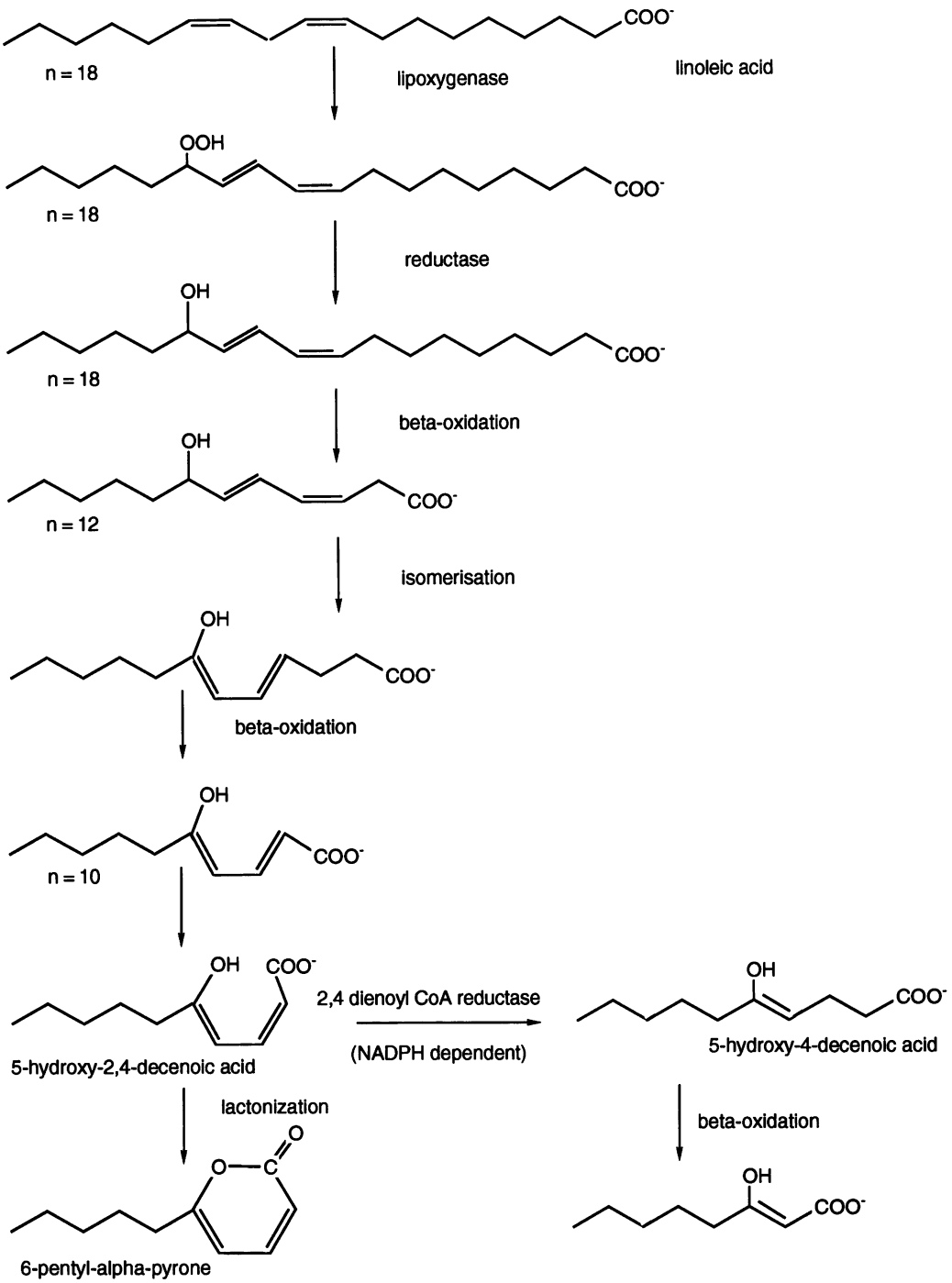

FIG. 4. Hypothetical pathway of 6PP generation from linoleic acid.

fatty acid is a natural component of *Trichoderma* lipids and is supposed to be a precursor of different flavor compounds (2, 24). In addition, labelled sodium mevalonate was added to fungal cultures to evaluate the possible implication of the isoprenic pathway in 6PP biosynthesis. Mevalonic acid is known to be the initial compound in sterol and volatile terpene biosyntheses from acetyl coenzyme A (3).

