Effect of Harvest Time, Temperature, Light, and Spore Inoculum Concentration on 6-*n*-Pentyl-2*H*-pyran-2-one Production by *Trichoderma* spp.

J. M. Cooney,* D. R. Lauren, D. J. Jensen, and L. J. Perry-Meyer

HortResearch, Ruakura Research Centre, Private Bag 3123, Hamilton, New Zealand

Selected strains of *Trichoderma* spp. were grown on a solid corn matrix under a variety of culture conditions and analyzed for the production of 6-*n*-pentyl-2*H*-pyran-2-one (6PAP). By optimizing harvest time, culture temperature, light conditions, and spore inoculum concentration, yields of 6PAP as high as 2000 mg/kg have been achieved. These results will provide an indication for future production studies potentially leading to the development of a bulk scale fermentation process.

Keywords: *Trichoderma; 6-pentyl-α-pyrone; 6PAP; metabolite; production; culture*

INTRODUCTION

Trichoderma spp. have in recent years received particular attention as agents for the biological control of several important fungal plant pathogens. Antibiosis is believed to be one of the mechanisms involved in disease control (Dennis and Webster, 1971a,b; Papavizas, 1985; Ghisalberti and Sivasithamparam, 1991), although lysis, competition, mycoparasitism, and promotion of plant growth have also been implicated (Henis, 1984; Papavizas, 1985; Chet, 1987; Baker, 1988; Lynch, 1990). Several studies have shown a correlation between the effectiveness of *Trichoderma* spp. to control plant diseases and their ability to produce pyrone metabolites such as 6-*n*-pentyl-2*H*-pyran-2-one (6PAP) (Figure 1) (Ghisalberti et al., 1990; Worasatit and Sivasithamparam, 1990; Worasatit et al., 1994). 6PAP has also been shown to give promising in vitro and in vivo control of several major phytopathogenic fungi that affect New Zealand grown crops including *Armillaria, Botrytis, Phytophthora,* and sapstain fungi (Cutler and Hill, 1994) and is currently being tested as a natural fungicide.

The ability of *Trichoderma* isolates to produce 6PAP has been found to differ between isolates of different species as well as between isolates of the same species (Dennis and Webster, 1971a,b). Our earlier studies have also shown that, for a particular isolate, culture conditions can have a major impact on the quantity of 6PAP produced (Cooney et al., 1997). We have identified several high-yielding strains of *Trichoderma* and have optimized 6PAP production by selection of solid media, liquid supplements, and harvest time.

6PAP can be prepared both synthetically in the laboratory and naturally via fermentation of *Trichoderma*, but it is a high-value compound and supplies are extremely limited. A number of syntheses of 6PAP have been reported in the literature (Nobuhara, 1968; Pittet and Klaiber, 1975; Dieter and Fishpaugh, 1988), and one method has been patented (Klaiber and Pittet, 1975, 1976). Generally the reported syntheses either utilize expensive reagents or require a complex number of steps, both of which preclude economic scaleup. Attempts within our organization to improve both the cost effectiveness and the reliability of synthetic methods have been unsucessful (G. Depree, personal communication). The literature also describes reports of the continuous removal of 6PAP from *Trichoderma viride* cultures by pervaporation through a hydrophobic membrane. Yields of 6PAP up to 1 g/L were reported (Bengtson et al., 1992). More recently, yields of 6PAP in the range 92−580 mg/L by *T. viride* cultures and using aqueous and two-phase extractive fermentation have been reported (Tekin et al., 1995).

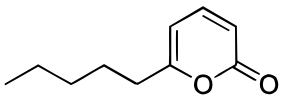

Figure 1. 6PAP, a secondary metabolite produced by *Trichoderma* spp.

This study continues from our previous work to optimize culture conditions for the production of 6PAP from *Trichoderma* cultures leading to the development of an economic bulk fermentation process.

MATERIALS AND METHODS

Apparatus. Culture samples were incubated in a Sanyo MIR 252 incubator (Japan) fitted with fluorescent lights operating continuously. Gas chromatography/flame ionization detection (GC/FID) analysis for 6PAP was carried out on a HRGC 5300 Mega Series Carlo Erba (Milan, Italy) instrument fitted with a Hewlett-Packard (Avondale, PA) HP-5 capillary column, 25 m × 0.20 mm i.d. (0.33 μm film thickness). Injector and detector temperatures were 250 °C. The GC oven temperature was held at 180 °C for 6 min, programmed at 30 °C/min to 230 °C, and then held for a further 10 min. The retention times of 6PAP and the internal standard, hexadecane, were 3.7 and 5.1 min, respectively.

