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RESEARCH

# Solid Formulation of *Trichoderma virens* for the Management of Banana Anthracnose Caused by *Colletotrichum musae*

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## ABSTRACT

*Trichoderma* spp. widely used as biocontrol agents for controlling a wide range of plant diseases. Banana anthracnose caused by *Colletotrichum musae* is a prominent, widely distributed postharvest disease. This study was carried out to assess the effectiveness of locally isolated *Trichoderma virens* against *C. musae* and the mass production of *T. virens* using locally available, low-cost solid substrates. *T. virens* was tested for the inhibition of *C. musae* isolate *in-vitro* and *in-vivo* by dual plating and inoculating into two varieties of ripened banana: *Kolikuttu* and *Cavendish*, respectively. For mass production, *T. virens* was inoculated into different solid substrates including scraped coconut waste, sawdust, tea waste, seeds from rice, finger millet, and maize, dried pieces of water hyacinth plant, paddy straw, and *Panicum maximum* leaves; regularly taken spore counts (cfu/g) and checked for viability by plating after 12 weeks of storage. The pathogen inhibition percentage by *T. virens* was 74.10%. Disease severity was 0% in *Kolikuttu* and 19% in *Cavendish* after 5 days of *T. virens* spore application ($1x10^7$ spores/ml). Significantly higher ($p \leq 0.05$) mean spore production resulted in rice seeds ($9.345x10^9$ spores/g) compared to the other substrates and the least resulted in sawdust ($1.808x10^9$ spores/g) at the 8th week after *T. virens* inoculation. Spores of *T. virens* were viable in all the tested substrates throughout the study period. The results conclude that *T. virens* is capable of controlling banana anthracnose and can be efficiently mass-produced by using rice seeds, dried pieces of *P. maximum* leaves, and finger millet seeds as substrates.

## INTRODUCTION

Bananas (*Musa* spp.) are the fourth most widely consumed food around the world and it is cultivated in more than 130 countries including Sri Lanka. Numerous abiotic and biotic stresses negatively impact the yield of bananas. Mainly postharvest diseases are responsible for short shelf life and it is the major drawback in banana exportation. Anthracnose caused by a fungal pathogen, *Colletotrichum musae* is one of such prominent and widely distributed pre- and post-harvest fungal diseases in the world (Stover and Simmonds, 1987; Udugama, 2002).

Commercial banana cultivars are also prone to anthracnose, which causes considerable postharvest losses accounting for 30-40% losses of marketable fruit (Ranasinghe et al., 2003; Perera et al., 1999). It directly influences the quality, nutritive aspects and marketable value of the fruits. At the early stage of the disease, irregularly shaped pale brown colour spots develop and eventually, the center of the spot may burst open. Anthracnose becomes severe with the occurrence of wounds or scratches on the banana fruits during handling and transportation. Chemical control is widely used to control the disease. Fungicides are applied as a dip or spray, and shortly after harvest is widely practiced (Thompson and Burden, 1995; Khan, 2001). The control of postharvest pathogens currently relies mainly on the application of fungicides due to the short time between treatment and consumption. However, there are strong public and scientific demands against the extensive use of agrochemicals to prevent residual toxicity, pollution and particularly fungicide-resistance development (Farzaneh et al., 2015; Abdel-Rahim and Abo-Elyousr, 2017). Therefore, it is required to minimize the use of synthetic chemicals and discover sustainable, non-chemical alternative methods for controlling postharvest diseases (Maqbool et al., 2010).

Further, there is an increased interest in bio-pesticides and biological control agents (BCAs) for effective control of plant pathogens ensuring environmental safety as well as human health. Biological control involves the use of naturally occurring non-pathogenic microorganisms that can reduce the activity of plant pathogens and thereby suppress diseases. However, adoption of these potential biological control methods is limited due to poor awareness of available products and their application, lack of durable products with low-cost substrates and packages, changes in the performance of products according to the crop and agro-climatic conditions, and lack of information on human, animal and plant safety (Keswani et al., 2014). However, different beneficial microorganisms have been commercially formulated with various solid, liquid or semi-solid substrates. The formulation is important for the introduced microorganism/mixture of microorganisms to compete and survive among the well-established existing microbes. Hence, the substrates utilized in formulations provide a better micro-environment for the BCAs to adapt to the new environment (Bashan et al., 2016).

Different antagonistic bacteria and some fungi have been tested in the world for their ability to control banana anthracnose disease (Kanapathipillai et al., 1988; Khleekorn and Wongrueng, 2014; Zhimo et al., 2017). *Trichoderma* species are among such microorganisms which are free-living, filamentous fungi that have a long history of successful application in controlling plant diseases. It enables to multiply rapidly on many substrates such as soil-decaying wood materials and as root colonizers (Grondona et al., 1997; Kolombet et al., 2008). Their aggressively competitive nature and diversified metabolic capability make them succeed in their habitats (Barari, 2016). *T. virens* is one of the most effective *Trichoderma* species which possess several control mechanisms to act against phytopathogenic organisms. These biocontrol mechanisms include competition with plant pathogens for nutrients or space, mycoparasitism, antibiosis, and production of lytic enzymes and secretion of secondary metabolites, inducing resistance in host plants and inactivation of pathogen's enzymes (Woo et al., 2014).

Although numerous commercial products primarily utilizing *T. virens* have been introduced worldwide to manage soil-borne

and foliar diseases across various horticultural crops (Lumsden et al., 1992; Samuels, 1996), Sri Lanka still lacks any officially registered commercial products featuring *T. virens*. Since banana anthracnose stands as a prominent disease leading to substantial losses in banana production in Sri Lanka, it is important to discover an effective, eco-friendly and sustainable approach to control it. Generally, spraying or dipping techniques are well-suited for the application of bio-pesticide to manage post-harvest diseases due to ensuring food safety, achieving uniform coverage, optimizing production efficiency, and minimizing physical damage to the produce. When utilizing the fungal biocontrol agents for disease management, utilization of solid formulations becomes essential for acquiring spores due to increasing spore production, preserving spore viability, ease of spore collection, and extending the shelf life. Further, substituting BCAs for fungicides allows for the certification of food as 'organic,' aligning with the worldwide trend that enhances the appeal and value of these agricultural products (Vilaplana et al., 2018). Considering all these facts, the present investigation was carried out as an attempt to employ locally isolated *T. virens* in the biocontrol of banana anthracnose disease caused by *C. musae* with a suitable low-cost substrate to adapt at the farmer level.

## METHODOLOGY

This study was conducted in 2021, at the Plant Pathology Laboratory, Faculty of Agriculture, *Rajarata* University of Sri Lanka, *Anuradhapura*.

### Isolation and confirmation of *Colletotrichum musae*

*C. musae* fungal isolates were obtained by using the method described by Anthony et al. (2004) from banana fruits having typical symptoms of various-sized black or brown sunken spots that may bear masses of salmon-coloured acervuli with their associated conidia on the ripened fruit peel (Ranasinghe et al., 2005; Thangamani et al., 2011). A pure culture of *C. musae* was prepared from a single spore culture and maintained on a potato dextrose agar (PDA) medium. The PDA discs of the pure fungal culture were preserved in sterile distilled water at 4 °C for further use. Morphological characteristics of the isolates were observed from the culture plates that were incubated for 7 days at 28 °C. The shape, size, and color of the conidia of the isolates were examined under the microscope and *C. musae* was identified based on the culture characteristics, spore morphology and molecular methods.

