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Methanol Extract and Nanocomposite of *Trichoderma* sp as a Potential Bio-Control against *Fusarium moniliforme* in Tomato (*Lycopersicon esculentum*)

Inovejas, R. C.[*] and Divina, C. C.

Department of Biological Sciences, Central Luzon State University, Science City of Muñoz, Nueva Ecija, Philippines.

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Abstract Nanotechnology has been cited as the foundation of a new advanced agriculture. It is a rapidly developing domain of research and practice. The potential uses and benefits of nanotechnology are enormous. These include plant disease management through the formulation of nanomaterials-based products. Use of pesticides and fungicides has been found to be effective against different plant pathogens but the large use of these chemicals can lead to serious problems, including environmental pollution and human health hazards. Therefore, alternative strategies are being widely employed. One potential strategy to counter attack pathogen is the use of bio-control agents. *Trichoderma* sp. is widely studied bio-control agent against plant pathogens because of their ability to reduce the population of soil borne plant pathogens including *Fusarium* sp. that causes wilt in tomato. The study determined the effectiveness of methanol extract and nanocomposite of *Trichoderma* sp. as a potential bio-control against *Fusarium moniliforme* in tomato through *in- vitro* and *in- vivo* conditions. *In-vitro* assays conducted were bi-culture and slide bi-culture while *in-vivo* assay was done using modified leaf assay. *In-vitro* results revealed that *Trichoderma* sp. colonized *F. moniliforme* and suppressed the growth by an average of 2.84 cm which resulted to damaged and deformed hyphae. In-*vivo* results showed that the methanol extract of *Trichoderma* sp. reduced the disease incidence and severity, while the nano*Trichoderma* extract did not. Given the results, it can be concluded that *Trichoderma* sp. had antagonistic property in controlling growth of *Fusarium moniliforme*.

Keywords: *Trichoderma* sp., *Fusarium moniliforme*, Nanotechnology

Introduction

Nanotechnology has been cited as the foundation of a new "advanced agriculture". It is a rapidly developing domain of research and practice. The potential uses and benefits of nanotechnology are enormous. These include plant disease management through the formulations of nanomaterials-based

[*] **Coressponding author:** Inovejas, R. C.; **Email:** rainhartinovejas04@gmail.com

products. Use of pesticides and fungicides has been found to be effective against different plant pathogens but the large use of these chemicals can lead to serious problems, including environmental pollution and human health hazards. Therefore, alternative strategies are being widely employed. One such practice is use of bio-control agents. *Trichoderma* sp. is the most widely studied bio control agents (BCAs) against plant pathogens because of their ability to reduce the population of soil borne plant pathogens (Papavizas, 1985) including *Fusarium* sp. that causes wilt in tomato. Tomato (*Lycopersicon esculentum*) is one of the important sources among all the vegetables throughout the world. It is a good source of vitamins and an important cash crop for small holders and medium-scale commercial farmers. But during cultivation, tomato crop is susceptible to various kinds of disease and disorders, among which *Fusarium* wilt is reported to cause one of the most severe diseases on tomato resulting in complete damage of crop causing yield loss. The yield loss due to this disease may be about 30% to 40%. Successful reductions of *Fusarium* wilt in tomato by the use of *Trichoderma* sp. has been widely practice and their successful antagonistic capacity on fungal plant pathogens is given by their different action modes, such as rhizophere competition, antibiosis, mycoparasitism, production of lytic enzymes and induction of host defense responses. Therefore, the aim of this study is to determine the ability of *Trichoderma* and its methanol extract and nano*Trichoderma* as a biological control agent.

Objectives: The general objective of this study was to evaluate the effectiveness of *Trichoderma* sp. and its methanol extract and nanocomposites against *Fusarium moniliforme* in tomatoes. This study aimed (1) to determine the interaction of *Trichoderma sp*. and *F. moniliforme* under *in-vitro* condition; and (2) to determine the effect of *Trichoderma* methanol extract and nano*Trichoderma* under *in-vivo*.

