MODE OF DISPERSAL AND VARIATION IN POPULATION OF WHITE ROOT FUNGUS *RIGIDOPORUS MICROPORUS* AS REVEALED BY MYCELIAL INCOMPATIBILITY

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Abstract

Management of the white root disease in Indonesia and other tropical regions has been developed without any knowledge of population of the causal fungus. We determined 33 mycelial compatibility groups (MCGs) out of 62 isolates collected from 11 plant species in South Sumatra and Bangka Island. Mycelial incompatibility in R. microporus is characterized by formation of demarcation line and/or sparse aerial mycelial growth along the zone of interaction of genetically different mycelia. Failure to anastomosis between incompatible mycelia induced abnormal, spiral-like of hyphal growth. Most of isolates colonizing the stumps were somatically compatible with isolates from either a nearby stump or living trees, which suggested the evidence of stump-to-tree or stump-to-stump clonal growth of the fungus. Each study site was occupied by at least one MCG which indicated that local population of R. microporus *consists of different genotypes (genetic individual) which spread clonally to form patches of mycelial networks. A territorial clone covered large area (up to 52 m across) that encompassed both old trees (*Hevea brasiliensis*,* Artocarpus integra *and* Arenga pinata*) and recent non-woody plants (*Languas galangal *and* Musa paradisiaca*). All of isolates occurring on different location have different MCG. This indicated that no evidence of clonal (vegetative) spread between separate locations. Most of isolates of R. microporus were strongly pathogenic on rubber seedlings. These isolates belonged to different MCG and showed strongly laccase activity as compared to the less pathogenic isolates.*

INTRODUCTION

Rigidoporus microporus (Sw.) Overeem synonym *R. microporus* (Klotzsch) Imazeki is white root rot fungus that cause significant losses in industrial plantations including rubber (Liyanage, 1997; Semangun, 2000) and black pepper (Suwandi, 2003).The fungus attacks roots and collar region of taproot causing white root disease and eventually kills trees at any growth stage. The disease attacks increase cumulatively as increased of rubber planting generations or even for other crops generated from rubber plantations, such as black pepper, cassava and acacia. In Bangka Island, where the black pepper and cassava are grown in ex-rubber area (field generated from rubber trees), the white root disease may cause total loss on those plants (Suwandi, 2003).

Management of the white root disease in Indonesia and other tropical regions has been developed without any knowledge of population genetic of the causal fungus. Studying population biology is the most important prerequisite to develop a

sustainable strategy and tactics in management of plant disease (McDonald, 1997). However, no studies have been reported dealing with population structure of *R. microporus* in any countries including Indonesia. Studying fungal population biology requires appropriate markers for identifying individual genotypes (clones or genets) in a population of the fungus (Anderson and Kohn, 1995). Mycelial incompatibility has been reported to be successfully used as a maker for identification of individual genotype in polypore fungi, i.e. *Ganoderma boninense* (Miller et al., 1999), *Helicobasidium mompa* (Aimi et al., 2002), and *Heterobasidion annosum* (Lygis et al., 2004). In this paper, macro- and microscopic of mycelial incompatibility in *R. microporus*, using of MCG to describe mode of dispersal, and relatedness of MCG with variation in pathogenicity and laccase activity are discussed.

MATERIALS AND METHODS

Collection, isolation and determination of mycelial compatibility

The study involved isolates represented population of *R. microporus* from rubber and non-rubber hosts in South Sumatra and Bangka Island (Table 1). Isolations were conducted as routinely performed in our laboratory (Phytopathology Laboratory, Sriwijaya University). Freshly collected basidiomes, rhizomorphs or decayed wood were cleaned from soil particles and wood debris by running tap water and wrapped in a paper towel wetted with water containing 30 mg/L benomyl and 100 mg/L streptomycin. The sample then was enclosed in a sealed PE plastic bag and keep in room temperature. An active growing mycelial strands out of the wrapped samples were planted on MEA (malt extract agar) supplemented with 30 mg/L benomyl and 100 mg/L streptomycin. Pure cultures were maintained at room temperature in slant of MEA embedded with rubber wood.

