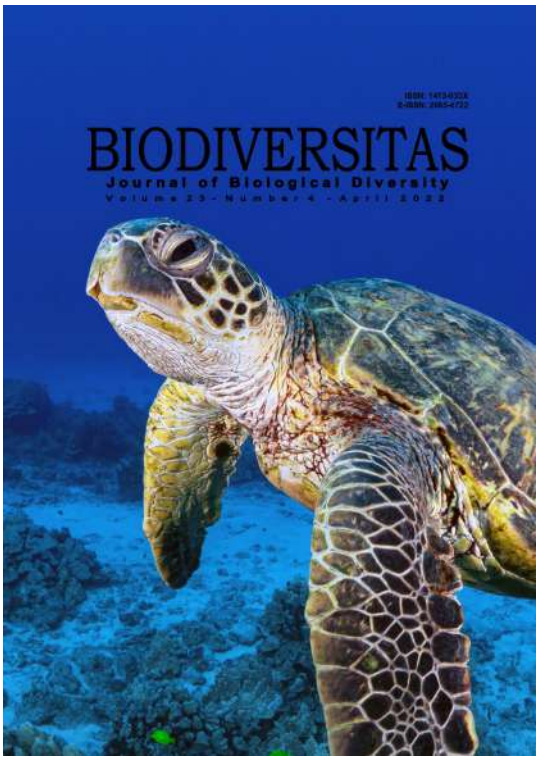

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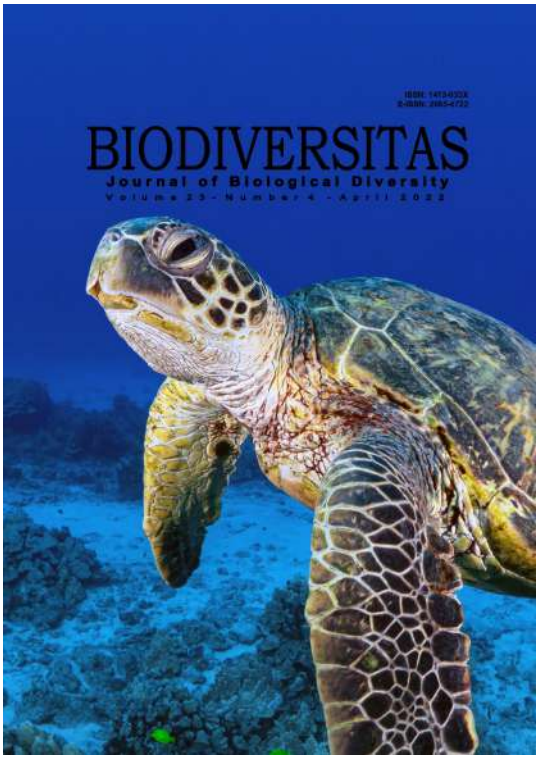

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Biological control of *Sclerotinia minor* attack on pyrethrum plants by *Trichoderma harzianum* in glasshouse experiment

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Abstract. *Ramona Y, Darmayasa IBG, Line MA. 2022. Biological control of* Sclerotinia minor *attack on pyrethrum plants by* Trichoderma harzianum *in glasshouse experiment. Biodiversitas 23: 3264-3269.* The aim of this research was to elucidate the efficacy of *Trichoderma harzianum* (isolate Td_{22}) grown in a ratio of 2:8 millet seeds and wood fiber waste (WFW) compost mixture to suppress *Sclerotinia minor* infection in pot trails on the pyrethrum plants (in 0.5 L pots). The pots were filled with soil and mixed with Td_{22}-grown WFW compost to obtain a concentration of 5% v/v. The *S. minor* (fungal pathogen) previously grown in millet seeds amounted at 2.0 g per pot, was then evenly inoculated at 2 cm below the surface of potting mix. Soil without compost amendment, amended with pathogen only, or without pathogen inoculation served as controls. All pots were acclimatized for 4 days in a shade house prior to transplanting (4 seedlings per pot) of pyrethrum seedlings (aged of 3 weeks). Eight replications per treatment were run for 8 weeks. The results showed that 5% v/v compost-grown Td_{22} provided 78% protection to pyrethrum plants at week 8. Each surviving plant in Td_{22}-treated pots also showed significantly higher average dry weight ($p<0.05$) than those planted in *S. minor* control treatment, indicating that Td_{22} has a potential to be developed as a novel fungal antagonist or a plant growth-promoting fungus.