Materials and methods

Pure culture

Pure culture of *Trichoderma* sp. was obtained from the Ramon Magsaysay Center of Agricultural Research and Environmental Studies (RMCARES), Central Luzon State, University, and the pure culture of *F. moniliforme* was acquired from the Philippine Center for Post-Harvest Development and Mechanization (PhilMech), Science City of Muñoz, Nueva Ecija, Philippines.

Preparation and extraction of nanoTrichoderma

Pure cultures of *Trichoderma* sp. were sub-cultured in PDA. Approximately 1000 bottles containing 25 mL of potato dextrose broth were prepared and 1x1 mm potato dextrose agar with pure culture of the fungus was transferred in each bottle. The fungus was cultured separately and incubated for 20 days. The mycelia mats were harvested by filtration. The mycelia of *Trichoderma* sp. were air dried under a shade for five days to avoid inactivation of the bioactive components. The fungal biomass was ground with electrical blender and then soaked in methanol for five days. The extracts were concentrated using rotary evaporator. Nano*Trichoderma* was prepared through electrospinning at the Faculty of Agricultural Technology, King Mongkut's Institute of Technology Ladkrabang, Bangkok, Thailand. *Trichoderma* methanol extract and nano *Trichoderma* were tested to determine whether they could inhibit the growth *F. moniliforme.*

In-vitro Screening of Antagonistic Property of Trichoderma sp. Against F. moniliforme

The antagonistic properties of *Trichoderma* sp. against *F. moniliforme* were determined using two methods. The pathogen and antagonist were cultured on PDA plates to determine mycelial growth and spore production inhibition.

Bi-culture Test

Bi-culture test was done following the methods of Soytong (1989). A virulent strain of *F. moniliforme* was used in bi-culture test with *Trichoderma* sp. An agar disc from the edge of radial growth of *F. moniliforme* in PDA plate was obtained using sterile cork borer. This was placed in one side of a potato dextrose agar (PDA) plate about 2.0 cm from the center. An agar disc of *Trichoderma* sp. was placed on the other side of the plate. For control treatment, the other plate contained pathogen only and the other have antagonist only. The bi-culture plates were incubated at room temperature for one week. Colony diameter was measured using digital vernier caliper and hyphal interaction was determined. Percentage of growth inhibition of pathogen was calculated using the formula below:

$$\% \text{ inhibition} = \frac{A - B}{B} \times 100$$

Where: A = diameter of pathogen in control plate
B = diameter of pathogen in bi-culture plate

Slide Bi-culture Test

Agar blocks of *Trichoderma* sp. and *F. moniliforme* was grown side by side with a distance approximately 1 cm in a glass slide. The slides were placed into a sterilized Petri dish lined with moist sterile filter paper as moist chamber. It was observed for almost one week under the compound microscope. Each treatment was replicated five times.

In-Vivo Assay of Trichoderma methanol extract and nanoTrichoderma

F. moniliforme was tested for pathogenicity in tomato leaves using Koch's postulates (Hung *et al*., 2013). *F. moniliforme* was sub-cultured on PDA dishes for 7 to 10 days at room temperature. Leaves from a 25-day-old tomato were plucked, cleaned by sterile water and wounded. The wounded leaves were inoculated with 0.5 cm diameter agar plugs of actively growing of pathogen. The inoculated leaves were placed in a modified growth chamber of microwavable container lined with moist sterilize tissue paper and kept at room temperature. After four days, the diameter of symptoms was recorded for evaluation of virulence. The three treatments used in the experiment and replicated five times were:
Treatment 1= leaves inoculated with pathogen only.
Treatment 2= leaves were inoculated with pathogen and *Trichoderma* methanol extract in opposite sides.
Treatment 3= leaves inoculated with pathogen and nano*Trichoderma*.

Results

In-vitro interactions of Trichoderma sp. and F. Moniliforme

The interaction of *Trichoderma* sp. and *F. moniliforme* was studied using the slide bi-culture and bi-culture. Description of the fungal microscopic relationship was done in slide bi-culture while the zone of colonization was measured in bi-culture.

