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Growth Response of Bacterial Antagonists in a Mix of Composted Wood Fibre Waste and Millet Seed under Sterile and Non-sterile Conditions

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Abstract: The potential use of composted wood fibre waste (WFW) for the cultivation of bacterial antagonists of *Sclerotinia minor* was examined with the result that a mix of millet seed (20% w/w) and WFW, suitably amended with nutrients, proved to be an ideal matrix for the growth of some of these bacteria. Densities in terms of cfu's ranged from 8.5 $\log_{10}$ cfu/g dw to 10.5 $\log_{10}$ cfu/g dw under sterile conditions after 14 days incubation. Lower population densities of the antagonists were achieved under non-sterile conditions in the compost: millet mix of between 7.9-9.3 $\log_{10}$ cfu/g dw at the same period. However, when applied in a pot (glasshouse) trial to protect against *S. minor,* the millet seed appeared to stimulate the growth of this pathogen resulting in a high incidence of attack of lettuce plants after 2-3 weeks. Although the percentage of healthy seedlings increased following application of compost mix grown antagonists (at a rate of 5% v/v) when compared to the control treatment, these values were not statistically significant ($p>0.05$) in most cases. Therefore, the use of millet seeds cannot be recommended as a nutrient supplement for the bacterial antagonist cultivation, if to be subsequently used to control fungal pathogens in the field.

Key words: *Sclerotinia minor, Pseudomonas corrugata, Lysobacter antibioticus*, wood fibre waste, compost, bacterial antagonists.

1. Introduction

Attempted manipulation of composting conditions or of mature compost for the cultivation of desired microorganisms has been slow to develop; particularly difficult is manipulation of non-sterile compost due to the high biological buffering provided by a diverse microbiota, combined with the problems posed by a microbial succession as temperatures change from mesophilic to thermophilic and back again, as occurs in the compost habitat. As reported by Nakasaki *et al*. in 1998, one of the first successful demonstrations of the directed cultivation of bacteria in compost followed the inoculation of pasteurized grass clippings with a *Bacillus subtilis* strain known to be an agent of biological control [1]. The bacterium grew to concentrations of 10^8 cfu/g dw before sporulating, the spores surviving subsequent high temperature composting to inhibit *Rhizoctonia* large patch disease of grass. Compost that was not inoculated showed no disease suppressive effects. Other less successful reports of bacterial inoculation of mature composts, particularly for the suppression of *R. solani*, have been made by Phae *et al*., Kok *et al*., Hoitink *et al*., and Rÿckeboer *et al*. [2-5].

A mix of composted or raw material of wood fibre waste (WFW) and millet seed in the ratio of 80:20 (w/w) was previously reported to be most favorable for growth of *Trichoderma* sp. (Td_{22}) with peak cfu and biomass (chitin-content) being reached after 14 days incubation [6]. The efficacy of the Td_{22} grown in this mix in protecting lettuces against *S. minor* attack in a

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series of glasshouse trials is described in Ramona and Line [6].

The present study investigated the use of composted WFW and millet seed as a possible carrier or substrate for the cultivation of selected bacterial antagonists previously isolated from the rhizosphere of crop plants.

2. Materials and Methods

2.1 Bacterial Antagonists

Several bacterial antagonists, such as *Pseudomonas corrugata*, *Bacillus polymyxa*, *B. megaterium*, *B. thuringiensis*, *B. mojavensis*, and *Lysobacter antibioticus,* isolated from various sources were investigated for growth response in the mix of composted WFW and millet seed (80:20 w/w). For long-term storage, these bacterial antagonists were cryo-preserved at -70℃ in Trypticase Soya Broth (TSB) medium supplemented with 30% glycerol.

2.2 Fungal Pathogen (S. minor)

The origin, method of cultivation, and storage of *S. minor* is described in Ramona [7].

2.3 WFW Compost Production

The origin and the method of composting of raw WFW are described in Ramona [7].

2.4 Inoculum Preparation

Selected bacterial antagonists were grown in a sterile trypticase soya broth (TSB) medium (Oxoid) following inoculation with loopfuls of 24-48 hour old TSA cultures. Flasks (500 mL capacity) of inoculated medium (100 mL) were incubated at 25°C under static conditions for 24-48 hours, until turbidity was achieved. Before use, the bacterial numbers were estimated by serial dilution plating (in triplicate) on trypticase soya agar (TSA) (Oxoid).

