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Use of Wood Fibre Compost for the Cultivation of *Trichoderma* sp. (Isolate Td_{22})

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Abstract: The main objective of this research was to investigate the ability of a *Trichoderma* sp. (Td_{22}), inhibitory to *Sclerotinia minor* Jagger, to grow and survive in mature wood fibre waste (WFW) compost of paper mill origin following nutrient amendment. The growth and survival of the fungus in the WFW compost was assessed by serial dilution plate count method followed by confirmation of the fungal identity using pectic enzyme analysis as described in Cruickshank and Pitt [1]. It was found in this study that the population densities of Td_{22} achieved under non-sterile conditions in the WFW compost following nutrient amendment was approximately in the range of 7.0 $\log_{10}$ cfu/g dw – 8.5 $\log_{10}$ cfu/g dw after 28 days, depending on pre-treatment. The efficacy of this WFW compost-grown Td_{22} for protection of lettuce from attack by *S. minor* was also demonstrated in glasshouse trials. This study indicates that cellulosic paper mill waste compost could provide an abundant low-cost growth medium for the large-scale cultivation of fungal antagonists, improving prospects for cost-competitiveness with chemical treatments.

Key words: Compost, biological control, *Trichoderma* sp., *Sclerotinia minor*.

1. Introduction

High concentration of pesticide usage often leads to increased pathogen resistance, resulting in ever higher pesticide applications in order to kill the same pathogen [2-4]. Furthermore, pesticide residue can have adverse effects on reproduction and developmental processes in a variety of animals including humans [5-7]. Due to these detrimental effects, reduced pesticide usage in agricultural practice has been championed [8-9] and one of the best broad spectrum pesticide/fumigants (methyl bromide) is no longer used in developed countries. Alternatives have been proposed, but none appears to be as effective [10]. In anticipation of such future reduction, Painuly and Dev [11] proposed some possible alternative control, such as biological control, use of pathogen-resistant plant varieties, and integrated pest management.

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Problems related to the large-scale production of biological control agents in low cost materials include difficulties in handling, transport, and storage. These problems have been largely overcome by maintenance of bacterial antagonists in carriers such as peat or vermiculite for periods of five months or more [12-14]. Cost of the cultivation medium, its transport, and field dispersal are critical factors in any assessment of biological control relative to chemical treatments, a problem sometimes exacerbated by the perceived need for proprietary media formulations.

Norske-Skog Paper Mill Limited produces approximately 33,000 tonnes of wood fibre waste (WFW) per annum, to be dumped as landfill [15].

This cellulosic waste was considered worthy of examination in view of its attributes of excellent water-holding and/or aeration capacity, its freedom from toxic elements, its free availability, and its potential utilization as a source of energy and carbon by cellulose-utilizing fungi, including the *Trichoderma* sp.

isolate Td_{22}.

Based on the above background, the potential use of wood fibre waste compost as a possible substrate for the low-cost cultivation of a fungal biological control agent (*Trichoderma* sp. isolate Td_{22}) was investigated, together with assessments of the efficacy of the compost-grown Td_{22} to protect lettuce seedlings from attack by *S. minor* in pot trials or glasshouse level experiments.

2. Materials and Methods

2.1 Isolate Td_{22}

Trichoderma sp., isolate Td_{22} was kindly provided by Dr. Dean Metcalf (DPIWE Tasmania). For long term storage, the fungus was maintained at 4 °C in sterilized and moist millet seeds.

2.2 Wood Fiber Waste

The WFW used in this study was the same as that used by Ramona and Line [15]. It is a mix of eucalypt and *Pinus radiata*, comprising holocellulose as its primary constituent with very low levels of metal contaminants. It was also deficient (from a recycling perspective) in N and P. In the present trial, composting of this material was for three months [followed the method specified by Jackson and Line [16] and Jackson [17]] until its toxicity to radish seeds was eliminated.

2.3 Inoculum Preparation

The active starters used in this experiment were prepared according to the methods as specified in Ramona [18]. The potency of this inoculum, measured in cfu, was determined by serial dilution plating (in triplicates) before being used to inoculate the WFW.

2.4 Assessment of Growth of the Td_{22}

The WFW compost (at a self-generated temperature of 55-60 °C) was air-dried in a glasshouse, mixed with 2% (w/v) Phostrogen® solution, and placed into 750 mL flasks (100 g quantities each). These flasks were then either briefly autoclaved for 5 minutes prior to inoculation (Compost A), or inoculated directly with inoculum (Td_{22} suspension described above) (Compost B) to give an initial density of 5.5 $\log_{10}$cfu/g compost. Incubation was at 25 °C for 4 weeks with periodic assay of cfu on Potato Dextrose Agar (PDA) for Td_{22} and on 0.1% Trypticase Soya Agar (TSA) for check for bacterial contaminants. The identity of the Td_{22} was confirmed by analysis of pectic enzymes using the method as described by Cruickshank and Pitt [1].

