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# Biodegradation of Cinnamates by White–Rot Fungus, *Phlebia Radiata*

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This study was attempted to elucidate the biochemical mechanism of cinnamate compounds labeled specifically $^{14}C$ in the different position. The pathways for cinnamate metabolism have been studied in detail for the white–rot fungus *Phlebia radiate* and the reaction sequence has been proposed. $^{14}CO_2$ release from carboxyl labeled cinnamate reached the maximum after 24 hrs on the medium with wheat straw and after 48 hrs on the media with glucose and spruce wood. In the case of the medium with cellulose the release of carbon dioxide increases linearility up to the 8$^{th}$ day of the cultivation. The evolution of carbon dioxide from aliphatic chain cleavage cinnamate showed very similar trend which demonstrated simultaneous process with the decarboxylation ones on all tested media. About 30% of carbon dioxide evolved from carboxyl–labeled cinnamate. The radioactivity in the mycelium was also above 10% in all cases and can be read as partially degraded or metabolized to the other components. The cellulose slightly repressed the decarboxylation of cinnamate in opposition to wheat straw and spruce wood, which doubled degradation. The maximum rate of decarboxylation was *ca.* 1.2% of the applied activity evolved as $^{14}CO_2$ per hour. According to identified metabolites, at first the substrates after decarboxylation, and aliphatic chain cleavage were demethylated in the position 4 following the demethylation in the position 3 and finally aromatic ring cleavage were observed in air and oxygen aeration on the media with glucose, cellulose, wheat straw and spruce wood.

## INTRODUCTION

The final step in the formation of lignin in the cell walls of vascular plants is a dehydrogenative polymerization of the monomeric precursors, *p*–coumaryl–, coniferyl– and sinapyl–alcohols (Freudenberg, 1965). The reaction is initiated by an oxidative enzyme (peroxidase or laccase), but the polymerization itself is currently viewed as being a process without enzymatic control over the distribution of structural units (Monties, 1989). The composition of the monomeric precursors of lignins depends on guaiacyl, guaiacyl–syringyl or guaiacyl–syringyl–*p*–hydroxyphenyl lignins depending on their origin: gymnosperms, angiosperms except grasses (Lewis and Yamamoto, 1990). Variations in the monomer composition of lignins also depend on the growth conditions, stresses and genetic variations (Monties, 1989). The sources of lignocellulose that occur in various forms in nature are so vast that they can only be compared to those of water (Bellamy, 1974). Lignocellulose is a compact, in part crystalline, complex. Polysaccharide components form microfibers and are densely packed in layers of lignin which protects them against the activity of hydrolytic enzymes and other external factors and which also serves as a stabilizer of the complex structure (Fengel, 1971). This structure endows plants with the necessary stiffness and performs in plants the function similar to a block of concrete with metal rods inside; its durability comes from the compact structure. There exists an analogy between metal rods inside prefabricated elements and cellulose fibres in lignin (Leonowicz *et al.*, 1997).

The biodegradation of the lignocellulose constituents, cellulose, hemicelluloses and lignin, is achieved by enzymatic activities. The conversion of cellulose and hemicelluloses into simple sugars has been studied for a long time. A large number of micro–organisms (bacteria, fungi and protozoas) make use of a whole string of hydrolases which are able to produce large quantities of mono– and di–saccharides from all polysaccharide components in lignocellulose. The degradation, however, is effected by the occurrence of polysaccharides in a complex with lignin because the latter forms a barrier against the microbial attack by hydrolytic enzymes. The lignin barrier also complicates cellulose production in the pulp and paper industry. For these ecological, economic and other reasons, research into the biotransformation of lignin has been carried out for decades (Leonowicz *et al.*, 1987; Eriksson *et al.*, 1990).

