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In Vitro Screening of Endophytic *Trichoderma* sp. Isolated from Oil Palm in FGV Plantation against *Ganoderma boninense*

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Abstract

The potential of several endophytic *Trichoderma* isolates, isolated from healthy oil palm roots was assessed against *Ganoderma boninense* (PER71), the causal agent of Basal Stem Rot (BSR) disease through *in vitro* screening. In the dual culture assay, all endophytic *Trichoderma* isolates were found to be capable of inhibiting the growth of PER71 mycelium, by causing more than 70% inhibition of the pathogen's radial growth (PIRG). *Trichoderma asperellum* M103 has caused the highest inhibition at 93.14%. The volatile compound produced by M103 also suppressed 65% of the pathogen's radial growth. On the other hand, *T. harzianum*, M108 was found to have the ability to produce non-volatile compounds that is toxic and it has effectively inhibited 98.18% of the growth of PER71. Collectively, *T. asperellum* M103 and *T. harzianum* M108 have the potential to inhibit the growth of *G. boninense* PER71. These isolates were shown to be viable options for the future management of *G. boninense* in the oil palm field.

Keywords

Endophytic *Trichoderma*, *Ganoderma boninense*, *In Vitro*, Oil Palm

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1. Introduction

Basal Stem Rot (BSR) disease in oil palm caused by *Ganoderma boninense* is one of the significant diseases which can affect oil palm yield in Malaysia's most productive area. With no effective control measures, the total acreage of oil palm plantations infected with BSR disease has continuously grown year after year [1]. Chemical solutions may only provide rudimentary control and are not favorable

due to their environmental impact [2]. As the world's third largest oil palm estate operator, FGV is dedicated to implementing a sustainable management system for pests and diseases, including BSR, in FGV oil palm estates.

Interest in using biological control agents (BCAs), especially *Trichoderma* sp. for disease management, apparently has gained much attention from agriculture industries. *Trichoderma* sp. which is commonly found in the soil and rhizosphere root ecosystem, provides a variety of benefits including controlling soil-borne pathogens through mycoparasitism, competition for nutrients and space, as well as the ability to produce antifungal metabolites that are toxic to a variety of plant pathogens [3] [4] [5]. On top of that, hundreds of *Trichoderma*-based products have been released in the market and become popular due to their being inexpensive and high viability after long-term storage. The products are also user-friendly and sustainable for the environment. *Trichoderma harzianum*, *T. virens*, and *T. hamatum* have shown potential effects against *G. boninense* both *in vitro* and in the field [6]. Recently, research has shown that *Trichoderma* sp., which is classified as an endophyte, has been successfully isolated from cocoa trees [7] and oil palm roots [8]. This characteristic gives the privilege for the microbes to colonize and sustain their population in the internal plant tissues without causing any harm to the plant. Due to this special characteristic, the endophytic *Trichoderma* would be sustained in the root region of the plant for a certain period and provide ample protection to the plant against pests and diseases.

However, information on the effect of endophytic *Trichoderma* sp. on *Ganoderma* sp. is still insufficient. Therefore, this study was conducted to isolate potential endophytic *Trichoderma* and evaluate their antagonistic effects against *G. boninense*, through dual cultures as well as the production of volatile and non-volatile compounds.

2. Materials and Methods

2.1. Root Sampling and Isolation

The sampling activities were conducted at FGV plantations, in Malaysia. The root samples were collected within the radius of the palm canopy and stored at 4˚C prior to the isolation process. Endophytic *Trichoderma* was isolated from healthy oil palm roots and cultured on Martin's Rose Bengal (MRB) agar, one of the selective media for *Trichoderma* isolation [9]. All the root samples were surface sterilized [10]. Root samples were cut into 10 cm lengths and cleaned under running tap water to remove the debris. About 0.5 cm was then removed from both tips. The remaining roots were cut into 3 cm lengths. Roots were surface sterilized in 10% sodium hypochlorite for two minutes, then in 50%, 70%, 90%, and 99% ethanol for 30 seconds each, before rinsing three times with sterile distilled water (SDW). The root samples were then allowed to dry on sterilized filter paper. Once dried, 1 cm of the ends of each section was discarded. The cuticle from the middle section was removed, cut into two sections of 0.5 cm each and

placed on MRB. Petri dishes were incubated at 28˚C ± 2˚C for 7 days. The distinguished individual colonies were subcultured onto Potato Dextrose Agar (PDA) (Oxoid) plates and were then incubated for 7 days. The pure cultures were finally maintained until further testing.