Reagents and Materials. All solvents were of analytical grade or better and were supplied by Mallinckrodt Baker (Paris, KY). Liquid supplements employed during the course of this study were solution 7 (Zeppa et al., 1990), containing yeast extract (1 g/L), casein hydrolysate (2 g/L), KH_2PO_4 (1.5 g/L), $MgSO_4 \cdot 7H_2O$ (1 g/L), and glucose (15 g/L); luria broth, containing bactotryptone, yeast, and NaCl (25 g/L); and yeast extract solution (10%). Casein hydrolysate and yeast extract were both obtained from DIFCO laboratories (Detroit, MI), while luria broth was supplied by Life Technologies (Paisley, Scotland). Inorganic salts and glucose were purchased from BDH Ltd. (Poole, England).

Trichoderma spore suspensions were prepared in sterile water using the surface growth from freshly grown malt 3.5

* Author to whom correspondence should be addressed (fax 64 7 838 5085; e-mail jcooney@hort.cri.nz).

S0021-8561(97)00086-1 CCC: $14.00

agar plates (malt agar, modified by adjusting the pH to 3.5 by the addition of lactic acid) (Atlas, 1993). Spore concentrations were determined using a Hawksley hemocytometer (depth 0.1 mm 1/400 mm^2).

***Trichoderma* Isolates.** A total of seven *Trichoderma* isolates, designated A, B, C, F, I, M, and O as in Cooney et al. (1997) and identified as *T. hamatum* Rifai (four), *T. harzianum* Rifai (two), and *T. koningii* Rifai (one) (supplied by R. A. Hill, HortResearch, Hamilton, New Zealand), were used during the course of this study.

General Culture Conditions. The *Trichoderma* isolates were grown in 200 mL conical flasks on sterilized solid media (10 g), treated with liquid supplement (5 mL) and water (3 mL), and inoculated with 1 mL of prepared spore suspensions with measured concentrations (unless otherwise stated) in the range 1×10^8 to 1×10^9 spores/mL. Flasks were incubated at 20 °C (unless otherwise stated).

Production of 6PAP with Time. Two isolates, A and B, were used to determine the optimum harvest time for culture samples grown on ground corn treated with liquid supplement solution 7. This had been established as the highest 6PAP-producing substrate and supplement combination in our earlier work (Cooney et al., 1997). Twenty replicates of each isolate were prepared and two of these analyzed every 2−3 days.

Effect of Temperature. Six isolates, A, B, C, F, I and M, were grown on ground corn supplemented with solution 7 and incubated at 15, 20, and 25 °C. The culture samples were extracted after 18 days and analyzed for 6PAP content. All culture samples were grown in duplicate.

Effect of Light. There were two experiments. In the first, isolates A and B were grown in the presence and absence of light on both ground corn and whole corn, each treated with one of three liquid supplements (water, 10% yeast extract solution, and solution 7 for isolate A or luria broth for isolate B). The "light" flasks were incubated as normal at 20 °C, while the "dark" flasks were wrapped in aluminum foil to exclude light before incubation alongside the "light" flasks. Isolate B culture samples were harvested after 11 days and isolate A culture samples after 18 days.

In the second experiment, both isolates were grown on the same solid and liquid supplement media as isolate A in the first experiment (i.e. both ground corn and whole corn each treated with water, 10% yeast extract solution, or solution 7). Reduced liquid levels were used for the whole corn cultures, i.e. liquid supplement (5 mL) and water (1 mL), to minimize rotting of the whole kernels. In this experiment, all culture samples were grown in duplicate and extracted after 17 days.

Effect of Spore Inoculum Concentration. There were two experiments. In the first, isolates A and O were grown on ground corn supplemented with solution 7 and inoculated using 1 mL of prepared spore suspensions of concentrations 1×10^8, 1×10^7, 1×10^6, 1×10^5, or 1×10^4 spores/mL. All samples were prepared in duplicate, and culture samples for both isolates were extracted after 21 days.