Wood–rotting basidiomycetes penetrate wood tissues in order to come into contact with the easily assimilable carbohydrate constituents of the lignocellulosic complex. The white–rot group of these fungi, which has a versatile machinery of enzymes co–operating with certain secondary metabolites of fungi, is capable of attacking the lignin barrier efficiently. These fungi use a multi–enzyme system including the so–called "feed–back" type enzymes to transform and degrade all structural elements of the lignocellulosic complex (polysaccharides

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and lignin). The currently known enzymes of white–rot fungi involved in wood degradation can be divided into three groups. The first can attack the wood constituents or their primary degradation products directly; this group includes the cellulase and hemicellulase complexes, laccase, different peroxidases, protocatechuate–3,4–dioxygenase, *etc*. The second group of enzymes, comprising among others arylalcohol oxidase and glyoxal oxidase, co–operates with the first group by providing $H_2O_2$ for the peroxidases, but these enzymes do not attack wood components directly. The third enzyme group represented by glucose oxidase and cellobiose:quinone oxidoreductase (cellobiose dehydrogenase) includes feed–back type enzymes which play a key role in joining the metabolic chains during the biotransformation of high–molecular mass wood constituents. All these enzymes, including laccase, can act separately or in co–operation (Leonowicz *et al*., 1999).

The lignin–degrading Basidiomycete *Phanerochaete chrysosporium* synthesizes *de`novo* from glucose *via* phenylalanine: veratryl alcohol, ferulic acid, 3,4–dimethoxycinnamic alcohol, and veratryl glycerol (Shimada *et al*., 1981; Shimada *et al*., 1989). The similar results were obtained in our previous paper for *Phlebia radiata* growing on the ADMS medium (Rogalski *et al*., 1996). Additionally, after the induction by ferulate and vanillate components synthesis *de`novo* was initially stopped (only the methylation process of vanillate and ferulate components were observed). Subsequently, between the $6^{th}$ and $8^{th}$ day of the growth the maximum of the synthesis *de`novo* was observed (Rogalski *et al*., 1992). However, in addition to oxidized products considerable amounts of coniferyl alcohol accumulated when the *Trametes* species was grown in the presence of ferulic acid. Similarly, when using anisic acid as a substrate, a rapid transformation of this acid into aldehyde and alcohole by certain wood destroying fungi was observed, with the preference to oxidative conversion as found by Shimazono *et al*. (1978). Phenolic acids can stimulate the production of cellulolytic enzymes such as hemicellulolytic ones (Rogalski *et al*., 1992; Longa, 1996).

The production of lignolytic enzymes can be induced by certain low molecular weight aromatic compounds, toxic aromatic compounds and lignin preparations (Rogalski *et al*., 1991; Rogalski *et al*., 1992). Having too big molecular weight, these enzymes cannot penetrate the undegraded plant cell wall (Evans *et al*., 1991; Fluornoy *et al*., 1991; Evans *et al*., 1994). On the other hand, there have been studied presenting the evidence that some of these enzymes (*e.g*. laccase) are able to bleach hardwood pulp by depolymerizing and solubilizing lignin in the presence of so–called mediator compounds (Bourbonnais and Paice, 1992; Bourbonnais *et al*., 1995; Call, 1994; Call and Mucke, 1997; Majcherczyk *et al*.,1999). Consequently, it has been suggested that many low–molecular weight compounds permeate wood cell walls and initiate decay. Examples of such substances include veratryl alcohol, oxalate, 3–hydroxyanthranilic acid, and Gt–chelators. They are produced as a result of fungal metabolism and their secretion enables fungi to colonize and degrade the wood cell wall structure more effectively than other organisms. The synthesis of veratryl alcohol was first observed in *Phanerochaete chrysosporium* (Lundquist and Kirk, 1978); oxalate and other organic acids were already found in culture liquids of a number of wood–rotting fungi in 1965, for example *Armillaria mellea, Fomes annosus,* and *Pleurotus ostreatus* (Takao, 1965). Later, their secretion was also shown for solid–state cultures (Galkin *et al*., 1998; Hofrichter *et al*., 1999). 3–Hydroxyanthranilic acid was isolated and identified from *Pycnoporus cinnabarinus* (Eggert *et al*., 1996 and Eggert *et al*., 1997), and a special phenolate derivative, the so–called Gt–chelator (molecular mass <1 kDa), was isolated from the brown–rot fungus *Gleophyllum trabeum* (Goodell *et al*., 1996). *Pseudomonas acidovorans, Bacillus subtilis* and *Rhodotorula rubra* metabolize ferulic acid to vanillin, vanillic acid and protocatechuic acids (Toms and Wood, 1970; Gurujeyalakshimi and Madahevan, 1987; Huang *et al*., 1993). Some microorganisms further convert vanillic acid to guaiacol and methoxyhydroquinone (Crawford and Olson, 1978; Buswell *et al*., 1979). Ferulic acid is also converted to methoxy derivatives of acetophenone (Jurkova and Wurst, 1993) to 4–hydroxy–3–methoxystyrene (Huang *et al*., 1993), to caffeic– and 3–methoxy–4–hydroxyphenylpropionic– acid by many microorganisms (Bache and Pfennig, 1981; Chesson *et al*., 1982; Grbic–Galic, 1985). A little work has been done to elucidate the biochemical mechanism by which substituted cinnamates are converted to any of these aromatic compounds. In the present paper the metabolism of cinnamic components labeled specifically $^{14}C$ in the different position has been reported.