2.2. Dual Culture Assay

A total of 14 endophytic *Trichoderma* isolates that was successfully isolated were screened for their antagonistic potential against *Ganoderma boninense* (PER71), obtained from Malaysian Palm Oil Board (MPOB) through dual culture assay [11]. Approximately 5 mm plug agar of PER71 was subcultured at 1 cm from the 90 mm Petri plate's circumference, while 5 mm plug agar of endophytic *Trichoderma* was inoculated opposite the PER71 agar plug (1 cm from circumference of Petri plate). Another Petri plate, which was only subcultured with PER71, was prepared as control. All the endophytic *Trichoderma* isolates in dual culture assay were incubated at 28˚C ± 2˚C for seven days. Percentage of inhibition radial growth (PIRG) was then measured based on the formula as below:

$$(\%)\text{PIRG} = \left[(\text{R1} - \text{R2})/\text{R1}\right] \times 100$$

where, R1 = radial growth of PER71 (control); R2 = radial growth of PER71 in dual culture plate.

The number of days required by endophytic *Trichoderma* to completely overgrow PER71 was recorded. The shortest number of days indicates a significant antagonistic effect in dual culture assay [12]. Their ability to overgrow and inhibit the growth of PER71 was then scored according to Bell's scale [13]. where:

R1 = 100% overgrowth;

R2 = 75% overgrowth;

R3 = 50% overgrowth;

R4 = locked at the point of contact.

To evaluate the potential of PER71 to proliferate, the inhibition zone between endophytic *Trichoderma* and PER71 in a dual culture assay was plugged and subcultured onto a new PDA. The ability of PER71 to grow on the new PDA was observed.

2.3. Scanning Electron Microscope

Scanning electron microscopy (SEM) observation was conducted at Quasi-S Technology Sdn. Bhd. The inhibition zone from plates with the highest PIRG was examined for their hyphal interaction activity through SEM. The plug agar at the inhibition zone was cut into 1 cm^3 and fixed in 1% gluteraldehyde fixative for 12 - 24 hours at 4˚C. Phosphate buffer was then used to wash the sample. The sample was serially dehydrated with acetone concentrations; 30%, 40%, 50%, 60%, 70%, 80%, 90% and 100% for 20 minutes. After that, the samples were dried and coated with gold before viewing through SEM.

2.4. Production of Volatile Compounds

The double plate method was used to test the production of volatile compounds

by endophytic *Trichoderma* candidates against *G. boninense* PER71. A 5 mm plug agar of all examined endophytic *Trichoderma* (5 days old) and PER71 (7 days old) were cultured on the center of PDA at 28˚C ± 2˚C. After 24 hours, the Petri plate covers of both *Trichoderma* and PER71 were removed. The plate containing endophytic *Trichoderma* culture was placed upside down on top of a plate containing PER71 culture. Both plates were tightly sealed together with parafilm to avoid any gap. The presence of gap between the plates may not only prohibit physical contact between the fungi but also prohibit the formation of volatile compounds by the tested *Trichoderma.* Another plate subcultured with PER71 alone was used as control. All culture plates were incubated at 28˚C ± 2˚C for 7 days to observe the PIRG.

