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Biotransformation of the *Trichoderma* metabolite 6-*n*-pentyl-2*H*-pyran-2-one by cell suspension cultures of *Pinus radiata*

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Abstract

Cell suspension cultures of *Pinus radiata* metabolize the antifungal *Trichoderma* secondary metabolite 6-*n*-pentyl-2*H*-pyran-2-one (6PAP) (**1**) via hydroxylation of the pentyl side chain. Examination of the culture medium following dosing studies with **1** revealed that 79–85% of this bioactive compound had been metabolised after 144 h. At that time, 34–40% of the metabolized dose was recovered as a series of monohydroxylated isomers of **1**, the principal metabolite being 5-(2-pyron-6-yl)pentan-5-ol (**7**).

Keywords: *Pinus radiata*; Cell suspension cultures; Biotransformation; Hydroxylation; Fungal metabolite; 6-*n*-pentyl-2*H*-pyran-2-one (6PAP)

1. Introduction

Fungi such as *Trichoderma* have been investigated as potential biocontrol agents (BCAs) because of their ability to reduce the incidence of disease caused by many common soil-borne plant pathogenic fungi. In New Zealand, due to export market demands, particular emphasis has been placed on finding natural product alternatives to synthetic pesticides and fungicides in timber crops such as *Pinus radiata*. To date, *Trichoderma* spp. treatments have proven effective against *Armillaria* infection of *Pinus radiata* in greenhouse and forest trials (Cutler & Hill, 1994), and have shown control of a range of soil-borne organisms causing disease on other *Pinus* species (Duda & Sierota, 1987; Kumar, 1993) and on a range of angiosperms (Corke, 1974; D'Ercole & Lugaresi, 1981).

Although the modes of action of these BCA organisms are not clearly understood, the biosynthesis of antibiotic compounds by some *Trichoderma* species is believed to contribute to their control function. In particular, production of the known antifungal compound, 6-*n*-pentyl-2*H*-pyran-2-one (6PAP) (**1**), is common amongst *Trichoderma* isolates identified as BCAs (Ghisalberti & Sivasithamparam, 1991). This compound (**1**) has been shown to be effective against several *P. radiata* pathogens in laboratory trials and against pathogens such as *Armillaria* in field trials (Cutler & Hill, 1994).

As a part of our efforts to understand the mode of action of **1** as a fungal biocontrol agent, we are investigating its interaction with the pathogen, plant and the environment. Recently, we reported that several pathogenic fungi in liquid culture were able to metabolize **1** to form a number of hydroxylated biotransformation products (Cooney, Lauren, Poole & Whitaker, 1997; Cooney & Lauren, 1999). The ability to oxidise compounds by hydroxylation is reported to be widespread among filamentous fungi (Hammer & Schauer, 1997) and it has been suggested that such transformations are part of a fungal strategy to eliminate metabolites that would otherwise be toxic (Kinderlerer, 1993).

The close association of plant roots and *Trichoderma* species, together with the recent demonstration that *Trichoderma* species may penetrate, and develop within, host plant tissues (Yedidia, Benhamou & Chet,

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1999), prompted an investigation of the ability of plant tissue to metabolize **1**. In this paper we report our preliminary findings using a *P. radiata* cell suspension culture as a model plant system.

2. Results and discussion

Incubation of **1** with suspension cells of *Pinus radiata* gave a series of monohydroxylated isomers of **1**, 5-(2-pyron-6-yl)pentan-1-ol (**3**), 5-(2-pyron-6-yl)pentan-2-ol (**4**), 5-(2-pyron-6-yl)pentan-3-ol (**5**), 5-(2-pyron-6-yl)pentan-4-ol (**6**), and 5-(2-pyron-6-yl)pentan-5-ol (**7**), and the carboxylic acid 3-(2-pyron-6-yl)propanoic acid (**2**). Two further biotransformation products retaining the pyrone ring, or otherwise having significant UV absorption (300 nm) were also noted (**X** and **Y**), but were not identified.