Keywords: Biocontrol, compost, millet seeds, *Sclerotinia minor*, *Trichoderma harzianum*

INTRODUCTION

Sclerotinia minor infection on various plants, such as lettuces, cabbages, and pyrethrum plants, are commonly found in countries with cool and moist climates (Tozlu et al. 2016). Hahm et al. (2017) reported that *Sclerotinia* sp. causes rot symptoms on basil plants in Korea. In Australia (Tasmania in particular), *S. minor* has also infected pyrethrum plants and causes significant profit loss annually (Macdonald 1995). Such infection on the same plant species was also reported in Kenya by Natrass (1950). Plants infected by *S. minor* commonly show typical symptoms, such as brownish spots on leaf and stem, and then advancing margins, wilting the whole plant and blighting, eventually die. Over the last three decades, such fungal pathogen has been controlled by applying chemical-based fungicides (Tyagi et al. 2020). Due to the many harmful effects of such chemical-based fungicides to our environment, their use in agricultural sectors has been reduced significantly worldwide, especially in developed countries. To avoid the application of excessive chemical-based fungicides, attention has recently been focused on the development of biological control approach, where cost competitiveness becomes important. There are some limitations on the application of biological control in large-scale agricultural sectors, such as difficulties in handling, storage, and delivery of biological control agents in the form of cell suspension. Farmers prefer solid or semi-solid preparations of biocontrol agents prior to their application in the field (Muñoz-Torres et al. 2021). To overcome this limitation, Metcalf (1997) used millet seeds as a supplement in the medium to improve the growth *Trichoderma koningii*. Application of such seeds as a growth supplement, however, was found to increase the production cost of this fungal antagonist, and therefore its large-scale production cost could not compete chemical-based fungicides. Therefore, research on alternative media is urgently required with a view to minimizing this production cost of biocontrol development. In our current research, the millet seeds were mixed with mature compost of WFW of paper mill origin to reduce the production cost of the biocontrol agent.

Ramona and Line (2002) reported that supplementation of millet at the rate of 20% w/w to compost or raw WFW successfully triggered the growth of *Trichoderma* spp. (isolate Td_{22}), which is antagonistic against *S. minor* and *S. sclerotiorum*. In such composition of millet and WFW compost, the fungal antagonist produced a spore density of ~1010 per gram dry weight of mixture after 14 days of incubation.

Based on the above rationale, the main aim of this study was to investigate the efficacy of Td_{22} grown in wood fiber waste compost and millet seed mixture to provide protection on pyrethrum plants from attack by *S. Minor*, with a view to developing a novel and eco-friendly method of plant-pathogen control, with reduced use of chemical-based fungicides.

MATERIALS AND METHODS

Isolates of the fungal pathogen (*Sclerotinia minor*) and fungal antagonist *Trichoderma* sp. (Td_{22})

The fungal antagonist isolate (*Trichoderma* sp. isolate Td_{22}) was kindly provided by Dr. Dean A Metcalf from his stock culture collection at the School of Agricultural Science, Tasmania University in Australia, while the pathogen (*S. minor*) was obtained from Microbiology Laboratory, Faculty of Mathematics and Sciences, Udayana University, Bali. The fungal pathogen (*S. minor*) was previously isolated from a lettuce farm in the Bedugul area (Bali) and maintained at the laboratory of Microbiology, Udayana University.

In vitro dual culture assay of Td_{22} against *S. minor*

The in vitro assay was conducted at the Integrated Laboratory for Biosciences and Biotechnology, Udayana University, to investigate the capability of the Td_{22} to antagonize *S. minor* prior to its application in pot trial experiments. Both fungi were challenged on sterile pectin agar (PA) plates. A plug (1 cm^2) of each fungus previously grown for 48 hours on the same medium was placed face to face on a plate (approximately 5 mm from the edge of the Petri dish) of PA and incubated for 7 days at 30ºC (until heavy mycelia was evident) with regular observations.

