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Occurrence and Characterisation of Mycoflora in Soil of Different Health Conditions Associated with White Root Rot Disease in Malaysian Rubber Plantation

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In Malaysia, rubber trees are planted for latex and it has contributed 5.7 % to the world's total production of natural rubber. However, nearly half of the rubber trees are lost to white root rot (WRR) disease caused by Rigidoporus microporus over the planting period. A total of 18 fungi belonging to 16 species distributed in 10 genera were isolated from the soil of three different tree health conditions in a rubber plantation at Rubber Research Institute of Malaysia (RRIM), Sungai Buloh. The objective was to characterise the fungi diversity with the health condition of rubber trees. Soil samples were collected from study site in August 2013 (1) White root rot (WRR) affected tree zone (ATZ) (2) Healthy tree zone (HTZ) and (3) Regrowth tree zone (RTZ). The soil mycoflora were isolated using soil dilution plate technique on potato dextrose agar (PDA). Identification and characterisation of the mycoflora were based on fungi morphology and PCR-based techniques. Results showed that RTZ zone had the greatest species diversity with 13 species of fungi isolated followed by HTZ zone with six species and ATZ zone with two species. The highest frequency of occurrence in the soils was shown by the genus Penicillium *(31.25%) and* Trichoderma *(18.75%), respectively. The study will be useful to understand the relationship of fungal distribution and trees health conditions. Among the isolates, species such as* Trichoderma spp. *and* Chaetomium cupreum *can be evaluated for the suppression of WRR disease.*

Keywords: Rubber; fungi diversity; white root rot disease; *Rigidoporus microporus*; PCR-based identification

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Malaysia, previously the world's top producer of natural rubber is now producing 668 613 tonnes or 5.7 % of the world's total production[1]. Natural rubber is a latex product tapped from rubber tree (*Hevea brasiliensis*). The Malaysian rubber industry started in early 1900 and it has been described as one of the greatest achievements of Western colonial enterprise. It formed the backbone of Peninsular Malaysia's economy along with tin industry. Malaysia national income was once driven by these two industries. The rubber acreage in Malaysia is currently around 1,000,000 ha[1].

Rubber trees grow well in climate of high surrounding relative humidity of more than 75% and rainfall of around 250 cm annually[2]. Malaysia and other tropical countries exhibit hot-wet climatic conditions (equatorial climate) which are favourable for rubber cultivation. Nonetheless, these conditions also promote the growth of multi-species fungal community in plantations. Brazil, as the native country of rubber has failed to establish commercial rubber plantations due to a devastating leaf disease known as South American Leaf Blight (SALB). The fungal pathogen *Microcyclus ulei* is fortunately confined to South and Central America[3].

The population and species of fungi present in rubber plantations are determined by a complex interaction of varying amounts of sunlight, temperature, moisture, soil pH and nutrient[4], soil being the most important[5–6]. The availability of water in the soil supports the development and activity of the fungi, as does the spatial distribution of nutrient.

Soil fungi can be categorised into three ecological groups based on how they obtain their energy (food source) (1) Natural decomposer (2) Mycorrhizal fungi and (3) Pathogenic or parasitic fungi. Saprophytes play an essential role in nutrient recycle. Mycorrhizae form mutualism with plants and assist in direct nutrient supply to the plants. Pathogenic fungi belonging to basidiomycetes class are major causative agents of rubber diseases[7–8]. For example, white root rot disease (WRR) causes juvenile trees to die if control measures are not taken[9].

The most notable and destructive root pathogen in rubber plantations is *Rigidoporus microporus* which occurs in all rubber plantations in the world[10]. WRR pathogen obtains its nutrient from the colonised living tissues of the host rubber by damaging and weakening them with toxins or by inhibiting their defence mechanisms[11]. The inoculation of WRR disease is the rhizomorphs, infected roots, dead stumps, or wood debris[12–14]. In order to control the disease, affected trees are drenched with a recommended fungicide. Similarly prophylactic drenching of fungicide is practised[15].