The time course for the biotransformation of **1** is shown in Fig. 1. Concentrations of the individual metabolites formed after incubation for 144 h are given in Table 1. After this sampling time observable metabolite levels began to decrease, suggesting further metabolism and cleavage of the pyrone ring. This presumably explains the difference between the total loss of **1** and the measurable amount of pyrone containing biotransformation products formed (34–40% of metabolized **1** at 144 h). Loss of **1** is not due to lactone hydrolysis in aqueous solution or volatilisation as the control flasks showed no such decrease of **1** concentration over time (Fig. 1). It is also possible that part of the missing dose may be retained intracellularly in the form of glycosidic conjugates.

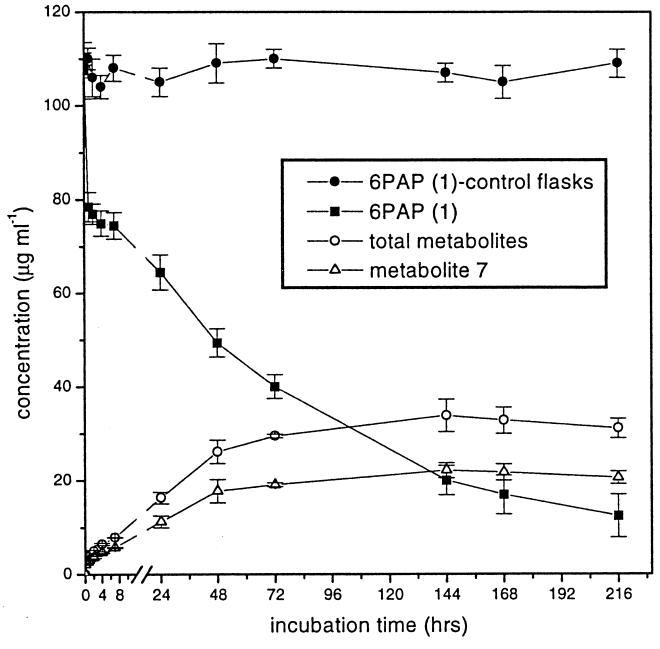


Fig. 1. Time course in the degradation of 6PAP (**1**) and appearance of metabolites (biotransformation products of **1**) by cell suspension cultures of *Pinus radiata* during an incubation period of 216 h (9 days).

Table 1
Concentration (µg ml^{-1}) of pyrone-containing compounds detected in *Pinus radiata* suspension cell cultures 144 h after dosing with 6PAP (**1**, 110 µg ml^{-1})

Flask	1 remaining	Pyrone-containing metabolites[a]								
		2	3	4	5	6	7	X	Y	Total
1	18.5	0.8	0.4	2.4	2.8	1.5	22.8	1.9	0.6	33.2
2	23.6	0.5	0.2	2.4	2.5	1.1	19.8	1.8	0.7	29.0
3	16.5	1.1	3.5	2.9	3.2	1.5	23.6	1.3	0.3	37.4
4	21.5	0.7	0.5	2.6	2.8	1.6	22.4	2.0	0.5	33.1

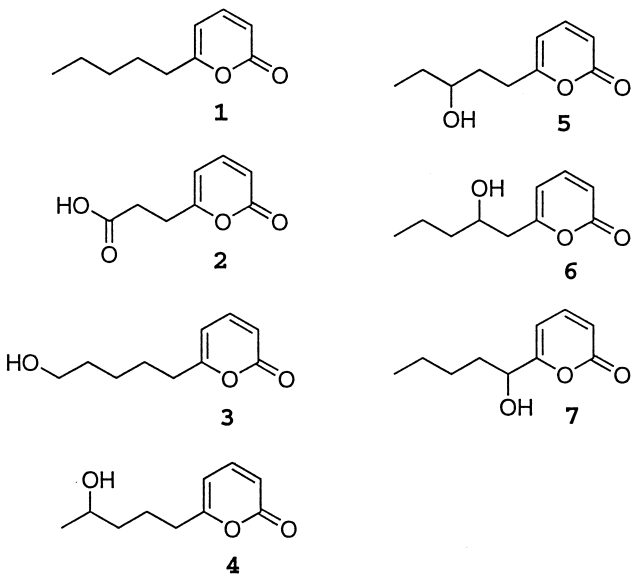
[a] Known biotransformation products retaining the pyrone ring or having significant UV absorption (300 nm).

