



A sustainable strategy for mass production of endophytic *Trichoderma virens* 159c and assessment of biological control properties via *in vitro* and *in vivo* assays

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Abstract

Trichoderma has been used as a biological control agent against plant pathogens and has since become a research focus for alternative chemical fertilizers. However, the use of potato dextrose agar as a carrier of *Trichoderma* for mass production is less suitable for field application due to its short shelf life and high cost. Therefore, this research attempts to utilize agricultural waste from oil palm to generate a high number of good-quality *Trichoderma virens* 159c spores and to evaluate its antagonistic activity against *Ganoderma boninense* which causes basal stem rot disease in the oil palm. The formulation, optimized with palm kernel cake and palm pressed fibre supplemented with nitrogen and carbon sources, showed a significant 2.6-fold increase in the total count of spores produced compared to the individual substrates. Enzyme analysis showed that the formulation produced higher levels of lytic and degrading enzymes, especially chitinase, which increased almost 3-fold compared to culturing only on PPF. Disease severity index (DSI) of oil palm seedlings using visual inspection and cross-section of the bole was evaluated for each treatment. The results showed that the plants inoculated with the *T. virens* 159c formulation had the lowest DSI, exhibiting only 18.3% visual DSI and 17.4% bole DSI, which was 37.3% and 37.1% significantly lower compared to the control, respectively. The present findings showed that the development of a sustainable *Trichoderma* formulation producing good-quality spores as a potential prophylactic treatment to slow down disease development in the oil palm caused by *G. boninense*.

Keywords – agriculture biomass – biological control agent – *Ganoderma* – mass production – *Trichoderma*

Introduction

Malaysia is the second largest palm oil producer in the world, where annual production in 2023 was 24.72 million tonnes of crude palm oil (CPO) with export earnings of oil palm products estimated at a new record high of RM 137.89 billion (Parveez et al. 2024). As the industry prepares for its fourth generation of replanting, it faces the dual challenges of managing the increasing amount of biomass waste and maintaining sustainable agriculture practices. The expansion of oil palm cultivation is accompanied by a significant rise in biomass residues such as oil palm trunks (OPT), fronds (OPF), palm pressed fibres (PPF), palm kernel cake (PKC), empty fruit bunches (EFB) and palm oil mill effluent (POME), which contribute to an overload that threatens environmental sustainability (Abdullah & Sulaiman 2013). The fundamental principles in waste management are to

reduce, reuse and recycle (Rice 2023). It is important to reduce the waste overload to maintain a sustainable agriculture. Some of the agricultural residues can be converted into organic fertilizers, animal feed, or even soil conditioners in the oil palm plantation through mechanical conversion. These conversions can be a sustainable approach as it might reduce the impact of waste to the environment by agro-industrial and livestock farming while improving the nutrient availability to plants and as soil conditioners (Jeyaraj & Sankarajan 2025, Shah et al. 2025). The use of organic fertilizer also improves soil organic content (SOC) with a larger yield trend along with contribution to carbon sequestration by favoring root development (Yan & Gong 2010).

With this, solid state fermentation (SSF) could be an economical and environmentally friendly approach to multiply important biocontrol fungi using agriculture residues (N. Mishra et al. 2016). SSF is one of the methods that is widely used and described as a process that takes place in a solid matrix. Moisture is required in the substrate to support the growth and metabolic activity of microorganisms (Thomas et al. 2013). It provides a stable product with an extended shelf life. This method has been used to produce metabolites such as enzymes, organic acids, antibiotics and other compounds that are useful in the industry (Angel et al. 2016, Kumar et al. 2021). Substrates that are commonly used in solid substrate fermentation are agricultural residues due to its high content of carbon source (Singh et al. 2009, Nigam & Pandey 2009).

The current study focuses on developing a formulation to mass produce endophytic *Trichoderma virens* 159c, which was first isolated by Sundram (2013b) from oil palm roots and its antagonistic activity has been extensively studied *in vitro* and *in vivo* towards *Ganoderma boninense* (Sundram et al. 2016, Angel et al. 2018, Paudzai et al. 2020). For *T. virens* 159c to be used as a prophylactic treatment for oil palm seedlings and high concentration of spores are required (Paudzai et al. 2020). However, the use of PDA as a carrier for *Trichoderma* is less suitable for field application due to its short shelf life and high cost (Doni et al. 2014).

Spores of *T. virens* 159c were mass produced using PDA where they were harvested with sterile distilled water (Sundram 2013a). However, there were a few limitations. Firstly, many PDA petri plates would be required to generate a large number of spores. Therefore, the cost of using PDA as a medium for spore production is high because PDA is expensive (Labchem 2026). Secondly, the quantity of spores that can be obtained from each plate is about $1-4 \times 10^8$ spores/ml per PDA plate (Sundram 2013a). Thirdly, it is a non-environmental-friendly method because a large quantity of petri plates will be discarded after the spores are harvested from the PDA. Finally, laborious work is required during preparation, including preparing the large number of PDA plates, subculturing the *Trichoderma*, and harvesting the spores. Consequently, it is important to develop a cheaper, easier and sustainable method for the mass production of *Trichoderma* spores. Utilization of agricultural waste as substrates creates an opportunity to manage waste and promote sustainable agriculture, especially wastes generated in the oil palm industry (Abdullah & Sulaiman 2013). Therefore, the present work aimed to evaluate the feasibility of using oil palm agriculture residues as substrates for a sustainable method to mass-produce *T. virens* 159c spores. Subsequently, effectiveness of the formulation was validated by evaluating the activity of the secreted enzymes, including the degradation enzymes (laccase) and lytic enzymes (β -glucanase and chitinase) by *T. virens* 159c during substrate utilization, and its effect on disease suppression towards *G. boninense*.