Wood fiber waste compost production

The composting process of WFW of paper mill origin, using the open windrow method, was conducted in a glasshouse for 3 months at ambient temperature (temperature range of 15-25°C) at the School of Agricultural Science, Tasmania University, Australia. Prior to composting, C:N:P:K ratio of the waste was adjusted to 35:1:0.6:0.1, respectively (Ramona 2003). Sample collection at WFW compost was conducted monthly and the samples were subjected to maturity evaluation (radish seed germination test) prior to use as a component of cultivation medium for the Td_{22} isolate. The 3 months old compost was used as a component of the Td_{22} medium as its toxicity was close to zero (support >90% radish seed germination following 5 days incubation). The assessment of compost maturity was measured according to the method as specified by Ramona (2003).

Preparation of Td_{22} inoculum

The compost previously made was used as the main component of the Td_{22} medium. This mature compost was mixed with millet seeds to obtain 20% w/w ratio. The moisture content of this mixture was adjusted to field capacity by applying the method of Ramona (2003). The mixture was then autoclaved at 121°C at 15 lbs for 15 minutes, cooled down to ambient temperature, then inoculated with mycelia plus spores of Td_{22} previously grown on pectin agar (PA), and incubated at 30°C for 2 weeks until heavy spores was evident.

Preparation of *S. minor* inoculum

The medium for the production of *S. minor* mycelia was prepared by autoclaving the wet millet seeds (2:1 ratio of millet seeds and distilled water, respectively) at temperature of 121ºC and 15 lbs for 15 minutes. The sterile millet seeds were then inoculated with 1 cm^2 plugs of 48 hours old *S. minor* mycelia previously grown on pectin agar medium and incubated at 25ºC for 1 week (until heavy mycelia of the pathogen was observed) prior to use in the glasshouse trials.

Efficacy of Td_{22} to protect pyrethrum plants from *S. minor* attack in a glasshouse experiment

The trial was conducted in 0.5 L capacity pots. Four variants of treatments used in the experiment were A0B0 (pyrethrum plants sown in soil, in the absence of both Td_{22} and *S. minor*); A0B1 (pyrethrum plants sown in soil inoculated with pathogen only and served as control treatment), A1B0 (pyrethrum plants sown in soil amended with Td_{22}-grown compost only), and A1B1 (pyrethrum plants sown in soil in the presence of both *S. minor* and Td_{22}-grown compost). Field soil was mixed with suppressive compost (compost with Td_{22} grown in it) to obtain 5% v/v compositions and then added into pots with appropriate treatments. The pathogen (*S. minor* grown on millet seeds) amounted at 2.0 g was inoculated evenly at approximately 2 cm from the surface of potting medium and acclimatized for 4 days in a shade house. Four pyrethrum seedlings of 3 weeks old (4 seedlings per pot) were then transplanted in these pots and maintained for 8 weeks with weekly observation on the seedling conditions (weekly recording on the healthiness of seedlings/plants). The trial was terminated at week 8 and all survive plant shoots were harvested, dried at 65ºC until their weight was relatively constant and then their dry weight was determined. The density of pathogen's sclerotia (number of *S. minor* sclerotia/g sample) in pots inoculated with *S. minor* only (A0B1) and those amended with both *S. minor* and Td_{22}-grown compost (A1B1) were also retrieved (using a 0.5 mm sieve) at week 8 by applying the method as described by Metcalf (1997). The effectiveness of Td_{22} to parasitise *S. minor* sclerotia was assessed by random sampling 20 retrieved *S. minor* sclerotia and plated into pectin agar medium with 60 μg/mL tetracycline.

Data analysis

The obtained quantitative data was statistically analyzed using Minitab software for windows version 12. When the significant difference was indicated at $p<0.05$, the results were further analyzed with the least significant difference (LSD) test.

RESULTS AND DISCUSSION

In the in vitro dual culture assay, both Td_{22} and *S. minor* initially showed approximately the same growth response on PA medium. After 24 hours of incubation at 30ºC, the mycelial tips of both fungi met at a certain point on the medium and the growth of both fungi slowed down on 3rd day of incubation. In prolonged incubation, mycelia of Td_{22} had overgrown on the *S. minor* mycelia, and had completely covered the whole surface of *S. minor* after 7 days of incubation (Figure 1). This likely indicated that Td_{22} parasitised the mycelia of *S. minor*.