Bi-culture Test

Bi-culture test was done to determine if *Trichoderma* sp. could colonize the agar lawn of *Fusarium moniliforme* as shown in Figure 1. The area of colonization of *Trichoderma* sp. and the pathogen were recorded within seven days of incubation as shown in Figure 2. The results revealed that the antagonist inhibited the pathogen average growth of 6.11 cm and first to cross the midline in which stopped the growth of *F. moniliforme* by an average of 2.84 cm. Table 1 shows the percentage of inhibition of *Trichoderma* sp. against *F. moniliforme* per replicates in bi-culture method. Results showed that *Trichoderma* sp. inhibited the growth of *F. moniliforme* with an average of 36.63%. Highest percentage of inhibition of *Trichoderma* sp. against *F. moniliforme* was obtained from R2 to an extent of 58.75% while the least percentage inhibition was obtained from R3 with 26.14%.

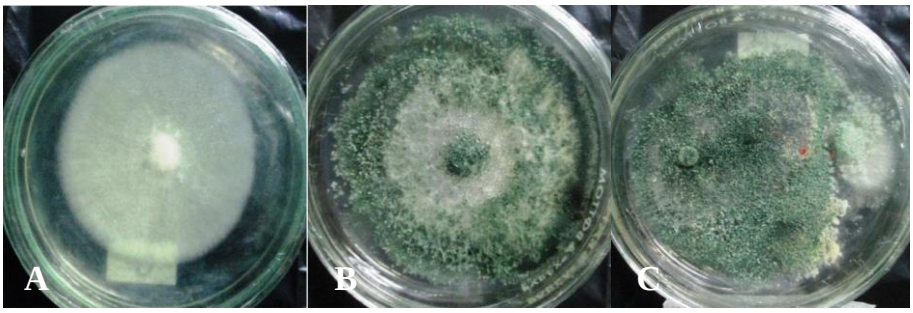
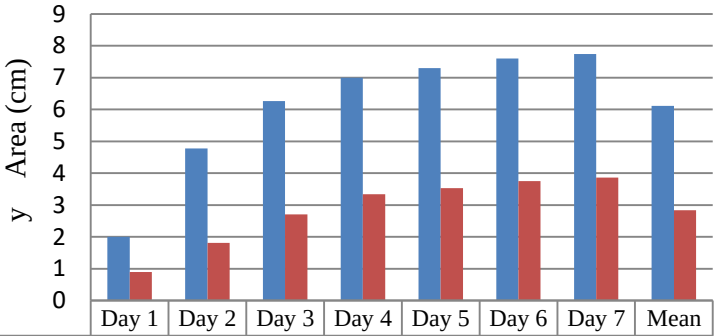


Figure1. *F. moniliforme* in control plate (A), *Trichoderma* sp. in control plate (B) and bi-culture of the two fungi (C).



	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Mean
Trichoderma sp.	2	4.77	6.26	7	7.3	7.6	7.74	6.11
Fusarium moniliforme	0.9	1.81	2.7	3.34	3.53	3.75	3.86	2.84

Figure 2. Daily average growth of *Trichoderma* sp. and *F. moniliforme* in bi-culture test.

Table 1. Percentage inhibition of *F. moniliforme* by *Trichoderma* in bi-culture test

Replicates	Percent inhibition over control
R1	32.62%
R2	58.75%
R3	26.14%
R4	33.09%
R5	32.50%
Mean	36.62%

Slide Bi-culture

Microscopic observations and interactions between *Trichoderma* sp. and *F. moniliforme* was done using slide bi-culture test. Fig. 3, A shows the growth *Trichoderma* sp. and *F. moniliforme* on slide culture. The presence of clear zone was observed and there were no physical interactions between the two fungi. Fig. 3, B shows normal conidia of *F.moniliforme* and Fig. 3 C, D, E, F show the photomicrographs of *Trichoderma* sp. and *F. moniliforme*. Some hyphae of the pathogen were broken when grown with *Trichoderma* sp. Differences were observed in morphology on comparing *F. moniliforme* under direct influence of *Trichoderma* sp. and free from *Trichoderma* sp. influence. *F. moniliforme* away from *Trichoderma* sp. showed normal and undamaged hyphae. There were no physical interactions between *Trichoderma* sp. and *F. moniliforme*, however *Trichoderma* sp. caused damaged the hyphae of *F. moniliforme*, thus it could be interfered that *Trichoderma* sp. used antibiosis as its antagonistic mechanism against *F. moniliforme.*