In the second experiment, isolate O was grown on ground corn supplemented with solution 7 and inoculated using 1 mL of prepared spore suspensions of concentrations 2×10^8, 2×10^7, 2×10^6, 2×10^5, 2×10^4, or 2×10^3 spores/mL. Four replicates at each spore concentration were prepared, and culture samples were extracted after 21 days.

Effect of Time and Spore Inoculum Concentration. To determine whether the spore inoculum concentration affected the time window in which to harvest culture samples for optimum 6PAP production, two isolates, A and O, were grown on ground corn supplemented with solution 7 and inoculated with spore suspensions of concentration 1×10^9 or 1×10^3 spores/mL. Twenty replicates of each isolate/inoculum concentration combination were prepared and duplicate samples analyzed every 2−3 days.

Extraction of Culture Samples. Culture samples were extracted by adding methanol/water (85:15, 50 mL) to the substrate representing 10 g of solid material. The samples were blended with a Polytron homogenizer for ca. 1−2 min so that no visible lumps remained and then allowed to stand for ca. 4 h. Each sample was filtered through a Whatman glass fiber filter (GF/A; 9 cm diameter). An aliquot of the extract solution (4 mL) was added to cyclohexane (2 mL) and buffered 10% saline solution (pH 6.5, 10 mL), then mixed and allowed to partition overnight. The cyclohexane layer was separated and dried by passing it through anhydrous Na_2SO_4 (ca. 1.5 g). An aliquot (1 mL) the dried cyclohexane fraction was spiked with a 22 ppm hexadecane internal standard solution (100 μL) and analyzed for 6PAP by GC/FID.

The partition step was used to provide a cleanup prior to GC/FID analysis and to transfer the analyte into a GC amenable solvent. The overall recovery of 6PAP was determined for ground corn and found to be in the range 67−74%. Results presented in this paper are as recorded, and for estimates of absolute yield values, a scaling factor of 1.4 should be applied.

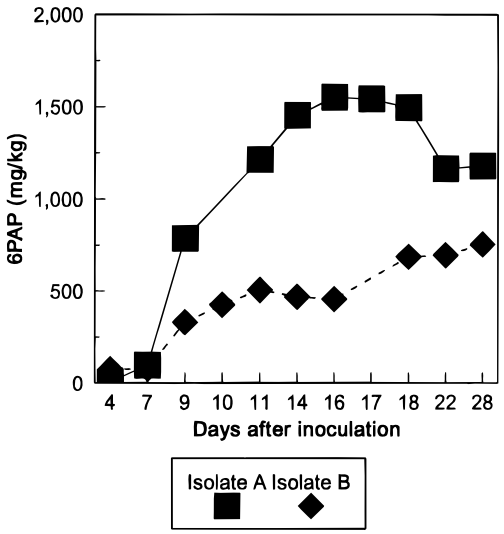

Figure 2. Production of 6PAP with time by *Trichoderma* isolates grown on ground corn supplemented with solution 7 for isolates A and B.

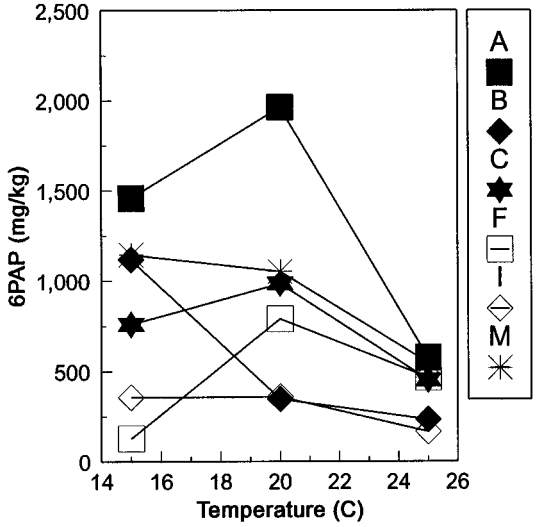

Figure 3. Effect of temperature on 6PAP production for six *Trichoderma* isolates (A−C, F, I, and M) grown on ground corn supplemented with solution 7.