For the molecular analysis, fungal genomic DNA extraction was done according to Mc Garvey and Kaper (1991) method with few modifications. Here the fungal mycelium was ground using the extraction buffer instead of liquid nitrogen. Polymerase Chain Reaction (PCR) was carried out with ITS1 (3' GCC GTA GGT GAA CCT GCG G 5') and ITS4 (3' GCC TCC GCT TAT TGA TAT GC 5') universal primer pair (White et al., 1990). PCR amplification was performed with a volume of 50 µl. Amplification consisted of an initial denaturation at 95 °C for 5 min; 40 cycles of denaturation at 95 °C for 1 min, annealing at 52 °C for 1 min, and extension at 72 °C for 1 min; and a final extension at 72 °C for 5 min; a GeneAmp PCR system 9600 thermal cycler (Perkin-Elmer Corp., Emeryville, Calif.) was used. The expected amplicon size after the PCR reaction was around 560 bp (Abang et al., 2002). The PCR products were visualized by gel electrophoresis in a 1.5 % agarose gel and sent to Macrogen Inc. for DNA sequencing. DNA sequence data of the studied isolate generated from this study was subjected to sequence similarity search using the NCBI_BLAST software (National Center for Biotechnology Information – The Basic Local Alignment Search Tool) for identification purposes.

### *Trichoderma virens* antagonism against *Colletotrichum musae*

A locally isolated and molecularly confirmed *T. virens* (Accession Number- MT256290.1) was used from this experiment onwards. The dual culture plate technique was used to determine the antagonistic effect of *T. virens*. Five-millimeter diameter gel plugs of *T. virens* and *C. musae* pure cultures were placed on the sterile PDA medium in opposite directions.

Four replicates were carried out while maintaining one control set without inoculating the *T. virens* and they were incubated at 28±1 °C for 14 days. The growth of both fungi was measured and the data were recorded daily in a regular manner. Antagonistic activity of *T. virens* was measured as zone inhibition and growth reductions of *C. musae* fungi. According to the following formula, the mycelial growth inhibition percentage was calculated (Bell et al., 1982).

*Percent inhibition of radial growth (PIRG)* = $\frac{R1-R2}{R1}$ x 100

R1= average of colony radius of the pathogen in the control plate
R2= average of the radial colony of the pathogen in the dual culture plate

**Screening of *Trichoderma virens* antagonism against banana anthracnose disease of *Kolikuttu* and *Cavendish* bananas**

**Preparation of fungal spore suspensions**

The fungal inocula of *T. virens* and *C. musae* were grown on a PDA medium until heavy sporulation for three weeks. Conidia were harvested by scrapping the colony surface with a glass slide and transferred through muslin cloth to sterile distilled water. Serial dilutions of *T. virens* and *C. musae* were prepared separately and the initial spore concentrations were directly counted using a haemocytometer (Doni et al., 2014).

**Application of *Trichoderma virens* spore suspension on the banana fruits inoculated with *Colletotrichum musae***

Commercially available *Kolikuttu* (a local variety) and Cavendish banana varieties were used to evaluate the biocontrol activity of *T. virens* against *C. musae.* Healthy unripe twenty bananas (at the harvesting maturity) were selected from each variety. The selected banana fruits were washed three times with sterile distilled water to remove dust particles and then the banana fruits were surface sterilized with 70 % ethanol. Three-millimeter-deep four wounds on each banana were done using sterilized needles. The treatments were comprised of different spore concentrations of *T. virens* as $10^5$, $10^6$, and $10^7$ spores/ml. In each concentration, 20 µl spore suspension was pipetted into each wound. The treated bananas were left to dry for 30 minutes and then an equal volume of *C. musae* spore suspension (concentration $10^6$ spores/ml) was applied. In control treatments, banana fruits were treated only with the pathogens. All the tested banana fruits were placed into square paper boxes at room temperature (28 °C). The percentages of lesion development on infected wounds were assessed five days after inoculation of the pathogen. The average lesion diameter was measured using a Vernier calliper. The severity percentage was recorded using the following equation (Khleekorn and Wongrueng, 2014; Adebesin et al., 2009).

*Percentage Disease Severity* = $\frac{LdA}{LdC}$ *x 100*

LdA = Average lesion diameter in wounds treated with antagonist, prior to inoculation with a pathogen
LdC = Average lesion diameter in control inoculated wounds

The statistical analysis of Variance (ANOVA) was performed using the MIXED model in SAS software program version 9.0. All means were separated using the LS means method at a 5% probability level.

**Mass culture preparation of *Trichoderma virens***

**Selection of suitable substrate**

Rice seeds, maize seeds, finger millet seeds, dried paddy straw, dried water-hyacinth plant pieces, sawdust, dried leaf pieces of *Panicum maximum* (*Mana*), tea waste, and scraped coconut waste were used as different substrates to grow *T. virens*. Each substrate was soaked in a bleach solution (15 %) for 2 hours and washed with distilled water thrice. Then, they were soaked in sucrose solution (10 %) and after 2 hours, excess water was removed and allowed to drain. Under aseptic conditions, each substrate with four replicates (100 g/replicate) was transferred to sterile polythene bags (8.0 x 6.5 $cm^2$) and sealed with

a sterile cotton ball and aluminium foil and autoclaved at 121 °C, 15 psi for 15 minutes. Three mycelium discs (diameter 9 mm) of *T. virens* (fully grown in PDA medium) per bag were inoculated and incubated at room temperature (28 °C) (Sachdev et al., 2018).

**Determination of spore concentration**

Starting from 14 days onwards, each substrate used was serially diluted with sterilized distilled water and the fungal spore concentration of each replicate was recorded at two-week intervals using a hemocytometer.

**Viability of *Trichoderma virens***

The viability of *T. virens* was studied after 24 weeks of inoculation into different substrates. Serially diluted spore suspensions from each substrate were plated using PDA media and incubated at 28 °C to observe the viability of *T. virens*.

**Isolation and confirmation of *Colletotrichum musae***

*C. musae* used in this study was isolated by the direct plating technique from banana fruits showing typical symptoms of anthracnose. The observed fungal colony formed white aerial mycelia and later turned into a salmon pink colour (Figures 1A and 1B). Generally, the mycelia of *C. musae* covered the whole culture plate in 7-8 days. Several black, acervulus-like masses developed on the mycelium after incubation for 10 days at 28 °C with dark-orange drops of conidia (Figure 1B). Figure 1C illustrates the spores of *C. musae* (under 1000x magnification) which were aseptate, hyaline, mostly ellipsoid in shape and ranging from 10–18 µm and 5–9 µm (average of 14.5–6.9 µm) in size. These microscopic and macroscopic features were similar to those described for species of *C. musae* isolated from banana fruits (Da costa et al., 2021).

The identity of the isolated pathogen was molecularly confirmed as *C. musae* (Table 01).

