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Biological Control of White Root of Rubber Trees Using *Chaetomium Cupreum*

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Abstract White root of rubber tress caused by *Rigidoporus microporus* brings about economic losses in rubber plantation. Sample collection was taken in the southern part of Thailand at Narathiwat, Trang, and Surat Thani provinces. Fifty isolates of *R. microporus* were isolated from infected roots and fruiting bodies. All isolates were proved for their pathogenicities on the rubber tree variety RRIM600. The symptom was observed within 3 months and all isolates were pathogenic on the rubber trees. The molecular phylogenetic study was confirmed to be a taxon of *R. microporus* by DNA sequencing at internal transcribed spencer (ITS) region. This was firstly recorded about ITS technique of *R. microporus*. The genetic variation among isolates of *R. microporus* was determined using ISSR. Cluster analysis based on ISSR characters grouped the isolates according to geographical origins and the results showed two main distinct groups, designated as A and B rooting from out group, *Ganoderma* sp. GM101. *Chaetomium cupreum* RY202 was tested for ability to inhibit the growth of *R. microporus*. The results showed that crude hexane extract from *Ch. cupreum* RY202 gave the highest inhibition of mycelial growth with inhibition of 82.0% and the effective dose (ED_{50}) of 170 µg/ml and crude ethyl acetate extract gave colony inhibition of 80.0 and 78.0% and the effective dose (ED_{50}) at 187 and 402 µg/ml, respectively. The bioactive compound from *Ch. cupreum* named rotiorinol showed ability to inhibit the growth of *R. microporus* and inhibited the mycelial growth at 250 and 500 µg/l with effective dose (ED_{50}) 26 µg/ml. *Ch. cupreum* RY202 was formulated as powder and oil form. These formulations were applied to inhibit the growth of *R. microporus*. The results showed that *Ch. cupreum* RY202 could reduce the white root disease of rubber trees with disease reduction in the treatment of powder and oil from of 60 and 80%, respectively.

Keywords: *Ch. cupreum* RY202, *R. microporus*

Introduction

The genus *Hevea* belongs to the family Euphorbiaceae. There are many species of *Hevea* such as *Hevea benthamiana* Muell-Arg., *H. brasiliensis* (Willd.) Muell.-Arg., *H. camargoana* Pires, *H. camporum* Ducke, *H. guianensis* Aubl., *H. microphyll* Ule, *H. nitida* Mart. ex Muell-Arg., *H.*

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pauciflora Muell-Arg., *H. rigidifolia* Muell Arg., and *H. spruceana* Muell Arg. etc. Among them, only three species yield usable rubber as follows:- *H. benthamiana*, *H. brasiliensis* and *H. guianensis*. However, *H.* b*rasiliensis* is the only species which is planted commercially and yielded the source of natural rubber (Wycherley, 1992; Orwa *et al*., 2009). Rubber tree was first found in the Amazon basin and it is a native plant of Brazil. However, Brazil was not the site of the successful commercialization of rubber (Law, 2009; FAO, 2001). It is introduced to many countries in Asia, such as China, India, Indonesia, Malaysia, Papua New Guinea, Sarawak, Sri Lanka, Thailand, and Vietnam as well as in Africa such as Cameroon, Côte d'Ivoire, Gabon, Liberia and Nigeria (Law, 2009). In Thailand, rubber trees were first introduced to Trang province from Malaysia by Phraya Ratsadanupradit Mahison Phakdi**,** Governor of Trang Province in 1899. It becomes one of the major crop in Thailand. The planting area of rubber tree has nowadays expanded all over the country. There are more than 80% of the rubber plantations are found in the southern regions. The south has fertile growing climate which result in highly conducive to rubber tree cultivation. This climate helps farmers achieve high yields roughly 1.76 tons of rubber per hectare. Since 1991, Thailand has become the world supplier of natural rubber production. In 2006, nearly 90% of natural rubber production or over 2,771,673 tons and estimated US$5.41 billion of natural rubber were exported. The rubber products are exported worldwide and produce significant revenue for the country. The leading export markets for Thai rubber are Japan, Malaysia, USA, China and South Korea. Ten percent of all the rubber produced in Thailand is used for domestic consumption. Of this portion, 65% is processed into value-added goods, such as tires and tubes for motorcycles, airplanes, cars and bicycles (46-51%), gloves (13-15%), rubber bands (8-10%), and elastic (8-9%). Rubber wood, a renewable resource that presents an attractive alternative to hardwoods timber, is an increasingly important product in the domestic market and now one of the major resources for making furniture for export (Anonymous, 2009).