2.5. Production of Non-Volatile Compounds

Culture filtrates methods against *G. boninense* PER71 were used to detect the production of non-volatile chemicals by endophytic *Trichoderma* isolates [14]. A 5 mm plug agar of tested endophytic *Trichoderma* (5 days old) was inoculated into a 250 mL conical flask containing 100 mL sterilized potato dextrose broth (PDB) (Oxoid). The inoculated flasks were shaken on an orbital shaker at 28 ± 2˚C for 7 days at 150 rpm. The harvested culture filtrate was first filtered through a filter paper and finally filtered through a filtrate membrane filter sized 0.45 µm. The final filtrate of endophytic *Trichoderma* was collected and mixed with double strength molten sterile PDA (50˚C) at ratio 1:1. The mixture of PDA and culture filtrate were then poured into a Petri plate and allowed to solidify. A 5 mm plug agar of PER71 was cultured on the center of a Petri plate agar containing the mixture of filtrate and PDA. A plug agar of PER71 was cultured on PDA without any filtrate as control. The cultured plates were then incubated at 28˚C ± 2˚C and PIRG was measured after 7 days.

2.6. Species Identification

The species of two isolates of endophytic *Trichoderma* that showed significant potential in suppressing *G. boninense* PER71 in the *in vitro* study was identified. Mycelia and spores of the isolates were scraped from the agar plates and inoculated in 100 mL PDB before incubated at 30˚C for five days. The five-day old mycelia were then filtered and pulverized in liquid nitrogen. Genomic DNA was isolated from pulverized fungal mycelia according to in-house protocol. Terra^{TM} PSR DNA polymerase (Clontech) was used for the amplification of internal transcribed spacer (ITS) of fungal genomic DNA using forward primer 5’ - TCC GTA GGT GAA CCT GCG G - 3’ and reverse primer 5’ - GGT GCG TGT TTC AAG AC - 3’. 16S rDNA was amplified from bacterial genomics DNA using forward primer 5’ - GGT TAC CCT ACG ACT T - 3’ and reverse primer 5’ - AGA GTT TGA TCM TGG CTC AG - 3’. PCR product was purified, then ligated in pGEM-T Easy Vector (Promega) and cloned in *E. coli.* Vectors carrying the insert were sent out to Apical Scientific Sdn. Bhd. for DNA sequencing ser-

vice. Nucleotide sequence was analyzed at SIRIM and then searched against Genbank database of the National Center of Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/) using Basic Local Alignment Search Tool (BLAST) programme [15]. Species identification was determined according to the homology search results from NCBI database. The nucleotide sequence of the two isolates was deposited to NCBI database. A phylogenetic tree was performed using MEGA (Molecular Evolutionary Genetics Analysis) version 9.0, generated through neighbour joining with bootstrap analysis using 1,000 replicates [16]. Published sequences available in NCBI were retrieved and used for phylogenetic analysis.

2.7. Experimental Design and Statistical Analysis

All the *in vitro* screening studies were conducted in Completely Randomized Design (CRD) with five replications. Data were analyzed with SAS® Software. The significant data was determined using Least Significant Different (LSD) test at 5% probability level. The percentage data were transformed into Arcsine transformation [17] before subjected to ANOVA.

3. Results

3.1. Isolation of Endophytic *Trichoderma*

A total of 14 endophytic *Trichoderma* isolates were successfully recovered from healthy oil palm root samples collected from various locations across the FGV plantation in Malaysia (**Table 1**). After 3 to 7 days of being incubated at 28 ± 2˚C, the colonies of endophytic *Trichoderma* have emerged on the MRB agar (**Figure 1**). The morphology of the mycelium initially appeared as white hyphae and later developed into green conidiation on the fifth day after plating [8].

3.2. Dual Culture Assay

The antagonistic effect of endophytic *Trichoderma* isolates to suppress the mycelium growth of pathogenic *G. boninense* PER71 was observed through a dual culture assay. All endophytic *Trichoderma* isolates were found to be capable of suppressing the mycelium growth of PER71 with PIRG between 70.86% to 93.14% after 7 days of incubation. Isolate M103 has recorded the highest PIRG at 93.14% (**Table 2**). The experiment was extended for another 7 days to observe their capability to overgrow the PER71 culture. Overall, all endophytic *Trichoderma*

Table 1. Number of endophytic *Trichoderma* isolates successfully isolated from oil palm heathy roots of FGV Plantation.