Materials & methods

Sporulation medium and inoculum development

Endophytic *T. virens* 159c was supplied by Plant Pathology and Biosecurity Unit, Biology and Sustainability Research Division, Malaysian Palm Oil Board, Malaysia (Sundram 2013b). The fungal species was cultured on potato dextrose agar (PDA) medium and incubated at 28 °C for 7 days. After sporulation process completes, spores were dislodged from the media by washing thoroughly with sterilized distilled water. The number of spores was calculated using a haemocytometer and adjusted to 10^7 spores/ml for application purposes in the *in vivo* assays.

***In vitro* assessment of best substrates and their formulation**

Wheat bran, rubberwood sawdust, palm kernel cake (PKC) and palm pressed fibre (PPF) from oil palm, were screened for their efficiency in producing mass inocula of *T. virens* 159c. The substrates were sterilized by autoclaving three times at 15 psi and 121 °C for 30 minutes and transferred to petri dishes (145/20 mm). An initial inoculum of five ml of *T. virens* 159c spore suspensions (10^7 spore/ml) were inoculated onto 20 g of sterilized substrates and incubated at 28 °C for 15 days. The experiment was performed in triplicate. Formulation optimization was subsequently carried out using the selected oil palm substrates. The optimized formulation consisted of PKC and PPF mixed at a 1:1 ratio (w/w), supplemented with 1.0% (w/w) yeast extract as an organic nitrogen source, 12.5% (v/w) molasses as an additional carbon source and 0.5% (w/w) lime soil dust as a pH-stabilizing mineral additive. Moisture content was adjusted to 50% (w/v) by adding 5 ml of sterile distilled water per 20 g of substrate mixture to adjust moisture content (50% w/v). The PKC + PPF mixture without supplementation served as the substrate control for comparison with the supplemented formulation. The spore concentration on different substrates was assessed by suspending the colonized substrate in 10 ml of sterile distilled water. The suspension was filtered through Whatman No. 1 filter paper and spore count was determined by Neubauer's Haemocytometer as spores/g for each substrate.

Storage stability and viability assessment of the formulation

It is crucial to ensure the microorganisms remain viable even after 12 months of storage to ensure that sufficient viable propagules are available at the time of application for successful colonization of the substrate and rhizophores. The storage stability of the optimized *T. virens* 159c formulation was evaluated under laboratory conditions (28 °C) over a 6 and 12-month periods. Freshly prepared formulation (month 0) was packed in the sterile polyethylene bags, sealed and stored at room temperature in the dark. Viable spore density was determined at 0, 6 and 12 months after production. For enumeration, 1 g of the formulation was aseptically transferred into 9ml of sterile distilled water and vortexed thoroughly to obtain 10^{-1} dilution. Serial dilutions were prepared up to 10^{-6} and 1 ml aliquots from appropriate dilutions were plated on Rose Bengal Agar (RBA) in triplicate.

The inoculated plates were incubated at 28 °C for 3 days and colonies exhibiting morphological characteristics of *Trichoderma* were counted. Viable count was expressed as colony-forming units per gram (CFU/g) of the formulation.

Extraction method of crude enzymes and determination of enzyme productivity

Extraction was carried out according to Naidu et al. (2020), with modifications. Ten grams of substrates containing *Trichoderma* were extracted with pre-chilled 0.05M sodium acetate buffer, pH 5.2 (1:10) and incubated at 28 °C for 18 hours at 200 rpm. The crude enzyme was filtered through Whatman No. 1 filter paper, and the supernatant was obtained by centrifugation at 12,000 g for 30 min. The supernatant was evaluated for the presence of lytic enzymes; β -glucanase and chitinase. β -glucanase activity was assayed (in triplicates) using a reaction system containing 250 μ L of a laminarin solution (1%) dissolved in 50 mM sodium acetate buffer, pH.5.0 and 0–125 μ L of enzyme solution. The reaction was allowed to proceed for 30 min at 37 °C and stopped by the addition of 1.5 ml of 3,5-dinitrosalicylic acid reagent. The reducing sugars formed were then determined spectrophotometrically at 550 nm. One unit of enzyme (U) was defined as the amount of protein necessary to produce one micromole of reducing sugars per min. Total chitinolytic activity was assayed by measuring the release of reducing saccharides from colloidal chitin. A reaction mixture containing 1 ml of extract, 0.3 ml of 1 M sodium acetate buffer (pH 4.6) and 0.2 ml of colloidal chitin was incubated at 40 °C. Following incubation, 0.75 ml of the upper layer of supernatant was transferred to a new tube and mixed with 0.25 ml of 1% solution of dinitrosalicylic acid in 0.7 M NaOH and 0.1 ml of 10 M NaOH. The mixture was heated at 100 °C for 5 min. Absorbance of the reaction mixture was measured at 582 nm it cooled to room temperature. A calibration curve with N-acetyl- β -D-glucosamine (NAGA) was used as the standard to determine reducing saccharide

concentration (Agrawal & Kotasthane 2012). The enzyme assays were repeated three times to obtain average data.

Efficacy of formulation with *Trichoderma virens* 159c against *Ganoderma boninense* PER71

Efficiency of the *T. virens* 159c formulation against *G. boninense* was tested through an artificially inoculated pathogenicity test using a seed germinating technique. The experimental design used in disease suppression trials was subjected to randomized complete block design (RCBD) with 90 replicates for each treatment. Each block has 30 replicates. The inoculum was prepared by using fresh rubber (*Hevea brasiliensis*) wood blocks of 6 × 6 × 3 cm with malt extract agar (MEA) that were sterilized at 15 psi at 121 °C. Once cooled, the sterilized block was inoculated with 7-day old *G. boninense* PER71 grown on PDA. The blocks were incubated in the dark at 28 °C for approximately eight weeks until fully colonized by *G. boninense* PER71 mycelium. The formulation was assessed in four different treatments: i) T1: positive control with only *G. boninense* PER71; ii) T2: compost; iii) T3: formulation colonized with *T. virens* 159c and iv) T4: formulation colonized with *T. virens* 159c with compost. *T. virens* 159c formulation was incorporated into soil mixture at the time of planting. *Ganoderma boninense* PER71 colonized blocks were placed beneath the soil layer, approximately 8 cm away from the surface in polybags containing soil mixture (3:1 v/v topsoil: sand). Single crossing of oil palm seeds was obtained from United Plantations Bhd., Perak, Malaysia. A booster application consisting of 10 ml × 10⁸ spores of *T. virens* 159c suspension was applied at four-week intervals (week 4, 8 and 12) for T3 and T4. After five months of infection, the seedlings were harvested and longitudinally sectioned to observe for internal symptoms based on bole decay of oil palm seedlings and expressed disease severity index (DSI) (Table 1–2). The percentage of DSI with symptoms in visual and bole tissue was calculated based on the index with modifications (Sundram et al. 2015, Naidu et al. 2018). The percentage using the formula as follows:

$$\text{Disease Severity Index (DSI)} = \frac{\sum(\text{rating} \times \text{number of infected plants})}{\text{total plants} \times \text{maximum rating}} \times 100\%$$

Table 1 Scale used to score disease severity index based on the foliar symptoms of oil palm seedlings inoculated with *Ganoderma boninense*.

Scale	Symptoms on oil palm
0	Leaves are green with no signs of infection. Absence of a white hyphal mass/button-like sporophore at the bole base.
1	Yellowing of the leaves observed. Presence of white hyphal mass/button-like sporophore at the bole area.
2	Necrosis of the leaves >25% – 50%. Presence of white hyphal mass/button-like sporophore at the bole area.
3	Necrosis of > 50% of the leaves. Presence of extensive white hyphal mass/button-like sporophore at the bole area.
4	Total necrosis and desiccation of the seedling,

Table 2 Scale used to score disease severity index based on root and bole tissues of oil palm seedlings inoculated with *Ganoderma boninense*.

Scale	Symptoms
0	Healthy, no internal rot
1	Initial infection stage, <10%
2	20% to 50% rotting of tissues
3	>50% rotting of tissues
4	>90% rotting of tissues

Evaluation of *Trichoderma virens* 159c for survival and their population in the rhizosphere soil of oil palm seedlings

Trichoderma virens 159c survival in the rhizosphere was evaluated according to Sundram (2005), with modifications. The soils from all treatments were sampled at 5 cm and 10 cm depth using a cork borer. The survival of *T. virens* 159c was enumerated via Rose Bengal Agar (RBA). Ten grams of soil sample was taken separately, resuspended in 10 ml of sterile water, and stirred well to get 10^{-1} dilution. One ml of the diluted soil sample was transferred to a test tube containing 9 ml of sterile distilled water to obtain 10^{-2} dilution and continued to dilution of 10^{-4} . One ml of the final dilution of each soil sample was aseptically pipetted into each sterile petri plate, to which 20 ml of sterilized and cooled RBA was poured and gently rotated for uniform mixing. The inoculated plates were inspected daily for the appearance of *Trichoderma* colonies, and the number of colonies was recorded with unit colony forming unit per gram (CFU/g).

Statistical analysis

Mean results for each parameter were subjected to ANOVA accordingly and tested for significance using LSD at $P < 0.05$ using SPSS software.

Results

Selection of suitable medium for sporulation and optimization of its formulation

Different agricultural wastes (PKC, PPF, wheat bran and rubberwood sawdust) and supplemented formulations were tested to determine the best formulation for the production of *T. virens* 159c spores. Significant differences in the number of spores produced by the different agricultural wastes (Fig. 1) suggested that *T. virens* 159c had the highest spore production in PKC (9.53×10^8 spores/g), followed by rubberwood sawdust, PPF and lastly, wheat bran.

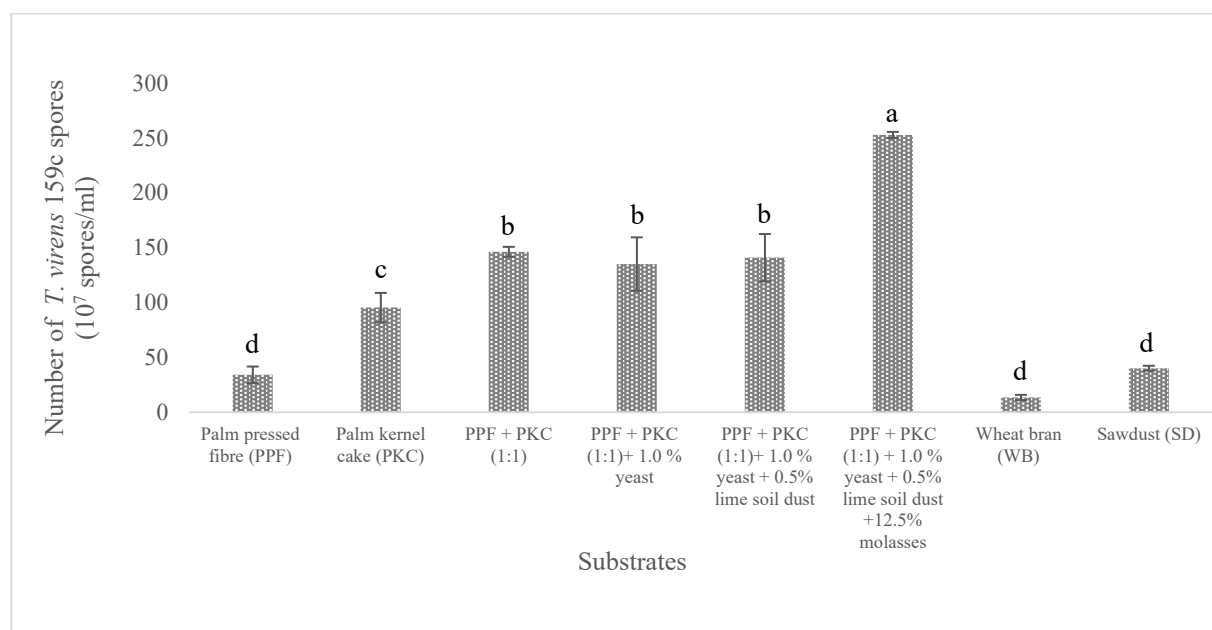


Fig. 1 – Productivity of endophytic *Trichoderma virens* 159c spores on different agricultural waste and comparison with modified formulation: palm pressed fibre (PPF); palm kernel cake (PKC); PPF + PKC (1:1); PPF + PKC (1:1) + 1.0% yeast; PPF + PKC (1:1) + 1.0% yeast + 0.5% lime soil dust; PPF + PKC (1:1) + 1.0% yeast + 0.5% lime soil dust + 12.5% molasses; wheat bran (WB); sawdust (SD). Different letters indicate statistically significant differences ($P < 0.05$).