Many diseases are known to attack *Hevea* trees which classified as 1) leaf diseases for example, leaf spot caused by *Botryodiplodia elactica*, *B. theobromae*, anthracnose caused by *Colletotrichum gloeosporioides*, bird's eye spot leaf caused by *Bipolaris heveae*, powdery mildew caused by *Oidium heveae*, Corynespora leaf disease caused by *Corynespora cassiicola*, leaf fall caused by *Phytophthora* sp. 2) stem diseases for example, black stripe caused by *Phytophthora botryosa* Chee., *P. palmivora* (Butler) Butler, die-back caused by *P. palmivora*, mouldy rot caused by *Ceratocystis fimbriata*, pink disease caused by *Pellicularis salmonicolor*, and 3) root diseases for example, white spongy rot caused by *Polystichus occidentalis* and *P. personii*, white root

disease caused by *Rigidoporus microporus* (Sw.) Overeem, brown root disease caused by *Phellinus noxius* (Corner) G.H. Gunn) and red root disease caused by *Ganoderma pseudoferreum* (Wakef) (Duke, 1983; Nandris *et al*., 1987; Nicole and Benhamou, 1991; Law, 2009). Among these diseases, root diseases are the most serious diseases especially white root disease (Nicole and Benhamou, 1991).

White root disease is controlled using an integration of cultural and chemical methods by clearing the land of old rubber tree to reduce the source of inoculum before replanting and after planting, cutting away diseased tissue and applying chemical fungicide. However, chemical fungicides have been known to have a negative effect on human health, cause environmental pollution, leave residues in the agricultural soil, and several plant pathogenic fungi have developed resistance (Deahl and Demuth, 1993). To avoid the negative effect or harmful of chemical use, biological control would therefore be taken as an alternative save and sound measure for controlling this disease by reducing the inoculum sources, as well as to inhibiting the disease spread.

The main objectives of this study were to collect, isolate and test for pathogenicity of white root rot pathogen, to confirm the species and study the pathogenic variability among isolates of the white root rot pathogen and to investigate the efficiency antagonistic fungi for controlling the white root disease and to test the efficiency of antagonistic fungi against white root rot pathogen in pot and field trials.

Results

Sample Collection, Isolation and Pathogenicity Test

White root disease was found in the rubber tree plantation in the South of Thailand where the climate is suitable for this disease. Sample collection was occurred at Narathiwat, Trang, and Surat Thani province. This disease can infect to young and the old trees. The visible symptom of white root disease was seen by changed in color the leaves from green to yellow. The yellowing leaves were observed on one or a few branches or whole canopy depend on the severity of disease (Fig. 1). The dead trees were also observed the causing agent produced the fruiting bodies at the collar of the dead stem. The fruiting bodies were broadly attached shelf and orange red brown in color. The fruiting bodies were normally produced in the rainy season. In humid condition, there were rhizomorph of the pathogen at the infected root. The rubber trees died in the large area if the disease severely occurred (Fig. 1E). Infected roots and

fruiting bodies of the pathogen were collected and taken to laboratory for isolation.

Isolation of pathogen

A total of collection as the infected root and fruiting bodies were isolated by tissue transplanting technique and direct isolation method. Fifty isolates were obtained from three provinces including 27 isolates from Narathiwat province as follows:- SND04, SND05, SND07, SND08, SND10, SNK02, SNK03, SNK04, SNK05, SNK06, SNK09, SNK10, SNP02, SNP05, SNP06, SNP08, SNS01, SNS02, SNS03, SNS04, SNS05, SNS06, SNS07, SNS08, SNS09, SNS10, SNS11, 8 isolates from Trang province as follows:- STR01, STR02, STR03, STR04, STR05, STR06, STR07, STR08, and 15 isolates from Surat Thani province as follows:- SSS01, SST01, SST02, SST04, SST05, SST06, SST07, SST08, SST09, SST11, SST12, SST13, SST14, SST15, SST16 (Table 1). All isolates were maintained on PDA slant and kept at room temperature (27-30 ^{0}C). These isolates were morphologically studied and test for their pathogenicities followed Koch's Postulation.