To determine mycelial incompatibility, isolates from the same sites were paired in all possible combinations. For each incompatibility group determined, 1-2 isolates were selected for pairings with isolates from other sites. Self-crosses were performed as controls**.** Isolates are grouped into the same mycelial compatibility group (MCG), when paired isolates grow together and merge to form a single culture. The interaction is determined as incompatible when paired culture produce a lysis zone and incompatible isolates then are grouped as different MCG. Each pairing was repeated at least twice. The number of SIGs and area it occupied in a field describes the role of basidiospores dissemination (sexual population) and the extent of clonal spread through rhizomorph in the fungus population.

Cytological characteristic of incompatible pairings

As most tropical tree fungi, information regarding cytological characteristic of *R. microporus* hyphae is still lacking. Cytological characteristic of hyphae in both compatible and incompatible pairings was observed using fluorescence microscopy and staining with fluorescent dye, DAPI (4', 6-diamidino-2-phenylindole).

Pathogenicity assay

Pathogenicity of isolates was tested on rubber seedlings in a pot system as routinely performed in our laboratory. Rubber seeds collected from plantings clone GT-1 were sown in sand culture and watered every day until germinate for 2 weeks. Seedlings then were transferred to clear plastic pot (volume 250 ml) and filled with mixture (in equal volume) of autoclaved soil and sand. Seedlings were grown in shading area for one month.

Inocula were prepared as colonized wood sticks. Autoclaved rubber wood sticks (1 X 0.5 X 5 cm) were inoculated with a-quarter plate size of 7-days-old mycelia of tested isolates and allowed to be colonized by the mycelia for 2 months. Inoculation was done by carefully inserting two sticks closely to taproot. Each isolate was inoculated on 10 seedlings. Assay was repeated at least twice. Disease severity was assessed 3 months after inoculation based on 9 scale of disease severity (Nandris et al., 1987). Pathogenicity is rated as weak, moderate and strong, when the average of severity value was less than 3.5, between 3.5 to 5.5, and more than 5.5, respectively.

Laccase activity

Laccase activity was assessed indirectly by dye decolorization method (Kiiskinen et al., 2004). Tested isolates were grown on malt extract agar (2%) containing 0.04% dye Remazol Brilliant Blue R (RBBR) (catalog no. R-8001, Sigma). Each isolate was grown on four plates. After 7 days incubation, the enzyme activity was examined by scoring the decolorization intensity. Test was repeated twice.

RESULTS AND DISCUSSIONS

In this study, we successfully isolated 62 field isolates of *R. microporus* associated with white root disease on 11 host plants from 4 locations in South Sumatra and 6 locations in Bangka Island (Table 1). Some isolates were found on non woody plants such as pineapple, banana and galangal (*Languas galangal*) and this finding is the first report on infection of white root fungus on non-woody, herbaceous plants.

Mycelial incompatibility in *R. microporus* is characterized by formation of demarcation line and/or sparse aerial mycelial growth along the zone of interaction of genetically different mycelia. In compatible interaction, paired isolates grew together and merged to form an apparently single colony. Intensity of incompatible responses was affected by culture media (Fig. 1E). Formation of zone of sparse aerial was obviously found in parings on malt extract agar (Fig. 1B). When paired on glucose yeast extract agar, yellowing pigment was frequently observed along the interaction zone (Fig. 1A). When colonized rubber wood sticks were paired on water agar, incompatibility was characterized by formation of compact and dense mycelia around the wood stick (Fig. 1C.). Addition of 10 g/L active charcoal on malt extract agar reduced incompatible responses (Fig. 1D).

Observation of hyphae after stained with DAPI using fluorescence microscopy showed that all hyphae including the hyphal tip of the fungus were composed by two nuclei (Fig. 2C), except for the oldest hyphae that have 4 or more nuclei (Fig. 2D). No difference in number of nuclei of incompatible pairings as well as were found in self and non-paired isolates. In incompatible pairings, we usually found abnormal or spiral-like hyphae, which typically produced at zone of interaction between two different hyphae (Fig.2A, 2B). Fusion between incompatible hyphae was not observed in all ten slide cultures tested.

