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Quantification and characterisation of *Trichoderma* spp. from different ecosystems

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Abstract

Basal stem rot of oil palm caused by *Ganoderma boninense* is of major economic importance. Observations of the low incidence of disease due to *Ganoderma* species in natural stands, suggest that the disease is kept under control by some biological means. *Trichoderma* spp. are saprophytic fungi with high antagonistic activities against soil-borne pathogens. However, their abundance and distribution are soil and crop specific. *Trichoderma* species have been found to be concentrated in the A1 (0–30 cm) and Be soil horizons (30–60 cm), although the abundance of *Trichoderma* was not significantly different between the oil palm and non-oil palm ecosystems. Characterisation of *Trichoderma* isolates based on cultural, morphological and DNA polymorphism showed that *T. harzianum*, *T. virens*, *T. koningii* and *T. longibrachiatum* made up 72, 14, 10 and 4% of the total *Trichoderma* isolates isolated. As *Trichoderma* species are present in the oil palm ecosystem, but at lower numbers and in locations different from those desired, soil augmentation with antagonistic *Trichoderma* spp. can be developed as a strategy towards integrated management of basal stem rot of oil palm.

Key words: basal stem rot, distribution, integrated management, oil palm, *Trichoderma* spp.

Introduction

The oil palm industry in Malaysia is threatened by basal stem rot (BSR), a disease commonly associated with areas where oil palms have been planted after coconut, especially on clay soils in coastal areas. Normally the disease progress is slow but this is not always the case, especially in the second-generation palms, where the disease progress can increase by 50%. Control measures such as clean clearing, tree surgery and fungicides have been found to be erratic and not to be long lasting. The success of biological control for many pathosystems has focused interest on exploring the potential of BSR control through manipulation of antagonistic microorganisms such as species of *Trichoderma*, towards a sustainable integrated management of the disease.

Rifai [1] distinguished nine species aggregates in the genus *Trichoderma*, and of these, *T. virens*, *T. harzianum* and *T. viride* have been reported as the most common biological control agents. Recently, macromolecular analyses based on nucleic acids have been used to differentiate between and within species aggregates of *Trichoderma* and can also be used to demonstrate the genetic diversity of individual isolates [2–4]. Preliminary attempts have been made to use macromolecular approaches to study genetic variations among *Trichoderma* isolates from the oil palm rhizosphere [5, 6].

There is limited information on the population dynamics of *Trichoderma*, particularly its survivability and proliferation in relation to soil type, soil depth and cropping history in the local ecosystem. As *Trichoderma* biocontrol agents are applied outside the plant, and act by competition,

mycoparasitism and possibly antibiosis, the ability of *Trichoderma* to disperse and to colonise roots will determine its effectiveness as a biocontrol agent. Thus, an understanding on the quantitative distribution of the fungus in different ecological niches is essential before it can be developed into biological formulations for field application. The following study was undertaken with the objectives of (i) quantifying the population dynamics of *Trichoderma* spp. from oil palm cultivated and non-cultivated ecosystems and (ii) to characterise the variation between species aggregates of *Trichoderma*.

Materials and methods

Soil sampling

Soil samples were collected from United Plantation Berhad Estate, Teluk Intan, Perak Darul Ridzuan. Soil samples were collected from six fields with different soil types, and from two ecosystems: oil palm cultivated and non-cultivated. The oil palm ecosystem consisted of four different fields: a BSR-infected field with a percentage of disease incidence (PDI) >30%, a healthy field with PDI <5%, an empty fruit bunch (EFB) mulched field and an inland oil palm field. The non cultivated ecosystem consisted of two fields: one of coastal jungle and one of inland jungle. Soil samples were collected with an auger from 10 representative palms chosen at random from each field. Soil samples were taken at two points 1.5 m away from base of palm, and at three different depth profiles: 0–30, 30–60 and 60–90 cm. Soil samples from the same soil depth were bulked into one sample and stored at 4 °C prior to isolation. Soil samples from the non-oil palm ecosystem were collected from 10 random sites at distances equivalent to planting points of oil palms.

The soil moisture content was determined by oven drying 10 sub-samples from each bulked sample. The H^+ ion activity was determined by adding 0.01 M $CaCl_2$ to the sample in the ratio 1:3 (v/v), which was then allowed to stand for 1 h [7].

Enumeration and isolation of Trichoderma *spp.*

Enumeration of *Trichoderma* spp. from soil samples was determined following the soil dilution plate technique on *Trichoderma* Medium E (TME) [8]. Ten sub-samples from each bulked sample were evaluated. Total counts (colony forming units, cfu) of the *Trichoderma* were transformed into average counts of cfu/g soil palm^{-1}. The data obtained were transformed and subjected to ANOVA analysis (SAS Institute Inc., Cary, NC, USA), and means were separated by Tukey's Studentised Range (HSD) Test at $P = 0.05$. Pure cultures were transferred onto fresh potato dextrose agar (PDA) for characterisation into species aggregates.