Pathogenicity test

All isolates of *R. microporus* were tested for their pathogenicities with 5-months rubber tree variety RRIM600. The disease incidence was determined at 90 days. Disease index (DI) was recorded as follows:- level 1 = healthy, green leaves, level 2 = 1-25% yellow leaves, level 3 = 26-50% yellow leaves, level 4 = 51-75% yellow leaves, level 5 = 76-100% yellow leaves.

Table 1. Pathogenicity test of *Rigidoporus microporus*

Virulent Group	Isolates	Disease Index (DI)[1]	Isolates	Disease Index (DI)
Low virulence	SND05	2.0bcd[2]	SND07	2.0bcd
	SNP05	2.0bcd	SNK04	2.0bcd
	SNK09	2.0bcd	SNK10	2.0bcd
	SNS01	1.3cd	SNS02	2.0bcd
	SNS04	1.3cd	SNS05	2.0bcd
	SNS06	1.0d	SNS08	2.0bcd
	SNS09	2.0bcd	SNS10	1.5cd
	STR01	1.0d	STR03	1.0d
	STR05	1.8bcd	STR06	1.0d
	STR07	1.8bcd	STR08	1.3cd
	SSS01	1.3cd	SST08	1.3cd
	SST09	2.0bcd	SST11	1.3cd
	SST14	2.0bcd		

Moderately virulence	SND04	3.8abcd	SNP02	3.0abcd
	SND10	2.8abcd	SNP06	3.0abcd
	SNP08	3.5abcd	SNK05	4.0abc
	SNK06	4.0abc	SNS03	2.5abcd
	SNS07	2.5abcd	SNS11	3.3abcd
	STR02	3.0abcd	STR04	2.8abcd
	SST01	3.0abcd	SST02	3.0abcd
	SST04	2.3abcd	SST05	4.0abc
	SST06	2.5abcd	SST07	2.3abcd
	SST12	2.5abcd	SST13	2.8abcd
	SST15	3.3abcd	SST16	4.0abc
High virulence	SND08	4.5ab	SNK02	5.0a
	SNK03	5.0a		

Morphological and Molecular Phylogenetic Study

The isolates of *Rigidoporus microporus* were studied the morphology on the PDA medium and their fruiting bodies.

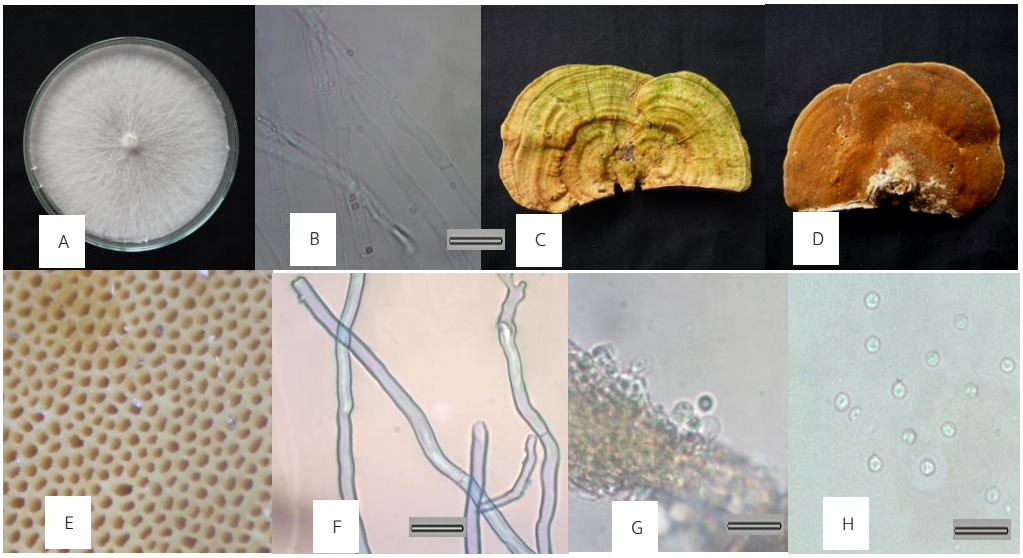

Fig. 1. The characteristic of *Rigidoporus microporus* SND04.
A = Colony on PDA at 6 days, B = hypha, C = fruiting body: upper surface, D = lower surface, E = pores at the lower surface, F = monomitic, generative hypha, G = hymenium and H = basidiospores. Bar. B, F, G, H = 10 μm