As the current study focused on the usage of oil palm agriculture waste, the combination of PKC and PPF (1:1) was investigated. Interestingly, the combination of PKC and PPF increased the

production to 1.46×10^9 spores/g (1.5-fold increase). Addition of 1.0% yeast, with or without 0.5% lime soil dust, did not further increase sporulation significantly. In contrast, supplementation of PKC and PPF with C and N sources (PI2020001604) increased the number of *T. virens* 159c spore production from 9.53×10^8 spores/g to 2.53×10^9 spores/g (2.6-fold increase). The appearance of *T. virens* 159c mass produced on the modified substrate formulation appeared to be the densest compared to individual substrates (Fig. 2). A long-term storage stability test was conducted to evaluate the viability of the formulation under ambient laboratory conditions. The initial number of viable spores were 10^9 . After 6 months of storage, the viable count decreased to 10^6 CFU/g and further declined to 10^4 CFU/g at 12 months of storage.

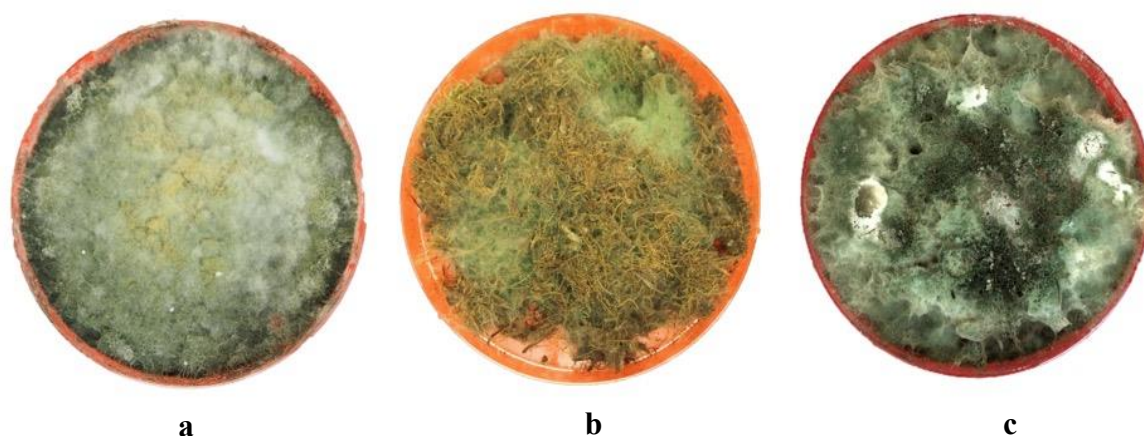


Fig. 2 – Endophytic *Trichoderma virens* 159c on oil palm waste. a Palm kernel cake (PKC). b Palm pressed fibre (PPF). c PKC and PPF supplemented with nitrogen and carbon sources.

Evaluation of lytic enzyme production from *Trichoderma virens* 159c in the substrates

Fig. 3 shows the chitinase and β -1,3-glucanase obtained in the solid-state fermentation using individual PKC, PPF, and formulated PKC and PPF as substrates for *T. virens* 159c. PKC as a substrate led to the highest production of laccase and MnP by *T. virens* 159c, followed by the formulated PKC and PPF, whereas *T. virens* 159c in formulated PKC and PPF showed the highest chitinase and β -1,3-glucanase production, followed by PKC as a substrate. The highest MnP of 8.22 $\mu\text{mol/ml/min}$ was obtained after 15 days of mass production with PKC as substrate, followed by 5.34 $\mu\text{mol/ml/min}$ with the formulated PKC and PPF as substrate. Laccase enzyme activity was also highest in PKC extract (138.05 $\mu\text{mol/ml/min}$) but it was only 98.87 $\mu\text{mol/ml/min}$ with formulated PKC and PPF extract. *Trichoderma virens* 159c releases β -1,3-glucanase enzyme when mass produced in the substrates, and the activity was the highest in the formulated PKC and PPF (581.52 $\mu\text{mol/ml/min}$), followed by PKC (551.84 $\mu\text{mol/ml/min}$). The chitinase enzyme produced by *T. virens* 159c also showed the highest activity in formulated PKC and PPF, 1248.62 $\mu\text{mol/ml/min}$, followed by PKC, 1225.74 $\mu\text{mol/ml/min}$. Nevertheless, no significant difference ($P > 0.05$) was observed between chitinase production of *T. virens* 159c obtained from PKC or formulated PKC and PPF. Based on the comparison of enzyme production levels, chitinase activity by *T. virens* 159c was highest on formulated PKC and PPF, followed by β -1,3-glucanase.

Effect of treatments on *Ganoderma* basal stem rot (BSR) disease suppression using seed germinating technique

The effectiveness of different treatments on the infection of oil palm seedlings by *G. boninense* was evaluated over five months. The disease symptoms included yellowing, necrosis of leaves and development of white hyphal mass and sporophores at the base of bole. DSI analysis revealed significant differences in disease suppression among the treatments (Fig. 4). Treatment T3, involving the application of *T. virens* 159c alone showed a marked reduction in disease severity. This treatment

resulted in a visual DSI of only 18.3% and a bole DSI of 17.4%. These values represented a substantial decrease of 37.3% and 37.1% in visual and bole DSI, respectively, compared to the untreated control (T1), which exhibited severe symptoms with 55.6% visual DSI and 54.5% bole DSI. Furthermore, the combination of *T. virens* 159 with compost (T4) also demonstrated effectiveness in reducing the infection symptoms by 28.4% in both visual and bole DSI. However, the T4 treatment did not further reduce the DSI as much as that observed in T3. These results highlight the possible protective effect of *T. virens* 159c against *G. boninense* with greater efficacy when used alone as compared to its combination with compost.