Number of Isolates	Location
5	Taib Andak, Johor
1	Ulu Belitong, Johor
4	Sahabat 17, Sabah
4	Pusat Penyelidikan Pertanian Tun Razak, Pahang

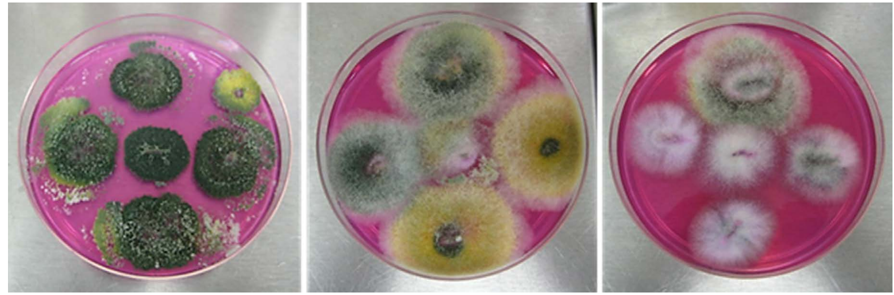

Figure 1. Endophytic *Trichoderma* isolated on MRB plate.

Table 2. Antagonistic effect of endophytic *Trichoderma* isolates on pathogenic *G. boninense* PER71 in dual culture assay.

Isolates	PIRG (%) ± SE (after 7 days)	Day of isolate overgrown the PER71	Bell's Ranking (after 9 days)	Recovery at inhibition zone on PDA
M103	93.14^{a} ± 2.19	9 days	R1	−ve
ETH2	92.29^{ab} ± 1.60	>14 days	R1	−ve
ETH7	88.86^{ab} ± 5.85	>14 days	R1	−ve
M108	87.99^{abc} ± 4.16	>14 days	R1	−ve
ETH11	85.28^{abc} ± 1.73	>14 days	R1	−ve
ETH12	83.77^{abc} ± 0.96	>14 days	R1	−ve
ETH1	83.71^{abc} ± 6.69	>14 days	R1	−ve
ETH4	80.29^{bcd} ± 1.72	>14 days	R1	−ve
ETH13	78.49^{bcd} ± 1.53	>14 days	R1	−ve
ETH5	77.71^{cd} ± 1.60	>14 days	R1	−ve
ETH6	70.86^{d} ± 4.16	>14 days	R1	−ve
ETH9	70.86^{d} ± 1.60	>14 days	R1	−ve
ETH10	70.86^{d} ± 1.60	>14 days	R1	−ve

Means in the same column with different alphabet (s) are significantly different ($p \leq 0.05$) according to LSD.

isolates are able to overgrow PER71 with Bell's Ranking at R1. Isolates M103 was determined as the fastest growing culture to overgrow PER71 after 9 days, compared to other isolates which only overgrow the pathogen at day 14 (**Figure 2**). PER71 was also not able to grow on the inhibition zone of all tested *Trichoderma* isolates.

3.3. Scanning Electron Microscope (SEM)

The mycoparasitism activity caused by isolate M103 on pathogenic PER71 was examined through Scanning Electron Microscope (SEM), specifically on the inhibition zone. The pictograph shows the mycelia of M103 had grown and coiled the mycelia of PER71, eventually penetrating the hyphae and lysed them

(**Figure 3**).