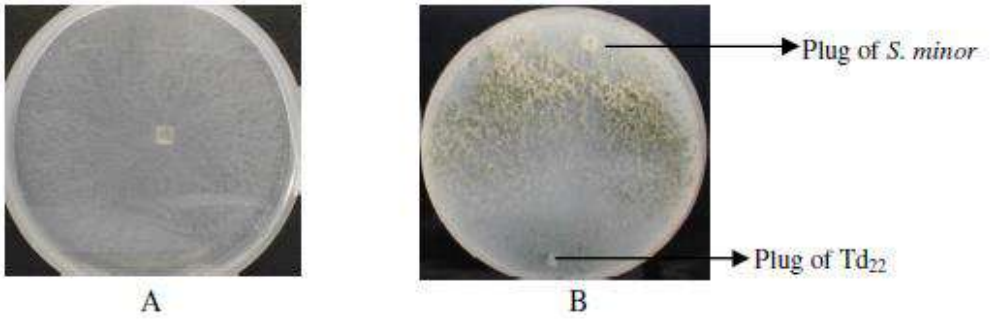

Figure 1. Dual culture assay to challenge *Trichoderma harzianum* (Td_{22}) and *Sclerotinia minor* on pectin agar medium. A. Normal growth of *S. minor* on pectin agar following 48 hours incubation at 30°C; B. Td_{22} mycelia totally overgrow the mycelia of *S. minor* on pectin agar after 7 days of incubation at 30°C

The ability of *Trichoderma* spp. to parasitize plant pathogenic fungi has widely been reported and reviewed extensively by researchers worldwide. Błaszczyk et al. (2014) mentioned various types of mechanisms, including hyperparasitism, by which this fungal antagonist control plant fungal pathogens. Similar results were also reported by Yusnawan et al. (2019), who studied the effectiveness of *Trichoderma* sp. to control several fungal pathogens, such as *Rhizoctonia solani* and *Fusarium* sp. in soybean and mung beans. In dual culture assay, *Trichoderma* isolate also overgrew on both the pathogens, indicating initial parasitism of *Trichoderma* sp. A recent study conducted by Nurzannah et al. (2022) also reported the hyperparasitic activity of *Trichoderma* species on fungal pathogens (*Ganoderma boninense*, the causative agent of basal stem rot in oil palm plants). The hyperparasitic properties of *Trichoderma* could be due to its ability to produce chitinase (Urbina-Salazar et al. 2018), an enzyme required to hydrolyze the main cell wall component of pathogenic fungi.

The efficacy of Td_{22} grown in the mixture of compost and millet seeds (20% w/w millet seeds) to reduce disease incidents due to *S. minor* attack in pyrethrum seedlings/plants is presented in Table 1. The Td_{22} grown in a mix of compost and millet seeds with a ratio of 8:2 provided significant protection ($p<0.05$) on pyrethrum seedlings/plants from attack by *S. minor* (Table 1). Over the period of the glasshouse trial, all seedlings/plants grown in pots, in the presence of Td_{22} and *S. minor* (A1B1), were found to survive in healthy conditions. In contrast, an increase in mortality was observed in pots inoculated with *S. minor* only (A0B1) (56±6.3% survivals on week 1 after sowing to 21.9±5.7% survivals at week 8). This indicated that 78% protection ($p<0.05$) was provided by the Td_{22} against *S. minor* after 8 weeks of maintenance in the glasshouse. Table 1 also shows that Td_{22} did not attack the pyrethrum plants, as 100% of seedlings/plants survival over this period of glasshouse trial were observed in pots inoculated with Td_{22} only, in the absence of *S. minor* (A1B0). The results shown in pots of nil control (A0B0) were as expected, where 100% of the seedlings/plants were found to be healthy during this pot trial up to week 8 (Table 1). The mechanisms by which this fungal antagonist (Td_{22}) controls plant pathogenic fungi may be through one or a combination of the following mechanisms: antibiosis and parasitism (Silva et al. 2019), induction of plant host systemic resistance (Yu et al. 2022), improvement of plant stress resistance (Hidangmayum and Dwivedi 2018), or competition (Oszust et al. 2020).

The effectiveness of *Trichoderma* sp. to control pathogenic fungi belonging to the genus *Sclerotinia* has been reported in various plants. Tancic-Zivanov et al. (2016) and Tozlu et al. (2016) found that (Td_{22}) fungal antagonists effectively control *S. sclerotiorum* in lettuce plantations. A similar result was also reported by Colak-Ates (2019), who found that *T. harzianum* protects lettuce plants from attack by *Sclerotinia sclerotiorum* with percentage protection of between 80 and 86%, when applied in the field in combination with *Coniothyrium minitans* at the rate of 4 kg/ha. This combination of biocontrol agents was also found to increase the lettuce yield by 13-34% when applied in combination with *C. minitans*. This indicates that biocontrol agents belonging to the genera of *Trichoderma* also has the capability to promote plant growth, in addition, to providing protection to plants from attack by fungal pathogens. Martanto et al. (2020) reported the efficacy of *T. harzianum* to control the causative agent of leaf rust disease in soybean. An increase in the weight of soybean seeds was also observed following the application of the fungal antagonist.