RESULTS AND DISCUSSION

Production of 6PAP with Time. The 6PAP time harvest results are shown in Figure 2 for the two main isolates of interest, A and B. For isolate A the level of 6PAP was minimal at day 4 but had increased rapidly by day 9. The measured concentration of 6PAP was maximum at about days 14−18 and then declined to a steady level from days 22 to 28. In comparison to the results obtained for isolate A on the same solid matrix supplemented with 10% yeast extract solution, namely

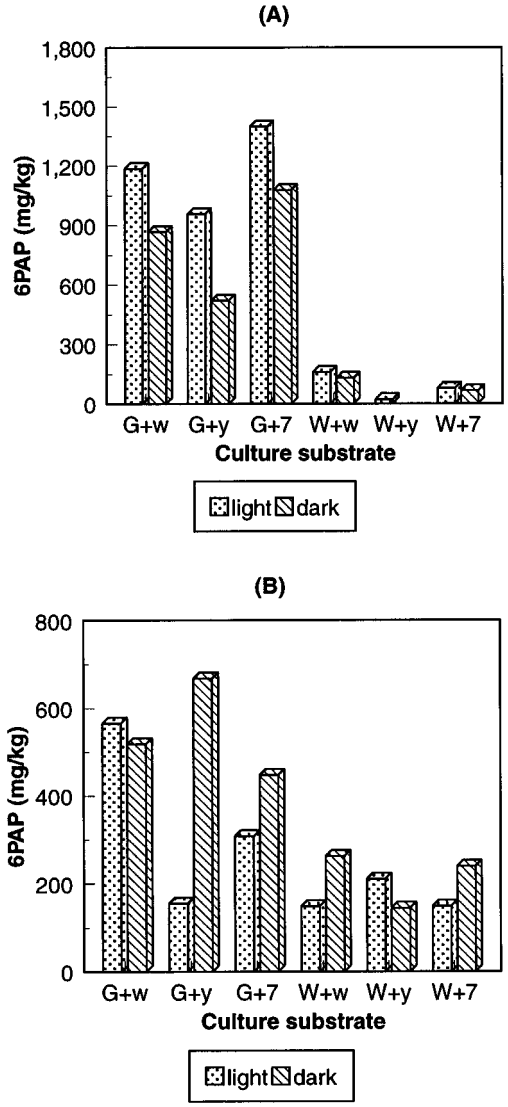

Figure 4. Effect of light on 6PAP production by *Trichoderma* isolates grown on ground corn (G) or whole corn (W) supplemented with either 10% yeast solution (y), solution 7 (7), luria broth (l), or water (w) for (A) isolate A and (B) isolate B.

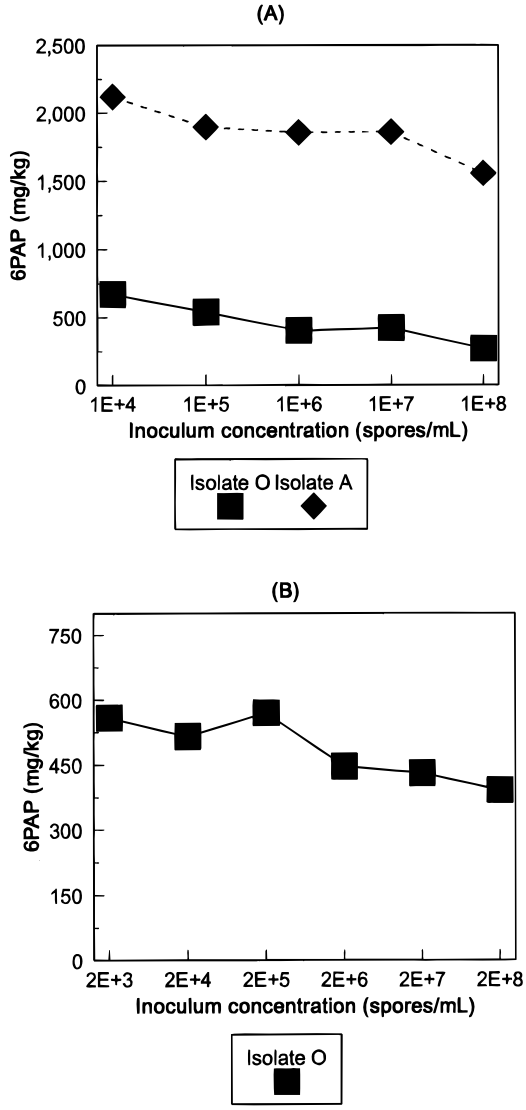

Figure 5. Effect of spore inoculum on 6PAP production for *Trichoderma* isolates grown on ground corn supplemented with solution 7 for (A) isolates A and O inoculated at concentrations over a range of 1×10^4 up to 1×10^8 spores/mL and (B) isolate O inoculated at concentrations over a range of 2×10^3 up to 2×10^8 spores/mL.

ca. 800 mg/kg maximum at day 19 (Cooney et al., 1997), it is apparent that, in addition to the higher 6PAP yields for solution 7, the harvest time window is wider and slightly earlier. The drop-off in 6PAP levels with extended incubation time is also less marked.