Recently, biological control is being investigated[16]. Numerous fungal species, (*Aspergillus niger, Chaetomium globosum, C. cochlioides, C. cupreum, Gliocladium catenulatum, Trichoderma hamatum, T. harzianum, T. virens* and *T. viride*[17–19]) have been reported to be effective in suppressing soil-borne diseases as well as promoting plant. The relationship between the degrees of soil suppressiveness to soil-borne pathogens and the abundance of soil microbial populations have been studied and documented[20]. Studies have shown that fungal species diversity is varied under healthy and diseased tree soil conditions (unpublished data), yet current understanding of fungal biodiversity in soil of rubber plantations in Malaysia is limited.

This preliminary study was undertaken to identify and characterise the fungal species collected from soil under different rubber tree health conditions using both morphological and PCR-based methods. The study could provide useful information regarding the role of soil mycoflora in the suppression of WRR

pathogen. Fungal communities are important components to the plantation ecosystem as they establish dynamic relationship with the plants and possible antagonists against host (rubber tree) pathogens. This will provide a sound measure to control the disease by reducing the infective sources, as well as inhibiting the disease spread in future.

EXPERIMENTAL

Sampling Site

RRIM Sungai Buloh is located in the district of Petaling in the state of Selangor, Malaysia at N03º 09.911' E101º 33.527'. The soil has sandy texture which is under Sungai Buloh soil series. There are three different categories of rubber tree health conditions in this rubber plantation (1) Rubber trees affected by WRR disease (2) Healthy rubber trees and (3) Regrowth of rubber trees after WRR infection.

Soil Sample Collection

The soil samples were collected from three different health condition zones in August 2013 (1) White root rot (WRR) affected tree zone (ATZ) (2) Healthy tree zone (HTZ) and (3) Regrowth tree zone (RTZ). Three replications of soil samples from each tree were collected using trowel with 10 cm depth of soil. Samples from the three zones were collected into a small sterilised polythene bags and brought to the Laboratory of Pathology, Faculty of Forestry, air dried for one day before proceeding to fungal isolation.

Isolation of Soil Mycoflora

The fungi were isolated using soil dilution plate method on PDA (Difco™, France). A dilution series of the soil samples were made up to 10^{-4} using 1 g of soil added into 9ml of distilled water[21]. Spread plating on PDA plates were performed with 200 µl from the serial dilutions of 10^{-3} and 10^{-4}. Three replicates were done for each dilution. The Petri dishes were incubated at 28 ± 2ºC for one week of fungal growth.

Morphological Identification of Soil Mycoflora

Fungi growing on the PDA plates were transferred by subculturing the edge of a single colony to a fresh PDA plate to ensure single species isolation. The fungal morphological characteristics were studied macroscopically based on the colony features (colour, texture and pigmentation) and microscopically (conidia, conidiophores and arrangement of spores) by staining the spores with a drop of lactophenol cotton blue on a microscope slide using a sterile needle.

The categorisation of fungal growth rate was based on the time taken by the fungus to cover the whole plate of Petri dish (1) Very fast = within 5 days (2) Fast = 5 to10 days (3) Medium = 10 to 15 days and (4) Slow = 15 to 20 days. The pure culture of each isolate was then identified to the genus level base on its macro and micromorphological characteristics by observation under the light microscope based on key identification as described by Stolk and Samson[22] and Tsuneo[23].

Genomic DNA Extraction, PCR and Sequencing

Genomic DNA of fungi was isolated according to the standard extraction procedures as in FastDNA® kit. The concentration and quality of the extracted genomic DNA

were determined using nanophotometer and agarose gel electrophoresis. A pair of primers was used to PCR amplify the internal transcribed spacer (ITS) regions ITS1 (5'TCCGTAGGTGAACCTGCGG 3') and ITS4 (5' TCCTCCGCTTATTGATATGC 3')[24].