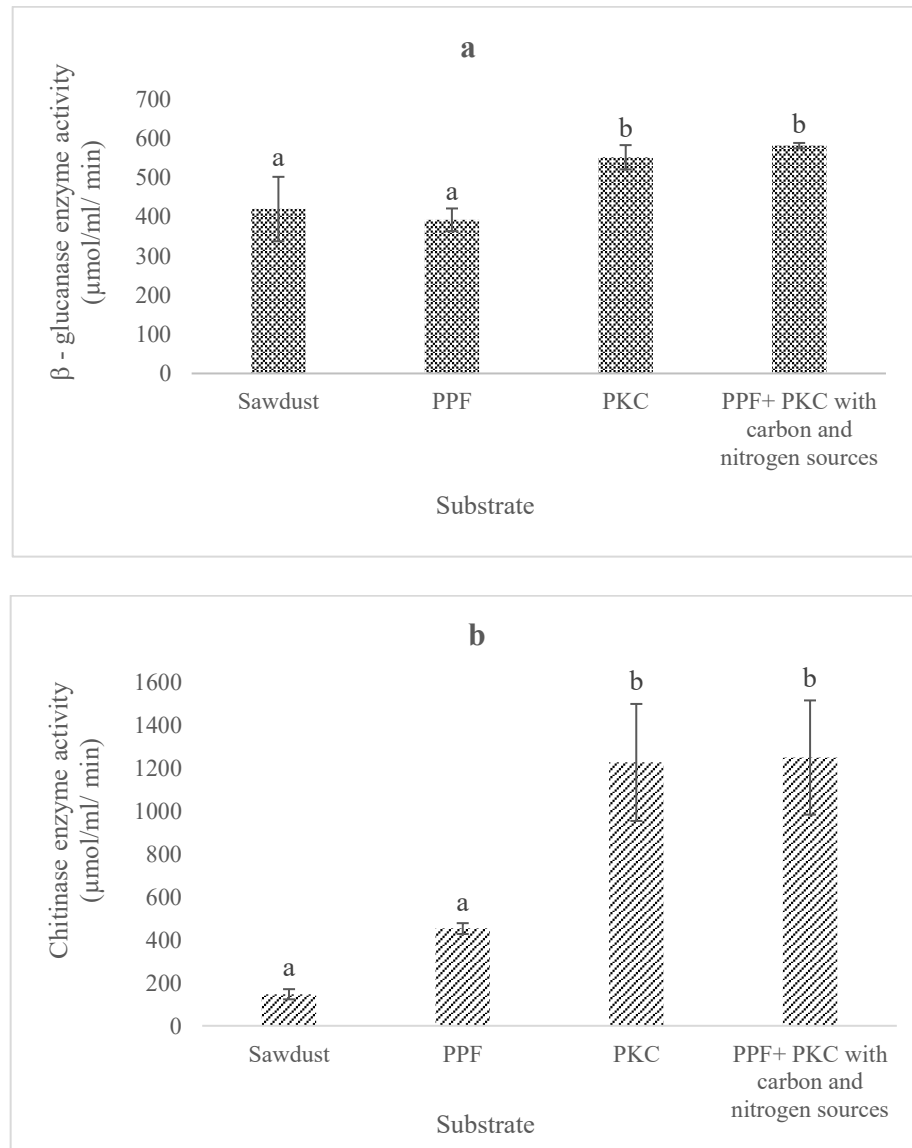


Fig. 3 – Comparison of enzyme productions by *Trichoderma* mass produced on PKC, PPF and formulated PKC and PPF. a β-1,3-glucanase enzyme. b chitinase enzyme. Different letters indicate statistically significant differences (P<0.05).

Evaluating survival of *Trichoderma virens* 159c in the rhizosphere of soil

Trichoderma virens 159c survival in the rhizosphere soil was significantly different at soil depths of 5 cm and 10 cm across different treatments over a period of 16 weeks (Fig. 5) and was calculated using serial dilution of 10^{-3} as the others were too many to count. The control (T1) and compost-only (T2) treatments showed negligible numbers of *Trichoderma* colonies at the sampling points, indicating the low amount of *T. virens* 159c in the treatments without inoculation.

For treatments involving *Trichoderma*, which was the T3 and T4, there was a noticeable increase in the *Trichoderma* spore population overtime, indicating successful colonization and persistence in the soil. At 5 cm depth, T3 led to a progressive increase of up to 1.85×10^5 cfu/g *Trichoderma* by the 16th week. Similarly, the *Trichoderma* with compost treatment (T4) demonstrated an initial low cfu count, which increased to 1.65×10^5 cfu/g by week 16 after inoculation. At a depth of 10 cm, the increase in *Trichoderma* was even higher. Treatment T3 exhibited a gradual increase from an initial 3.16×10^4 cfu/g to 1.76×10^5 cfu/g by week 16, while T4 showed a rise from 5.44×10^3 to 1.57×10^5 cfu/g in the same period. These trends suggested that *T. virens* 159c not only survives but multiplies when applied as a fortified organic compost with higher survival rates of *Trichoderma* noted in the treatment without compost.