3.4. Production of Volatile Compound

Endophytic *Trichoderma* isolates that have recorded PIRG higher than 80% in dual culture assay were further tested for their ability to produce volatile compounds. A total of 9 isolates were determined producing volatile compounds, indicated by the successful suppression of *G. boninense* PER71 growth on plates. The PIRG recorded was in the range of 36.00% to 65.00% after seven days of incubation (**Table 3**). The highest suppression was 65%, caused by the volatile compound produced by M103, while the lowest suppression was recorded from the volatile compound produced by M108, with PIRG of 36.00%. This study shows that the production of volatile compounds may be varied from the different isolates of endophytic *Trichoderma* which eventually produce different levels of suppression on PER71.

3.5. Production of Non-Volatile Compound

The antibiosis effect of non-volatile compounds secreted by the endophytic *Trichoderma* isolates against *G. boninense* PER71 were determined via cultures filtrated assay. The percentage of inhibition caused by the tested isolates was

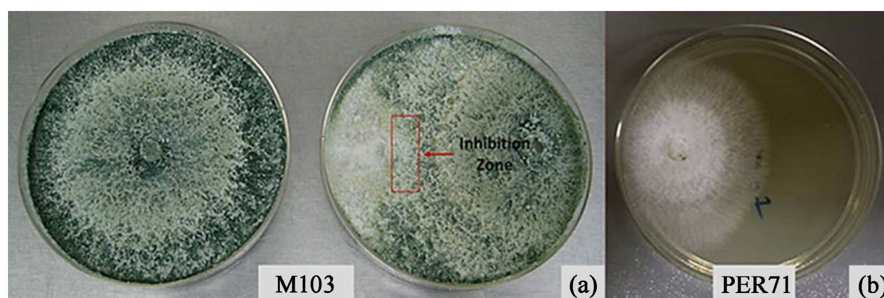

Figure 2. (a) Isolate M103 overgrowth on the culture of *G. boninense* PER71 in dual culture plate. (b) PER71 culture alone act as a control.

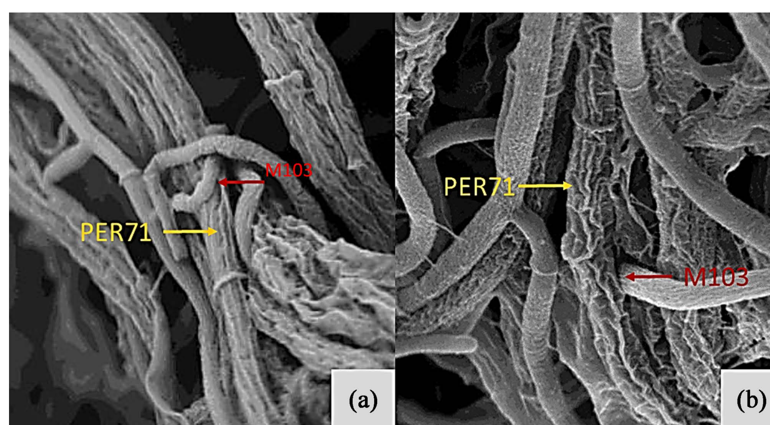

Figure 3. Pictograph of inhibition zone (between M103 and *G. boninense* PER71) via SEM. Mycelia of M103 (red arrow) coiled (a) and penetrated (b) the mycelium of PER71 (yellow arrow). Mycelium of PER71 had lysed due to penetration of M103.

Table 3. Antagonistic effect of endophytic *Trichoderma* against *G. boninense* PER71 through production of volatile compounds.

Isolates	PIRG (%) ± SE (after 7 days)
M103	65.00^{a} ± 1.43
ETH11	63.90^{ab} ± 1.98
ETH12	56.68^{abc} ± 2.66
ETH7	55.90^{cb} ± 1.30
ETH14	54.00^{cd} ± 1.15
ETH2	46.70^{de} ± 3.87
ETH1	46.40^{de} ± 1.68
ETH4	41.60^{ef} ± 1.33
M108	36.00^{f} ± 0.69

Means in the same column with different alphabet(s) are significantly different ($p \le 0.05$) according to LSD.

Table 4. Effect of culture filtrated produced by endophytic *Trichoderma* isolates against *G. boninense* PER71 after 7 days of incubation.