Taq PCR Master Mix Kit (Qiagen, Germany) was used to prepare PCR master mix by following the manufacturer's instruction and the following programme was used. Initial denaturation at 94ºC for 3 min, followed by 35 cycles of denaturation at 94ºC for 30s, annealing at 55ºC for 10s, extension at 72ºC for 1 min and final extension at 72ºC for 10 minutes. The PCR products were checked by agarose gel electrophoresis and purified for direct DNA sequencing. Sequences for each of the isolates were identified using Basic Local Alignment Search Tool (BLAST) searches (http://www.ncbi.nlm.nih.gov/BLAST). All new sequences data of those isolated fungi were deposited in the GenBank nucleotide sequence databases.

RESULTS

Morphological Identification and Characterisation of Soil Mycoflora

The fungal species obtained from the first culture plates of the serial dilution (10^{-2}, 10^{-3} and 10^{-4}) of RTZ, HTZ and ATZ soil samples were 18, 10 and 5 (in total 33), respectively. Among these, 18 pure isolations were choose and successfully grouped into ten morphogroups according to their morphological characteristics, such as colony colouration, pigment formation, texture and growth of mycelia colony on PDA media (*Table 1*) and also microscopic view (*Table 2*). The remaining of the other 15 species isolations were not selected as they have the same morphology as in the 18 pure isolations that have been chosen to be identified.

Molecular Identification of Soil Mycoflora

There are 14 fungal isolations from RTZ soil, three isolations from HTZ and one isolation from ATZ were selected and purified for DNA extraction. The sequencing results showed that these fungal isolates were closely affiliated to 10 different genera (*Penicillium, Trichoderma, Fusarium, Gongronella, Cunninghamella, Purpureocillium, Chaetomium, Talaromyces, Myrothecium*, and *Rhodosporidium*) as shown in *Table 3*.

The most abundant fungal species was *Penicillium* (31.25%) with at least five species identified, *i.e. P. rolfsii, P. janthinellum, P. citrinum, P. oxalicum* and *P. specie*, followed by *Trichoderma* species (*T. reesei, T. asperellum* and *T. koningiopsis*) which represents 18.75%. Other genera (*Fusarium, Gongronella, Cunninghamella, Purpureocillium, Chaetomium, Talaromyces, Myrothecium* and *Rhodosporidium*) were represented by a single identified isolate (6.25%) with 50% in total.

Soil Fungal Distribution in Different Health Zone of Rubber Tree

A total of 16 species (13 species from RTZ soil, 6 species from HTZ soil, and 2 species from ATZ soil) belonging to ten genera were isolated from the soil samples collected from three different rubber tree health zones as shown in *Table 4*.

The data showed that 13 species of fungi have been successfully isolated from RTZ soil where the most dominant species was *Penicillium* sp. followed by *Trichoderma* sp. which marked the highest fungi population compared to the other zones. From these 13 fungi species isolations, 11 species were grouped under phylum of Ascomycota (*Penicillium, Fusarium,*

TABLE 1. FUNGAL MACROSCOPIC MORPHOLOGICAL IDENTIFICATION

Fungal isolation ID	Macroscopic morphological characteristics				
	Colour	Texture	Reverse	Growth rate	Health zone
IS(a)	Greyish white	Cotton	White	Very fast	ATZ
NI(b)	Greyish white	Cotton	White	Very fast	HTZ
NI(g)	White to purple	Moist, filamentous	Creamy	Medium	HTZ
NI(i)	White	Cotton wood	Creamy	Slow	HTZ
RH(a)	White to light green	Cotton	Yellow pigment	Very fast	RTZ
RH(b)	Light olive and reddish in mature	Felted	Red pigment	Medium	RTZ
RH(d)	Greyish yellow	Effuse, powdery	Creamy	Fast	RTZ
RH(e)	Greyish yellow to greyish red	Effuse, powdery	Yellowish	Fast	RTZ
RH(f)	Dark green	Effuse, powdery	Pale yellow	Fast	RTZ
RH(g)	Whitish green to dark green	Floccose	Pale yellow	Very fast	RTZ
RH(i)	Yellowish white	Cotton	Creamy	Medium	RTZ
RH(j)	Yellowish green to army green	Effuse, powdery	Creamy	Fast	RTZ
RH(k)	White	Floccose	Creamy	Medium	RTZ
RH(L)	Pink vinaceous	Effuse, powdery	Creamy	Medium	RTZ
RH(m)	Yellowish white to green	Cotton	Pale yellow	Medium	RTZ
RH(n)	White	Cotton	Creamy	Medium	RTZ
RH(p)	White	Cotton	White	Fast	RTZ
RH(r)	Salmon orange	Smooth and watery	Salmon orange	Fast	RTZ