## MATERIALS AND METHODS

### Organism and cultural condition

*Phlebia radiata* Fr no 79 [ATCC 64658] was isolated at the Department of Microbiology, University of Helsinki (Hatakka and Uusi–Rauva, 1983) and was maintained on 2% (w/v) malt agar slants. The preparation of inoculum was performed according to (Hatakka and Uusi–Rauva, 1983). After the 6–day growth in 28 °C the mycelial mats were collected and homogenized in a Warning Blender. After the inoculation with 4% (v/v) of the homogenate, 100 ml conical flasks, each containing 10 ml of ADMS–LN medium with 1% glucose, 1% cellulose, 1% wheat straw or 1% spruce wood as a carbon source, were incubated stationary at 28 °C. On the $3^{rd}$ day of growth cinnamic acid in the concentration of 1 mM and about 1 kBq of cinnamate isotopes specifically labeled on different positions were added to each inoculated flask. The flasks were then fitted with polypropylene stoppers (Kartel, Italy). Radiorespirometric methods to collect evolving $^{14}CO_2$ and the assay of radioactivity were used according to Hatakka and Uusi–Rauva (1983). Sterile synthetic air (20% oxygen) or pure oxygen were used for aeration and $^{14}CO_2$ collection purposes. Culture flasks (10 ml sample) were taken in duplicate every 24 h. Cultures were filtrated by Whatman No. 4 filter paper on

a glass filter (Schott No. 4, Duran, FRG), and the filter paper plus mycelium was combusted as described by (Hatakka and Uusi–Rauva, 1983) to determine the mycelial $^{14}C$–activity. The radioactivity was counted in liquid scintillation counters (LKB–Willa Oy, Finland and Beckman type LS 5000TD, Germany).

**Chemicals**

Carboxyl–labeled p–hydroxy cinnamic acid ($^{14}COOH$ ($C_1$)–cinnamate; $35.7\times10^3$Bq/mg), methoxyl–labeled dimethoxy cinnamate in position –3 and 4 (3–$O^{14}CH_3$–cinnamate; $27.3\times10^3$Bq/mg; 4–$O^{14}CH_3$–cinnamate; $34.3\times10^3$Bq/mg), and *p*–hydroxycinnamate labeled in aliphatic chain in $C_3$ position ($C_3$–cinnamate; $36.8\times10^3$Bq/mg) were kindly supplied by Dr. Konrad Haider and Prof. Jerzy Trojanowski, Inst. für Pflanzenernchrung und Bodenkunde, Bundesforschungsanstalt fűr Landwirtschaft, Braunschweig, Germany.