Characterisation of Trichoderma *isolates*

The characterisation of 135 isolates into species aggregates was made on the basis of cultural and morphological characters [1, 9–13] and DNA polymorphism [5, 6].

Results

Enumeration of Trichoderma *isolates*

The abundance of *Trichoderma* was not significantly different between the cultivated oil palm and the uncultivated ecosystems. *Ganoderma* infected fields where the percentage disease incidence was >30% showed a higher isolation frequency (9.5×10^3 cfu/g air-dried soil) of *Trichoderma*. In the non-cultivated ecosystems, inland soil appeared to harbour higher populations (10.9×10^3 cfu/g air-dried soil) of *Trichoderma* (Table 1). This was significantly different from coastal soil where the frequency of isolation was only 1.1×10^3 cfu/g air-dried soil. Soil pH and moisture did not influence the distribution and abundance of *Trichoderma* in all the fields sampled. Generally for all ecosystems and fields sampled, the two upper soil horizons (Al and Be) supported higher populations of *Trichoderma* in the range of 2.1×10^3 to 10.2×10^3 cfu/g air-dried soil and 0.8×10^3 to 13.4×10^3 cfu/g air-dried soil, respectively. The distribution of *Trichoderma* isolates decreased with the depth of the soil. Fields cultivated with oil palms and the non-cultivated inland field provided good habitats for the proliferation of *Trichoderma*. When the field was mulched with empty fruit bunches there was a significant increase in the isolation frequency and the depth profile for *Trichoderma* (Table 2).

Table 1. Frequency of isolation of *Trichoderma* (cfu/g air-dried soil) in relation to soil pH and moisture content from oil palm cultivated and non-cultivated ecosystems

Ecosystem	Field	cfu/g air-dried soil ($\times 10^3$)	pH	Moisture content (%)
Oil palm	Infected coastal (PDI > 30%)	9.5 ab	3.7 b	35.5 b
	Healthy coastal (PDI > 5%)	6.7 ab	3.3 c	40.7 a
	Healthy inland (PDI 0%)	5.3 ab	3.6 b	25.4 c
	EFB mulched (PDI 0%)	6.8 ab	3.2 c	28.5 c
Non-oil palm	Inland	10.9 a	4.0 a	14.3 d
	Coastal	1.1 b	3.3 c	38.4 ab

The quantitative estimation of these three parameters within the same field was expressed as average of accumulation from three soil horizons (PDI, percentage of disease incidence; EFB, empty fruit bunch).
Means with the same letters within column do not differ significantly according to HSD ($P < 0.05$).

Table 2. Comparison of quantitative distribution of *Trichoderma* (cfu/g air-dried soil) between fields within the same horizon (PDI, percentage of disease incidence; EFB, empty fruit bunch).

Ecosystem	Field	Horizon		
		Al (0–30 cm)	Be (30–60 cm)	Ga (60–90 cm)
Oil palm	Infected coastal (PDI > 30%)	10.2 a	13.4 a	4.9 bc
	Healthy coastal (PDI > 5%)	8.1 ab	9.5 ab	2.6 cd
	Healthy inland (PDI 0%)	6.3 abc	2.0 b	7.6 ab
	EFB mulched (PDI 0%)	4.5 bc	6.5 ab	9.1 a
Non-oil palm	Inland	9.1 a	17.0 a	6.6 abc
	Coastal	2.1 c	0.8 b	0.3d

Means with the same letters within column do not differ significantly according to HSD ($P < 0.05$).

Characterisation of Trichoderma *isolates*

Characterisation based on cultural, morphological and DNA polymorphism showed that *T. harzianum*, *T. virens*, *T. koningii* and *T. longibrachiatum* made up 72, 14, 10 and 4% of the total *Trichoderma* isolates obtained. The colony appearances and cultural characteristics of each of the *Trichoderma* species aggregate based on mycelial growth, sporulation patterns, pigmentation and colour of conidia correlated well with the data generated from the RAPD analysis. Two random primers (OPC-11 and OPC-15) were used to profile 97 isolates of *T. harzianum*, 19 isolates of *T. virens*, 14 isolates of *T. koningii* and 5 isolates of *T. longibrachiatum* [6]. These two primers resulted in the amplification of amplicons from the genomic DNA of *T. harzianum*, *T. koningii and T. longibrachiatum* and can be used as molecular marker to distinguish species aggregates of *Trichoderma* from oil palm ecosystems. However, no comparable amplicons were found for *T. virens*. The genetic relatedness between the different species aggregates based on cluster analysis was also very low, with a dissimilarity index of 85%.