**RESULTS AND DISCUSSION**

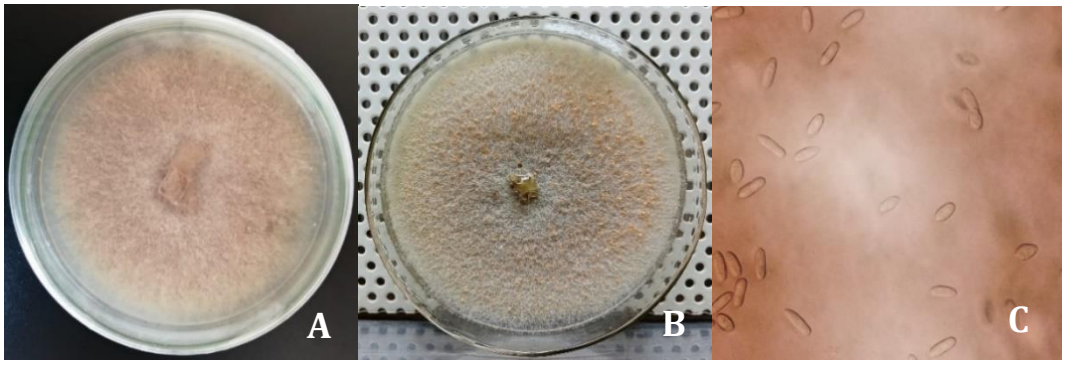


**Figure 01. Macroscopic and microscopic features of *Colletotrichum musae*: (A) Fully grown culture on PDA medium after 7 days of incubation (B) Fully grown culture on PDA medium after 21 days of incubation (C) Spore morphology**

**Table 01. Confirmation of the identity of the PCR products through DNA sequencing and homology search.**

| Sample | Description | E value | Query cover | Identity | Accession No. |
|---|---|---|---|---|---|
| RUSL_08 | *Colletotrichum musae* 18S rRNA gene, 5.8S rRNA gene, 28S rRNA gene (partial), internal transcribed spacer 1 (ITS1) and internal transcribed spacer 2 (ITS2), strain BBA 62471 | 0.0 | 86% | 98.30% | MG386643.1 |

**Screening for antagonism of *T. virens* against *C. musae***

Figure 02 depicts the growth inhibition of *C. musae* by *T. virens* after the 3rd, 5th and 7th days after inoculation. *T. virens* colony displayed a more rapid growth rate compared to *C. musae* and actively suppressed the growth of the latter. The radial growth inhibition percentage was 74.10±8.9 % in dual culture plate assay on PDA. This observation indicated a competition between the biological control agent (*T. virens*) and the pathogen (*C. musae*) in terms of nutrients and space.

According to Bell's classification (Bell et al., 1982), the results show that *T. virens* is an efficient antagonist against *C. musae.* Our results agree with those reported previously for different *Trichoderma* species (Qualhato et al., 2013; Li et al., 2018; Da costa et al., 2021). Da costa et al. (2021) stated that *T. piluliferum* significantly inhibits the mycelial growth of *C. musae* by 62 % through volatile metabolites when compared with the control. The metabolites produced by *Trichoderma* spp. have ability to inhibit a wide range of plant pathogens, including *Alternaria alternata*, *A. brassicae*, *A. solani*, *Fusarium oxysporum*, *F. solani*, *Rhizoctonia solani*, *Sclerotium rolfsii*, and *S. sclerotiorum* (Lopes et al., 2012; Qualhato et al., 2013; Meena et al., 2017).

The effect of three different concentrations of spore suspensions of *T. virens* i.e.: $1\times10^5$, $1\times10^6$ and $1\times10^7$ spores/ml against the pathogenicity of *C. musae* is shown in Table 02. According to the results, the lesion diameter was significantly reduced by the *T. virens*, particularly at concentrations of $10^6$ and $10^7$. Spore concentration $10^5$ resulted in a minimal or no significant reduction in lesion diameters indicating that the activity is dose-dependent. Also, the results suggest that the reduction of lesion diameter was not affected by the variety and there was no variety-spore concentration interaction. Figure 03 illustrates how the disease severity of *C. musae* varies on both *Kolikuttu* and Cavendish banana fruits at different levels of *T. virens* spore concentrations. The disease severity had been reduced on *Kolikuttu* by 0 % on the 5th day, 2.08 % on the 6th day and 14.35 % on the 7th day after inoculation and in Cavendish, it was 19 % on the 5th day, 48 % on the 6th day and 54 % on the 7th day after inoculation (Table 03 and Figure 03).

Among the three concentrations of spore suspensions, 1 x $10^7$ spores/ml treatment had the highest inhibition ability against *C. musae* than other treatments on the 5th day after inoculation (Table 03). *Kolikuttu* showed lower disease severity than Cavendish under given conditions. Initial anthracnose lesions on banana peel in treated samples occurred lately compared to the control samples. It is evidence of the ability of *T. virens* application that increases the vigour and the shelf life of banana fruits.

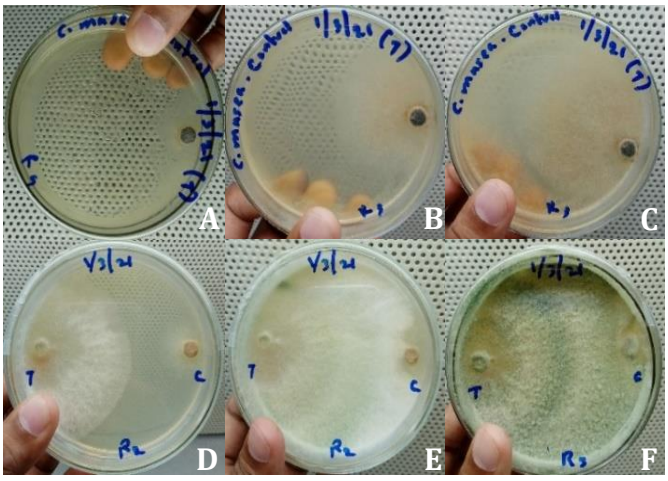


**Figure 02. The colony growth of *C. musae* (Control) at (A) 3rd, (B) 5th, (C) 7th day after inoculation and the colony growth of *T. virens* against *C. musae* at (D)3rd, (E)5th, (F) 7th days after inoculation**

**Table 02. Diameter of anthracnose lesions of *Kolikuttu* and *Cavendish* banana fruits at 5th, 6th and 7th days after inoculation with *Colletotrichum musae* and *Trichoderma virens*.**

| Treatment | Lesion Diameter (cm) | | | | | |
|---|---|---|---|---|---|---|
| | 5th day | | 6th day | | 7th day | |
| | *Kolikuttu* | *Cavendish* | *Kolikuttu* | *Cavendish* | *Kolikuttu* | *Cavendish* |
| *C. musae* only (Control) | $8^{ab}$ | $10.69^{a}$ | $18^{a}$ | $18^{a}$ | $24.81^{a}$ | $22.25^{ab}$ |
| *C. musae + T. virens* 1 x $10^7$ (C1) | $0^{d}$ | $2.12^{cd}$ | $0.37^{c}$ | $8.69^{b}$ | $3.56^{e}$ | $12.56^{d}$ |
| *C. musae + T. virens* 1 x $10^6$ (C2) | $1.6875^{cd}$ | $3.75^{bcd}$ | $9.625^{b}$ | $12.44^{b}$ | $16.69^{cd}$ | $15.44^{cd}$ |
| *C. musae + T. virens* 1 x $10^5$ (C3) | $6.875^{abc}$ | $9.25^{ab}$ | $17.81^{a}$ | $17.81^{a}$ | $23.56^{ab}$ | $19.5^{bc}$ |
| Standard error | 1.9199 | | 1.727 | | 1.9278 | |
| Source of variation | | | < 0.05 | | | |
| Concentration (C) | 0.0006 | | <.0001 | | <.0001 | |
| variety (V) | 0.1032 | | 0.027 | | 0.8199 | |
| C x V | 0.9984 | | 0.0647 | | 0.0044 | |

*Means in a column with the same letter are not significantly different (at p < 0.05) according to DMRT; each value represents mean of three replicates