Isolates	PIRG (%) ± SE (after 7 days)
M108	98.18^{a} ± 1.05
ETH4	97.36^{a} ± 1.74
ETH14	68.82^{b} ± 2.61
ETH2	66.18^{b} ± 1.51
ETH7	44.18^{c} ± 1.34
ETH12	36.99^{c} ± 0.60
ETH11	35.18^{c} ± 1.29
M103	16.09^{d} ± 0.84

Means in the same column with different alphabet(s) are significantly different ($p \le 0.05$) according to LSD.

from 16.09% to 98.18% (**Table 4**). In this assay, M108 has produced a toxic non-volatile compound, which caused 98.18% inhibition on PER71 on plates, which was the highest PIRG recorded compared to other isolates. Contrarily, M103 which was found to be the most outstanding isolate in previous dual culture assay and production of volatile compound experiments, has produced a non-volatile compound that only caused the lowest inhibition on PER71 (16.09%).

3.6. Species Identification

The ITS sequence data of isolates M103 and M108 were compared with several possible homologous sequences from NCBI GenBank database (**Table 5**). Based on the sequence analysis, isolates M103 and M108 isolates were identified as

Table 5. Details on Internal Transcribed Spacer (ITS) sequence data used in this study.

Isolates	Strain ID	GenBank Accession Number
T. asperellum	P68	KX146490
T. asperellum	BHU-BOT-RYRL16	KR856222
T. asperellum	TR-1	KU203320
T. asperellum	H531	KY978581
T. asperellum	jz-3	MG171157
T. asperellum	Tr48	AJ230669
T. asperellum	M103	MF363050
T. asperellum	G	KF723005
T. asperellum	T76-14	LC158827
T. viride	CUMB VP-PN4-12	MT138567
T. viride	LCM799.01	MF495372
T. viride	LESF115	KT278861
T. longibrachiatum	QT22040	KY29918
T. longibrachiatum	T44	FJ459963
T. longibrachiatum	XZN209-1	MF116270
T. longibrachiatum	34915eDRJ	MF782825
T. longibrachiatum	AB2	MF164178
T. harzianum	M108	MH266422
T. harzianum	GMS	MH333257
T. harzianum	CKP01	KC330218
T. harzianum	Cul-2018	MK673510
T. harzianum	24	AJ224006
T. harzianum	TRS65x	KP009228
T. virens	GV29-8	XM014094808
T. virens	GPRI-FUN34/YaaH	BK006704

Trichoderma asperellum and *T. harzianum* at 99% similarity with NCBI Genbank accession number given in the phylogenetic studies of sequence analysis data. The generated neighbour joining based on the phylogenetic tree shows that endophytic *Trichoderma* are clustered together according to their species (**Figure 4**).

4. Discussion

Trichoderma sp. is commonly abundant in soil [18], rhizosphere of host plants [19] and recently detected as endophytes [8] [20]. In this study, a total of 14 endophytic *Trichoderma* isolates, isolated from healthy oil palm roots were assessed