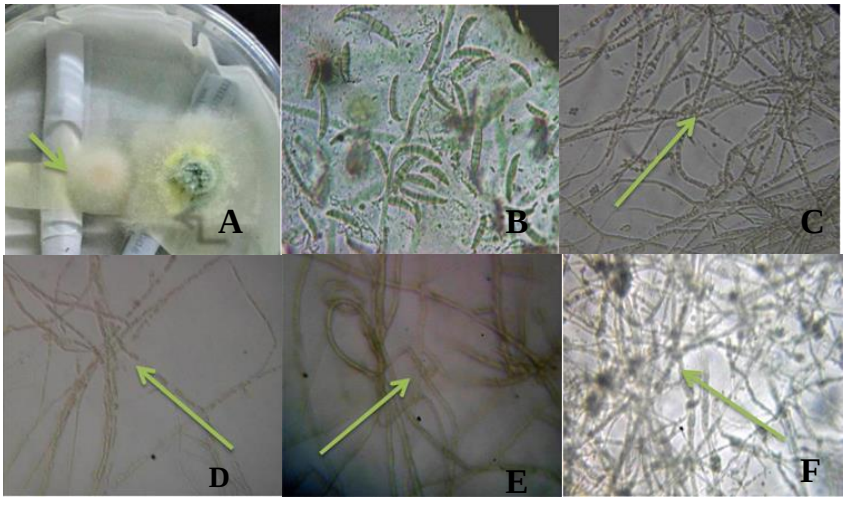


Figure 3. Slide bi-culture of *F. moniliforme* and *Trichoderma* sp. (A), normal conidia of *F. moniliforme* (B) Normal hyphae of *F. moniliforme* (C) and abnormal hyphae (D) Nomal hypha of *Trichoderma* sp. (E) and during heavy sporulation (F)

In-Vivo Assay of Trichoderma methanol extract and nano Trichoderma Based Biological Fungicides

To further determine the effect of *Trichoderma* sp. methanol extract and nano*Trichoderma* against *F. moniliforme* the extracts were both tested under *in-vivo*. Severity of disease was recorded after four days of incubation. Results showed that inoculated leaves with pathogen alone expressed severe infection with the typical sign of symptom like leaf chlorosis, the diseased leaves wilted and dried up. In contrast, control leaves were completely free from disease as shown in Figure 4(A, B). It revealed that the methanol extract of *Trichoderma* sp. had the capability to reduce the disease incidence as shown in Figure 4(C), the wilting stops and the leaves did not completely dry-up. In contrast, the treatment with nano*Trichoderma* did not prevent the disease instead the leaves were completely burned (Fig. 4D).