2.5 Carrier Preparation and Inoculation under Sterile Conditions

Preparation of the composted WFW and millet seed (80:20 w/w) mix is described in Ramona and Line [6]. Prior to sterilization, the pH of the nutrient-amended mix was adjusted to approximately neutral by addition of $CaCO_3$. On cooling, the mix was inoculated with suspensions of the antagonistic bacteria at the rate of 10% **(**v/w**)** to give an initial density in the mix of between 5.9 $\log_{10}$ cfu/g dw and 8.0 $\log_{10}$ cfu/g dw. Flasks were incubated at 25 ℃ with periodic assay for growth of the bacterial antagonists following dilution plating onto TSA.

2.6 Carrier Preparation and Inoculation under Non-sterile Conditions

The preparation of the mix was the same as that previously described above, however prior to inoculation the mix was pre-incubated at 60 ℃ for one week (to simulate hot-composting conditions) with a view to minimize the density of indigenous mesophilic microbiota. On cooling, the mix was inoculated with suspensions of bacterial antagonists. The inoculated mix was incubated at 25 ℃ under static condition with periodic assay for growth of the bacterial antagonists following dilution plating onto TSA. The identity of the antagonists following re-isolation was confirmed on the basis of their colony morphologies. When necessary, determination of cellular morphology by Gram staining and of biochemical reactions, such as the ability to hydrolyse casein or starch, was also undertaken.

2.7 Glasshouse Trial

A glasshouse trial was conducted to investigate the efficacy of the bacterial antagonists grown under sterile conditions in a mix of composted WFW and millet seed (80:20 w/w) in suppressing challenge by *S. minor*. Soil (sampled from NW Tasmania) was mixed with 5% (v/v) suppressive WFW mix, dispensed into pots of 1.5 L capacity, and inoculated with *S. minor* cultured on millet seeds, placed at 20 mm below the surface of the mix at the rate of 2 g millet-inoculum per pot. Pots inoculated with *S. minor* only and pots without *S. minor* inoculation but containing suppressive compost amendment served as controls. All pots were left under irrigated conditions in a shade house for one week prior

to sowing. Ten lettuce seeds were sown per pot with four replicates per treatment. After sowing, the pots were maintained in the shade house for four weeks. The numbers of germinated seeds were recorded one week after sowing, prior to thinning to five seedlings per pot. Numbers of healthy seedlings were recorded at weekly intervals thereafter. The data obtain in this trial was analysed by using analysis of variance (ANOVA).

3. Results

3.1 Growth of Bacterial Antagonists in the Mix of Composted WFW and Millet Seed (80:20 w/w) under Sterile Conditions

The results of the bacterial growth response in the mix of composted WFW and millet seed under sterile conditions are presented in Fig. 1. All tested bacterial antagonists were found to grow well in the WFW mix with increases of between 2.5 and 4.4 orders of magnitude (depending on the bacterial species) in density generally occurring in the first 14 days. Following prolonged incubation (up to 56 days), the population density of these antagonists plateaued between 8.4 $\log_{10}$ cfu/g and 10.1 $\log_{10}$ cfu/g (Fig. 1).

3.2 Growth of Bacterial Antagonists in the Mix of Composted WFW and Millet Seed (80:20 w/w) under Non-sterile Conditions

The growth of the bacterial antagonists in a mix of WFW and millet (80:20) under non-sterile conditions is shown in Fig. 2.

The growth rate of these bacterial antagonists appeared to be suppressed when grown under non-sterile conditions, indicated by a lower cfu, when compared to that recorded under sterile conditions (Fig. 2). In some cases, the cfu of the antagonist fell by more than one order of magnitude over the same period of incubation time. The *L. antibioticus* consistently showed the best growth response in this mix (both under sterile and non-sterile conditions), although its growth was somewhat suppressed when grown under non-sterile condition over the same period of time (Figs. 1 and 2). All the tested antagonists however, reached a density of more than 7 $\log_{10}$ cfu/g dw in the mix under these conditions after 28 days incubation.