**HPLC analysis**

HPLC was carried out using a Shimadzu liquid chromatograph equipped with high pressure pumps LC–9A, a gradient system, and diode array UV–visible spectrum detector SPD–M6A connected to an IBM Pentium Ⅱ data system. The reversed phase column was a Novopack–$C_{18}$ column (3.9 mm×150 mm; Waters Assoc. Inc.). All analyses were run with gradient elution by using HPLC gradient grade acetonitrile (Merck) and 1 mM $H_3PO_4$ (pH 3.2). The gradient was partially linear, with acetonitrile (v/v) increase as follows: 11% (0 min); 25% (5 min); 25% (6 min); 40% (8 min); 60% (11 min); and 60% (15 min) as in Lundell *et al*. (1990).

## RESULTS AND DISCUSSION

The dynamics of $^{14}CO_2$ release by *P. radiata* was measured in a standing culture containing as the sole carbon source: a) unlabeled *p*–hydroxycinnamic acid and separately, carboxyl–, or $C_3$–labeled cinnamate; B) unlabelled dimethoxycinnamic acid and separately, 3–methoxy– or 4–methoxy cinnamate.

Fig. 1A demonstrates that $^{14}CO_2$ release from carboxyl labeled cinnamate reached the maximum after 24 hrs on the medium with wheat straw and after 48 hrs on the media with glucose and spruce wood. In the case of the medium with cellulose the release of carbon dioxide increases linearility up to the 8$^{th}$ day of the cultivation. The evolution of carbon dioxide from aliphatic chain cleavage cinnamate (Fig. 1B) showed very similar trend which demonstrated simultaneous process with the decarboxylation ones on all tested media. The processes of the demethylation of 4–methoxy group (Fig. 1D) and demethylation of 3–methoxy group (Fig. 1C) were next observed in all cases. Among these processes only