In a more recent study, Sriwati et al. (2022) reported the role of the peroxidase enzyme to improve the resistance of the local varieties of patchouli plants in addition to plant growth induction by *T. harzianum*. Growth induction by Td_{22} on pyrethrum seedlings/plants was also observed in the present study. This is indicated by the significantly higher average dry weight of the plants ($p<0.05$) at week 8 in the Td_{22} amended treatments (A1B0 and A1B1) when compared to control treatment (A0B1) (Table 2). The Td_{22} amended treatments also produced relatively higher plant dry weight than that of nil control (A0B0), although these results are not significant ($p>0.05$) statistically (Table 2 and Figure 2). Growth promotion of pyrethrum plants was also observed this is probably due to the physiological effects of the fungus (Td_{22}) on the plants. Sood et al. (2020) and Alfiky and Weisskopf (2021) mentioned in their review that *Trichoderma* sp. may promote the growth of its plant hosts by improving uptake of Mg^{2+} ions from soil and improving nutrient solubilization and absorption.

Table 1. Efficacy of *Trichoderma harzianum* (Td_{22}) grown in 8:2 w/w ratio of compost and millet seeds mixture to suppress *Sclerotinia minor* infection on pyrethrum seedlings/plants

Treatments*	Percentage of survive seedlings/plants (%)			
	Week 1	Week 2	Week 4	Week 8
A0B0	100.00±0.00 a	100.00±0.00 a	100.00±0.00 a	100.00±0.00 a
A0B1	56±6.3 b	28.3±5.7 b	21.9±5.7 b	21.9±5.7 b
A1B0	100.00±0.00a	100.00±0.00a	100.00±0.00a	100.00±0.00a
A1B1	100.00±0.00a	100.00±0.00a	100.00±0.00a	100.00±0.00a

Note:* A0B0 (pyrethrum plants sown in soil, in the absence of both Td_{22} and *S. minor*); A0B1 (pyrethrum plants sown in soil inoculated with pathogen only and served as control treatment); A1B0 (pyrethrum plants sown in soil amended with Td_{22}-grown compost only), and A1B1 (pyrethrum plants sown in soil in the presence of both *S. minor* and Td22-grown compost). Values in Table 1 are averages of 8 replicated pots with 4 pyrethrum seedlings/plants per pot. Values ± standard error followed by the same letter in the same column are not significantly different ($p>0.05$), based on the LSD test following ANOVA.

Table 2. Average dry weight of pyrethrum plants at week 8 (trial termination).

Treatments	Average dry weight per plant (g)*
A0B0	1.07 ± 0.1 ab
A0B1	0.72 ± 0.12 b
A1B1	1.14 ± 0.07 a
A1B0	1.11 ± 0.12 a

Note: * A0B0 (pyrethrum plants sown in soil, in the absence of both Td_{22} and *S. minor*); A0B1 (pyrethrum plants sown in soil inoculated with pathogen only and served as control treatment); A1B0 (pyrethrum plants sown in soil amended with Td_{22}-grown compost only), and A1B1 (pyrethrum plants sown in soil in the presence of both *S. minor* and Td_{22}-grown compost). Each value is an average of 8 replicates ± standard error, except A0B1 (average of 5 replicates, with plants in other pots having died). Values followed by the same letter(s) are not significantly different at $p<0.05$ using the LSD test following ANOVA

Table 3. Relative abundance of *Sclerotinia minor* sclerotia at week 8 (trial termination)

Treatments*	Average density of *Sclerotinia minor* sclerotia per pot*
A0B0	ND
A0B1	108±11.74 a
A1B1	58.3±8.58 b
A1B0	ND

Note: * A0B0 (pyrethrum plants sown in soil, in the absence of both Td_{22} and *S. minor*); A0B1 (pyrethrum plants sown in soil inoculated with pathogen only and served as control treatment); A1B0 (pyrethrum plants sown in soil amended with Td_{22}-grown compost only); and A1B1 (pyrethrum plants sown in soil in the presence of both *S. minor* and Td_{22}-grown compost). Values in Table 3 are averages of 8 replicates ± standard error. Values followed by the same letter are not significantly different using the LSD test following ANOVA. ND: not determined.