**Table 03. Disease severity of anthracnose on *Cavendish* and *Kolikuttu* banana fruits at 5th, 6th and 7th days after inoculation with *C. musae* and *T. virens.***

| Treatment | Percentage Disease Severity | | | | | |
|---|---|---|---|---|---|---|
| | 5th day | | 6th day | | 7th day | |
| | *Kolikuttu* | *Cavendish* | *Kolikuttu* | *Cavendish* | *Kolikuttu* | *Cavendish* |
| *C. musae + T. virens* 1 x $10^7$ (C1) | $0^{b}$ | $19.88^{b}$ | $2.08^{c}$ | $48.26^{b}$ | $14.36^{d}$ | $56.46^{c}$ |
| *C. musae + T. virens* 1 x $10^6$ (C2) | $21.09^{b}$ | $35.08^{ab}$ | $53.47^{b}$ | $69.10^{ab}$ | $67.26^{bc}$ | $69.38^{ab}$c |
| *C. musae + T. virens* 1 x $10^5$ (C3) | $85.94^{a}$ | $86.55^{a}$ | $98.96^{a}$ | $98.96^{a}$ | $94.97^{a}$ | $87.64^{ab}$ |
| Standard error | 18.9967 | | 10.5305 | | 8.9455 | |
| Source of variation | | | < 0.05 | | | |
| Concentration (C) | 0.003 | | <.0001 | | <.0001 | |
| variety (V) | 0.47 | | 0.0291 | | 0.0965 | |
| C x V | 0.8747 | | 0.1137 | | 0.0249 | |

*Means in a column with the same letter are not significantly different (at p < 0.05) according to DMRT; each value represents mean of three replicates

Similar to the results of the present investigation, several previous studies have proven the biocontrol ability of different microorganisms particularly bacteria and fungi against banana anthracnose disease caused by *C. musae*. Fu et al. (2010) reported that bacterial strain B106 (1 x $10^8$ spore/ml) controlled the banana anthracnose disease at the postharvest stage by 48.6 %. Similarly, Zhimo et al. (2017) demonstrated that the banana fruits treated with *Candida tropicalis* YZ27, 36h before pathogen inoculation had 96 % anthracnose disease inhibition. An inoculation test conducted by the Chuang and Yang (1993) stated that four bacterial isolates (TN-S221, TN-Y21, TN-Y511, TP-Tu311) and five yeast isolates (Y11-1, Y11-2, Y23-10, Y24-7, Y24-8) were consistently effective to inhibit anthracnose lesion expansion on ripe banana fruit by 22-43 % and 32-42 %, respectively.

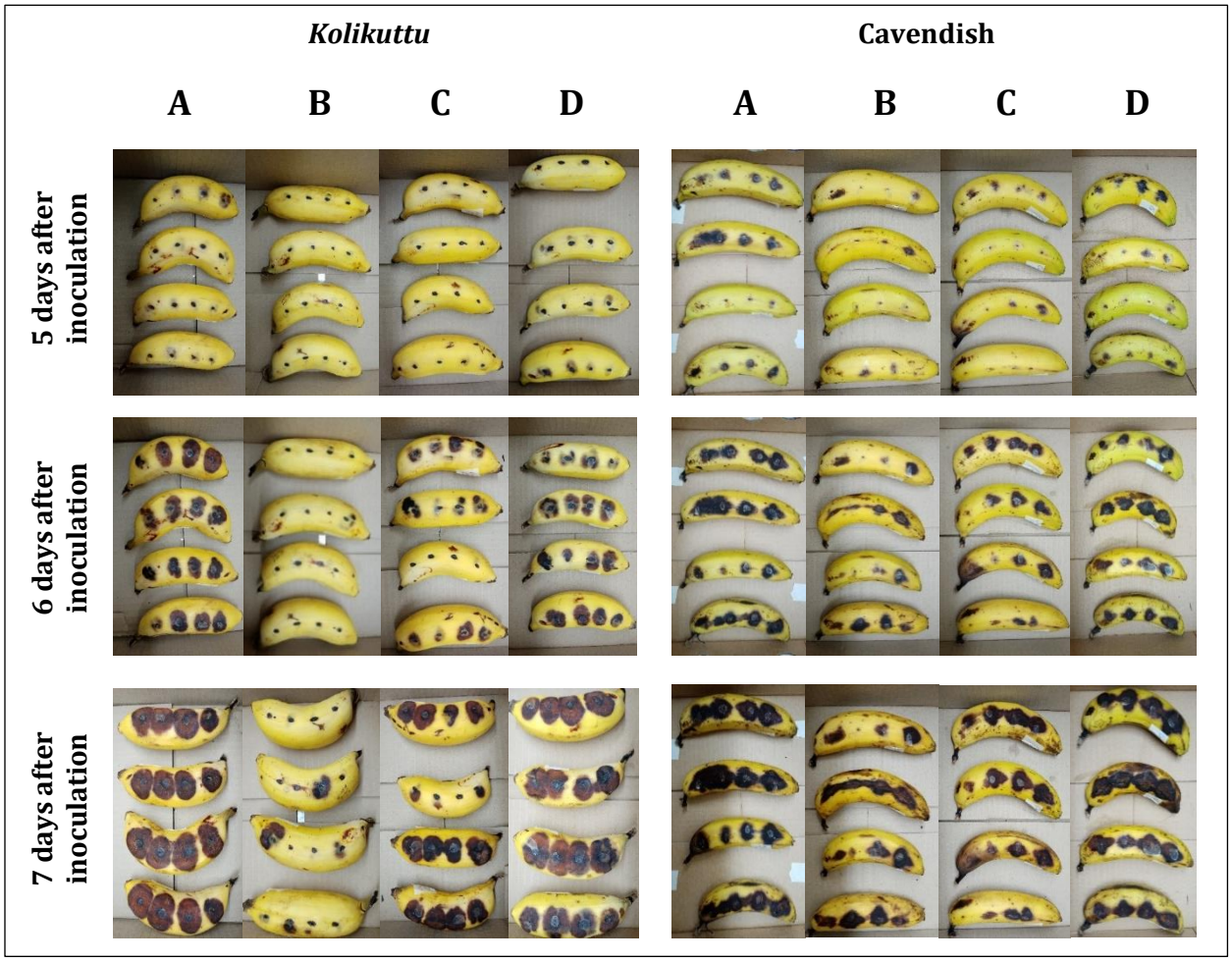


**Figure 03. Anthracnose disease severity of *C. musae* on Cavendish and *Kolikuttu* fruit peel. [(A) *C. musae* only (1 x $10^6$ spore/mL), (B) *T. virens* (1 x $10^7$ spore/mL) + *C. musae* (1 x $10^6$ spore/mL), (C) *T. virens* (1 x $10^6$ spore/mL) + *C. musae* (1 x $10^6$ spore/mL), (D) *T. virens* (1 x $10^5$ spore/mL) + *C. musae* (1 x $10^6$ spore/mL)]**

### Mass culture preparation of *Trichoderma virens*

### Selection of suitable substrates

Solid formulations are vital in the mass production of fungal spores because they offer a stable, manageable, and consistent environment for spore development and preservation. Figure 04 illustrates the mean sporulation of *T. virens* in different substrates over 12 weeks to determine the best substrate for mass production of *T. virens*. The data revealed that significantly ($p \leq 0.05$) higher mean spore production of *T. virens* was on rice seeds (9.345x$10^9$ spores/g) with compared to the other substrates as finger millet (8.117x$10^9$ spores/g), *P. maximum* (*Mana*) (6.023x$10^9$ spores/g), scraped coconut waste (4.132x$10^9$ spores/g), paddy straw (3.432x$10^9$ spores/g), Tea waste (3.15x$10^9$ spores/g), maize seeds (1.655x$10^9$ spores/g), water hyacinth (1.604x$10^9$ spores/g), and sawdust (1.808x$10^9$ spores/g).

