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Dry Preparation of *Trichoderma harzianum* for Controlling *Rhizoctonia* damping-off in *Brassica rapa*

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Abstract: Preparation of *Trichoderma harzianum* (UPM40) with compost (OPTCD) as the food-base carrier was evaluated for the control of *Rhizoctonia* damping-off in leaf mustard (*Brassica rapa* L.). Seeds sown in *Rhizoctonia*-infested soil treated with UPM40 dry preparation and fungicide (Brassicol®) gave significantly higher seedling emergence. The losses in emergence were only 2.83 and 3.71%, respectively, as compared to 21.99% in the untreated soil. The Disease Incidence (DI) expressed as the Area Under the Disease Progress Curve (AUDPC) was highest for the seedlings in the untreated soil and lowest for the seedlings in the UPM40- and fungicide-treated soils. The local isolate *T. harzianum* (UPM40) dry preparation was therefore effective in protecting the seeds and seedlings of *B. rapa* against *R. solani* pre- and post-emergence damping-off. No significant differences in the mean dry weights between the *Trichoderma*- and chemically-treated soils were observed in the *Rhizoctonia*-infested soils. However, in the non-infested soils, the mean dry weight of plants was significantly higher for the treatment with UPM40 (23.88 g) followed by that with fungicide (20.88 g) and then the control (16.78 g).

Key words: Dry formulation, *Trichoderma*, *Rhizoctonia* diseases, *Brassica rapa*

Introduction

In Peninsular Malaysia, *Brassica rapa* L. (leaf mustard), is the most popular leafy vegetable with 2,402 ha under it in 1999 (Department of Agriculture Malaysia, 1999). Intensive cultivation of leaf mustard makes it vulnerable to soil-borne pathogens such as *Rhizoctonia* that cause damping-off in many vegetable crops (Sokhi, 1994). *Rhizoctonia solani* Kuhn is an important seed- and soil-borne plant pathogen associated with seed rot and seedling decay in vegetables (Chan, 1999), thus reducing germination and causing pre- and post-emergence damping-off in seedlings, resulting in a poor plant stand. Practically all farmers rely intensively on chemicals. Indiscriminate and excessive use of chemicals for seed and soil treatment has caused the development of pathogen resistance to pesticides, pesticide contamination of produce, poisoning and a rise in the crop production costs. The use of biological control agents, however, is hampered by the lack of suitable formulations and application methods (Papavizas and Lewis, 1981). Soil amendments with an energy source, such as organic materials, to encourage beneficial microorganisms may also reduce or eliminate fungistasis (Lockwood, 1977; Papavizas and Lewis, 1983) and stimulate proliferation of the antagonists.

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Trichoderma harzianum (UPM40), an indigenous fungal isolate, has been shown to actively attack *R. solani* *in vitro* (Daghman, 2004). To have the same efficacy of the biocontrol agent in the field as in the laboratory, a proper formulation and good application techniques are needed. This study was to evaluate the efficacy of *Trichoderma* (UPM40) dry preparation, applied as an additive to the soil mixture, in controlling *R. solani* diseases in *B. rapa*. Mature compost prepared from Oil Palm Trunk and Chicken Dung (OPTCD) was incorporated as the food base-carrier in the formulation (Daghman, 2004).

Materials and Methods

Preparation Fermentor Biomass of UPM40

T. harzianum (UPM40) was obtained from the Plant Protection Laboratory, Faculty of Agriculture, Universiti Putra Malaysia and confirmed to have antagonistic properties against *R. solani* (Daghman, 2004). The fungal culture was maintained on Potato Dextrose Agar (PDA) as the biocontrol candidate to be tested in this study. Production of the fungal biomass was carried out in 2 L bench-top fermentor flasks with a working volume of 1.5 L sterilized culture medium comprising a mixture of Rice Flour (RF) and molasses (ML) (1:4 RF:ML%). The medium was inoculated with 15 mL spore suspension (15×10^8 spores mL^{-1}) of UPM40 and maintained at 30°C in the incubator shaker (500 rpm) for 72 h. The biomass obtained (conidia and mycelia) were stored at -20°C for 24 h and then freeze-dried over 48 h, ground to fine powder and labeled as the dried-fermentor biomass of UPM40.

Preparation of Rhizoctonia Solani Inoculum

R. solani was isolated from infected leaf mustard and a pure culture maintained on PDA. *Rhizoctonia* inoculum was raised in a sterilized wheat grain medium (100 g/bag) incubated at room temperature ($28 \pm 2^\circ\text{C}$) for 2 weeks. The medium was then mixed thoroughly in the soil mixture at 3% of the total soil weight. The artificially infested soil was incubated for four days for the pathogen to establish. The population of *R. solani* in the soil was evaluated initially (4 random samples per tray), that is, immediately after the 4 days' incubation and at the end of the experiment 36 days later (Rodriguez-Kabana *et al.*, 1980).