Molecular phylogenetic study

The molecular phylogenetic study was confirmed the taxon of *R. microporus* by DNA sequencing and ISSR technique. Seven ISSR primers

showed multi band patterns in each isolate. The primers amplified a total of 34 bands from 41 isolates tested including out group. The average number of bands per primers was 4.6. Band size ranged from 200-2500 bp. Some DNA banding profiles generated by ISSR-PCR with primer (GA) 6GC (A), (GA)8T (B), (TG)8GA (C), (GA)8YG (D), (GT)8YC (E), GGGC(GA)8 (F), and (CGA)5 (G). An UPGMA analysis based on total ISSR characters difference was carried out to group the 40 isolates of *Rigidoporus microporus* with the outgroup *Ganoderma* spp. GM101. A dendrogram resulting from a cluster analysis showed two main distinct groups, designated as A and B rooting from outgroup *Ganoderma* spp. GM101. All isolates obtained from Surat Thani and Trang province were grouped together as "A" and all of isolates obtained from Narathiwat province were grouped together as "B". The results indicated that isolates from Trang province were grouped together and in the same way as isolates from Surat Thani province were grouped together. There was a high similarity (0.88 – 1.00) within the isolates from Trang province, similarity (0.78 – 1.00) within the isolates from Surat Thani province, and similarity (0.76 – 1.00) within the isolates from Narathiwat province. There also was a high similarity in the range of 0.76 - 0.90 between isolates from three provinces.

Screening Biological Control Agent

The promising antagonistic fungi were obtained from baiting and soil plate technique from Surat Thani and Narathiwat provinces. Identification was mainly referred to Domsch and Gams (1993). All promising antagonistic fungi were tested for their antagonistic abilities to control the growth of *R. microporus*. The test was carried out in the laboratory using dual culture technique. The most aggressive isolate, *R. microporus* SNK02 was used in this experiment. The effective antagonistic fungi were screened according to percentage of the growth inhibition (PGI).The results showed that eighteen isolates of antagonistic fungi could inhibit the colony of *R. micropors* at 10 days with PGI over 50% as follows:-*Ch. cupreum* RY202 (57.5). It is noticed that dual culture antagonistic test provide a premising result as promising antagonist. The other screening techniques of antagonistic crude extract and bioactive compound tested must be further considered to decide the most effective isolate to develop as biofungicide used to control white root disease.

Bioactivity crude extract test

The effective isolate of *Ch. cupreum* RY202 was used to extract metabolites which was tested for its ability to control the growth of *R. microporus*. The effect of crude extracts at the concentration of 1,000 μg/ml to

inhibit the growth of *R. microporus* and found here were a significant differ in colony diameter and percentage of colony inhibition at $P = 0.01$. Crude extract gave the percentage of colony inhibition over 50% as follows:- crude hexane, crude ethyl acetate, crude methanol from *Ch. cupreum* RY202 The results also showed that all three kinds of crude which extracted from *Ch. cupreum* RY202 could inhibit the growth of *R. microporus*. Crude hexane from *Ch. cupreum* RY202 gave the highest results to inhibit the growth of pathogen with colony diameter and percentage of colony inhibition of 0.9 cm and 82%, respectively. Crude hexane extract from *Ch, cupreum* RY202 gave the best result to inhibit the growth of pathogen with ED_{50} value of 170 µg/ml and crude ethyl acetate extract from *Ch, cupreum* RY202 with ED_{50} value of 187 and 402 µg/ml, respectively. The effect of crude extracts of these biological control agents and Dual culture test, antagonistic crude extract test and bioactive compound test, *Chaetomium cupreum* RY202 was selected to formulate as powder and oil form according to the methods of Assoc. Prof. Dr. Kasem Soytong (unpublished data) for testing their ability to control *R. microporus* in the pot experiment.

Bioactive compound test

The characteristic of rotiorinol from *Ch. cupreum* was red and amorphous powder and its structure. The results showed that, the effect of rotiorinol on mycelial growth of *R. microporus* was significantly different at $P = 0.01$. The mycelium of *R. microporus* could not grow at concentrations of rotiorinol at 100 and 250 µg/l. The fresh weight, dry weight after treated with rotiorinol at 100 and 250 µg/ml were 0.05, 0.03, 0.004 and 0.002 g, respectively. The percent fresh weight and percent dry weight inhibition after treated with rotiorinol at 100 and 250 µg/ml were 97.83, 97.21, 99.32 and 99.05%, respectively. Base on the result, rotiorinol could inhibit the growth of *R. microporus* and ED_{50} was 26 µg/ml (Table 2).