Table 1. Source and characteristics of *Rigidoporus microporus* isolates

Isolates	Locality[1]	Host Plant	MCG[2]	Pathogenicity[3]	Laccase[4]
Sbs1	Sembawa, SS	Rubber	1	nt[5]	nt
Sbs2	Sembawa, SS	Rubber	1	nt	nt
Sbs3	Sembawa, SS	Rubber	2	nt	nt
Sbt4	Sembawa, SS	Rubber	2	nt	nt
Sbs5	Sembawa, SS	Rubber	3	nt	nt
Sbt6	Sembawa, SS	Rubber	3	nt	nt
Sbs7	Sembawa, SS	Rubber	4	nt	nt
Sbs8	Sembawa, SS	Rubber	4	nt	nt
Sbt9	Sembawa, SS	Rubber	5	nt	nt
Sbs11	Sembawa, SS	Rubber	6	nt	nt
Sbs12	Sembawa, SS	Rubber	6	nt	nt
Sl1	Sembawa, SS	Rubber	6	7.4 (strong)	+++
Sl4	Sembawa, SS	Rubber	6	nt	+++
Sl6	Sembawa, SS	Rubber	6	nt	+++
Sl9	Sembawa, SS	Rubber	6	nt	+++
Sl10	Sembawa, SS	Rubber	6	nt	+++
Sbs13	Sembawa, SS	Rubber	7	6.8 (strong)	nt
Sbs16	Sembawa, SS	Rubber	7	nt	nt
Sb17	Sembawa, SS	Rubber	8	nt	+++
Sbl17	Sembawa, SS	Rubber	8	8.4 (strong)	+++
Sb10	Sembawa, SS	Rubber	9	7.1 (strong)	+++
S1	Sembawa, SS	Rubber	10	8.6 (strong)	nt
S12	Sembawa, SS	Rubber	10	7.1 (strong)	nt
S2	Sembawa, SS	Rubber	11	nt	nt
S3	Sembawa, SS	Rubber	12	nt	nt
S6	Sembawa, SS	Rubber	13	5.9 (strong)	nt
S9	Sembawa, SS	Rubber	14	nt	nt
S11	Sembawa, SS	Rubber	15	6.6 (strong)	nt
M32	Muara Enim, SS	Rubber	16	6.6 (strong)	nt
M34	Muara Enim, SS	Pineapple	16	6.3 (strong)	nt
M35	Muara Enim, SS	Rubber	16	6.6 (strong)	nt
M36	Muara Enim, SS	Rubber	16	6.7 (strong)	nt
M37	Muara Enim, SS	Rubber	16	7.3 (strong)	nt

Isolates	Locality[1]	Host Plant	MCG[2]	Pathogenicity[3]	Laccase[4]
Dd1	Inderalaya, SS	*Erythrina variega*	17	7.6 (strong)	+++
Ddb1	Inderalaya, SS	*Erythrina variega*	18	7.6 (strong)	nt
Ddb2	Inderalaya, SS	*Erythrina variega*	18	nt	nt
Dd4	Inderalaya, SS	*Erythrina variega*	19	6.7 (strong)	nt
Dd5	Inderalaya, SS	*Erythrina variega*	19	nt	nt
Klb31	Balunijuk, Bangka	Coconut	20	7.9 (strong)	nt
Kmb30	Balunijuk, Bangka	*Vernicia montana*	21	6.2 (strong)	+++
Kml30	Balunijuk, Bangka	*Vernicia montana*	21	nt	++
Kmb1	Balunijuk, Bangka	*Vernicia montana*	22	7.5 (strong)	+++
Cpb1	Balunijuk, Bangka	*Artocarpus integra*	23	nt	++
Cpb1-2	Balunijuk, Bangka	*Artocarpus integra*	23	4.7 (moderate)	+++
Cpb2	Balunijuk, Bangka	*Artocarpus integra*	23	4.6 (moderate)	nt
Ldb	Balunijuk, Bangka	Black pepper	23	5.1 (moderate)	++
U2	Balunijuk, Bangka	Cassava	23	3.9 (moderate)	+
Enb1	Balunijuk, Bangka	Sugar palm	23	5.1 (moderate)	nt
Enb1-2	Balunijuk, Bangka	Sugar palm	23	4.6 (moderate)	nt
LB	Balunijuk, Bangka	Galangal	23	6.1 (strong)	+++
Krb	Balunijuk, Bangka	Rubber	24	5.3 (moderate)	++
Psb	Balunijuk, Bangka	Banana	25	5.1 (moderate)	nt
Ldpb	Payak Benua, Bangka	Black pepper	26	3.9 (moderate)	+
Ubpb	Payak Benua, Bangka	Cassava	27	6.0 (strong)	nt
Ldca	Cengkong, Bangka	Black pepper	28	7.2 (strong)	
Ubca	Cengkong, Bangka	Cassava	29	4.9 (moderate)	+++
Krkc	Kace, Bangka	Rubber	30	6.1 (strong)	nt
Ubkc	Kace, Bangka	Cassava	31	7.0 (strong)	nt

