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Potential for the Large-Scale Production Of a Biocontrol Fungus In Raw and Composted Paper Mill Waste

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The growth and survival of a *Trichoderma spp*. (Td_{22}) antagonistic to *Sclerotinia minor* Jagger and *Sclerotium cepivorum* Berk was studied in raw wood fibre waste (WFW) of paper mill origin and in mature compost of this material. In nutrient-amended, sterilized WFW or WFW compost (both supplemented with 20% w/w millet seed), the biocontrol fungus reached densities in the order of 10^{10} colony forming units (cfu)/g after 14 days incubation. Lower population densities of Td_{22} were achieved under non-sterile conditions in the compost:millet mix of between 10^7-10^9 cfu/g after 28 days, depending on pretreatment. Viable spore density of Td_{22} in raw WFW amended with nutrients and 20% w/w millet seed reached approximately 10^{10} cells/g after 14 days incubation. This study indicates that cellulosic paper mill waste could provide an abundant low-cost growth medium for the large-scale culture of this or other biocontrol fungi.

Introduction

Problems of storage of microbial antagonists to plant pathogens have been largely overcome with the demonstrated maintenance of bacterial antagonists in matrices such as peat and vermiculite for 5 months or more (e.g. Vidhyasekaran *et al.* 1997; Gazoni *et al.* 1998). However the large-scale growth of these antagonists can be problematic because of the high cost of growth media, particularly where a large inoculum of biocontrol agent is needed. Cost of the cultivation medium is presently a critical factor in any assessment of merit relative to chemical treatments, a problem sometimes exacerbated by the perceived need for proprietary media formulations.

The use of compost to suppress plant diseases has been extensively examined, with recent reviews of the topic by e.g. Craft & Nelson (1996) and Hoitink, *et al.* (1997). Use of compost as a growth-medium for microbial antagonists rather than a storage material or an agent of non-specific inhibition, is however, very recent. An indication of the potential for manipulating the microbiota of compost to advantage was provided by Ramamurthy *et al.* (1996), who demonstrated that composting of eucalypt sawdust inoculated with the mushroom fungus *Volvariella* resulted in a product which enhanced the growth of wheat seedlings. Likewise Nakasaki *et al.* (1998) successfully cultivated a biocontrol *B. subtilis* to high population levels in composted grass clippings, with subsequent demonstration of the effectiveness of the modified compost against *Rhizoctonia* large patch disease of turf grass.

A crucial factor for the large-scale production of microbial antagonists in compost or other growth media, is their cultivation and survival in cell densities sufficient for end-use effectiveness, with Kodiak® (containing *Bacillus subtilis* effective against *Rhizoctonia spp*. and *Fusarium spp*. in cotton) being one of the few such products marketed in the USA (Brannen and Kenedy 1997).

Td_{22} (Metcalf, 1997a) is a *Trichoderma sp*. isolate known to give excellent field control of *Sclerotinia minor* Jagger and *Sclerotium cepivorum* Berk, the causative agents of lettuce drop and white-rot of onions respectively. Td_{22} is normally cultivated on auto-

claved moist millet seeds or on citrus pectin agar. Although its efficacy in field experiments has been amply demonstrated, its large-scale cultivation for agricultural application has been limited by cost considerations.

In the present study, raw and composted WFW was investigated for possible use as a base medium for Td_{22} cultivation and carriage. Cellulosic waste was considered worthy of examination in view of its attributes of good water-holding/aeration capacity and its potential utilization as a source of energy and carbon by Td_{22}.

Materials and Methods

Fletcher Challenge Mill WFW

Norske Skog Paper Mills Limited, Tasmania, landfills approximately 33,000 tonnes of WFW p.a. The material is of mixed eucalypt and *Pinus radiata* origin, comprising holocellulose as its primary constituent, with very low levels of metal contaminants and being deficient (from a recycling perspective) in N and P. In concentrated form it is phytotoxic to seeds, although the potential for its recycling to the field following composting was demonstrated by Jackson and Line (1997).

A compost was produced from this material by amending mill waste with single-superphosphate, urea and KNO_3 to give a C:N:P:K ratio of 35:1:0.6:0.1. This mix was composted at ambient temperatures for three months, at which time no residual toxicity was apparent to radish seed germination and growth.

Growth of Td_{22} Inoculum

Td_{22} inoculum was grown in sterile citrus pectin broth medium (pH 4.5) comprising (gL^{-1} distilled water): $NH_4H_2PO_4$, 0.9; $(NH_4)_2HPO_4$, 2.0; $MgSO_47H_2O$, 0.1; KCl, 0.5; citrus pectin, 10.0 (Metcalf, 1997b). Flasks (500mL) of inoculated medium (100 mL) were shaken for 7 days in a water bath at 25°C. Before use the potency of this inoculum, measured in cfu on pectin agar (the above medium solidified with 1.5% w/v agar), was determined by serial dilution plating (in triplicate).