### Spore concentration

Graphical illustration in Figure 05 indicates the variation in spore count of *T. virens* with time in different substrates. Over the studied period, *T. virens* has shown a distinct pattern of sporulation in different substrates.

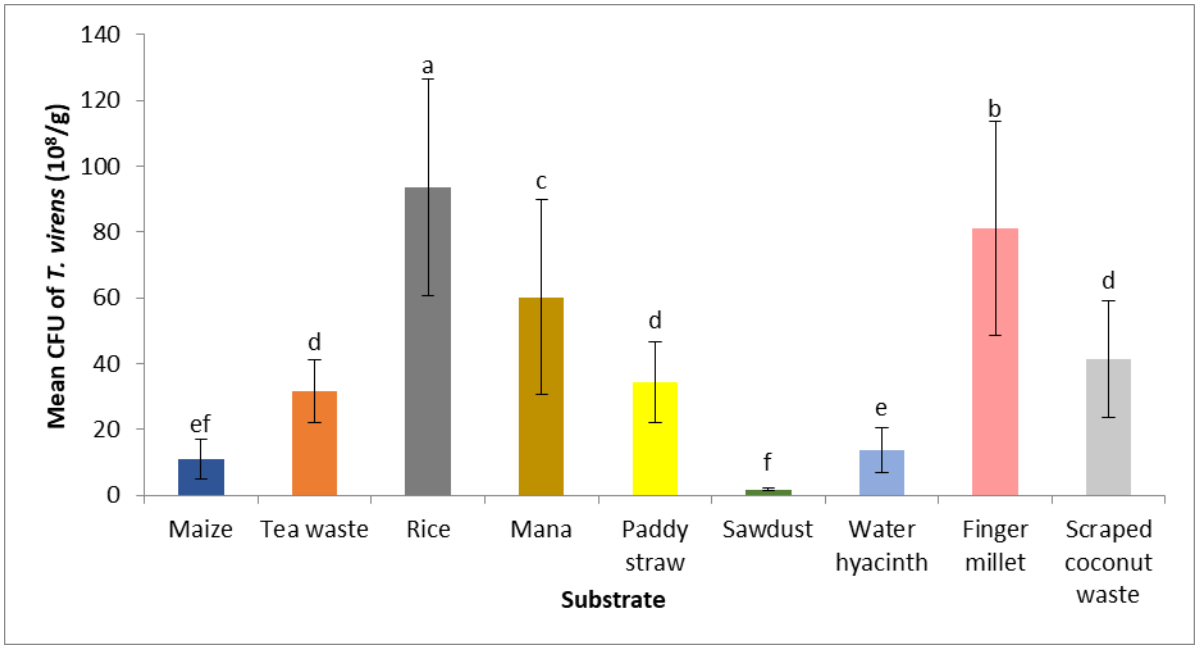


**Figure 04. Mean CFU of *T. virens* in different substrates throughout the study period (12 weeks).** *(Error bars indicate 95% confidence intervals. Means denoted as same letters are not significantly different at $p < 0.05$ according to DMRT)

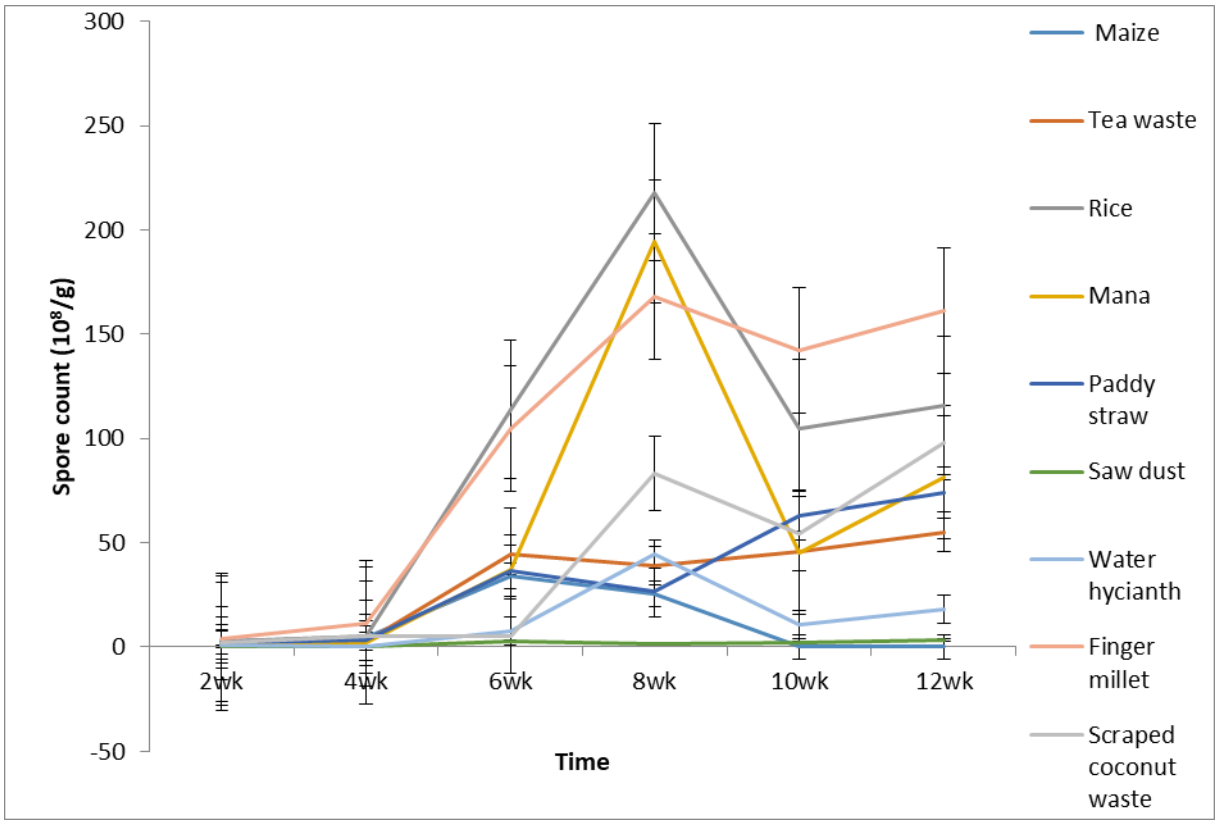


**Figure 05. Variation of *T. virens* sporulation in different substrates** *(Error bars indicate 95% confidence intervals. Means denoted as same letters are not significantly different at $p < 0.05$ according to DMRT)

In each substrate, the spore formation was initialized around the 4th week after inoculation. In the majority of instances, there was a significant rise in spore production starting from the 4th week to the 8th week. Following this, spore quantities decreased until the 10th week. Subsequently, new cycles of sporulation commenced in each substrate (Figure 05). The fact that the persistence of *T. virens* in the growth phase even beyond the 10th week of incubation indicates that prolonging the incubation period could lead to a further rise in spore production. During the 4-8 weeks period, rice, finger millet and *P. maximum* (*Mana*) exhibited an exponential increase in spore production in contrast to the other substrates. Although, spore production in each substrate reduced in the 10th week, finger millet and rice seeds showed a lower reduction compared to *Mana* and other substrates. Therefore, finger millet and rice would be better for the multiplication of *T. virens* among the tested substrates.