Discussion

The isolation frequency of *Trichoderma* spp. was higher in the *Ganoderma* infected field (PDI > 30%) than in other fields sampled within the oil palm cultivated ecosystem. *Trichoderma* was also significantly more abundant in the mature field with a high BSR incidence, than in younger palms with low disease incidence [5]. These findings could be due to the presence of oxidisable organic matter from the decaying roots and root exudates excreted during the infection process providing a substantial supply of nutrients, and so allowing *Trichoderma* to proliferate. The quantitative estimation of *Trichoderma* in the EFB mulched field

was also not significantly different between fields within the cultivated ecosystems, however it was interesting to note that within this field, *Trichoderma* abundance increased significantly ($P = 0.05$) in relation to the soil depth. This may be due to the decomposition of EFB mulch through microbial activity, and this may have altered the soil texture. The organic matter makes the soil less heavy by reducing the coherence of soil particles and so increases both air and water flow rates through fine textured soil and produces larger soil pores [14]. The increase in permeability of water flow permits more dissolved nutrients to be leached to the deeper soil profile and these could serve as nutrients for *Trichoderma* growth.

The change in ecosystem from non-cultivated to cultivated does not appear to have influenced the distribution and abundance of *Trichoderma* spp. in the soil. The pH of the soil was in the range of pH 3 and pH 4, and as *Trichoderma* is favoured by acidic soil, it could be assumed that the soil reaction could be a factor in determining the abundance and distribution of *Trichoderma* as observed in this study. Soil moisture has also been shown to have an effect on the composition of major groups of soil microflora, mainly *Trichoderma*, *Aspergillus* and *Penicillium* [15]. This may explain the higher distribution of *Trichoderma* in the inland jungle soil as compared with the coastal soil, as fluctuations in moisture content are more prominent in the coastal soil of the Selangor series.

Although the mean population of *Trichoderma* was found to be higher in the BSR infected field (PDI > 30%), with *T. harzianum* and *T. virens* as the predominant species, the incidence of *Ganoderma* disease was still high. This is contrary to the hypothesis that if *Trichoderma* is a potential antagonist of *Ganoderma* [16–18], higher *Trichoderma* populations should result in lower BSR incidence. However in this case the population of antagonistic *Trichoderma* was lower than the effective magnitude of 10^6 cfu/g soil proposed by Papavizas [19], and so an opportunity may exist to introduce specific antagonistic microorganisms into the oil palm rhizosphere. Although, *T. harzianum* and *T. virens* are the most commonly cited species [20] of biocontrol agents against plant pathogenic fungi [4], different isolates within the same species aggregates can show different degrees of adaptation to soil types, biotic and abiotic factors, methodology, time of application and rhizosphere competency. Howell and Stipanovic [21] found that one group of *T. virens* designated as 'Q' strains were effective against *Rhizoctonia solani* but inactive against *Pythium ultimum*. A second group within *T. virens* designated as 'P' strains, was strongly active against *P. ultimum* but was inactive against *R. solani*. Koch [22] showed that in the control of *R. solani* on peas, *T. harzianum* strain PV 5736-89 was significantly more effective than the strain KRL-AG2, but there were no significant differences between these strains when tested against *P. ultimum*. Different isolates of *Trichoderma* within the same species aggregate have also been shown to have different antagonistic activities to *Ganoderma* when tested in dual culture *in vitro* [5]. Therefore, in order for soil augmentation to be successful, a highly effective biocontrol strain must be obtained and this should be able to compete and persist in the environment in which it will operate, and to be able to colonise and proliferate on newly formed roots well after application.

Species aggregates in *Trichoderma* can be differentiated based on their macroscopic (colour of conidia, sporulation patterns and density) and microscopic (structure and arrangement of phialides, conidial size and shape) features, however, this is time consuming and sometimes confusing. The PCR amplification with random primers OPC-11 and OPC-15 was found to be stable and reproducible in our study on *Trichoderma* isolates for oil palm cultivated and non-cultivated ecosystems, and confirmed the taxonomic grouping of all the isolates. The macromolecular approach was found to be quicker and more reliable, and PCR products could be developed as molecular markers in studies of this nature, even though the RAPD technique has its own limitations.

The greatest practical implications for the control of *Ganoderma* in oil palm are: (i) during/soon after planting and establishment period and (ii) later in the planting cycle, and so control of BSR has to be approached in a more holistic manner. Biological control would be effective during the early phases of planting and establishment, and then could be followed by chemicals for sustainable control. Sanitation and good cultural practices apply to all phases of the growth cycle.

Even though biological control agents might not be the key answer to managing BSR, they can however fulfill important roles where there is lim-

ited success with other methods of control. *Trichoderma* survives as chlamydospores under unfavourable conditions, and these are fairly resistant to common fungicides and herbicides. Research on the manipulation of resident antagonistic species through soil amendments, other than augmentation, should be undertaken simultaneously with attempts to define the optimum delivery and application methods so that the biocontrol agent will grow well and be able to achieve their purpose.

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