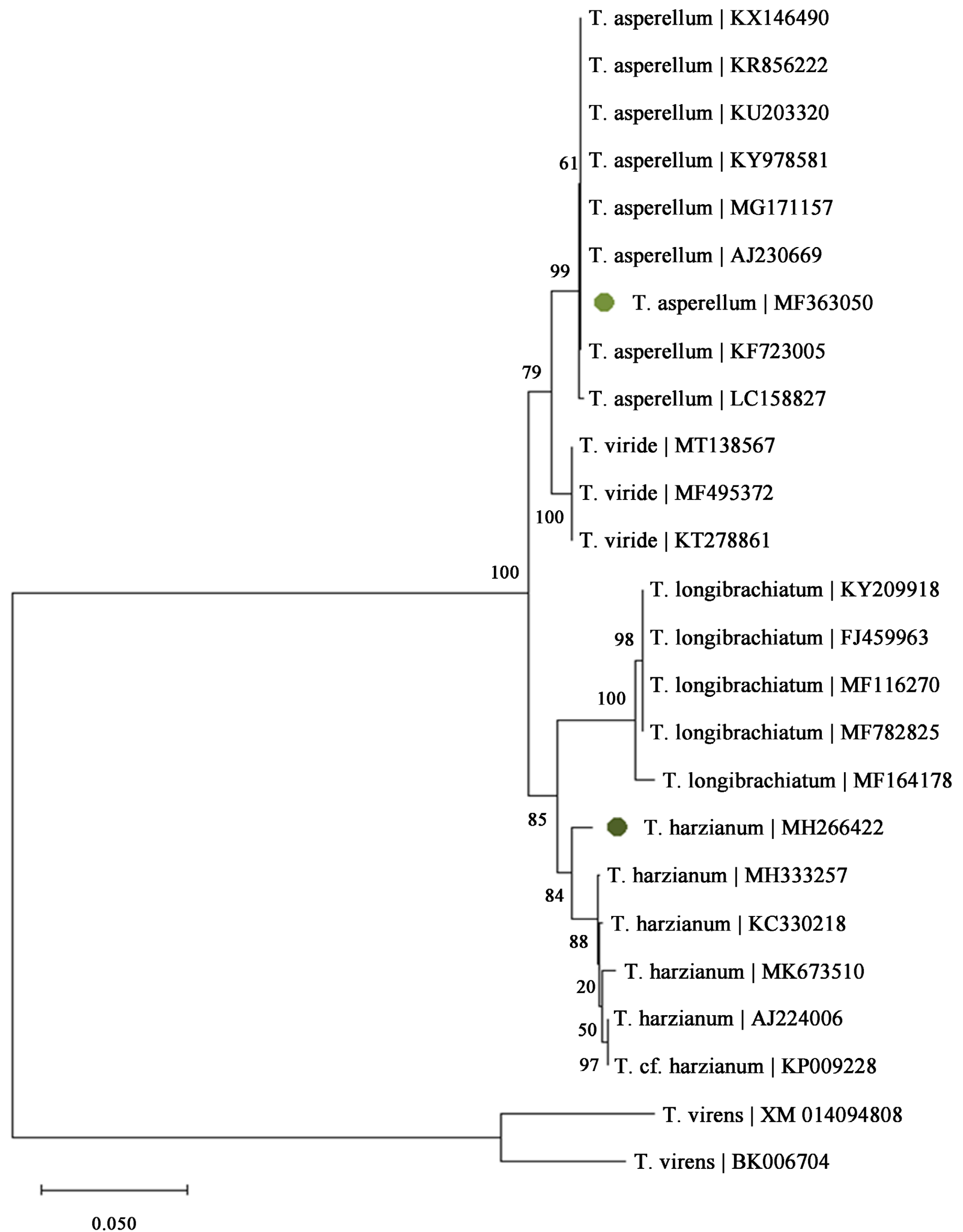

Figure 4. Phylogenetic tree of endophytic *Trichoderma* isolates from the ITS region using neighbour-joining analysis. The p-distance model was used as the nucleotice substitution model. The numbers of branches are bootstrap support values obtained from 1000 bootstrap replications. M103 and M108 are indicated in green label.

for their potential as biological control agent (BCA) against *G. boninense* PER71. All the tested isolates have the ability to suppress more than 70% of PER71 mycelia in the dual culture assay. *Trichoderma asperellum* M103 was the most effective inhibitor in this assay. It has overgrown the mycelium of PER71 within only 9 days, compared to other isolates which took a total of 14 days to completely