The sporulation of *Trichoderma* is primarily affected by various factors, including elevated nutrient levels, water absorption capacity, physical structure, texture, porosity, and the ability to create hydrogen bonds, as well as the C: N ratio of the substrates which enable the fungus to access the nutrients easily (Khan et al., 2011; Mshari, 2019). Furthermore, Pang and collaborators in 2006 demonstrated that a substrate with a loose and less dense structure promotes the diffusion of oxygen, the absorption of nutrients, and the assimilation by fungal mycelia. As a result, substrates like finger millet, rice, *P. maximum*, paddy straw, and water hyacinth employed in the current study exhibited significant growth and sporulation of *T. virens*, likely due to their loose and unconsolidated texture. The C: N ratio stands as a crucial factor that influences fungal spore production (Elson et al., 1998). Moreover, Li et al. (2017) and Sankina et al. (2017) indicated that insufficient nitrogen hampers hyphal growth and sporulation of fungi. Consequently, the substrates containing a high C: N ratio led to sluggish mycelium growth and reproduction rates. Hence, the sawdust substrate employed in this study displayed the lowest mean spore count, potentially attributed to a significant nitrogen deficiency. Further, wood materials typically possess varying quantities of extractives or secondary metabolites such as resins, terpenes, phenols, quinones, and tannins (Umezawa and Higuchi, 1991) which often have protective biological and anti-microbial activities (Torssell, 1997). The presence of such fungicidal properties within the sawdust may inhibit fungal colonization within the substrate (Asdaq et al., 2022). In this investigation, utilized Mango wood to obtain the sawdust that could harbor compounds with such antifungal properties that might also be contributed to the decrease in spore count.

Moreover, previous studies suggest that the time of optimum spore production of *Trichoderma* on different substrates is strain-specific owing ability to utilize carbon and nitrogen as a source of nutrition differently (Kumar et al., 2014). Sachdev et al. (2018) reported that sugarcane bagasse was observed to support maximum growth ($20.08\times10^7$ spores/g) of *T. lixii* TvR1 after two weeks of incubation. The conidial growth of *T. harzianum* was recorded as $10.80\times10^8$ cfu/g and $3.73\times10^8$ cfu/g on rice bran and sugarcane bagasse respectively, after three weeks of incubation (Tewari and Bhanu, 2004). Further, Siddhartha et al. (2017) demonstrated that the growth of *T. harzianum* was recorded higher in coir pith ($43\times10^{10}$ cfu/g) after 8 weeks of incubation. Similarly, in the present study, the substrates except the paddy straw, tea waste, maize and saw dust gained the highest spore production after 8 weeks of inoculation. In comparison with the lignin content, herbaceous plants contain higher amounts of cellulose (Klinke et al., 2004). Since *Trichoderm*a is a cellulosic fungus, cellulose-rich herbaceous plant materials such as *P. maximum*, paddy straw and water hyacinth would be better substrates for sporulation, growth and development for long periods.

During the present study, finger millet substrate also demonstrated remarkable efficacy in fostering fungal growth and sporulation. In terms of nutritional composition, finger millet generally contains substantial amounts of nutrients such as carbohydrates (72-79.5 %), protein (5.6-12.7 %), and elevated amounts of ash (1.7-4.13 %),

calcium (162-487 mg %) and iron (3.61-5.42 mg %) contents which enable enhanced fungal colonization (Kim et al., 2011; Singh and Raghuvanshi, 2012). Various physical forms of substrates can also be associated with the usability of nutrients. Compared to other grains like rice (2.5 mm), wheat (3 mm), and legumes (8–13 mm), finger millet grains are notably smaller (approx. 2 mm in diameter) (Jenkins et al., 1998). Hence, the small size of finger millet grain offers a significantly larger surface area per unit volume, providing ample room for fungi to proliferate and spore production. When the aggregated millet grains formed by the fungal mycelial networks are suitably broken into pieces once or twice during cultivation, the substrate is properly aerated. The extent of aeration is generally influenced by both mycelial growth levels and the physical attributes of the grains (Kim et al., 2011). These advantageous physical characteristics may contribute to the potential of finger millet grains as a well-suited substrate to yield substantial spore production. Another advantage lies in practical application in that the mycotized finger millet grains can be directly utilized as a granular product for field application due to the small grain size without additional processing. Generally, bio-fungicides have a short shelf-life due to their susceptibility to degradation at high temperatures, indicating a low percentage of conidial germination following exposure to high-temperature conditions (Devi et al., 2005; McClatchie, 1994). Nevertheless, fungal conidia could be exposed to high-temperature conditions during both product distribution in the market and subsequent application on crops (Burges, 2012). Kim et al. (2010) demonstrated the potential of millet grains as a substrate for generating conidia with enhanced thermos-tolerance in a mass-production system. Therefore, the robust growth and heightened survival rate during storage enable finger millet as an appropriate carrier material for effectively marketing *T. virens* as biocontrol agents on a commercial scale.

**Viability of *Trichoderma virens***

In the present study, the survival and viability of *T. virens* were evaluated up to the 12$^{th}$ week after inoculation because the colony count gradually increased and started to reduce to a constant level within that time. Figure 6 demonstrates the mycelium growth of re-inoculated *T. virens* from each substrate after 12 weeks of storage. Hewavitharana et al. (2018) have described that the colony counts of *Trichoderma* gradually increased and started to reduce to a constant level within 180 days. Since the viability of *Trichoderma* can change after 180 days, further studies are required to confirm the maximum growth and higher survival stabilization of *T. virens* in different substrates.

**CONCLUSION**

Based on the present study, the identity of the banana anthracnose-causing pathogen (*C. musae*) was confirmed morphologically and through DNA sequencing. Among the substrates used for the mass preparation of *T. virens,* rice seeds, *P. maximum* leaves and finger millet seeds showed significantly ($p < 0.05$) higher mean spore concentrations at 8$^{th}$ weeks after inoculation. *T. virens* could survive and be viable on the substrates up to 12 weeks after inoculation. Based on the spore concentration, viability, and easiness of handling; rice seeds, *P. maximum* leaves, and finger millet seeds were efficient as locally available low-cost substrates for the mass production of *T. virens* at room temperature of 28 $^0$C with sustaining viable spores.

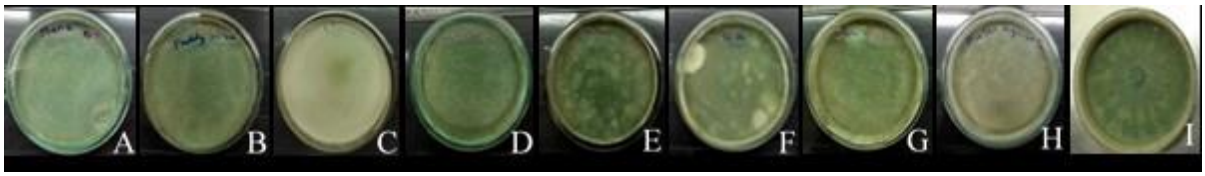


**Figure 06: Viability of *T. virens* spores obtained from different substrates after 12 weeks of storage at room temperature (28 $^0$C): A- *P. maximum,* B- Paddy straw, C- Maize, D- Coconut waste, E- Rice, F- Tea waste, G- Saw dust, H-Water hyacinth, I- Finger millet**

**REFERENCES**

Abang, M. M., Winter, S., Green, K. R., Hoffmann, P., Mignouna, H. D. and Wolf, G. A. (2002). Molecular identification of *Colletotrichum gloeosporioides* causing yam anthracnose in Nigeria. *Plant Pathology*, *51*(1), 63-71.