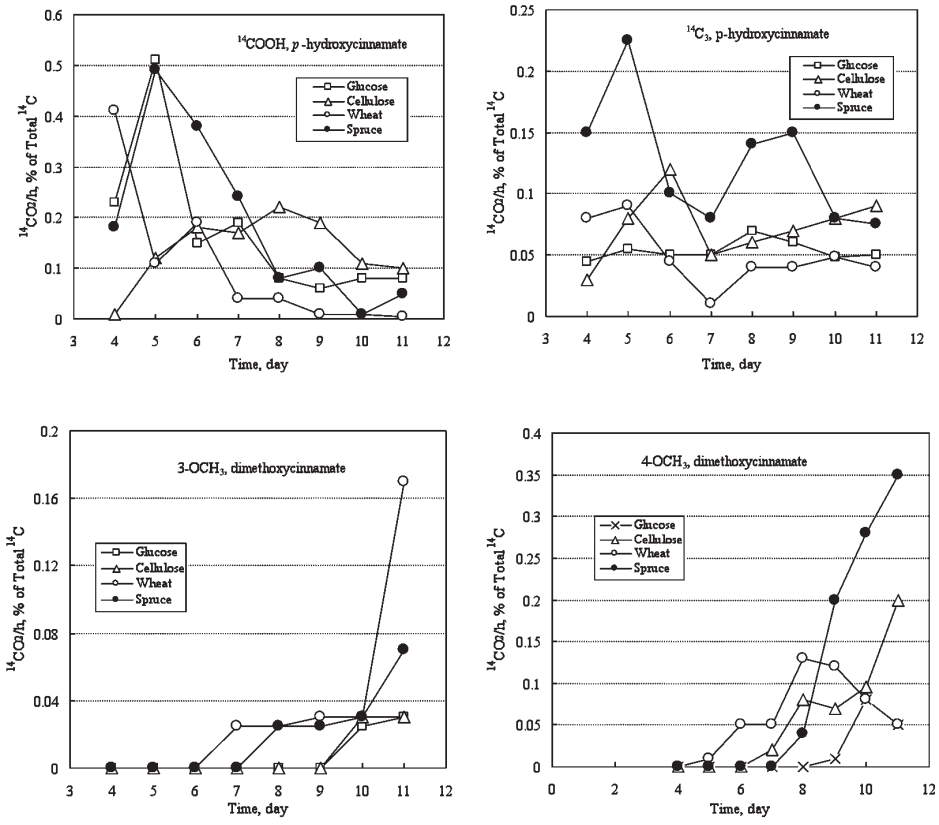


**Fig. 1.** $^{14}CO_2$ evolution from ($^{14}COOH$)*p*–hydroxycinnamate(**A**); ($^{14}C_3$)*p*–hydroxyninnamate(**B**); [3–$OCH_3$] dimethoxycinnamate(**C**); (4–$OCH_3$) dimethoxycinnamate(**D**) by *Phlebia radiate* grown in air aeration on 1% glucose (□–□); 1% cellulose (△–△); 1% wheat straw (○–○) and 1% spruce wood (●–●) as the sole carbon source.

decarboxylation seemed to occur in one step: all other patterns showed more or less periodical behaviour as in the degradation of veratric acid (Rogalski *et al*., 2000).

The release of carbon source from methoxyl groups does not occur directly but instead via methanol and formaldehyde formation (Frick and Crawford, 1983; Eriksson *et al*., 1990). In *Phanerochaete chrysosporium* three enzymes are probably involved in this pathway: (i) alcohol oxidase which transforms methanol to formaldehyde; (ii) formaldehyde dehydrogenase which transforms formaldehyde to formate; and (iii) formate dehydrogenase which oxidizes formate to carbon dioxide (Buswell, 1986; Eriksson and Nishida, 1989). Theoretically, the same transformation can be achieved by alcohol dehydrogenase but the enzyme isolated from the mycelium of *Sporotrichum pulverulentum* (*P. chrysosporium*) has no activity towards methanol (Rudge and Bickerstaff, 1986).

The recovery of $^{14}C$–labeled elements coming from $^{14}C$ labeled aromatic acids isotopes is presented in Table 1. Such a high recovery of $^{14}C$ isotopes above 93% demonstrated that the used apparatus system was very tight. It can be observed from the above that about 30% of carbon dioxide evolved from carboxyl–labeled cinnamate. The radioactivity in the mycelium was also above 10% in all cases and can be read as partially degraded or metabolized to the other components.

The degradation of cinnamate compounds in oxygen atmosphere were presented in (Fig. 2). As it was shown the mechanism of this component degradation was very similar to that in the case when the fungus grew in air atmosphere. The only difference is that the level of evolved radioactivities was double when the experiment was performed in oxygen.

The cellulose slightly repressed the decarboxylation of cinnamate in opposition to wheat straw and spruce wood, which doubled degradation. The maximum rate of decarboxylation was *ca*. 1.2% of the applied activity evolved as $^{14}CO_2$ per hour. The degradation of radio–labeled veratric acid (Rogalski *et al*., 2000), vanillic acid (Rogalski *et al*., 2003) and ferulic acid (Rogalski *et al*., 2003a) gave the same following sequence. At first the substrates after decarboxylation, and aliphatic chain cleavage were demethylated in the position 4 following the demethylation in the position 3 and finally aromatic ring cleavage was observed. The results from the *Fusarium oxysporium* indicated that after decarboxylation first in the position 3 and then in 4 occurred (Targoński *et al*., 1986). They also found that the presence of cellulose in culture medium decreases the rate of aromatic acid breakdown. In the case of *P. radiata* cellulose as a carbon source also decreased the rate of degradation except in the case of *para*–demethylation of veratric acid (Rogalski *et al*., 1996).