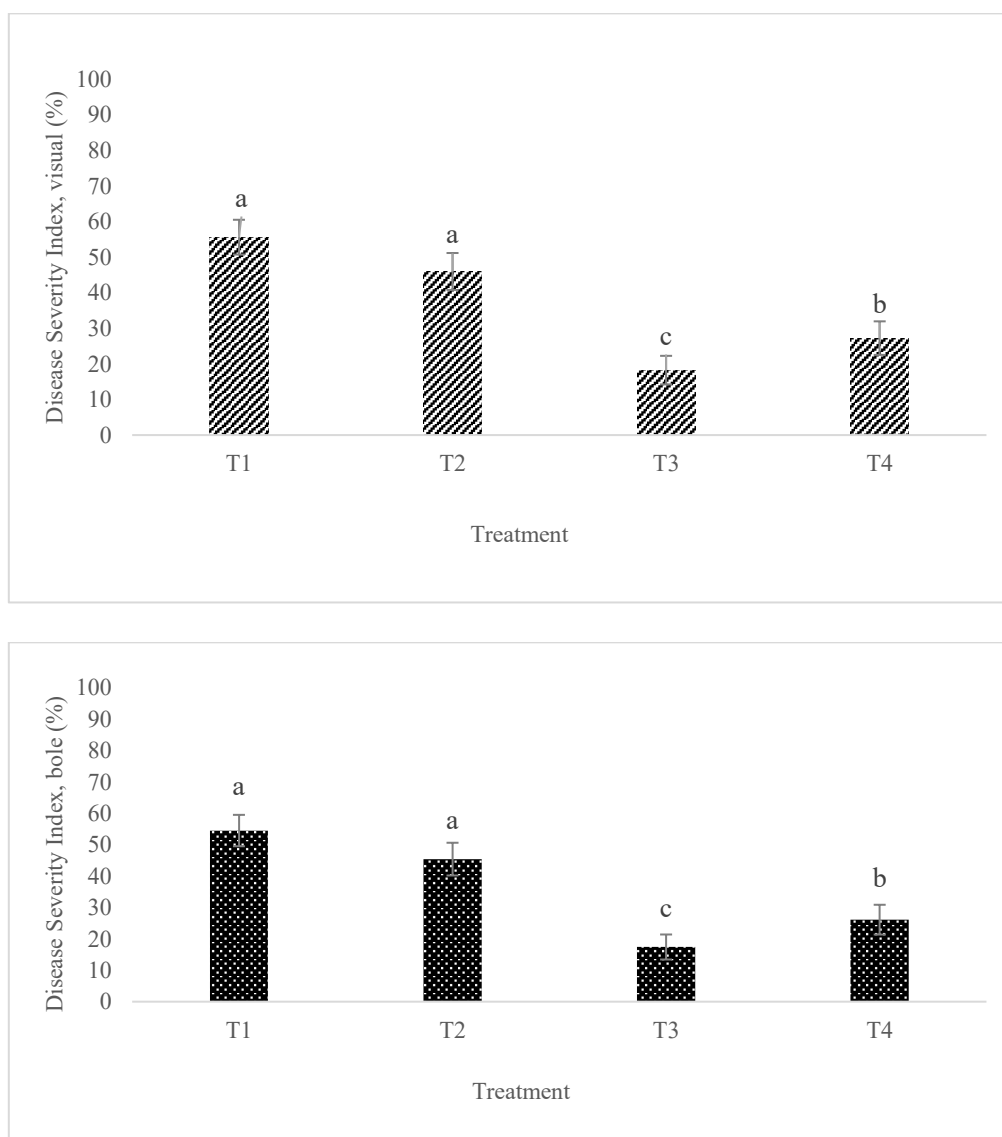


Fig. 4 – Percentage disease severity index (DSI) of *Ganoderma*-infected oil palm for visual (upper) and bole symptoms (lower) after different treatments: i) T1: positive control with only *G. boninense* PER71; T2: compost; T3: formulation colonized with *T. virens* 159c and T4: formulation colonized with *T. virens* 159c with compost. Different letters indicate statistically significant differences ($P < 0.05$).