Table 2. Effect of rotiorinol to inhibit *Rigidoporus microporus*

Concentrations (µg/ml)	Fresh weight (g)	Fresh weight inhibition (%)	Dry weight (g)	Dry weight inhibition (%)	ED_{50} (µg/ml)
0 (Control)	4.64a[1]	-	0.156a	-	
10	3.26b	29.02c	0.107b	31.37c	
50	2.35c	49.03b	0.091c	42.02b	26.00
100	0.05d	97.83a	0.004d	97.21a	
250	0.03d	99.32a	0.002d	99.05a	
c.v. (%)	15.46	9.72	5.72	3.95	

[1]Mean of four replications. Mean followed by a common letter are not significantly different when compared to Duncan's Multiple Range Test (DMRT) at $P = 0.01$.

Efficacy of Biofungicide to Control White Root of Para Rubber

The pot experiment was conducted to test the efficiency of *Chaetomium cupreum* RY202 in the powder and oil form for control *R. microporus*. The experiment was done for five months. Data collection as disease index (DI) was periodically recorded every 4 weeks. Infected root colonization was also be observed and recorded. DI was categorized as follows:- level 1 = healthy, green leaves, level 2 = 1-25% yellow leaves, level 3 = 26-50% yellow leaves, level 4 = 51-75% yellow leaves and level 5 = 76-100% yellow leaves. The results showed that the rhizomorph of the *R. microporus* at the basal stem of rubber tree appeared in the treatment of inoculated with *R. microporus*, whereas there were no any rhizomorph at the basal stem of rubber trees in others treatment at 60 days. Disease index at 150 days of non-treated one (T1), inoculated with *R. microporus* (T2), treated with *Ch. cupreum* RY202 in powder form (T3), treated with *Ch. cupreum* RY202 in the oil form (T4), treated with *Ch. cupreum* RY202 in the powder form and *R. microporus* (T5), treated with *Ch. cupreum* RY202 in the oil form and *R. microporus* (T6) and treated with fungicide (sulfur) and *R. microporus* (T7) were 1, 5, 1, 1, 2, 1, and 1, respectively. Base on the results, inoculated with *R. microporus* gave the most severity of symptom followed by treated with *Ch. cupreum* RY202 in the powder form and *R. microporus* with 1-25% yellow leaves (DI = 2). However, there were no significant different in treatments of treated with *Ch. cupreum* RY202 in the powder form and *R. microporus* when compared with treatment of fungicide (sulfur) and *R. microporus* (Table 3). There was no disease in the treatment of *Ch. cupreum* in the oil form and *R. microporus* (T6). Disease index was reduced when applied biofungicides in the powder and oil form at 60 and 80%, respectively (Table 4).

Table 3. Disease index of white root disease

Treatments	Disease Index (DI)[1]
	150 days
non-treated (T1)	1b
R. microporus (T2)	5a
Ch. cupreum in powder form (T3)	1b
Ch. cupreum in the oil form (T4)	1b
Ch. cupreum in the powder form and *R. microporus* (T5)	2b
Ch. cupreum in the oil form and *R. microporus* (T6)	1b
Sulfur and *R. microporus* (T7)	1b

[1]DI:- level 1 = healthy, green leaves, level 2 = 1-25% yellow leaves, level 3 = 26-50% yellow leaves, level 4 = 51-75% yellow leaves and level 5 = 76-100% yellow leaves.
[2]Mean of four replications. Mean followed by a common letter are not significantly different when compared to Duncan's Multiple Range Test (DMRT) at $P = 0.01$.