The HPLC data confirm the sequence of the methabolic steps presented above with isotopes. The hypothetical methabolic pathways were presented in Fig. 3. The transformation of cinnamic acids occurs *via* 3,4–dimethoxyvinylobenzene or 3–methoxy, 4–hydroxyvilylobenzene to vanillic acid, which is methabolized fur-

**Table 1.** Distribution of $^{14}C$–acitvities in 11–day–old *Phlebia radiata* cultures grown on various carbon sources in air conditions. The fungus was cultivated in the presence of $^{14}C$–labeled compounds and unlabeled cinnamic and coumaric acids (1 mM)

| Carbon source | Type of label [1kBq/culture] | $^{14}CO_2$ evolved [%] | $^{14}CO_2$ mycelium [%] | $^{14}C$ in culture filtrate [%] | Total $^{14}C$ [%] |
|---|---|---|---|---|---|
| 1% glucose | *p*–OH [$^{14}$COOH]–cinn[a)] | 33.99±3.11 | 9.96±0.42 | 53.24±3.40 | 97.10±5.11 |
| | *p*–OH [$^{14}C_3$]–cinn | 12.16±1.92 | 22.72±0.62 | 63.72±1.90 | 98.60±2.90 |
| | dimethoxy [3–O$^{14}CH_3$]–cinn | 0.98±0.10 | 15.08±0.21 | 83.34±2.10 | 99.41±2.00 |
| | dimethoxy [4–O$^{14}CH_3$]–cinn | 4.46±0.51 | 10.23±0.62 | 84.11±3.22 | 98.81±2.90 |
| 1% cellulose | *p*–OH [$^{14}$COOH]–cinn | 28.55±1.63 | 15.48±1.23 | 52.87±1.72 | 96.89±2.80 |
| | *p*–OH [$^{14}C_3$]–cinn | 14.03±2.11 | 23.83±0.96 | 59.84±3.90 | 97.72±4.14 |
| | dimethoxy [3–O$^{14}CH_3$]–cinn | 1.43±0.37 | 32.54±1.62 | 64.43±4.11 | 98.40±3.14 |
| | dimethoxy [4–O$^{14}CH_3$]–cinn | 13.51±1.67 | 12.59±2.00 | 70.80±2.80 | 96.90±3.79 |
| 1% wheat straw | *p*–OH [$^{14}$COOH]–cinn | 19.35±2.00 | 12.60±0.93 | 65.55±3.12 | 97.52±4.33 |
| | *p*–OH [$^{14}C_3$]–cinn | 9.17±0.99 | 30.66±1.67 | 54.87±2.70 | 94.70±3.66 |
| | dimethoxy [3–O$^{14}CH_3$]–cinn | 5.30±0.76 | 32.69±2.22 | 58.61±4.31 | 96.61±4.72 |
| | dimethoxy [4–O$^{14}CH_3$]–cinn | 11.54±1.33 | 9.73±1.37 | 76.63±2.20 | 97.90±3.91 |
| 1% spruce wood | *p*–OH [$^{14}$COOH]–cinn | 37.29±3.23 | 14.65±2.17 | 41.86±2.72 | 93.81±5.40 |
| | *p*–OH [$^{14}C_3$]–cinn | 24.59±3.00 | 30.80±1.73 | 39.21±3.20 | 94.61±5.70 |
| | dimethoxy [3–O$^{14}CH_3$]–cinn | 2.27±0.83 | 40.95±0.98 | 53.68±2.10 | 96.43±2.00 |
| | dimethoxy [4–O$^{14}CH_3$]–cinn | 19.96±2.61 | 21.47±1.63 | 53.97±1.90 | 95.43±3.71 |