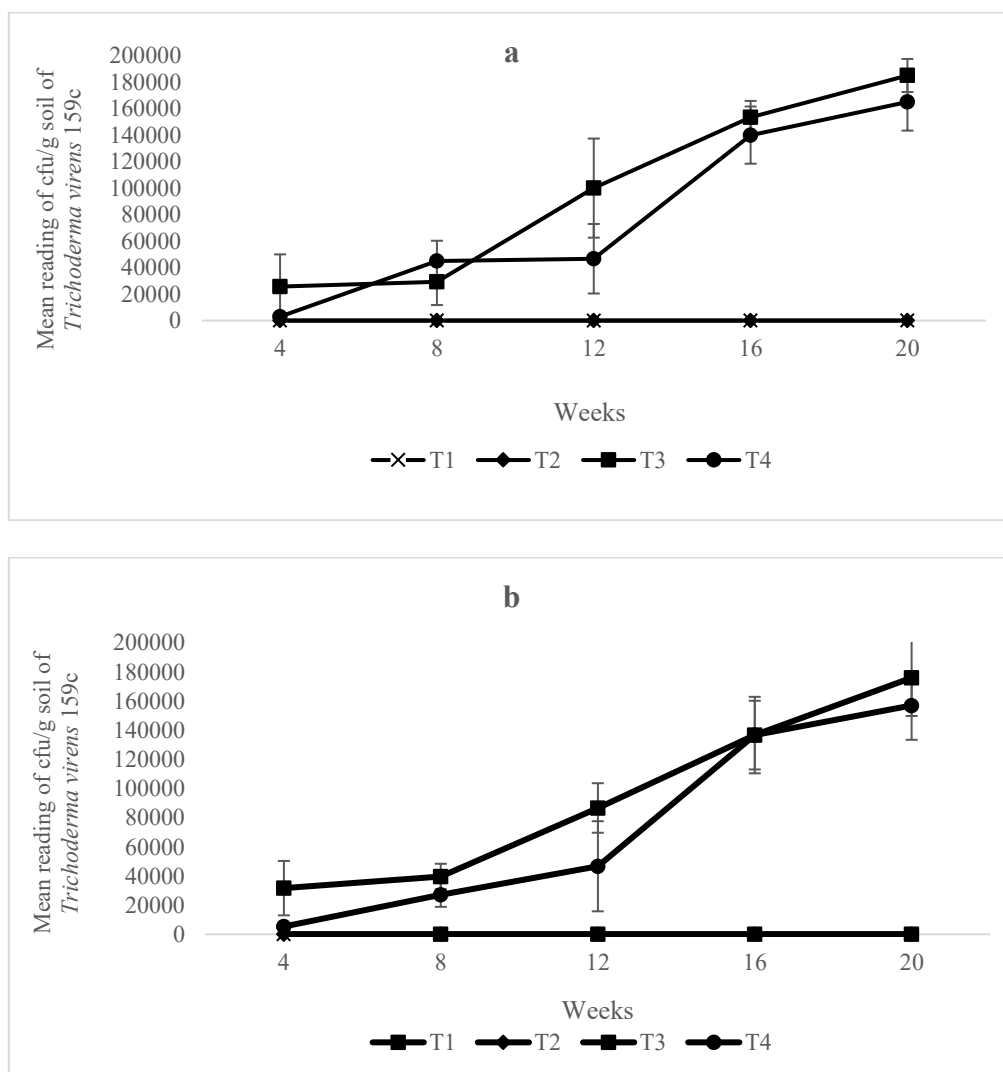


Fig. 5 Mean reading of cfu/g soil of *Trichoderma virens* 159c at a 5 cm. b 10 cm depth between 4 to 20 weeks after inoculation.

Discussion

Trichoderma is a potent biocontrol agent and is recognized as a highly rhizosphere competent microbe (Sundram 2013b). It can colonize and proliferate on the growing root surface over a considerable period. Other than being a biological control agent, it is also able to act as natural decomposition agent reported by several studies (Sharma et al. 2016). The rate of the decomposition process increases when *Trichoderma* was inoculated in agricultural residues (Amira et al. 2011, Sharma et al. 2016). The current findings focus on the mass production of *Trichoderma* spores, suggesting that PKC is the main substrate that contributes to the production of a high number of *Trichoderma* spores. Substrate PKC contains 16% fibre and a high phosphorus to calcium ratio (Aspar 2001). It is also a good source of C, making it as a cheaper SSF substrate for different strains of microorganisms. (Ong et al. 2004, Ramachandran et al. 2007). Meanwhile, PPF is a lignocellulose biomass that is able to serve as an alternative substrate for the mass production of *T. virens* due to its high content of fibre and C source (47.2%) (Kolade et al. 2006). The loose and less compact texture of PPF also allows oxygen diffusion, nutrient absorption and assimilation by fungal mycelia (Pang et al. 2006). The properties of PPF are an added advantage to the modified formulation, which can further enhance the growth of *T. virens* 159c. Rich C and N supplementation is important to further enhance the growth of microorganisms' spore production in the mass production process (S. Mishra et al. 2016).

Nonetheless, both PKC and PPF are deficient in N content. Organic N sources are able to provide the required vitamins, micronutrients and intermediate compounds for fungi. It also acts as a stimulator and precursor that is essential for optimum growth (Hashem et al. 2015). The pH stability is also important and a stable pH 5 is suitable for the proliferation of *T. virens* 159c. The formulation for the mass production of *T. virens* 159c was modified and optimized. The advantage of this modified formulation is that its initial pH ranges of pH 5.0–5.3, favours *T. virens* 159c growth. This study showed an excellent boost in the productivity of *T. virens* 159c spores by combining both agriculture residues with minimal supplementation of nutrients. Besides being a food substrate promoting the mass production of *T. virens* 159c spores, this modified formulation is also able to act as a carrier material for bioremediation of the soil. One of the obstacles to the formulation is the loss in viability of the propagules over time. PKC and PPF offer a supportive matrix that aids the colonization and growth of *Trichoderma*. The fibrous and porous nature provides a large surface area for fungal attachment and proliferation, which is essential to establish a significant population of the biological control agent (Ogawa et al. 2024). In addition, these materials contain residual oils and other nutrients that can serve as energy source for microbial growth, which enhances the viability and longevity of *Trichoderma* spores. It is important to ensure that the formulation is able to sustain the viable nature of biological control agents (Kumar et al. 2014).

This enzymatic study is important to further support the use of oil palm waste as a substrate for mass production of *Trichoderma*. Selecting an appropriate substrate is a crucial aspect of SSF as the solid material will act as a physical support and nutrient source for the microorganisms (de Castro et al. 2015). The physical and chemical characteristics of the substrates are important parameters for enzyme production in the SSF (Qasim et al. 2024). The findings showed that PPF as a substrate alone is not a suitable substrate for the mass production of *T. virens* 159c as it produces less spores and was inefficient in producing beneficial enzymes compared to the use of PKC and optimized formulation. The findings in this study further substantiate that PKC is a promising substrate that generally contains 50.3% carbohydrates, 14.8% rich-protein content, 16.7% crude fibre, and 7.9% of oil (Othman & Halim 2020) and its highly nutrient-dense. These fibrous substrates are rich in complex polysaccharides and structural components, which stimulate *Trichoderma* to upregulate and secrete cell-wall-degrading enzymes during SSF process (Ahmed et al. 2009). The significant increase in chitinase and β -1,3-glucanase production observed in PKC and optimized formulation can be attributed to the synergistic effects of enhanced fungal biomass. Previous comprehensive studies on the optimization of enzymes by fungi under solid-state cultivation show that an increase in biomass yield can contribute to the secretion of extracellular enzymes (Chen et al. 2016, Naidu et al. 2020). This optimised formula significantly increases spore production by 3-fold and efficiently produces lytic enzymes (chitinase and β -1,3-glucanase) at the same time. The significant increase in both enzymes in PKC and the optimized formulation can be attributed to the specific lignocellulosic and nutritional profiles of the substrate, which act as direct environmental triggers for complex genetic regulation. The production of hydrolytic enzymes in *Trichoderma* is controlled by a complex regulatory network involving transcription factors, signalling pathways and environmental cues (Horta et al. 2018). Synthesis-specific enzymes such as chitinase, rely on environmental stimuli such as carbon source availability. These external cues possibly activate essential signalling cascades (Galello et al. 2024), which drive enzyme gene transcription. Once the regulatory network is activated by the available optimized nutrients from the agricultural waste, *Trichoderma* releases the enzymes. PKC with highly accessible carbon and nitrogen profiles contributes to the secretion of enzymes induced during SSF will assist in antagonistic effects against pathogens in the future (Gajera & Vakharia 2012). Both chitinase and β -1,3-glucanase are two key cell wall-degrading enzymes involved in the remote sensing of *Trichoderma* to other fungi to initiate mycoparasitism (Harman et al. 2004). Ultimately, the optimized formulation does more than simply support rapid fungal growth, it also stimulates *T. virens* 159c to produce high levels of cell-wall-degrading enzymes before it is applied in the field.