Efficacy of UPM40 Dry Formulation in the Control of Rhizoctonia Diseases

The freeze-dried fermentor biomass of UPM40 was incorporated in OPTCD in the ratio 1:10 w/w fungal biomass: compost. A soil mixture was prepared by thoroughly mixing topsoil, peat grow and sand in 3:2:1 v/v ratio. The soil mixture was placed in aluminum trays (90 × 60 × 30 cm) with each tray containing 25 kg soil mixture. The formulated biomass was added to the soil mixture at 2% of the total soil weight. Random soil samples were taken from the aluminum trays (4 random samples from each tray) to determine the initial antagonist populations expressed as colony forming units (cfu) per g^{-1} air-dried soil and at 36 days after sowing (Papavizas and Lewis, 1981). *B. rapa* seeds were primed by soaking in distilled water for 24 h at room temperature to give them an advantage in moisture imbibition to enhance their germination. The treatments were soils treated with UPM40 dry preparation (T_1) and fungicide (Brassiccol® 200 mg L^{-1}) (T_2) and untreated soil (control, T_3). The primed seeds were then sown in the soils.

The experimental design was factorial with the trays (replicates) arranged in a Completely Randomized Design on glasshouse benches. Each treatment was replicated four times with each replicate consisting of 30 seeds. The plants were watered daily and organic fertilizer

(chicken manure) applied as recommended for *B. rapa*. The emerging seedlings were counted at day 4 after sowing and the percentage emergence calculated from the total number of seeds sown.

Seeds that failed to emerge or which had rotted were recorded as having pre-emergence damping-off. The incidence of *Rhizoctonia* post-emergence damping-off was recorded based on the symptoms of wilt, lesions, collapsed hypocotyls or dead seedlings. Re-isolation from the infected seeds and plant parts on Potato Dextrose Agar (PDA) was done to confirm the casual pathogen. The Disease Incidence (DI) was calculated based on the foliar-associated symptoms assessed every two days, according to the formula (modified from Campbell and Madden, 1990):

$$\text{Disease incidence (\%)} = \frac{\text{Number of seedlings infected}}{\text{Number of seedlings assessed}} \times 100$$

The reduction in DI, as a measure of the effectiveness of the treatments in suppressing the disease progress, was assessed using the data plotted as disease progress curves and the Area Under the Disease Progress Curve (AUDPC) calculated based on:

$$\text{AUPDC} = \sum_i^{n-1} \left[\frac{Y_i + Y_{i+1} + 1}{2} \right] [t_{i+1} - t_i]$$

Whereby,

$$\begin{aligned} Y &= \text{Disease incidence} \\ t &= \text{days} \end{aligned}$$

Slopes of the disease progress curves were obtained by transforming the DI data using a monomolecular model (Monit) by Campbell and Madden (1990). The effect of the dry formulation of *Trichoderma* on plant vigor was determined by the difference in dry weight increase of the seedlings and dry weight increase of the roots in infested vs. non-infested soils. The antagonist population on the roots of the seedlings was also assessed at 36 days after sowing in the *Rhizoctonia*-infested and non-infested soils.

Results and Discussion

The UPM40 dry preparation and chemically-treated soils both suppressed the activity of *R. solani* in the *Brassica* rhizosphere and subsequently, the disease development. *Brassica* seeds sown in *Rhizoctonia* non-infested soil with (T₁) and without UPM40 (T₃) gave emergence of 88.33 and 83.33%, respectively, not significantly different from that of the chemically-treated soil (T₂; 90.00%). However, when the seeds were sown in *Rhizoctonia*-infested soil, T₁ and T₂ gave significantly better emergence at four days after sowing than T₃. Infected seeds had rotted and failed to germinate. The percentage reduction in emergence in the *Rhizoctonia*-infested soil from the control (T₃) was 2.83 and 3.71%, respectively. This indicated that UPM40 dry preparation was effective in protecting the seeds against *Rhizoctonia* pre-emergence damping-off. As the emerged seedlings continued to grow, they were further subjected to *Rhizoctonia* infection resulting in post-emergence damping-off. Typical symptoms observed on the seedlings were water-soaked lesions on the hypocotyls, which subsequently collapsed, resulting in death of the seedlings. Re-isolation from the infected seeds and plant parts on PDA plates consistently produced pure cultures of the pathogen *R. solani*, thus confirming its pathogenicity on *Brassica rapa*.