Abdel-Rahim, I. R. and Abo-Elyousr, K. A. (2017). Using of endophytic *Saccharomycopsis fibuligera* and thyme oil for management of gray mold rot of guava fruits. *Biological control*, *110*, 124-131.

Adebesin, A. A., Odebode, C. A., Ayodele, A. M. (2009). Control of Postharvest Rots of Banana Fruits by Conidia and Culture filtrates of *Trichoderma asperellum*. *Journal of Plant Protection Research, 49*, 302–307.

Anthony, S., Abeywickrama, K., Dayananda, R., Wijeratnam, S. and Arambewela, L. (2004). Fungal pathogens associated with banana fruit in Sri Lanka, and their treatment with essential oils. *Mycopathologia, 157*(1), 91-97.

Asdaq, S.M.B., Nayeem, N., Alam, M.T., Alaqel, S.I., Imran, M., Hassan, E.W.E. and Rabbani, S.I. (2022). *Tectona grandis* Lf: A comprehensive review on its patents, chemical constituents, and biological activities. Saudi Journal of Biological Sciences, 29(3), 1456-1464.

Barari, H. (2016). Bio-control of Tomato fusarium wilt by *Trichoderma* species under *in vitro* and *in vivo* conditions. *Cercetari Agronomice in Moldova, 1*(165), 91-98.

Bashan, Y., Kloepper, J.W., de-Bashan, L.E. and Nannipieri, P. (2016). A need for disclosure of the identity of microorganisms, constituents, and application methods when reporting tests with microbe-based or pesticide-based products. *Biology and Fertility of Soils, 52*(3), 283-284.

Bell, D.K., Wells, H. D. and Markham, C. R. (1982). *In vitro* antagonistism of *Trichoderma* species against six fungal plant pathogens. *Phytopathology, 72*, 378–382

Burges, H.D. (2012). *Formulation of microbial biopesticides: beneficial microorganisms, nematodes and seed treatments*. Springer Science & Business Media.

Chuang, T.Y. and Yang, H.R. (1993). Biological control of banana anthracnose. *Plant Pathology Bulletin*, *2*, 71-77.

Da costa, A.C., de Miranda, R. F., Costa, F. A. and Ulhoa, C. J. (2021). Potential of *Trichoderma piluliferum* as a biocontrol agent of *Colletotrichum musae* in banana fruits. *Biocatalysis and Agricultural Biotechnology*, 34, 102028.

Devi, K.U., Sridevi, V., Mohan, C.M. and Padmavathi, J. (2005). Effect of high temperature and water stress on in vitro germination and growth in isolates of the entomopathogenic fungus *Beauveria bassiana* (Bals.) Vuillemin. *Journal of invertebrate pathology*, *88*(3), 181-189.

Doni, F., Isahak, A., Che Mohd Zain, C.R., Mohd Ariffin, S., Mohamad, W., Nur'ashiqin, W. and Wan Yusoff, W.M. (2014). Formulation of *Trichoderma* sp. SL2 inoculants using different carriers for soil treatment in rice seedling growth. *Springerplus, 3*(1), 1-5.

Elson, M. K., Schisler, D. A. and Jackson, M. A. (1998). "Carbon-to- nitrogen ratio, carbon concentration, and amino acid composition of growth media influence conidiation of *Helminthosposition solani*,"*Mycologia* 98, 406-413

Farzaneh, M., Kiani, H., Sharifi, R., Reisi, M., Hadian, J. (2015). Chemical composition and antifungal effects of three species of *Satureja* (*S. hortensis*, *S. spicigera*, and *S. khuzistanica*) essential oils on the main pathogens of strawberry fruit. *Postharvest Biology and Technology*, *109*, 145–151.

Fu, G., Huang, S., Ye, Y., Wu, Y., Cen, Z. and Lin, S. (2010). Characterization of a bacterial biocontrol strain B106 and its efficacies on controlling banana leaf spot and postharvest anthracnose diseases. *Biological control*, *55*(1), 1-10

Grondona, I., Hermosa, R.M., Tejada, M.D., Gomis, P.F., Mateos, P.D., Bridge, E. and

Garcia-acha, I. (1997). Physiological and biochemical characterization of *Trichoderma harzianum* a biological control agent against soil borne fungal plant pathogens. *Applied and Environmental Microbiology 63*(8), 3189.

Hewavitharana, N., Kannangara, S.D.P and Senanayake, S.P. (2018). Isolation, identification and mass production of five *Trichoderma* spp. On solid and liquid carrier media for commercialization. *International Journal of Applied Sciences and Biotechnology, 6*(4), 285-293.

Jenkins, N.E., Heviefo, G., Langewald, J., Cherry, A.J. and Lomer, C.J. (1998). Development of mass production technology for aerial conidia for use as mycopesticides. *Biocontrol News and information*, *19*, pp.21N-32N.

Kanapathipillai, V.S., Ahmad, R. and Mahamad, M.I. (1988). The effect of sterile filtrates of *Trichoderma* spp. and *Penicillium* spp. and 4KGy irradiation of the spore germination of *C. musae*. *Movement of pests and control strategies*. 283-282.

Keswani, C., Mishra, S., Sarma, B.K., Singh, S.P. and Singh, H.B. (2014). *Applied Microbiology and Biotechnology, 98*(2), 533–544.

Khan, S., Bagwan, N.B., Iqbal, M.A. and Tamboli, R.R. (2011). Mass Multiplication and Shelf life of Liquid Fermented final Product of *Trichoderma viride* in Different Formulations. *Advance in Bioresearch, 2*(1), 178-182.

Khan, S.H., Aked, J. and Magan, N. (2001). Control of the anthracnose pathogen of banana (*Colletotrichum musae*) using antioxidants alone and in combination with thiabendazole or imazalil. *Plant Pathology*, *50*, 601-608.

Khleekorn, S. and Wongrueng, S. (2014). Evaluation of antagonistic bacteria inhibitory to *Colletotrichum musae* on banana. *Journal of Agricultural Technology, 10*(2), 383-390.

Kim, J.S., Je, Y.H. and Roh, J.Y. (2010). Production of thermotolerant entomopathogenic *Isaria fumosorosea* SFP-198 conidia in corn-corn oil mixture. *Journal of Industrial Microbiology and Biotechnology*, *37*(4), 419-423.

Kim, J.S., Kassa, A., Skinner, M., Hata, T. and Parker, B.L. (2011). Production of thermotolerant entomopathogenic fungal conidia on millet grain. *Journal of Industrial Microbiology and Biotechnology*, *38*(6), pp.697-704.

Klinke, H.B., Thomsen, A.B. and Ahring, B.K. (2004). Inhibition of ethanol-producing yeast and bacteria by degradation products produced during pre-treatment of biomass. *Applied microbiology and biotechnology*, *66*, pp.10-26.

Kolombet, L.V., Zhigletsova, S.K., Kosareva, N.I., Bystrova, E.V., Derbyshev, V.V., Krasnova, S.P. and Schisler, D. (2008). Development of an extended shelf-life, liquid formulation of the biofungicide *Trichoderma asperellum*. *World Journal of Microbiology and Biotechnology*, *24*(1), 123-131.

Kumar, S., Thakur, M. and Rani, A. (2014). *Trichoderma*: Mass production, formulation, quality control, delivery and its scope in commercialization in India for the management of plant diseases. *African Journal of Agricultural Research, 9*(53), 3838-3852.