Assessment of Growth of Td_{22} Under Sterile Conditions

Growth of Td_{22} was assessed in raw WFW, WFW composted for 3 months, millet seed, as well as mixes of WFW plus millet seed and composted WFW amended with either potato starch or Phostrogen® (a NPK-minerals formulation produced by Phostrogen Ltd, Australia). Unless stated otherwise these materials (100g in 750mL flasks, 3 replicates/treatment) were brought to field capacity (amounting to approximately 1.5L/kg for the 80:20 WFW/millet mix) with a solution containing (gL^{-1} distilled water): NH_4NO_3, 5.0; K_2HPO_4, 2.0; $MgSO_4.7H_2O$, 0.2; $CaCl_2.2H_2O$, 0.01; $FeCl_3$, 0.01, and autoclaved at 121°C for 30 minutes on each of two consecutive days. All flasks were inoculated with 10mL of Td_{22} suspension described above to give an initial density of between 3.7–5.5 $\log_{10}$ cfu/g dry mix. Inoculated mixes were incubated at 25°C for 4 weeks with periodic assay of growth of Td_{22} following dilution plating onto Oxoid Potato Dextrose Agar (PDA).

The relative biomass of Td_{22} under sterile conditions was assessed using the assay for chitin described by Chen and Johnson, 1983. Microscopic estimation of spore numbers in the various mixes was made using a haemocytometer. All estimates of cfu/g, spores/g or chitin biomass/g are given on a dry weight basis (100°C to constant weight).

Assessment of the Growth of Td_{22} Under Nonsterile Conditions

Air-dried WFW compost (dried to ambient moisture content under glasshouse conditions) was brought to field capacity with 2% w/v Phostrogen® solution and 100g quantities placed into 750mL flasks. The compost was then either autoclaved at 121°C for 5 minutes prior to inoculation, or directly inoculated with Td_{22} suspension described above to give 5.4 $\log_{10}$ cfu/g compost. Flasks were incubated at 25°C for 4 weeks with periodic assay of cfu on PDA for Td_{22} and on Trypticase Soy Agar (TSA) for bacteria [TSA comprising (gL^{-1} distilled water): Oxoid Trypticase Soy Broth Powder, 3.0; Oxoid yeast extract, 1.0; Davis agar, 15.0]. The identity of representative Td_{22} colonies was confirmed by isoenzyme analysis of pectic enzymes as described by Cruickshank and Pitt, 1987.

Glasshouse Assay of Effectiveness of WFW-Grown Td_{22}

Effectiveness of the 14-day culture of Td_{22} in an 80:20 w/w WFW compost:millet seed mix in protecting against *Sclerotinia minor* challenge was assessed in a pot-trial. Treatments of 10% and 20% v/v culture in soil were used with appropriate controls. Each treatment comprised five replicate pots. *S. minor* inoculum was mixed 20mm beneath the soil surface prior to adding 5 lettuce seeds per pot. Plants were assessed for health at weekly intervals for 4 weeks.

Statistical Analysis

Analysis of variance (ANOVA) of data obtained from this study was carried out using Minitab software for Windows. The significance of differences between means ($p < 0.05$ in all cases where significance is reported) was tested using the least significant difference (LSD) test following ANOVA.

Results

Growth of Td_{22} in Various Mixes under Sterile Conditions

Td_{22} grew well in all mixes tested except in 100% millet seeds, in which cfu fell after 14 days of incubation (Figure 1). The best growth response was observed in either raw or composted WFW supplemented with nutrients and 20% (w/w) millet seed, plateauing at 14 days incubation (at 10.3 and 9.9 $\log_{10}$ cfu/g respectively). Both results were significantly higher than those for a nutrient supplemented compost control after 14 or 28 days. Likewise, nutrient-supplemented WFW compost containing 50% w/w millet seed or 1% w/w starch did not improve the growth (cfu) of Td_{22} over the unamended control. Replacing the mineral nutrient supplements to WFW compost with 2% w/v Phostrogen® resulted in a very similar growth response (cfu) by Td_{22} (mix A cf. Mix F).

Haemocytometer assay of spore numbers in the raw 80%WFW:20% millet seed mix (showing the best growth of Td_{22}) indicated these to be of the same order of magnitude as the cfu determined from dilution plate counts (Figure 2). Total estimated spore numbers in other mixes were significantly lower than the cfu.