[a)] cinn=cinnamic acid

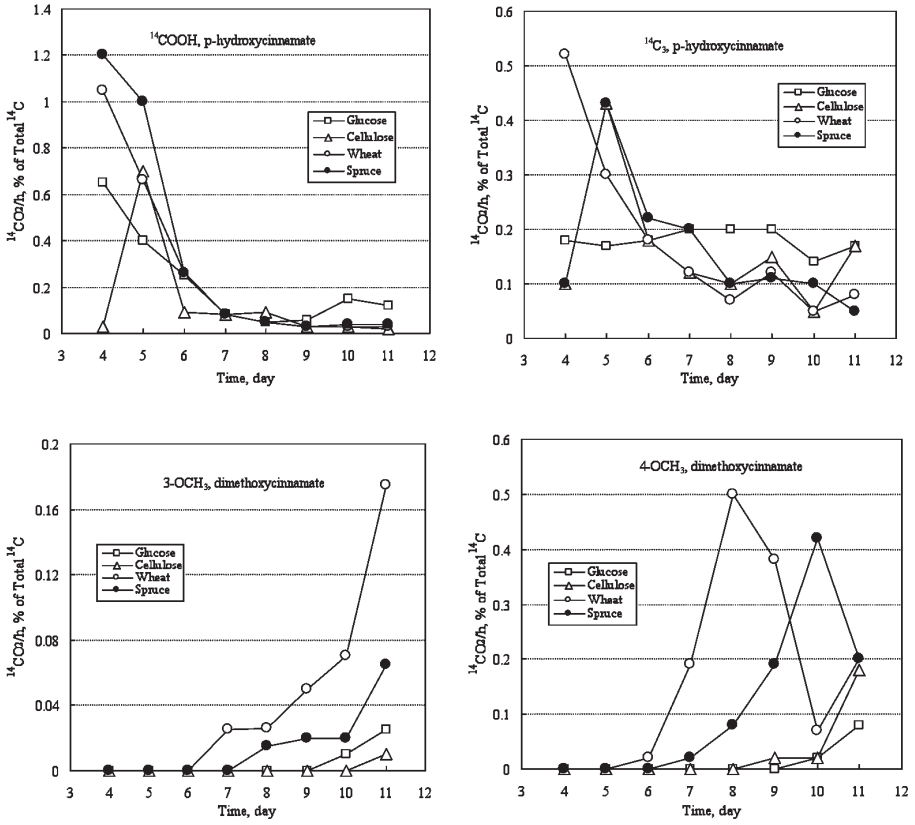


**Fig. 2.** $^{14}CO_2$ evolution from [$^{14}$COOH)*p*–hydroxycinnamate(**A**); [$^{14}C_3$]*p*–hydroxycinnamate(**B**); [3–OCH$_3$] dimethoxycinnamate(**C**); [4–OCH$_3$] dimethoxycinnamate(**D**) by *Phlebia radiate* grown in oxygen aeration on 1% glucose (□–□); 1% cellulose (△–△); 1% wheat straw (○–○) and 1% spruce wood (●–●) as the sole carbon source.

**Table 2.** Distribution of $^{14}$C–acitvities in 10–day–old *Phlebia radiata* cultures grown on various carbon sources in oxygen conditions. The fungus was cultivated for 10 days in the presence of $^{14}$C–labelled compounds and unlabeled cinnamic and coumaric acids (1 mM)