Li, H., Zhang, Z., Li, M., Li, X. and Sun, Z. (2017). Yield, size, nutritional value, and antioxidant activity of oyster mushrooms grown on perilla stalks. Saudi Journal of Biological Sciences, 24(2), pp.347-354.

Li, Y.T., Hwang, S.G., Huang, Y.M. and Huang, C.H. (2018). Effects of *Trichoderma asperellum* on nutrient uptake and Fusarium wilt of tomato. *Crop Protection, 110*, 275-282.

Lopes, F.A.C., Steindorff, A.S., Geraldine, A.M., Brandão, R.S., Monteiro, V.N., Júnior, M.L., Coelho, A.S.G., Ulhoa, C.J. and Silva, R.N. (2012). Biochemical and metabolic profiles of *Trichoderma* strains isolated from common bean crops in the Brazilian

Cerrado, and potential antagonism against *Sclerotinia sclerotiorum*. *Fungal Biology, 116*(7), 815-824.

Lumsden, R.D. and Locke, J.C. (1989). Biological control of damping-off caused by *Pythium ultimum* and *Rhizoctonia solani* with *Gliocladium virens* in soilless mix. *Phytopathology*, *79*(3), pp.361-366.

Maqbool, M., Ali, A., Ramachandran, S., Smith, D.R. and Alderson, P.G. (2010). Control of postharvest anthracnose of banana using a new edible composite coating. *Crop Protection, 29*(10), 1136 – 1141.

Mc Garvey, P. and Kaper, J.M. (1991). A simple and rapid method for screening transgenic plants using the PCR. Biotechniques, 11(4), 428-432.

McClatchie, G.V., Moore, D., Bateman, R.P. and Prior, C. (1994). Effects of temperature on the viability of the conidia of *Metarhizium flavoviride* in oil formulations. *Mycological Research*, *98*(7), 749-756.

Meena, M., Swapnil, P., Zehra, A., Dubey, M.K. and Upadhyay, R.S. (2017). Antagonistic assessment of *Trichoderma* spp. by producing volatile and non-volatile compounds against different fungal pathogens. *Archives of Phytopathology and Plant Protection, 50*(13-14), 629-648.

Mshari, A. (2019). Ability of Manufacturing of Bioformulations using *Trichoderma* spp. for Biological Control of Some Plant Pathogenic Fungi. *Journal of Global Pharma Technology, 11*, 08-13

Pang, P.K., Poppe, L., Szakacs, G. and Ibrahim, C.O. (2006). Xylanase production by a local isolate, *Trichoderma* spp. FETL c3-2 via solid state fermentation using agricultural wastes as substrates. *Malaysian Journal of Microbiology*, *2*(1), 7-14.

Perera, O.D.A.N., Basnayake, B.M.K.N.K. and Karunaratne, A.M. (1999). Physicochemical characteristics, popularity and susceptibility to anthracnose of some local banana cultivars. *Journal of the National Science Foundation of Sri Lanka, 27*(2), 119-130.

Qualhato, T.F., Lopes, F.A.C., Steindorff, A.S., Brandao, R.S., Jesuino, R.S.A. and Ulhoa, C.J. (2013). Mycoparasitism studies of *Trichoderma* species against three phytopathogenic fungi: evaluation of antagonism and hydrolytic enzyme production. *Biotechnology letters, 35*(9), 1461-1468.

Ranasinghe, L., Jayawardena, B., Abeywickrama, K. (2005). An integrated strategy to control post-harvest decay of Embul banana by combining essential oils with modified atmosphere packaging. *International Journal of Food Science and Technology, 40*(1), 97-103.

Ranasinghe, L.S., Jayawardena, B. and Abeywickrama, K. (2003). Use of waste generated from cinnamon bark oil (*Cinnamomum zeylanicum* Blume) extraction as a postharvest treatment for Embul banana. *Food, Agriculture and Environment, 1*(2), 340-44.

Sachdev, S., Singh, A. and Singh, R.P. (2018). Optimization of culture conditions for mass production and bio-formulation of *Trichoderma* using response surface methodology. *3 Biotech, 8*(8), 1-8.

Samuels, G.J. (1996). *Trichoderma*: A review of biology and systematics of the genus. *Mycological Research*, 100: 923-935.

Sankina, O.V., Chernysh, A.P. and Sankin, A.S. (2017). Usage of farm animal waste for biogas production. In *IOP Conference Series: Earth and Environmental Science*, *66*, 1, 012034.

Siddhartha, N.S., Amara, K.V., Ramya, Mol K.A., Saju, K.A., Harsha, K.N., Sharanappa, P., Kumar, K.P. (2017). Evaluation of substrates for mass production of *Trichoderma harzianum* and its compatibility with Chlorpyrifos + Cypermethrin. *International Journal of Current Microbiology and Applied Sciences,* 6(8):3628–3635. https://doi.org/10.20546/ijcmas.2017.608.437

Singh, P. and Raghuvanshi, R. S. (2012). Finger millet for food and nutritional security. African *Journal of Food Science, 6*(4), 77-84.

Stover, R.H. and Simmonds, N.W. (1987). Bananas (No. 3. Ed.). *Longman Scientific and Technical*.

Tewari L, Bhanu C (2004). Evaluation of agro-industrial waste for conidia based inoculum production of biocontrol agent: *Trichoderma harzianum*. *Journal of Scientific and Industrial Research,* 63:807–812

Thangamani, P.R., Kuppusamy, P., Peeran, M.F., Gandhi, K. and Raguchander, T. (2011). Morphological and Physiological Characterization of *Colletotrichum musae* the Causal Organism of Banana Anthracnose. *World Journal of Agricultural Sciences, 7*(6), 743-754.

Thompson, A. K., and Burden, O. J. (1995). Harvesting and fruit care. In *Bananas and plantains*, 403-433. Springer, Dordrecht.

Torssell, K.B.G. (1997). Natural product chemistry: A mechanistic. *Biosynthetic and Ecological Approach, Second Edition, Apotekarsocieteten-Swedish Pharmaceutical Society (Stockholm)*, 313-347.

Udugama, S. (2002). Septoria leaf-spot disease of banana *Mycosphaerella eumusae*: a new record for Sri Lanka. *Annals of the Sri Lanka department of Agriculture*, *4*, 337-343.

Umezawa, T. and Higuchi, T. (1991). Chemistry of lignin degradation by lignin peroxidases. In: Leatham GF, Himmel ME (eds) Enzymes in biomass conversion. ACS Symposium Series, Washington, 236–246.

Vilaplana, R., Pazmino, L., Valencia-Chamorro, S. (2018). Control of anthracnose, caused by *Colletotrichum musae*, on postharvest organic banana by thyme oil. *Postharvest Biology and Technology*, 138, 56–63. https://doi.org/10.1016/j.postharvbio.2017.12.008.

White, T.J., Bruns, T., Lee, S.J.W.T., Taylor, J., Innis, M.A., Gelfand, D.H. and Sninsky, J.J. (1990). PCR protocols: a guide to methods and applications.

Woo, S. L., Ruocco, M., Vinale, F., Nigro, M., & Lorito, M. (2014). *Trichoderma*-based products and their widespread use in agriculture. *Open Mycol. J*, *8,* 71-126.

Zhimo, V. Y., Dilip, D., Sten, J., Ravat, V. K., Bhutia, D. D., Panja, B., & Saha, J. (2017). Antagonistic yeasts for biocontrol of the banana postharvest anthracnose pathogen *Colletotrichum musae*. *Journal of Phytopathology*, *165*(1), 35-43.