Trichoderma, Chaetomium, Talaromyces and *Purpureocillium*) and the remaining was classified under Zygomycota (*Gongronella butleri*) and Basidiomycota (*Rhodosporidium paludigenum*).

Under HTZ, all the fungi species identified belonged to Ascomycota (*T. koningiopsis, F. oxysporum, Myrothecium verrucaria, P. citrinum*, and *P. janthinellum*) except for *G. butleri*. Whereas only two species (*Cunninghamella echinulata* and *G. butleri*) belonging to Zygomycota were isolated from ATZ. Overall, the fungi identified from this study area included: 13 Ascomycota, 2 Zygomycota and 1 Basidiomycota. The detail distribution of species in the soil of three health zones is presented in *Table 4*.

TABLE 2. FUNGAL MICROSCOPIC MORPHOLOGICAL IDENTIFICATION

Genera (Fungal Isolate ID)	Microscopic morphological characteristics
Penicillium RH(d), RH(e), RH(f), RH(i), RH(m), RH(n)	Hyphae septate; Conidiophores well developed, hyaline, spore aggregate in a row; Conidia in chain, 1-celled, globose; Penicillus well developed.
Trichoderma RH(a), RH(g), NI(b)	Conidiophores hyaline, irregularly branched, well developed; Spore mass formed at apical parts of conidiophores; Conidia hyaline, 1-celled.
Fusarium RH(k), NI(g)	Conidia more than 2-celled, lunate with a foot cell. Macroconidia are fusiform, slightly curved, pointed at the tip, mostly three septate, basal cells pedicellate, Microconidia are abundant, never in chains, mostly non-septate, ellipsoidal or cylindrical, straight or curved.
Talaromyces RH(j)	Ascomata without distinct walls or covered with hyphal networks; Asci borne in chains ; Conidial state Penicillium, but belonging to other series, green, greyish or olive-brown; Penicilli typically biverticillate-symmetrical, often fractional, or monoverticillate, occasionally reduced to solitary phialides, green; mesophilic.
Cunninghamella IS(a)	Sporangiophores vesicles formed and connected to sporangia; Sporangia formed directly on vesicle; Sporangiospores one per sporangium (conidium).
Gongronella RH(p)	Sporangia globose, with apophysis; Apophysis globose.
Myrothecium NI(i)	Conidia one-celled, branched, aggregate in a mass; Conidiophores well developed.
Chaetomium RH(b)	Ascocarps hairy, ostiolate or papillate; Ascospores 1-celled, Asci 4- to 8- spores.
Purpureocillium RH(L)	Conidia hyaline, 1-celled; Phialides swollen at basal part, tapering into a thin distint neck; Conidia divergent chains, ellipsoid to fusiform.
Rhodosporidium RH(r)	Short chains of budding cells; Short lengths of true mycelium may be present; True mycelium bearing spheroidal teliospores; Teliospores were in clusters of two to four, intercalary; rarely terminal on the mycelium.