The major biotransformation product found throughout the duration of the experiment was compound **7**. Metabolites **3**, **4**, **5** and **7** could be detected in low concentration 5 min after administration of **1**. Metabolites **2**, **6**, **X** and **Y** were not detected until 24 h later. With the exception of **2** and **3**, individual metabolite levels typically reached a maxima after 144 h incubation. Metabolite **3** reached its highest concentration after 72 h (2.3–3.5 µg ml^{-1}) but then rapidly declined with time. The carboxylic acid, **2**, continued to accumulate slowly, but still made up only a very small percentage of the total metabolite concentration by the final sampling time (216 h). HPLC analysis of the culture medium of the undosed *Pinus* cell and media control showed no peaks at the same retention times as any of the metabolites observed.

We have previously reported the isolation and characterisation of the biotransformation products (**2**–**7**) from studies investigating the fungal metabolism of **1** by selected plant pathogens (Cooney et al., 1997; Cooney & Lauren, 1999). That plant tissue is also able to metabolize **1** to give many of the same biotransformation products may have important implications for biocontrol strategies. While we have shown that these oxidised metabolites are considerably less toxic to a fungal pathogen than **1** in germination assays (Cooney et al., 1997), we have not extended these experiments to plant tissue. Biotransformation of foreign compounds by plant tissue via hydroxylation however has been widely reported (Hamada, Fuchikami, Ikematsu, Hirata, Williams & Scott, 1994; Ruhland, Engelhardt & Wallnöfer, 1996; Zweimüller, Antus, Kovács & Sonnenbichler, 1997; Park & Kim, 1998), and is believed to be part of a defense reaction of the plant cells mediated by a cytochrome dependent P-450 monooxygenase enzyme.

Our studies suggest that while **1** alone may be effec-

tive as a short-term fungal biocontrol agent, its capacity to provide sustained disease control is likely to be limited by the ability of both pathogen and plant to efficiently metabolize it to products less toxic to the pathogen. Therefore, for long-term disease control the establishment and growth of **1**-producing strains of *Trichoderma* spp. in living tissue may provide the most effective system for delivery of **1** over an extended period of time to the target sites. There is also the possibility of other antifungal metabolites produced by *Trichoderma* assisting in a synergistic role.



3. Experimental

3.1. General experimental procedures

Analytical HPLC was performed on a Prodigy 5 ODS-2 column (4.6 × 150 mm), oven temperature 35°C, using a UV detector set at 300 nm. The initial mobile phase, MeOH-1% aq. HOAc (20:80) at 1 ml min^{-1}, was held for 15 min, then programmed using a linear solvent gradient to MeOH-1% HOAc (60:40) over 5 min, and held for 8 min before resetting to the original conditions.

3.2. Pinus radiata cell culture

P. radiata D. Don cell cultures were grown as previously described (Hotter, 1997) except that GF16 seed was obtained from Proceed New Zealand, cell and callus cultures were grown at 23 ± 1°C, and cell cultures were shaken at 100 rpm.

3.3. Transformation of 6PAP (1) by suspension cells of Pinus radiata

Five flasks comprising 25 ml of suspension media containing 12 ml of cells and 2 control flasks containing 25 ml of media were shaken at 23°C at 100 rpm. An aq. solution of **1** was prepared and added to four of the suspension cell flasks and the 2 media control flasks to give a concentration of 110 μg ml^{-1}. The fifth suspension cell flask acted as an undosed *Pinus* cell and media control to determine whether any interfering compounds were produced by the *Pinus* cells. Subsamples (150 μl) were taken from each flask 5 min, 1 h, 2 h, 4 h, 7 h, 24 h, 48 h, 72 h, 144 h, 168 h and 216 h after this addition. Each subsample was diluted with 150 μl of MeOH, filtered to remove suspended material, and analysed by HPLC for **1** and transformation products. The retention times for **1** and its biotransformation products were; 4.2 min (**2**), 8.3 min (**X**), 10.8 min (**Y**), 12.9 min (**4**), 13.8 min (**3**), 16.3 min (**5**), 21.6 min (**6**), 24.1 min (**7**) and 27.8 min (**1**). Metabolites were identified by co-elution with standards prepared from compounds **2–7** that had been previously isolated from experiments investigating the fungal biotransformation of **1** (Cooney et al., 1997; Cooney & Lauren, 1999).

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