overgrown the pathogenic fungi. The results showed that *T. asperellum* M103 is an excellent competitor which successfully competes for space and nutrients due to its ability to grow faster than PER71. Similar findings have been reported, showing *T. asperellum* isolate which grows faster than *G. boninense* (UPM13) [21] and *F. oxysporum* f. sp. *lycopersici* [22]. Isolate PER71 was also not able to grow once the mycelia from the inhibition zone was subcultured onto a new PDA. This could be due to the presence of either antibiotic, metabolite compounds or lytic enzyme produced by *T. asperellum* M103 or mycoparasitic mechanism that occurred in the dual culture assay which contributed to the antagonistic effect against *G. boninense* [21]. The direct interaction between *Trichoderma* and pathogen in dual culture assay is called mycoparasitism [12]. The interaction can be clearly observed through SEM specifically in the area of inhibition zone. The hyphae of *T. asperellum* M103 usually coil, penetrate the mycelium of PER71 and finally lysed them. The lysis occurs probably due to secretion of lysis enzymes or metabolites and these are the common process of mycoparasitim by *Trichoderma* sp. [23].

The inhibitory effect of pathogenic *Ganoderma* PER71 in the presence of headspace gases on agar in the double plate method assay indicated the production of unidentified volatile substances by *Trichoderma* sp. [12]. Volatile compounds that are produced by *T. asperellum* M103 had caused the highest suppression on the growth of PER71 compared to other tested isolates. The volatile compounds produced by different species of *Trichoderma* had impeded the hyphal growth of pathogen on agar plates depending on the species of *Trichoderma* [24]. *Trichoderma* sp. have been shown to produce a broad array of volatile compounds [25], containing antifungal properties such as alkyl pyrones produced by *T. harzianum* [26] [27] and 2-ethylhexanol, 1-nonanol, 6-PP, and 2-methyl-1-butanol by *T. asperellum* (T76-14) [28].

Generally, isolates that caused the least inhibition on PER71 in both dual culture and double plate assays have exhibited excellent inhibition ability towards the pathogen through the culture filtrate method. Interestingly, this study discovered that *T. harzianum* M108 produced a toxic, non-volatile compound that is sufficient enough to almost totally suppress the growth of PER71 via the culture filtrate method. M108 recorded the highest PIRG value at 98.18%. On the other hand, *T. asperellum* M103 is suspected to produce less toxic, non-volatile compound as it only caused 16.09% inhibition on PER71 growth. However, the isolate has showed excellent inhibition performance on the pathogen in both dual culture assay and double plate methods. Cell free culture filtrate of *Trichoderma* which contains the non-volatile compounds has been used to demonstrate the possible antibiosis effect in biocontrol. For example, compound 6-pentyl-α-pyrone [29], non-volatile compound produced by *T. harzianum,* is proven toxic to the grapevine trunk disease [30] and Fusarium wilt in cucumber [31].

This trial generally discovered that different isolates of *Trichoderma* sp. may

produce various amounts and toxicity of volatile and non-volatile compounds, as they have caused different levels of inhibition on the pathogen. Even though both isolates M103 and M108 have shown the potential ability to suppress the growth of PER71 *in vitro*, the results are still insufficient to declare that these isolates are suitable biological control agents to be used in the field [32] [33]. Therefore, it is important to conduct both *in vitro* and *in vivo* studies to gain comprehensive and conclusive results as screening tools for the selection of potential biocontrol agents [32].

5. Conclusion

Trichoderma asperellum M103 and *T. harzianum* M108 were found to effectively inhibit the growth of *G. boninense* PER71 *in vitro* through a dual culture test with more than 85% PIRG and other different mechanisms involved. Based on the outstanding results, M103 and M108 would have huge potential as biocontrol agents (BCA) against *G. boninense* in oil palm. Besides the excellent efficacy, the endophytes characteristic of the isolates may be the ideal privilege for the BCA. The characteristic would shelter them from environmental changes and sustain their competency as BCA in the rhizosphere. Their presence in the roots of host plants also may aid in the prevention of soil-borne plant diseases as well as provids other advantages to the plants. In conclusion, combating PER71 with endophytic *T. asperellum* M103 and *T. harzianum* M108 could be the key to overcoming BSR disease in oil palm.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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