(Stolk and Samson, 1972; Tsuneo, 2010)

TABLE 3. ISOLATED AND IDENTIFIED FUNGI WITH RELATIONSHIP TO THE GENUS OR SPECIES AND ITS IDENTITY PERCENTAGE FROM NCBI WEBSITE

Genera	Fungal species	Isolate ID	GenBank Accession number	Fungal species and Genbank Accession number (% Identity)
Trichoderma	*Trichoderma reesei*	RH(a)	KM246746	*Trichoderma reesei* KJ767092 (100%)
Chaetomium	*Chaetomium cupreum*	RH(b)	KM246747	*Chaetomium cupreum* JQ676206 (97%)
Penicillium	*Penicillium rolfsii*	RH(d)	KM246748	*Penicillium rolfsii* KJ767061 (99%)
Penicillium	*Penicillium janthinellum*	RH(e)	KM246749	*Penicillium janthinellum* AJ608945 (99%)
Penicillium	*Penicillium citrinum*	RH(f)	KM246750	*Penicillium citrinum* KF530863 (100%)
Trichoderma	*Trichoderma asperellum*	RH(g)	KM246751	*Trichoderma asperellum* KF723005 (99%)
Penicillium	*Penicillium janthinellum*	RH(i)	KM246752	*Penicillium janthinellum* HQ839782 (100%)
Talaromyces	*Talaromyces verruculosus*	RH(j)	KM246755	*Talaromyces verruculosus* HQ607919 (99%)
Fusarium	*Fusarium oxysporum*	RH(k)	KM246753	*Fusarium oxysporum* KJ774041 (100%)
Purpureocillium	*Purpureocillium lilacinum*	RH(L)	KM246754	*Purpureocillium lilacinum* KC478538 (100%)
Penicillium	*Penicillium oxalicum*	RH(m)	KM246756	*Penicillium oxalicum* KC344971 (99%)
Penicillium	*Penicillium* sp.	RH(n)	KM246757	*Penicillium* sp. JQ776535 (100%)
Gongronella	*Gongronella butleri*	RH(p)	KM246758	*Gongronella butleri* JN943049 (99%)
Rhodosporidium	*Rhodosporidium paludigenum*	RH(r)	KM246763	*Rhodosporidium paludigenum* HQ670676 (99%)
Trichoderma	*Trichoderma koningiopsis*	NI(b)	KM246760	*Trichoderma koningiopsis* EU718083 (99%)
Fusarium	*Fusarium oxysporum*	NI(g)	KM246761	*Fusarium oxysporum* JF776163 (99%)
Myrothecium	*Myrothecium verrucaria*	NI(i)	KM246762	*Myrothecium verrucaria* HQ607996 (100%)
Cunninghamella	*Cunninghamella echinulata*	IS(a)	KM246745	*Cunninghamella echinulata* KF170217 (98%)

(http://www.ncbi.nlm.nih.gov/BLAST)

TABLE 4. DISTRIBUTION OF SOIL MYCOFLORA UNDER THREE DIFFERENT RUBBER TREE HEALTH ZONES OF RUBBER PLANTATION AT RRIM SUNGAI BULOH, MALAYSIA

Health Zone	Phylum Classification Ascomycota	Zygomycota	Basidiomycota
WRR affected tree zone (ATZ)	-	*Cunninghamella echinulata*, *Gongronella butleri*	-
Healthy tree zone (HTZ)	*Trichoderma koningiopsis*, *Fusarium oxysporum*, *Myrothecium verrucaria*, *Penicillium citrinum*, *Penicillium janthinellum*	*Gongronella butleri*	-
Re-growth tree zone (RTZ)	*Trichoderma reesei*, *Trichoderma asperellum*, *Fusarium oxysporum*, *Chaetomium cupreum*, *Purpureocillium lilacinum*, *Talaromyces verruculosus*, *Penicillium janthinellum*, *Penicillium rolfsii*, *Penicillium citrinum*, *Penicillium oxalicum*, *Penicillium* sp.	*Gongronella butleri*	*Rhodosporidium paludigenum*

DISCUSSION

In soil fungi community, *Aspergillus* and *Penicillium* appear to be the common soil inhabitants. The present result showed that *Penicillium* spp. appeared to be the dominant species inhibits in the soil while no species of *Aspergillus* has been identified. This is in contradiction to studies by Singh, Chesters, Farrow and Ghulam[25–28] where *Aspergillus* spp. are the dominant fungi in tropical soils while *Penicillium* spp. are abundant in temperate soils.