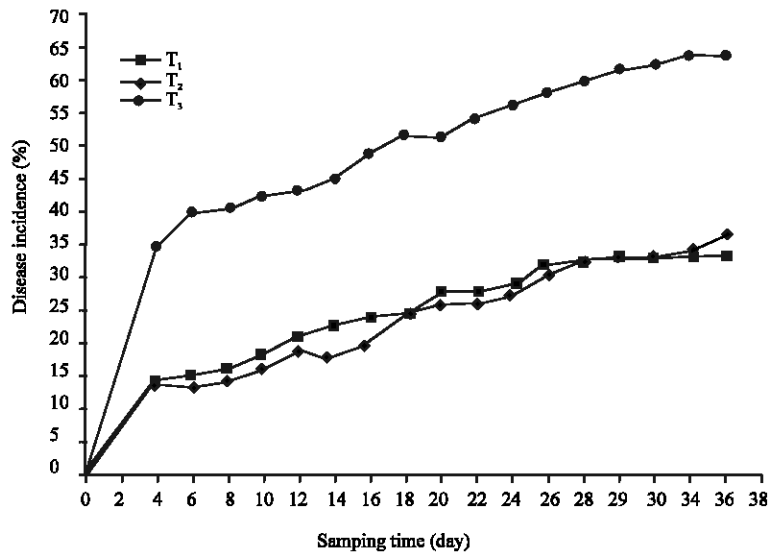


Fig. 1: Effect of UPM40 dry formulation on the incidence of *Rhizoctonia* damping-off in *B. rapa* seedlings from *Rhizoctonia*-infested soil. (T₁=UPM40-treated; T₂ = fungicide-treated; T₃ = control)

The Disease Incidence (DI) was used to indicate the effectiveness of UPM40 dry formulation in suppressing the damping-off. The DI was rated based on symptoms that developed on the hypocotyls, stems and leaves. It was highest in T₃ and lowest in T₁ and T₂ (Fig. 1). UPM40 dry preparation was able to control post-emergence damping-off based on its DI being significantly lower (30%) than that for the control (T₃; 65%), 36 days after sowing. Brassicol®, applied as soil drench (T₂; 25%), was as effective as UPM40, but the continuous application of fungicide will not only have an adverse effect on the environment but, more importantly, also on the produce. The disease development, as expressed by the AUDPC values of infected seedlings, at 36 days after sowing was 3616.7 units/square for T₃ and 2347.7 units/square and 2433.9 units/square for T₁ and T₂, respectively. The disease developed faster in the seedlings in the non-treated soil with an epidemic rate (r_m) of 0.871 unit/day as compared to T₁ ($r_m = 0.655$ unit/day) and T₂ ($r_m = 0.718$ unit/day) (Table 1). Since *R. solani* is pathogenic to many vegetable crops, the use of UPM40 dry formulation can be an alternative control strategy against damping-off to obtain a good plant stand and quality produce.

UPM40 has been proven to be strongly antagonistic against *R. solani* in *in vitro* dual culture and colony degradation tests with a percentage inhibition in radial growth (PIRG) of 82.72% (Daghman, 2004) and its potential as biocontrol agent against *R. solani* has been clarified in this study. The mode of antagonism is possibly through competition and mycoparasitism. Competition occurs when the antagonist reduces the pathogenic activity by consuming some/most of the limited resources, such as organic and inorganic nutrients, oxygen and space (Graham and Mitchell, 1999) and denying them to the pathogen. UPM40 overgrew a colony of *R. solani* within 7 days of co-incubation in the laboratory and antagonistic activity of *T. harzianum*, *T. viride* and *G. virens* has been reported previously against *R. solani* (Kim and Roh, 1987; Mathew and Gupta, 1998). *T. harzianum* had also

Table 1: Epidemic rates and areas under the disease progress curve (AUDPC) for *Rhizoctonia* damping-off under the different soil treatments at 36 days after sowing

Treatment	Epidemic rate (slope) (Unit/day)	AUDPC (Units/Square)
T ₁	0.655b	2347.7b
T ₂	0.718b	2433.9b
T ₃	0.871a	3616.7a

Means in the same column with the same letter are not significantly different by the LSD test ($p = 0.05$) on their transformed values. Each value is the mean of four replicates, T₁ = UPM40-treated, T₂ = fungicide-treated T₃= control AUDPC= Area Under Disease Progress Curve

Table 2: Mean dry weights of plants from the different treatments at 36 days after sowing

Treatment	Mean plant dry weight (g)	
	<i>Rhizoctonia</i> -infested soil	Non-infested soil
T ₁	12.828a	23.88a
T ₂	11.733a	20.88ab
T ₃	8.130b	16.78b
% Reduction (T ₁ from Control)	36.62	29.73
% Reduction (T ₂ from Control)	30.71	24.40