2.5 The Efficacy of WFW Compost-Grown Td_{22}

Lettuce seeds were sown in pots containing field soil amended with suppressive compost (Compost A) at levels of 2.5, 5.0, 10 and 20% v/v.

The spore density of the antagonist (Td_{22}) in the compost was 9.21 $\log_{10}$ spores/g wet weight of compost. Lettuce pathogen (*S. minor*) grown on millet seed was evenly inoculated approximately 20 mm below the soil surface at the rate of 2.0 g inoculum per pot. Soil without compost amendment, amended with pathogen only, or without pathogen, served as controls. Five replicate pots per treatment, each containing five seeds, were maintained for four weeks in a shade house. Pots were irrigated as required and the germinated seeds counted one week after sowing. Disease incidence on the lettuce seedlings was recorded from two weeks after sowing.

2.6 Data Analysis

The data obtained from this experiment was statistically analyzed by using analysis of variance (ANOVA) using Minitab software for Windows. The significance of differences between means was further tested using the least significant different (LSD) test at $P < 0.05$ following ANOVA.

3. Results

3.1 The Growth and Survival of Td_{22} in WFW Compost

The growth and survival of Td_{22} in compost over 28

days following different pre-treatments is presented in Fig. 1. Significant increases in cfu of Td_{22} in steam-treated or untreated composts were observed in the first seven days, after which time the populations remained relatively constant at approximately 8.5 log_{10}cfu/g dw in compost A or approximately 7.0 log_{10}cfu/g dw in compost B.

The identity of Td_{22} cultivated in non-sterile compost was confirmed by both morphological characteristics and isozyme profile (Fig. 2).

The initial bacterial populations (contaminants) in the mixes depended on the method of preparation. The population of the mesophilic bacteria in compost A was recorded at approximately one order of magnitude lower than that in compost B after two weeks incubation. However after four weeks of incubation, the growth rate of the indigenous mesophilic bacteria in compost A exceeded that of compost B, to result in a population of 9.3 log_{10} cfu/g in compost A, in comparison with 8.5 log_{10} cfu/g in compost B.

3.2 The Efficacy of Td_{22}-Grown Compost to Protect Lettuce from S. minor Attack

The Td_{22}-grown compost was found to be non toxic as between 88-100% of the lettuce seeds were observed to germinate in soil amended with 5-20% (v/v) of this compost. No inhibition of seed germination was evident even higher rate of Td_{22}-grown compost was applied 4 weeks after sowing.

The effectiveness of the Td_{22}-grown compost

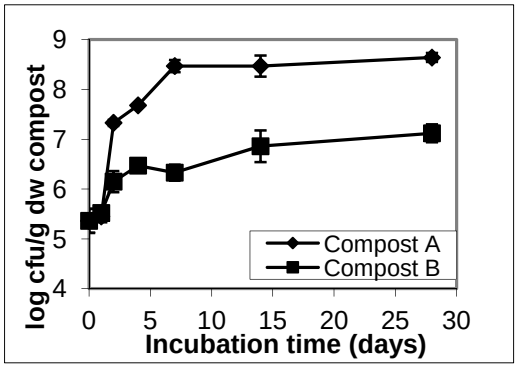

Fig. 1 Population density of *Trichoderma* spp. (isolate Td_{22}) in three months old WFW compost, either briefly treated for 5 minutes at 121 °C prior to inoculation (Compost A), or directly inoculated following re-wetting (Compost B). Both compost types were brought to approximately field capacity with 2% (w/v) Phostrogen® solution (~350 mL/L). Each value in the graph is an average of three replicate determinations ± standard error.

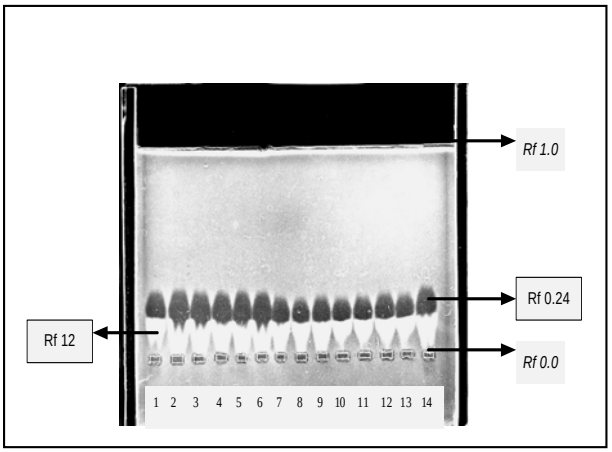

Fig. 2 Contact print following polyacrylamide-gel electrophoresis to confirm survival of Td_{22} in three-month old WFW compost. Dark bands indicate polygalacturonase and light bands indicate pectinesterase. Wells 1, and 9 – 14 contained the control reference, Td_{22}, while wells 2 – 8 contained re-isolated fungi from compost B on day 7 of sample collection.