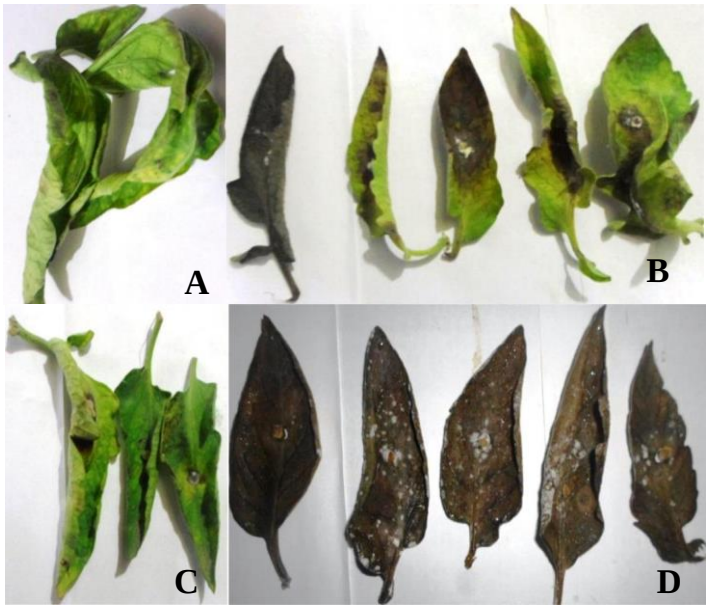


Figure 4. Treatments under *in-vivo* assay. Control leaves (A) and leaves with pathogen alone (B) within four days of incubation Treatments under *in-vivo* assay. *Trichoderma* methanol extract (C) and nano*Trichoderma* (D) within four days of incubation.

Discussion

Bi-culture Test

Based on the results, *Trichoderma* sp. supressed the growth of *F. moniliforme* which supports the study of Sharma P. (2011) that antibiosis, myco-parasitism and competition for nutrition and space and dominance are the mechanisms involved in biocontrol of pathogens by *Trichoderm*a. They stated that the complete course of interaction between *Trichoderma* and *Fusarium* as observed on the bi-culture plates are the initial phase marked by interaction without mycelia contact; the intermediate phase in which *Trichoderma* may or may not be able to overcome the inhibitory effect of *Fusarium*; and, the final phase where *Trichoderma* parasitizes *Fusarium,* which happens only when *Trichoderma* was able to overcome inhibitory metabolites of *Fusarium.*

Slide Bi-culture Test

Slide Bi-culture test found out that *Trichoderma* sp. caused damaged in the hyphae of *F. Moniliforme* thus it could be interfered that *Trichoderma* sp. used antibiosis as its antagonistic mechanism against *F. moniliforme.* Antibiosis occurs during interactions involving low-molecular-weight diffusible compounds or antibiotics produced by *Trichoderma* strains that inhibit the growth of other microorganisms**.** According to Vey *et al.* (2001), *Trichoderma* strains produce volatile and nonvolatile toxic metabolites that impede colonization by antagonized microorganisms; among these metabolites are: harzianic acid, alamethicins, tricholin, peptaibols, antibiotics, 6-penthyl-α-pyrone, massoilactone, viridin, gliovirin, glisoprenins, heptelidic acid and others have been described. There are also examples of antibiotic-overproducing strains, such as gliovirin over producing mutants of *T. virens*, which provide control similar to that of the wild-type, and of gliovirin-deficient mutants (Chet *et al.*, 1997).

In-Vivo Assay of Trichoderma methanol extract and nanoTrichoderma Based Biological Fungicides

The *Trichoderma* methanol extract may have reacted with the cells of the plant and had the capability to stop the manifestation of the disease. This corresponds with the study of Harman *et al.* (2004) that strains of *Trichoderma* added to the rhizosphere protect plants against numerous classes of pathogens,

e.g. those that produce aerial infections, including viral, bacterial and fungal pathogens, which points to the induction of resistance mechanisms similar to the hypersensitive response (HR), systemic acquired resistance (SAR), and induced systemic resistance (ISR) in plants. The higher protective effects may be because some compounds in the extract that may have induced the plant defense response before the inoculation of *F. moniliforme*. It has been reported that metabolites from *Trichoderma* spp. can induce plant resistance by eliciting the synthesis of phytoalexins and pathogenesis-related proteins (Dana *et al*., 2001; Harman *et al*., 2004). The plant defense system could be systemically boosted by metabolites produced by *T. harzianum*. More recently, it was reported that *T. harzianum* can induce the expression of defense response genes in potato roots.

Plant genes respond to pathogens and elicitors. For this reason, plant defense mechanisms do not necessarily require stimulation by the living organism. The addition of Trichoderma metabolites that may act as elicitors of plant resistance, or the expression in transgenic plants of genes whose products act as elicitors, also results in the synthesis of phytoalexins, PR proteins and other compounds, and in an increase in resistance against several plant pathogens, including fungi and bacteria (Dana 2001; Elad *et al*., 2000), as well as resistance to hostile abiotic conditions (Harman *et al*., 2004).

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