[1] SS : South Sumatra
[2] MCG : Mycelial compatibility group
[3] Pathogenicity on rubber seedlings, based on 9 scale disease severity,
0 = no rhizomorph on root and
9 = plant died after 3 month inoculation.
Weak = disease severity less than 3.5; moderate = disease severity 3.5-5.5;
strong = disease severity more than 5.5.
[4] Measured as decolorization intensity of 0.04% Remazol Brilliant Blue R (RBBR),
+ = less intense and +++ = more intense.
[5] nt = not tested.

This phenomenon was different with incompatibility in other polypore fungi such as *H. mompa* that formerly fused before cell death due to failure of anastomosis (Aimi et al., 2002).

A total 33 mycelial compatibility groups (MCGs) were determined among the isolates (Table 1). Most of isolates colonizing the stumps were somatically compatible with isolates from either a nearby stump or living trees, which suggested the evidence of stump-to-tree or stump-to-stump clonal growth of the fungus (Fig. 3). Each study site was occupied by at least one MCG which indicated that local population of *R. microporus* consists of different genotypes (genetic individual) which spread clonally to form patches of mycelial networks. A territorial clone covered large area (up to 52 m across) that encompassed both old trees (*Hevea brasiliensis*, *Artocarpus integra* and *Arenga pinata*) and recent non-woody plants (*Languas galangal* and *Musa paradisiaca*).

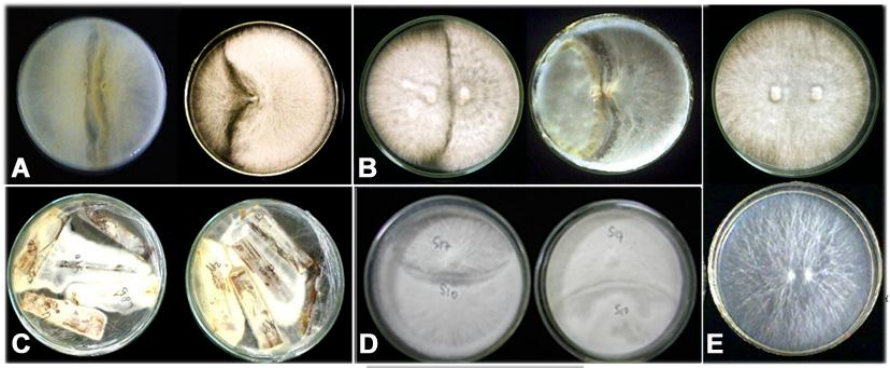

Figure 1. Macroscopic mycelial interaction between isolates of *Rigidoporus microporus*