There was a more pronounced difference in the 6PAP production time profile for isolate B when using solution 7 compared to earlier results obtained with a 10% yeast extract solution supplement (Cooney et al., 1997). While 6PAP was again detected after 4 days and appeared to plateau at 400−500 mg/kg from days 10 to 14, from days 15 to 28 the level of 6PAP increased to about 900 mg/kg (Figure 2). This is in contrast to the marked decline in 6PAP levels to below 100 mg/kg recorded over this time interval when 10% yeast extract solution was employed as the supplement (Cooney et al., 1997).

Effect of Temperature. Figure 3 shows comparative 6PAP yield data for six *Trichoderma* isolates cultured at 15, 20, and 25 °C. Three of the isolates, A, C, and F, produced best at 20 °C, while isolate B performed markedly better at 15 °C. Production data by isolates M and I were similar at 15 and 20 °C. For all of the isolates examined, the 6PAP yield declined at 25 °C despite fungal growth being initiated earlier and more luxuriantly at this temperature.

Effect of Light. Results for the first experiment showing comparative effects of light and dark on 6PAP production are presented in Figure 4A for isolate A and in Figure 4B for isolate B cultured on both ground corn and whole corn media. Harvest times for the two isolates were chosen in accord with the time windows suggested from earlier harvest studies carried out using a ground corn matrix and 10% yeast extract solution supplement (Cooney et al., 1997). For isolate A yields were always higher when the *Trichoderma* was cultured in the light on ground corn for each of the three liquid supplements. Whole corn "light" cultures also gave higher levels of 6PAP than those cultured in the dark. The trends are more difficult to determine for isolate B, but indications are that this isolate produces slightly more 6PAP when cultured in the dark. For both isolates, production of 6PAP on whole corn was poor compared with the yields obtained on ground corn. Whether this is due to the breaking of the epidermis, allowing access to a more readily metabolized nutrient source, or just increased surface area for the latter was not investigated. It is also interesting to note that for both isolates, yields on whole corn supplemented with

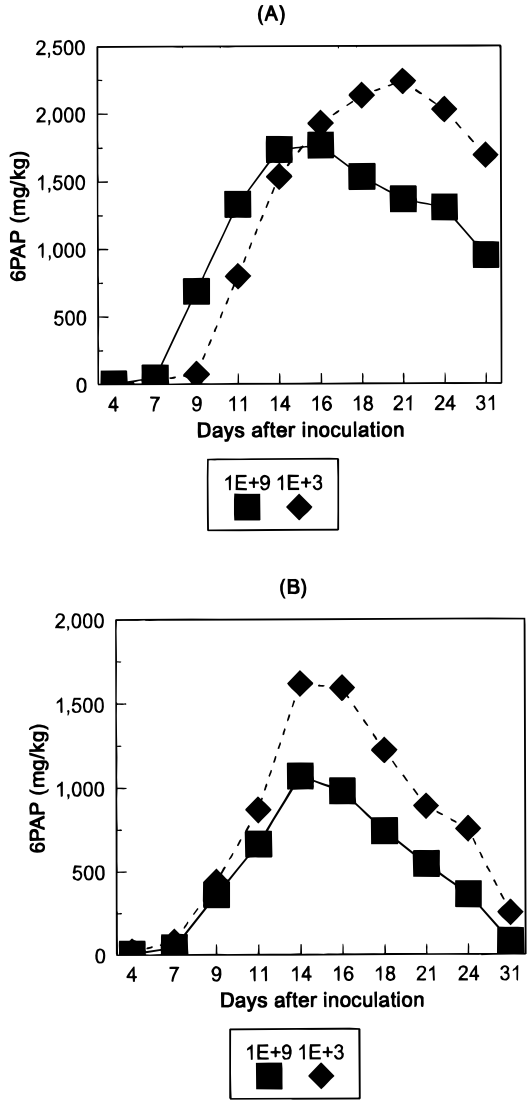

Figure 6. Production of 6PAP with time by *Trichoderma* isolates grown on ground corn supplemented with solution 7 and inoculated with spore suspensions of concentration 1×10^3 or 1×10^9 spores/mL for (A) isolate A and (B) isolate O.