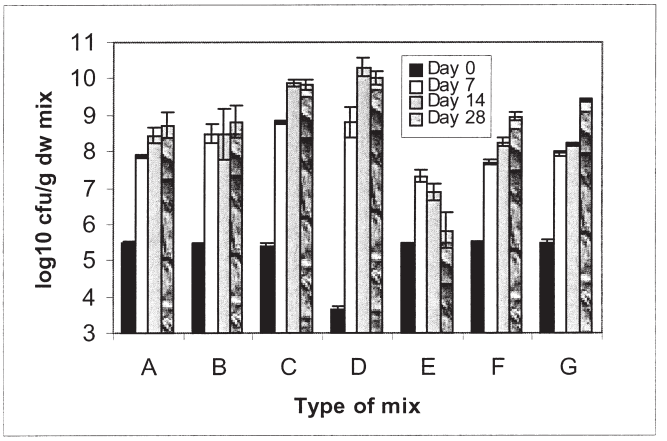

Figure 1. Growth of Td_{22} in various mixes under sterile conditions. Each value is an average of 3 replicates ± standard error. Initial and final pH levels (respectively) are indicated in brackets
Mix A: WFW compost (4.6-5.4)
Mix B: 50% WFW compost + 50% millet seed w/w (4.8-7.5)
Mix C: 80% WFW compost + 20% millet seed w/w (5.3-5.5)
Mix D: 80% Raw WFW + 20% millet seed w/w (5.0-5.5)
Mix E: Millet seed (5.6-7.8)
Mix F: WFW compost re-wetted with 2% w/v Phostrogen® solution (4.9-5.6)
Mix G: WFW compost amended with 1% w/w potato starch (4.7-5.4).

Biomass of Td_{22} in the various mixes after prolonged incubation (10 weeks) as determined from chitin contents, indicated levels of approximately 50-60 mg/g mix except for mixes F and G, which contained about 35 mg/g mix.

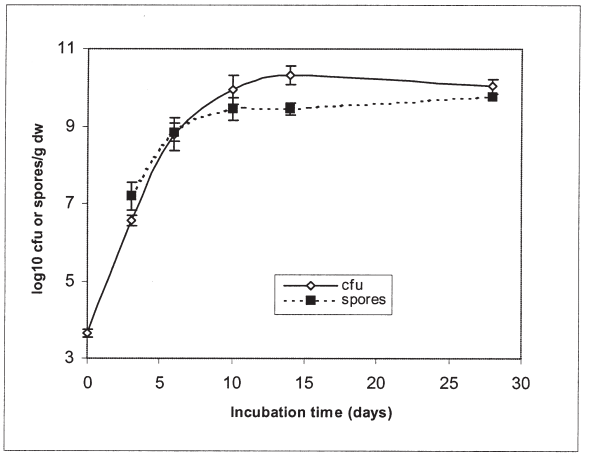

Figure 2. Growth of Td_{22} in raw WFW:millet (80:20% w/w) mix measured as cfu/g or spore density/g. Each value is an average of 4 replicates ± standard error.

Growth of Td_{22} in Nonsterile Compost

The growth of Td_{22} was followed for 28 days in non-sterile, steam-treated or air-dried composts, equivalent in other respects to mix F (Figure 1), after which time 8.6 $\log_{10}$ cfu/g and 7.1 $\log_{10}$ cfu/g were recorded in the respective mixes. The identity of

Td_{22} in the air-dried compost was confirmed by both morphological characteristics and isoenzyme profile. Over the same period the populations of mesophilic bacteria in the mixes reached 9.3 and 8.5 $\log_{10}$ cfu/g respectively.

Glasshouse Assay of Effectiveness of WFW-Grown Td_{22}

No toxicity to the germination (100%) or growth of lettuce seedling was observed in mixes of 10% or 20% v/v of composted WFW-millet culture mixed with soil. Challenge with *Sclerotinia minor* resulted in 100% mortality in soil-only pots after 4 weeks, compared with 8% mortality in the 10% v/v WFW-culture:soil mix and 0% mortality in the 20% v/v WFW-culture:soil mix.

Discussion

This investigation has demonstrated the potential use of WFW as a growth medium for the large-scale cultivation of spores of a biocontrol fungus, Td_{22}, known to be particularly effective against *Sclerotinia minor* and *Sclerotium cepivorum*, with cfu reaching 10^{10}/g culture in 14 days. Composting the WFW was initially thought to be advantageous to avoid deleterious effects on plant seeds (the original material was toxic to radish seedlings) and to provide nutrient balance. However it appears to be unnecessary for the purpose described; nutrient-amended, raw WFW-Td_{22} culture after 14 days gave comparable cfu to that of the composted equivalent, with no toxicity to radish seed germination evident at a high rate of application (20% v/v). After 14 days incubation the biocontrol agent in the raw WFW medium appeared to be largely converted to spores, auguring well for long-term survival in cultivation medium prior to application.

Except for mixes F and G, similar chitin contents were found in all mixes after 10 weeks incubation indicating similar hyphal abundance at this time, with presumably similar potential for spore production. From a production potential however the cfu produced in the different mixes after 14 to 28 days is of greater interest.

Air drying compost in the glasshouse for 3 weeks or brief steam-treatment (rather than sterilizing) (Nakasaki *et al.* 1998) with a view to minimizing the indigenous biota prior to inoculation with Td_{22} gave encouraging results, with two to three orders of magnitude increase in cfu. Possibly better alternatives such as solarisation (McLean *et al.* 2001) known to be effective for eliminating pathogens in glasshouse soil have yet to be examined.

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