The occurrence of dominant species in the sandy soil of Sungai Buloh series belong to the genus *Penicillium*. This corresponds well with earlier investigation by Fatma[29] where *Penicillium* is the most frequent species found in sandy soil samples. The occurrence of genus *Aspergillus* is recorded in high organic matter area[30]. Likewise, the organic matter in Sungai Buloh series (sandy soil) is low[31]. One of the reasons contributing to the absence or low *Aspergillus* species occurrence in the soil of Sungai Buloh series is the amount of organic matter. *G. butleri* was the species found to be distributed in all the three zones which strongly indicated that it is a common soil fungus in Malaysia. The same occurrence has been reported in the studies of Eicker[32] on Zululand soils and Varghese[33] on Malaysian soils.

The species *C. echinulata* and *G. butleri* were the only two species successfully isolated from ATZ soil. As ATZ is infected by WRR disease, there are some chemical treatments

(*e.g*. propiconazole 25.2% a.i, Group 3 fungicide) implemented to the surrounding environment to control the disease. Chemicals that are often used in conventional agriculture practices to control plant diseases and pests and soil disturbance have shown a significant effect on soil microbial diversity[34–35]. Hence, the soil microbial community of affected tree is low due to the application of chemical fungicides. The survival of the two species *C. echinulata* and *G. butleri* in the soil of ATZ could be due to propiconazole resistance (Moderate-risk category for resistance), as some naturally occurring fungi will develop resistance towards this chemical fungicide.

The fungi species belonging to the genus *Penicillium, Fusarium, Trichoderma, Chaetomium, Purpureocillium, Talaromyces, Gongronella*, and *Rhodosporidium* have been successfully isolated from the soil of RTZ. Among these fungal species, *Penicillium* is the genera that have the ability to produce fungal and bacterial antibiotics[36]. Moreover, there are certain species under the genus *Trichoderma* are believed to have antagonistic characteristic against some diseases caused by other fungi[37–40]. According to Jayasuriya and Thennakoon[10], the isolations of *Trichoderma* spp. from rubber planting area are found to be antagonistic against WRR pathogen. *Trichoderma* species are generally known to be aggressive competitors and they produce high level of phialospore in the rhizosphere[10]. The mechanisms of invasion by *Trichoderma* against WRR pathogen also involved the antifungal metabolite activities which have made it a promising biological control agent[16]. The characteristics of inhibition by *Trichoderma* spp. against WRR pathogen were best demonstrated in the RTZ soil where a new tree branch grew from the collapsed rubber tree which was previously infected by WRR disease.

Besides, the bioactive compound from *C. cupreum* named rotiorinol has shown the ability to inhibit the growth of *R. microporus*[41]. Therefore, the distribution of various fungal species in RTZ and majority species from the category of potential biocontrol agent were the reason the rubber tree in this soil (RTZ) survived with new branching even when its main stem has been affected by WRR and collapsed. This study revealed the important role of soil microbial associated with the tree health condition and the species diversity under different rubber tree health zones. Besides, the *Trichoderma* spp. and *C. cupreum* species isolates are found to be potential biocontrol agents against WRR pathogen as supported by the previous studies in Jayasuriya and Thennakoon[10], and Soytong and Kaewchai[41]. This study has documented the first information on range of mycoflora in soil of different health conditions associated with WRR disease occurrence.

CONCLUSION

A detailed identification and description of fungal community structure and candidate taxa that might influence soil fungi and different tree health condition interactions in rubber tree plantation was established. It is found that fungal species in the different health zones varied although some species (*G. butleri, F. oxysporum, P. citrinum* and *P. janthinellum*) were present in the soil of two zones. There were also four fungi species (*T. reesei, T. asperellum, T. koningiopsis* and *C. cupreum*) that have been identified to be used as potential biocontrol agents against WRR pathogen. This study revealed that soil fungal diversity was closely related with health condition of the tree, demonstrating that higher fungal diversity in the soil might suppress the WRR disease occurrence.

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