3.3 Glasshouse Trial

The efficacy of the suppressive mixes to protect lettuce seedlings/plants from attack by *S. minor* is shown in Table 1. The germination rate of the lettuce seeds in pots containing the antagonist, pathogen, or a combination of antagonist and pathogen-treated pots was high (ranging from 87.5 to 92.5%). Germination rates were not significantly different (P>0.05) when compared to the nil control (A0B0) showing 92.5% germination (Table 1). Generally, the percentage of healthy seedlings in the pots treated with mix-grown antagonists was relatively higher than that in the control treatment pots (A0B1), although in most cases the results were not statistically significant (P>0.05) (Table 1). *Lysobacter antibioticus* (A2B1) and *Pseudomonas corrugata* (A5B1) significantly protected the seedlings up to week two, with 35% and 40% healthy seedlings respectively, while only 15% survival was found in the control treatment (Table 1). As more plants became infected with prolonged incubation, differences in the percentage of healthy plants between treatments and the control became statistically insignificant (P>0.05) (week 4, Table 1). It would appear from this trial that the growth of the pat-

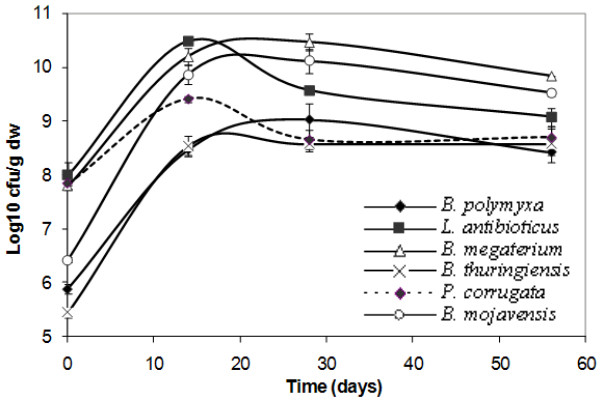

Fig. 1 Growth response of six bacterial antagonists in a mix of 80% composted WFW and 20% millet seed under sterile conditions. Each value shown is an average from four replicate mixes ± standard error.

Table 1 Germination rate of lettuce seedlings in suppressive mixes, and the effectiveness of the mix-grown antagonists in protecting against S. minor. Each value is an average obtained from four replicate pots ± standard error.

Treatments*	Germination rate†	Healthy seedlings (%)¥	
		Week 2	Week 4
A0B0	92.5 ± 2.2	100 ± 0.0 a	100±0.0 a
A0B1	90 ± 3.7	15 ± 5.0 b	5 ± 5.0 bc
A1B1	92.5 ± 2.2	20 ± 8.2 bc	15 ± 5.0 b
A2B1	92.5 ± 2.2	35 ± 5.0 c	15 ± 9.6 bc
A3B1	87.5 ± 2.2	35 ± 9.6 bc	10 ± 5.6 bc
A4B1	95 ± 2.6	25 ± 5.0 bc	0 ± 0.0 c
A5B1	92.5 ± 4.3	40 ± 8.2 c	20 ± 8.2 b
A6B1	87.5 ± 2.2	20 ± 8.2 bc	10 ± 5.8 bc

†The germination rate of lettuce seed was not statistically different at $P<0.05$ in all treatments; *A0B0: nil control (neither pathogen nor antagonist were inoculated); A0B1: pots inoculated with *S. minor* only (control treatment); A1B1, A2B1, A3B1, A4B1, A5B1, and A6B1: Pots inoculated with both pathogen (*S. minor*) and *B. polymyxa, L. antibioticus, B. megaterium., B. thuringiensis, P. corrugata,* or *B. mojavensis*, respectively; ¥Values in the same column followed by the same letter are not significant statistically at $P<0.05$.

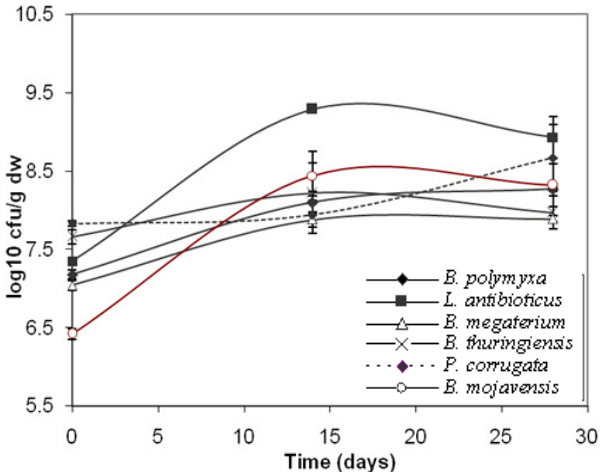

Fig. 2 Growth of six bacterial antagonists in a mix of 80% composted WFW and 20% millet seed under non-sterile conditions. Each value shown is an average from four replicate mixes ± standard error.

hogen (*S. minor*) was stimulated in the soil by the millet seed of the mix, resulting in a more aggressive attack by the pathogen on the lettuce plants than would otherwise be the case.