An increase in Mg^{2+} uptake by plants after interaction with *Trichoderma* spp. (Halifu et al. 2019) lead to stimulation of chlorophyll formation in plant leaves because this ion is a major chlorophyll constituent (Hasanah et al. 2020). This ion has also been reported to be involved in catalyzing enzymatic activity as well as in regulating genes engaged in photosynthesis (Tränkner et al. 2018). The growth stimulation of pyrethrum plants is presented in Figure 2 and Table 2. This could be partly due to the interaction of Td_{22} with the plants, leading to an increment of Mg^{2+} uptake from the potting mix medium, although this hypothesis needs to be further elucidated.

On the completion of the pot trial (week 8 after sowing), the relative abundance of *S. minor* sclerotia in all pots inoculated with *S. minor* (A0B1) and those inoculated with both *S. minor* and compost-grown Td_{22} were retrieved using a 0.5 mm mesh sieve. The density of *S. minor* sclerotia in the pots inoculated with *S. minor* only (A0B1) was found to be almost twice higher than that of treatment A1B1 (pyrethrum plants sown in soil in the presence of both *S. minor* and Td_{22}-grown compost), which was statistically significant at $p<0.05$ (Table 3). This suggested that inhibition of pathogen or parasitism and death of sclerotia occurred in the presence of Td_{22}. The latter possibility was supported by the finding of Ramona and Line (2002), who reported that 92.5% of the sclerotia retrieved from the pathogen/Td_{22} treatment (A1B1) were parasitized by Td_{22}, even though 15.6% of those retrieved from the pathogen-only treatment (A0B1) were also found to be parasitized by the fungus (this probably attributable to splash contamination). The efficacy of fungal antagonists (belong to the genus of *Trichoderma*) to parasitize sclerotia of pathogenic fungi has also been reported by many studies, such as by Smolińska and Kowalska (2018) and Colak-Ates (2019).

The use of WFW compost mixed with millet seeds (at the ratio of 8:2 w/w) presented in the present study could be applied for large-scale production with moderate cost to avoid liquid cultures, as reviewed by Leggett et al. (2011). The Td_{22} grown in composted WFW and incubated for at least 2 weeks produced a high density of spores (Ramona and Line 2002). Application of Td_{22} in the form of spores appeared to be more beneficial because it reduces concerns relating to its viability in the field. In the form of spore, the fungal antagonist is relatively more resistant than the mycelial form against environmental stress (Huang and Hull 2017).

The formulation (compost-grown Td_{22}) was found to be excellent in controlling *S. minor* attack on pyrethrum plants in the glasshouse experiment (Table 1 and Figure 2). The effectiveness of a similar formulation (compost-grown fungal antagonists) to control plant pathogens has also been reported by Ivayani et al. (2018) and Joos et al. (2020). The survival of a *Trichoderma harzianum* strain in a compost medium used as its carrier was also extensively reviewed by Joos et al. (2020).

Figure 2. The conditions of the pyrethrum plants at week 8 prior to termination for dry weight measurements. From left to right: A0B1 (pyrethrum plants sown in soil inoculated with pathogen only and served as control treatment); A1B1 (pyrethrum plants sown in soil in the presence of both *Sclerotinia minor* and Td_{22}-grown compost); A0B0 (pyrethrum plants sown in soil, in the absence of both Td_{22} and *S. minor*); and A1B0 (pyrethrum plants sown in soil amended with Td_{22}-grown compost only)

The present investigation clearly demonstrated that compost-grown *Trichoderma harzianum* (isolate Td_{22}) applied at the rate of 5% significantly provided protection to pyrethrum plants from attack by *S. minor*. When compared to the control pots (pots inoculated with *S. minor* only), compost-grown Td_{22} provided 78% protection at eight weeks after transplantation ($p<0.05$) in addition to improving the growth of pyrethrum plants. No toxicity was evident to the pyrethrum plants when compost-grown Td_{22} only was applied at this concentration. This indicates that *Trichoderma harzianum* (Td_{22}) has the potential to be developed into a novel fungal antagonist or a plant growth-promoting fungus.

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