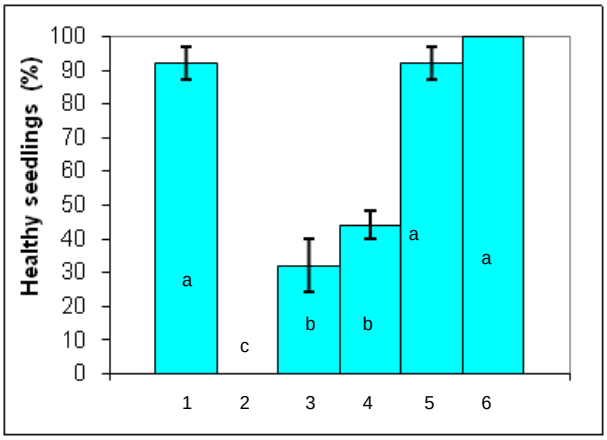

Fig. 3 Protection of lettuce seedlings from attack by *S. minor*. Each value in the graph ± standard error bar is an average of 5 replicates. Bars with the same letter are not statistically significant at $P < 0.05$.

1. Nil pathogen and antagonist addition; 2. Pots amended with *S. minor* only; 3. Pots amended with 2.5% compost-grownTd_{22} and *S. minor*; 4. Pots amended with 5% compost-grown Td_{22} and *S. minor*; 5. Pots amended with 10% compost-grown Td_{22} and *S. minor*; 6. Pots amended with 20% compost-grown Td_{22} and *S. minor*.

The effectiveness of the Td_{22}-grown compost

amendment to protect lettuce seedlings from attack by *S. minor* is presented in Fig. 3. As indicated in Fig. 3, the rate of amendment of the compost/fungus was proportional to disease control, ranging from 32% provided by 2.5% (v/v) amendment to 100% protection provided by 20% (v/v) amendment at four weeks after sowing. This was statistically significant when compared to the control treatment (pots inoculated with *S. minor* only) where 100% mortality was observed at four weeks after sowing (Fig. 3).

4. Discussion and Conclusion

Air drying compost in a glasshouse for three weeks or brief steam-treatment (rather than sterilizing) with a view to minimizing the indigenous microbiota, prior to inoculation with Td_{22}, gave encouraging results. The cfu of this fungus cultured under non-sterile conditions increased by two to three orders of magnitude, although they were always lower than those obtained under sterile conditions (Fig. 1). The cfu were found to be higher in the briefly autoclaved compost (compost A) than those in the directly inoculated compost (compost B) following re-wetting. Application of heat treatment to the WFW compost (by briefly autoclaving at 121 °C) may have changed its composition. However the most probable reason for the better growth of Td_{22} in this medium was the near-elimination of competition by mesophilic bacterial survivors in compost A relative to compost B. Similar results were also demonstrated by Nakasaki et al. [19] who found a reduced growth rate of a strain of *Bacillus subtilis* following inoculation of this antagonist into non-sterile (as compared with sterile), but freshly-cut grass clipping compost. This reinforces the notion that competition between the indigenous microbiota and the inoculated antagonists is important in the directed establishment of such antagonists in a compost matrix.

The ability of *Trichoderma* spp. to produce cellulase and pectolytic enzymes illustrated in Figure 2 has been reported previously by Metcalf [20], Oksanen et al. [21], Domingues et al. [22], and Lee et al. [23]. These and other enzymes are important in the degradation of complex carbon sources contained in WFW and the ability to produce them is advantageous when in competition for available major energy sources.

Assay of isoenzyme profiles (e.g. pectolytic enzyme profiles as undertaken in the present study) of fungal isolates provided a convenient tool to differentiate Td_{22} from other isolates of *Trichoderma* spp. following re-isolation from non-sterile samples. This assay has been applied by many workers [24-26] to distinguish their fungal isolates from other related fungi.

The effectiveness of *Trichoderma* sp. (Td_{22}) grown in wood fibre waste compost to protect against *S. minor* in glasshouse trials has been demonstrated in the present study. Application of compost-grown Td_{22} at rates up to 20% (v/v) was non-toxic (relative to controls) to lettuce seed in all treatments (Fig. 3).

The utilization of composted WFW as we describe could be achieved in large scale at moderate cost while avoiding the problems of liquid cultures as outlined by Hadar et al [27]. Application of compost-grown Td_{22} after allowing growth for two weeks or more appeared to be advantageous, because it was mostly in the form of spores (unpublished data). This reduces concerns relating to viability in the field, because fungal spores will be relatively more resistant than mycelia to environmental stress [28].

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