4. Discussion

The growth of the selected bacterial antagonists in three-month old WFW compost amended only with 0.5% w/w Phostrogen® was very poor (results not shown). A modification on this compost [amending it with 20% millet seed, re-wetting it with nutrient solution (NH_4NO_3-BMS) to approximately field capacity, and adjusting the pH to approximately neutral] resulted in excellent growth responses of the antagonists (Fig. 1). In some cases, the cfu-density reached by the bacterial antagonists under sterile conditions was comparable to that reached by the Td_{22} in the same mix [6]. This indicated that the mix of composted WFW and millet seed in the ratio of 80:20 (w/w) was also suitable for the cultivation of bacterial biological control agents. The presence of millet seed in the mix appeared to provide a ready source of available carbon and nitrogen, particularly in the initial stages of bacterial growth. WFW alone, which mostly consists of cellulose, remained largely undegraded by the inoculated antagonists, none of which was cellulolytic. The C:N ratio of raw WFW is approximately 218 [8], however, following three months composting with appropriate nitrogenous amendment the C:N ratio was determined to be approximately 40 [6]. Millet seed has been estimated to contain 1.7% w/w N [9]. Hence its combination with WFW at the rate of 20% (w/w) would contribute further (if low) nitrogen supplementation for the growth of the bacterial antagonists.

The growth of bacterial antagonists under non-sterile conditions was somewhat reduced in terms of cfu/g mix when compared with that recorded under sterile conditions (Figs. 1 and 2). A similar or greater effect of using non-sterile conditions was observed for the fungal agent Td_{22} [6], with cfu/g mix falling by one to two orders of magnitude under non-sterile conditions. A similar reduction in cell numbers under non-sterile conditions was also reported by Nakasaki *et al*. [1] who reported the diminished growth of a *B. subtilis* strain in the presence of indigenous contaminants in a grass clipping compost substrate. Therefore, minimizing the density and/or diversity of indigenous microbiota prior to inoculation with antagonists is seen to be advantageous in achieving maximal numbers of inoculated antagonists.

Pre-treatment of the mix at 60 ^{o}C for seven days (simulating hot-compost conditions) was found to reduce the diversity of the isolated indigenous bacteria, although the density of the total bacterial loading (cfu on TSA at 60 °C) was not significantly reduced. This was not of concern, since the biota would be strongly dominated by thermophiles, which would be expected to compete poorly against the mesophilic biological control inoculants at temperatures of ~ 20 °C.

As previously noted, the millet seed used in the cultivation of the bacterial antagonists also appeared to stimulate the growth of the fungal pathogen (*S. minor*) in the pot trial. Apparently the complex carbohydrate-components of the millet were either beyond the metabolic capacity of the biological control bacteria to degrade, or (more likely) in excess of requirements over the period of antagonist-cultivation, leaving these components as substrates for the fungal pathogens. Therefore the use of millet seed as a nutrient supplement for the bacterial antagonists cultivation, to be subsequently used to control fungal pathogens in the field, cannot be recommended.

The finding that the use of millet/cellulose-medium (containing Td_{22}) as 10% or 20% inocula of potting media was antagonistic to the growth of *S. minor* is in qualified agreement (bearing in mind the above comment) with a report by Metcalf [10] that *S. cepivorum* was suppressed by *Trichoderma koningii* in a composition of 100% millet seed. The use of other carbon sources rather than millet seeds, such as casein or starch for the cultivation of bacterial biological control agents was reported by Ramona and Line [6].

5. Conclusions

Modification of WFW compost, by amending with millet seed and adjusting the pH to neutral made it suitable as a growth medium for inoculated bacterial antagonists. However such amendment also appeared to provide an excellent nutrient source for the fungal pathogen (*S. minor*) on soil application. Therefore, the use of millet seed as a nutrient source for the bacterial antagonists is precluded. Although pre-treatment of the mix at 60 ^{o}C for one week successfully eliminated most of the mesophilic bacteria, the residual microbiota was still capable of some degree of suppression of the growth of inoculated bacterial antagonists relative to sterile counterparts following incubation at mesophilic temperatures. The possibility remains for the use of radical temperature shift (60 °C to 25 °C) to minimize the indigenous competition with desired inoculated biota on subsequent cultivation in bulk media.

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