Means in the same column with the same letter are not significantly different by the LSD test ($p \leq 0.05$) on their transformed values. Each value is the mean of four replicates, T₁ = UPM40-treated, T₂ = fungicide-treated, T₃= control

Table 3: Average colony forming units (cfu) of UPM40 recovered from the rhizosphere and rhizoplane of *B. rapa*, 36 days after sowing in *Rhizoctonia*- infested and non-infested soils

Treatment	cfu's g wet soil ($\times 10^3$)	
	Rhizosphere (Initial)	Rhizoplane (36 days)
<i>Trichoderma</i> in <i>Rhizoctonia</i> - infested soil	0.189 b	8.81 a
<i>Trichoderma</i> in <i>Rhizoctonia</i> non-infested soil	14.71 b	296.82a

Means within the same row with the same letter are not significantly different by the LSD test ($p \leq 0.05$). Each value is the mean of four replicates

been reported to suppress diseases by other soil-borne fungi (Mukherjee *et al.*, 1995; Begum *et al.*, 1998; Jinantana and Sariah, 1998).

The ability of *Trichoderma* to promote the growth of *B. rapa* was also assessed based on the total dry weight increase of the plants. In the *Rhizoctonia*-infested soils, there was no significant difference in the total dry weights between the UPM40- and fungicide-treated soils, suggesting that both treatments were equally effective in controlling the damping-off. However, the *Rhizoctonia*-infested and non-infested soils amended with the dry preparation of UPM40 (T₁) produced significantly higher total dry weights than the untreated soil (T₃) (Table 2). Their percentages reductions were 36.62 and 29.73% from their respective controls. This could have been due to strong competition by the pathogen for food and space which reduced the antagonist populations in the rhizosphere and rhizoplane of the seedlings. The results were similar to those of Chan (1999) who reported that *T. virens* significantly increased the plant stand and height, suggesting its possible role as a plant growth promoting fungus. Windham *et al.* (1986) also reported that *T. harzianum* produced a growth regulating factor that increased the rate of germination and stem dry weight increase in tomato. It has been postulated that in the biocontrol of pathogens in the rhizosphere, nutrient release from the soil and organic matter and enhanced plant hormone production may partially explain the *Trichoderma*-stimulated growth responses (Inbar *et al.*, 1994).

Enhancement of root growth was also observed in the plants grown in the UPM40-treated soil with a root dry weight of 9.12 g, followed by those of the chemically-treated (8.39 g) and untreated (5.72) soils which facilitated the uptake of water and nutrients. This resulted in a better crop health

leading to a better stand, yield and, possibly, even better nutritive value of the produce. Cheah and Page (1997) found that *Trichoderma* effectively reduced club root and increased the fresh top weight of Chinese cabbage. *Trichoderma* also reduced root rot in diseased avocado seedlings and stimulated root regeneration in the treated plants (Sait, 2000).

The ability of UPM40 to proliferate and survive in the rhizosphere and rhizoplane of *B. rapa* was estimated based on the cfu's of *Trichoderma* recovered using the *Trichoderma* selective media (TME). The population of UPM40 in the rhizosphere had decreased significantly by day 36 in the *Rhizoctonia*-infested and non-infested soils (Table 3). The decrease in propagules of UPM40 could have been due to competition by the indigenous microorganisms as the soil mixture used was not sterilized. Competition by indigenous microbes is likely to be a major determinant for successful establishment of introduced inoculants in the rhizosphere to a level sufficient for disease suppression. However, recovery of UPM40 was significantly higher from the roots than the rhizosphere, suggesting that it was migrating from the rhizosphere to the roots, colonizing the germinating roots and living on the root exudates. Plant roots are also a major source of carbon and energy for microorganisms in their production of exudates. Competition for the nutrients, primarily carbon, nitrogen and iron, may result in biological control of soil-borne plant pathogens (Sivan and Chet, 1989). For soil-borne diseases, a *Trichoderma* population in the soil mixture in the magnitude of 10^4 - 10^6 was reported to have a beneficial effect on plant growth and disease suppression (Papavizas, 1985).

In addition, the biomass of UPM40 used in this study comprised mainly of conidia and mycelium although the chlamydospores are thought more important in the survival and proliferation of the biocontrol candidate. Therefore, using a biomass of chlamydospores may enhance the effectiveness of UPM40 in controlling *Rhizoctonia* damping-off in *Brassica*. Studies on the distribution of UPM40 as root colonists with time and space are needed to describe and predict the root colonization process. This would enable more effective management of the rhizosphere population for better biological control of *Rhizoctonia* diseases and enhanced plant growth. It should be followed up with locality trials under a range of conditions since this study is a pioneer research in the use of local isolate of *T. harzianum* for the control of *Rhizoctonia* damping-off in *Brassica*.

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