| Carbon source | Type of label [1kBq/culture] | $^{14}CO_2$ evolved [%] | $^{14}CO_2$ mycelium [%] | $^{14}$C in culture filtrate [%] | Total $^{14}$C [%] |
|---|---|---|---|---|---|
| 1% glucose | *p*–OH [$^{14}$COOH]–cinn[a)] | 38.39±4.21 | 3.79±0.41 | 56.32±2.21 | 98.00±5.41 |
| | *p*–OH [$^{14}C_3$]–cinn | 34.12±2.94 | 9.61±0.41 | 53.37±3.20 | 97.11±5.10 |
| | dimethoxy [3–O$^{14}CH_3$]–cinn | 0.39±0.09 | 8.29±0.52 | 90.72±1.58 | 99.40±1.88 |
| | dimethoxy [4–O$^{14}CH_3$]–cinn | 2.47±0.55 | 6.27±0.37 | 90.16±1.70 | 98.90±2.00 |
| 1% cellulose | *p*–OH [$^{14}$COOH]–cinn | 28.76±1.99 | 7.00±0.41 | 61.84±2.41 | 97.60±3.65 |
| | *p*–OH [$^{14}C_3$]–cinn | 29.22±2.11 | 6.97±0.33 | 58.51±2.90 | 94.70±4.21 |
| | dimethoxy [3–O$^{14}CH_3$]–cinn | 0.16±0.06 | 16.49±0.52 | 76.65±2.11 | 93.30±2.33 |
| | dimethoxy [4–O$^{14}CH_3$]–cinn | 5.66±1.14 | 13.97±0.41 | 75.77±1.90 | 95.40±2.10 |
| 1% wheat straw | *p*–OH [$^{14}$COOH]–cinn | 48.52±2.75 | 7.50±0.40 | 40.88±1.41 | 96.91±3.11 |
| | *p*–OH [$^{14}C_3$]–cinn | 34.20±2.40 | 13.55±0.45 | 49.95±1.90 | 97.72±3.00 |
| | dimethoxy [3–O$^{14}CH_3$]–cinn | 8.01±1.00 | 14.63±0.50 | 76.66±2.22 | 99.31± 2.00 |
| | dimethoxy [4–O$^{14}CH_3$]–cinn | 32.58±1.60 | 11.48±0.31 | 53.74±1.60 | 97.80±2.62 |
| 1% spruce wood | *p*–OH [$^{14}$COOH]–cinn | 61.55±1.72 | 5.73±0.40 | 30.82±1.20 | 98.10±1.99 |
| | *p*–OH [$^{14}C_3$]–cinn | 48.78±1.22 | 14.85±0.51 | 33.07±1.83 | 96.71±2.88 |
| | dimethoxy [3–O$^{14}CH_3$]–cinn | 2.45±0.20 | 20.89±0.46 | 75.86±2.20 | 99.21±2.00 |
| | dimethoxy [4–O$^{14}CH_3$]–cinn | 21.95±2.64 | 16.41±0.60 | 60.14±2.11 | 98.50±3.40 |

[a)] cinn=cinnamic acid

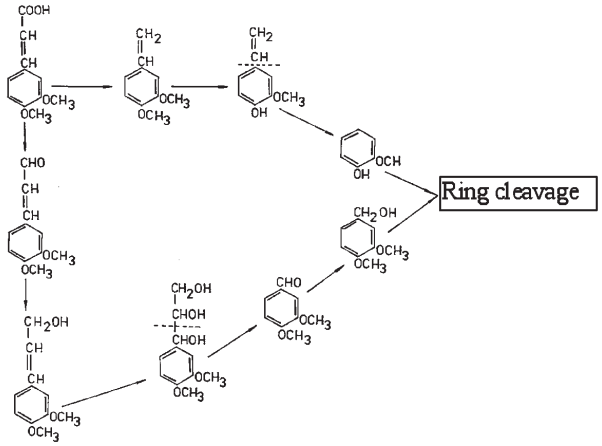


**Fig. 3.** Pathways for cinnamic acid metabolism by *Phlebia radiate*.

ther as described earlier (Rogalski *et al*., 2003). In the case of *Lentinus edodes* the degradation of 3,4–dimethoxycinnamate results in ferulic acid or 3,4 dimethoxycinnamic aldehyde (Crestini and Sermanni, 1994). *Trametes versicolor* growing on the media with low nitrogen and high oxygen oxidized 3,4–dimethoxycinnamic alcohol to veratrylglycerol; the cleavage between $C_\alpha$ and $C_\beta$ was next observed giving veratraldehyde and veratryl alcohol (Kamaya and Higuchi, 1984). The peroxidase from *Nicotina tabacum* can rapidly oxidize sinapic acid to 4–coumaric acid, ferulic acid and coniferyl alcohol (Takahama *et al*., 1996). Fungal laccase from *Pycnoporus oryzae* oxidized sinapic acid to ferulic– and *p*–coumaric acid (Takahama, 1995), whereas laccase from *Trametes versicolor* transform sinapic acid to dimmers by the reaction of coupling of two phenoxy radilals and latter to 2,6 dimethoxy–*p*–benzoquinone (Lacki and Duvnjak, 1998).

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