Table 4. Disease reduction of white root disease

Treatments	Disease Index (DI)[1]
	150 days
Ch. cupreum in powder form	80
Ch. cupreum in the oil form	80
Ch. cupreum in the powder form and R. microporus	60
Ch. cupreum in the oil form and R. microporus	80
Sulfur and R. microporus	80

Discussions

White root disease of rubber trees were proved to be seriously occurred in Thailand, especially in the south such as Trang, Narathiwat and Surat Thani provinces as stated by Guyot and Flori (2002) that the disease epidemic was expended to many countries, e.g. India, Indonesia, Maleysia, Sri Lanka, West and Central Africa. The causing agent is *Rigidoporus microporus* causing white root which Hood (2006) stated that *R. microporus* mostly occur in monsoon climate and *Heterobasidion annosum* causing agent of root disease in the temperate zones. According to white root disease, it is observed that *R. microporus* can infect all stage of plant from seedling to mature trees. The visible symptom of white root disease was clearly shown yellowing leaves on one or a few branches or whole canopy depend on the severity of disease, and finally the tree die. It is also shown that the pathogen can infect the roots by rhizomorph growing from the stumps or infected woody debris remaining in the ground and by contacting with the infected root as also stated by the works of Nandris *et al*. (1987) and Guyot and Flori (2002). The isolates of *Rigidoporus microporus* were studied the morphology on the PDA medium and studied their fruiting bodies. The colony on PDA showed white and flat. The hypha of this fungus showed hyaline, septum, no clamp connection, and possess many branches. This result was similar to the report of Nandris *et al*. (1987) who stated that the fungus formed many white and flattened mycelium but the colony on malt medium formed superficial, white mycelial felt.

The phylogeny study was still needed for confirmation the species and studying the pathogenic variability among isolates of *R. microporus***.** In this study, the phylogenitic tree by ITS revealed that *R. microporus* was in the same branch as *R. ulmaroius*, *Oxyporus*, *Heterobasidion*, and *Laetiporus*, *Melanoporia* defined from out group *Auricularia delicata*. This result is similar to the work of Ryvarden (1991) stated that the *Rigidoporus* is in the same group as *Melanoporia*, *Nigrofomes*, *Heterobasidion*, *Oxyporus*, *Leucophellinus*, *Laetiporus*, *Flavodon* and *Irpex*. However, there is no report on determination of genetic variation among pathogen populations of white root disease pathogen.

In this study, 40 isolates of *R. microporus* were tested with ISSR primers to determine the distribution of genetic diversity which represents the difference rubber tree planting areas Base on the results, the isolates which obtained from three provinces were separated into two groups. It was clearly that there was a relationship between the geographical distributions and clustering of isolates. This result similar to those by Yu *et al.* (2006) stated that ISSR marker of *Melamspora larrici-populina* could divide tested isolates in to northern and western population. On the contrary, Rodrigues *et al* (2004) stated that ISSR-PCR analysis which separated strains of *Guignardia mangiferae* into three groups but not corresponded either to the host or to the geographic origin. It is suggested that ISSR markers could be still a good choice for DNA fingerprint for easy handle and rapid investigation.

All isolated fungi which obtained from soil were tested for their abilities to inhibit the growth of *R. microporus* by dual culture. *Chaetomium cupreum* RY202 was selected to be a good antagonistic fungus against *R. microporus* because it could inhibit the growth of *R. microporus* over 50% in dual culture and grown over the colony of pathogen within 30 days. This is indicated as the first report using *Ch. cupreum* against white root pathogen *in vitro*. The other criteria to select *Ch. cupreum* RY202 was proved by its bioactivity test of crude extract and bioactive compound. Base on the result, crude extracts from *Ch. cupreum* gave the highest results on growth inhibition of *R. microporus* with the effective dose (ED_{50}) of 170 µg/ml. The bioactive compound produced from *Ch. cupreum* named rotiorinol was confirmed its ability to inhibit the growth of *R. microporus* with ED_{50} of 26 µg/ml. This result showed promising biological control potential of *Ch. cupreum* RY202 that could be developed to be biological fungicide to test *in vivo* as stated by Soytong (2005) and Kanokmedhakul *et al*. (2006) that rotiorinol, a bioactive compound from *Ch. cupreum* CC3003 has a potent to inhibit the growth of *Phytophthora parasitica*, *P. palmivora*, *Colletotrichum gloeosporioides*.

In this study, *Ch. cupreum* RY202 was formulated as powder and oil form according to the work of Soytong *et al*. (2001). The biofungicide was tested to inhibit the growth of *R. microporus* and revealed that the disease incidence was not significantly different from sulfur treatment. The results implied that the biofungicide from *Ch. cupreum* RY202 could reduce white root disease of rubber trees. It is suggested that biofungicide produced from *Ch. cupreum* RY202 may have a potential biofungicide to control white root of rubber trees in the field. Further more, this biofungicide will further evaluate in the field trials.

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