The faster incorporation of labeled linoleic acid in *T. harzianum* than in *T. viride* may be explained by the intracellular lipid compositions of the species. Indeed, the $C_{18:2}$ fatty acid content, after incubation on lipid accumulation medium, is higher in *T. harzianum* than in *T. viride*, even with glucose as the sole carbon source. Most of the labelled fatty acid was incorporated by *T. harzianum* during the first hours of incubation, whereas in *T. viride* cells a progressive intracellular incorporation was observed. Little radioactivity was measured in the aqueous phase during incubation. This suggests that either the exogenous fatty acid was not β-ox-

idized or the acetate obtained by β-oxidation was quickly metabolized to another lipid form.

Analysis of aroma compounds demonstrated that [U-^{14}C]linoleic acid was quickly incorporated into the 6PP molecule. However, the isotopic incorporation of radioactivity from labelled linoleic acid (<0.2%) into 6PP was weak in comparison to γ-decalactone production from ricinoleic acid (30%, wt/wt) by *Tyromyces sambuceus* (9) or δ-dodecalactone production from linoleic acid derivatives (20%, wt/wt) by *Cladosporium suaveolens* (2). We supposed that there could exist a limiting step in the 6PP biosynthesis by *Trichoderma* spp. from linoleic acid. Indeed, studies on δ-decalactone (saturated form of 6PP molecule) production by *Sporobolomyces odorus* showed that the best yields were obtained when cells were incubated with 13-hydroxy-9,11-octadecadienoic acid (1). This hydroxy acid was obtained by soya lipoxygenase action on linoleic acid. Several lipoxygenase activities in fungi have been detected (8). Lipoxygenases of *Fusarium oxysporum* have been well studied, and the hydroperoxide derivatives have been identified (12); a ratio of 70:30 was found at pH 9 between 9- and 13-hydroperoxide with purified enzyme and linoleic acid as the substrate. As in jasmonic acid biosynthesis elucidated by Vick and Zimmerman (21), 6PP biosynthesis in *Trichoderma* spp. could have a first step that depends on the formation of 13-hydroperoxide from linoleic acid. The lipoxygenase reaction is followed by β-oxidation and isomerization to form 5-hydroxy-2,4-decenoic acid (Fig. 4). The lactonization occurs if a further β-oxidation cycle is not promoted by a saturase activity such as 2,4-dienoyl-coenzyme A reductase (NADPH dependent) (5). Thus, the low rate of 6PP formation from linoleic acid by these fungal strains could be explained by a limited formation of 13-hydroperoxide derivatives.

The incorporation of [5-^{14}C]sodium mevalonate by both strains seems to be accomplished in two steps. Labelled compound is first introduced into the cells and then incorporated into NL. Contrary to our expectations, radioactivity was also found in FFA and PL fractions when fungal cells were incubated with labelled sodium mevalonate. The incubation of both fungal strains with [5-^{14}C]sodium mevalonate resulted in a lower production of labelled 6PP than in experiments with labelled linoleic acid. This lower isotopic incorporation should not be due to poor intracellular availability of mevalonate since, as shown in Fig. 2, this compound is constantly present in fungal cells.

One explanation for mevalonate utilization in 6PP synthesis by fungal cells might be the possible existence of a mevalonate shunt pathway. Degradation of mevalonate to acetate with subsequent polymerization of acetate to fatty acids has been demonstrated in plants (19) and animals (15). Although it has never been reported in fungi, the high intracellular mevalonate concentration in our fungal cells should favor this degradation to acetate. A comparison of radioactivity incorporation into the 6PP molecule during the first 24 h shows that the rate from [U-^{14}C]linoleic acid was 18-fold more important than that from [5-^{14}C]sodium mevalonate. The incorporation of radioactivity decreases after 24 h in *T. harzianum*; this fact can be explained by the utilization of linoleic acid as a carbon source for growth.

In conclusion, the production of labelled 6PP biosynthesized from labelled linoleic acid confirms the involvement of lipid metabolism in the production of this lactone. Lipoxygenase activity could be the limiting step in 6PP formation from linoleic acid. It could be the origin of weak radioactivity incorporation yields in 6PP obtained from [U-^{14}C]linoleic acid. The importance of the linoleic acid end chain was demonstrated when strains were incubated with [1-^{14}C]linoleic acid and no labelled 6PP was found. Thus, the involvement of the β-oxidation pathway in 6PP production by *Trichoderma* spp. was confirmed.

ACKNOWLEDGMENTS

L. Serrano-Carreon is grateful to CONACYT (Consejo Nacional de Ciencia y Tecnologia) of Mexico and CEFI (Centre d'Etudes et de Formation d'Ingénieurs) of France for financial support.

We thank Marie Claude Monnot for skillful technical assistance.

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