10% yeast extract solution were particularly poor in both the presence and absence of light.

Results for the second experiment were effectively the same as for the first experiment except that, for isolate B, results were improved for the whole corn treatments, although 6PAP levels were still generally <50% of those for the ground corn treatments.

Effect of Spore Inoculum Concentration. Results for the first experiment showing 6PAP yield data for isolates A and O inoculated at a range of spore inoculum concentrations are presented in Figure 5A. There is a trend toward increased levels of measured 6PAP as the spore count of the inoculum is decreased. This effect was more apparent for isolate O than for isolate A. At the lowest spore inoculum concentration, for which the highest 6PAP levels are recorded, the visible fungal growth on the solid matrix was very sparse, particularly for isolate O. These results imply that a dense luxuriant mat of fungal growth is not a necessary prerequisite for high levels of secondary metabolite production, an observation that was also noted for the temperature study.

Results for the second spore inoculum concentration experiment presenting 6PAP production levels for isolate O only are shown in Figure 5B. While the trend found in the first experiment is still valid, the effect is not as marked in this experiment as in the earlier one.

Effect of Time and Spore Inoculum Concentration. The 6PAP time harvest results for high and low spore inoculum cultures are presented in Figure 6, parts A and B for isolates A and O, respectively.

For isolate A the measured levels of 6PAP at the low inoculum concentration reached a maximum at day 21 as compared to day 16 for the high spore inoculum cultures. Again, the maximum recorded levels of 6PAP are higher for the low spore inoculum cultures, although it can be seen from Figure 6A that this increase was not achieved until about day 16, when maximum production occurred for the higher spore concentration. In effect, the 6PAP concentration profile for the low spore inoculum cultures has been shifted to the right, representing a time delay in maximum 6PAP production of almost a week.

For isolate O, the 6PAP concentration profile remained unchanged with respect to time, with both high and low spore inoculum cultures recording maximum 6PAP levels at day 14. As observed in the earlier experiments, 6PAP was afforded in higher concentration for the low spore inoculum cultures.

The time harvest window for isolate O was narrower than for isolate A and occurred slightly earlier. For isolate A, the spore inoculum concentration affected both the optimum harvest time and the measured 6PAP concentration, while for isolate O, only the 6PAP concentration was affected.

The results of our studies indicate that solid state fermentation of *Trichoderma* on a corn medium is capable of supplying 6PAP in good yield. Attention to isolate selection, harvest time, culture temperature, light conditions, and spore inoculum concentration can enhance metabolite production, resulting in yields of 6PAP as high as 2000 mg/kg. These data, combined with the results from earlier production studies that identified ground corn as a preferred growth medium (Cooney et al., 1997), will provide a basis for future *Trichoderma* bulk scale fermentation studies.

ACKNOWLEDGMENT

We thank Tania Trower and Colin Malcolm for technical assistance.

LITERATURE CITED

Atlas, R. M. *Handbook of Microbiological Media*; Parks, L. C., Ed.; CRC Press: Boca Raton, FL, 1993.

Baker, R. *Trichoderma* spp as plant growth regulators. *CRC Crit. Rev. Biotechnol.* **1988**, *7*, 97−106.

Bengtson, G.; Boeddeker, K. W.; Hanssen, H. P.; Urbasch, I. Recovery of 6-pentyl-α-pyrone from *Trichoderma viride* culture medium by pervaporation. *Biotechnol. Tech.* **1992**, *6*, 23−26.

Chet, I. *Trichoderma*—application, mode of action, and potential as a biocontrol agent of soilborne plant pathogenic fungi. In *Innovative Approaches to Plant Disease Control*; Chet, I., Ed.; Wiley: New York, 1987; pp 137−160.