The pathogenicity assay results provide the evidence to support the use of *T. virens* 159c as a viable biocontrol agent against the *Ganoderma* disease in oil palm. The significant reduction in DSI

in treatments involving *Trichoderma* (T3 and T4) compared to the control (T1) and compost-only (T2) treatments highlight the importance of integration into sustainable agricultural practices. This supports previous studies by Harman et al. (2004) and Sundram (2005) that the *Trichoderma* species could effectively antagonize pathogenic fungi through various mechanisms, including mycoparasitism, secretion of antimicrobial compounds and enhancement of plant defence mechanisms. Sundram (2013b) also previously demonstrated the efficacy of *T. virens* through *in vitro* assay results. Angel et al. (2018) showed that *T. virens* 159c was capable of producing antifungal and antimicrobial compounds that suppressed *Ganoderma*'s growth in an *in vitro* study. Another study conducted by Paudzai et al. (2020) proved that the inoculation of this *Trichoderma* strain were able to promote the vegetative growth and development of oil palm seedlings. The application of *Trichoderma* is vital to be carried out before or concurrently with the infection of *Ganoderma*. This to provide a protection booster to the oil palm against *Ganoderma* infection. A similar effect is expected when the palms are planted in the field and treated at the planting hole during the replanting phase. The close alignment between visual DSI and bole DSI observed in this trial is highly characteristic of the developmental stage of the oil palm seedlings. As the harvesting time is on the 5th month, the basal bole in 5-months old seedlings is small, in which minor tissue decay could disrupts its limited vascular system. Therefore, immediately translates to visible external symptoms such as external leaf yellowing and necrosis. This developmental dynamic is supported by previous nursery assessments, which demonstrated a 96% correlation ($R^2 = 0.9664$) between external visual symptoms and internal progressive bole decay in the oil palm seedlings (Sundram et al. 2015).

Compost can enhance the biological activity of biological control agents by providing essential nutrients and creating a more favorable microenvironment (Tóthné Bogdányi et al. 2021), providing the necessary elements for microbial growth. However, the optimal balance of *Trichoderma* and compost requires further investigations to maximize both survival and biocontrol efficacy. Previous studies have shown that the type of organic matter and its decomposition stage can significantly influence the microbial interactions and biocontrol outcomes (Raza et al. 2023). The interaction between compost and microbial biological control agents can vary depending on the compost's physical and chemical properties (Whipps 2001).

The current study extends these findings by demonstrating not only *Trichoderma*'s efficacy in reducing disease severity but also its ability to survive and proliferate in the rhizosphere, which is crucial for sustained biocontrol activity. Absence or very low recovery of *T. virens* 159c in the control (T1) and compost-only (T2) treatments across all sampling points was likely due to the treatments not being inoculated with *T. virens* 159c, thus the fungi were low in amount in the rhizosphere. Second, the detection method involved serial dilution to 10^{-3} , which may have further limited the recovery of any residual or naturally occurring *Trichoderma* spp. that were present in lower concentrations. Without an external inoculum or enrichment, *Trichoderma* populations were not sufficiently abundant to be detected under the conditions used.

The survival of *Trichoderma* in the soil rhizosphere was witnessed by the increased cfu/g in treatments T3 and T4 over the 20-week period, suggesting it can establish a robust presence that is likely to contribute to its effectiveness in suppressing *G. boninense*. The increased colony count indicates active growth and colonization, factors essential for the prolonged biocontrol of pathogens in the soil environment (Whipps 2001). This is particularly significant as there will be competition with the native microflora, as well as the harsh soil conditions that can impede the growth and maintenance of the biological control agent.

Incorporating effective *Trichoderma* formulation into the management strategies of oil palm nurseries or field could significantly reduce the reliance on chemical fungicides. Reduction in chemical inputs not only minimizes environmental and health risks but also aligns with global sustainability efforts, potentially improving market acceptance for crops produced under these practices (Chojnacka 2024). Further research will focus on the application rates of *T. virens* 159c formulation to enhance its efficacy and also should include spore germination rate to better characterize spore quality and predict field efficacy of the formulation. Investigating the interactions between different types of compost and *Trichoderma* could lead to more effective disease control

strategies. Long-term field trials are important to validate the efficacy of *Trichoderma* formulation in diverse environmental conditions and to assess their impact on crop health and productivity over extended periods.

Conclusions

Besides saving time and cost, this discovery supports the zero-waste policy where the by-products produced by the oil palm industry can be utilized effectively as a substrate for the mass production of *Trichoderma*. PKC and PPF formulations have shown excellent potential as substrates for spore production. The modified formulation developed using PKC and PPF as the main carbon source will improve the sustainability of the oil palm industry through effective waste management, by minimizing and recycling the waste. Optimum usage of the waste products generated from the palm oil mills will not only create an opportunity to reduce environmental pollution but also provide an inexpensive substrate for the mass production of *Trichoderma* for future commercialization efforts.

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Conflict of Interest

None

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Accessibility of data

Trichoderma virens 159c (KT363921)

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