Cooney, J. M.; Lauren, D. R.; Jensen, D. J.; Perry-Meyer, L. J. Effect of solid substrate, liquid supplement and harvest time on 6-*n*-pentyl-2*H*-pyran-2-one (6PAP) production by *Trichoderma* spp. *J. Agric. Food Chem.* **1997**, *45*, 531−534.

Cutler, H. G.; Hill, R. A. Natural fungicides and their delivery systems as alternatives to synthetics. In *Biological Control of Postharvest Diseases Theory and Practice*; Wilson, C. L., Wisniewski, M. E., Eds.; U.S. Department of Agriculture: Kearneysville, WV, 1994; pp 135−152.

Dennis, C.; Webster, J. Antagonistic properties of species-groups of *Trichoderma*. I. Production of non-volatile antibiotics. *Trans. Br. Mycol. Soc.* **1971a**, *57*, 25−39.

Dennis, C.; Webster, J. Antagonistic properties of species-groups of *Trichoderma*. II. Production of volatile antibiotics. *Trans. Br. Mycol. Soc.* **1971b**, *57*, 41−48.

Dieter, R. K.; Fishpaugh, J. R. Synthesis of α-pyrones from vinylogous thiol esters and α-oxo ketene dithioacetals. *J. Org. Chem.* **1988**, *53*, 2031−2046.

Ghisalberti, E. L.; Sivasithamparam, K. Antifungal antibiotics produced by *Trichoderma* spp. *Soil Biol. Biochem.* **1991**, *23*, 1011−1020.

Ghisalberti, E. L.; Narbey, M. J.; Dewan, M. M.; Sivasithamparam, K. Variability among strains of *Trichoderma harzianum* in their ability to reduce take-all and to produce pyrones. *Plant Soil* **1990**, *121*, 287−291.

Henis, Y. Biological Control. In *Current Perspectives in Microbial Ecology*; Klug, M. J., Reddy, C. A., Eds.; American Society for Microbiology: Washington, 1984; pp 353−361.

Klaiber, E. M.; Pittet, A. O. Process for preparing substituted alpha-pyrones. U.S. Pat. 3,873,574, March 1975.

Klaiber, E. M.; Pittet, A. O. Keto-ester composition. U.S. Pat. 3,946,054, March 1976.

Lynch, J. M. Fungi as antagonists. In *New Directions in Biological Control: Alternatives for Suppressing Agricultural Pests and Diseases*; Liss: New York, 1990; pp 243−253.

Nobuhara, N. Syntheses of unsaturated lactones III. Flavorous nature of some δ-lactones having the double bonds at various sites. *Agric. Biol. Chem.* **1969**, *33*, 1264-1269.

Papavizas, G. C. *Trichoderma* and *Gliocladium*: biology, ecology and potential for biocontrol. *Annu. Rev. Phytopathol.* **1985**, *23*, 23−54.

Pittet, A. O.; Klaiber, E. M. Synthesis and flavour properties of some alkyl substituted α-pyrone derivatives. *J. Agric. Food Chem.* **1975**, *23*, 1189−1195.

Tekin, A. R.; Oener, M. D.; Kaya, A. Production of coconut-like aroma by *Trichoderma viride* in aqueous and two-phase fermentation. *Turk. J. Eng. Environ. Sci.* **1995**, *19*, 247−251.

Worasatit, N.; Sivasithamparam, K. Fungi associated with mycelium of *Rhizoctonia solani* in bare patches of wheat. *Phytopathology* **1990**, *80*, 1049 (Abstr.).

Worasatit, N.; Sivasithamparam, K.; Ghisalberti, E. L.; Rowland, C. Variation in pyrone production, lytic enzymes and control of rhizoctonia root rot of wheat among single-spore isolates of *Trichoderma koningii*. *Mycol. Res.* **1994**, *98*, 1357−1363.

Zeppa, G.; Allegrone, G.; Barbeni, M.; Guarda, P. A. Variability in the production of volatile metabolites by *Trichoderma viride*. *Ann. Microbiol.* **1990**, *40*, 171−176.

Received for review January 27, 1997. Revised manuscript received April 28, 1997. Accepted April 29, 1997.[⊗] Funding from the NZ Foundation for Research Science and Technology in the form of a Postdoctoral Scholarship for J.M.C. is gratefully acknowledged.

JF970086O

[⊗] Abstract published in *Advance ACS Abstracts,* June 1, 1997.