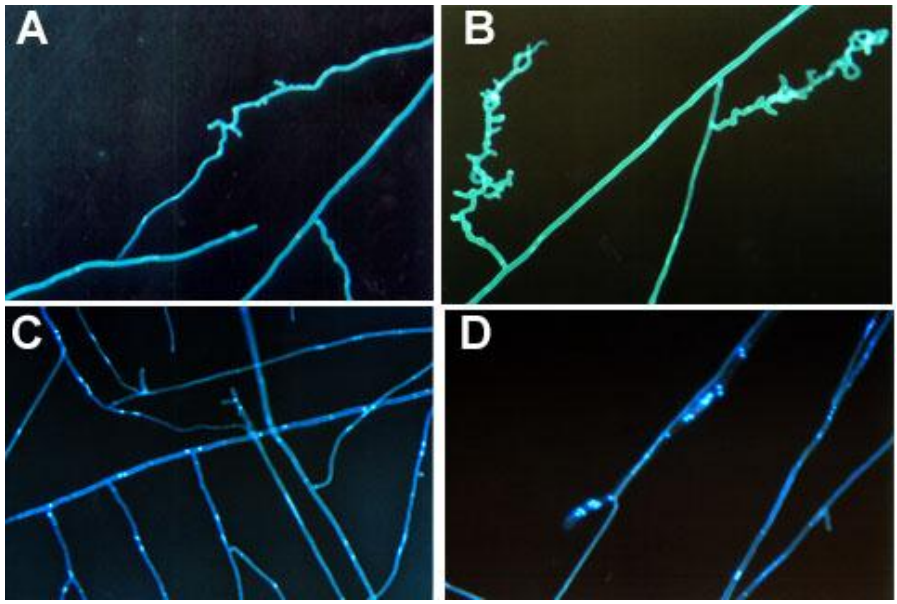

Figure 2. Hyphae of *Rigidoporus microporus* after stained with DAPI (400 X)
A and B : formation of abnormal braching system in incompatible pairing.
C : binucleate of young hyphae.
D : old hyphae with numerous nuclei.

All of isolates occurring on different location have different MCG (Table 1). This indicated that no evidence of clonal (vegetative) spread between separate locations. Therefore, dissemination of the fungus through mycelia colonized rootstocks was not unlikely occurs.

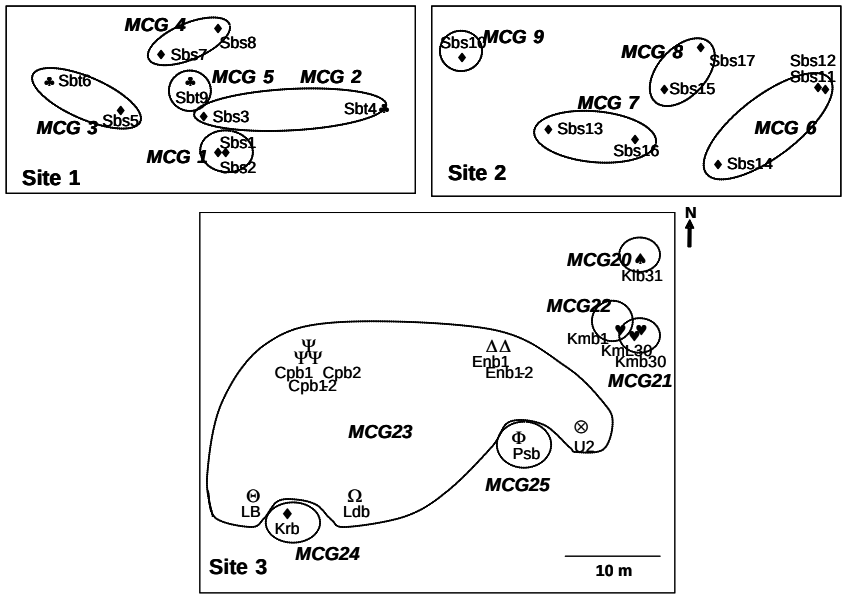

Figure 3. Distribution map of mycelial compatibility groups (MCGs) of *Rigidoporus microporus* existing in a rubber plantation (site 1 and 2) and ex-rubber field (site 3) Line enclose isolates of each SIG and not intended to indicate the exact SIG boundaries;

- ♣ = basidiomes on living rubber tree;
- ♦ = basidiomes on stumps of rubber tree;
- Θ = basidiome on *Languas galangal*;
- Ω = rhizomorph on black pepper;
- Ψ = basidiome on *Artocarpus integra*;
- Φ = basidiome on banana;
- Δ = basidiome on sugar palm (*Arenga pinata*);
- ⊗ = rhizomorph on cassava;
- ♥ = basidiomes on tung tree (*Vernicia montana*);
- ♠ = basidiome on coconut.

Isolates of *R. microporus* collected from South Sumatra were strongly pathogenic on rubber seedlings (Table 1) as they caused necrotic on most part of taproot and killed the test plants. Some moderately pathogenic isolates were found on either stumps or diseased plants in ex-rubber fields planted with black pepper in Bangka Island. Interestingly, most of these isolates belong to MCG 23, the oldest and the most widely distributed MCG in this study. A moderately pathogenic isolate of this MCG, U2 grew slower than strongly pathogenic LB. It was hypothesis that reduced pathogenicity in this isolates may be associated with infection of dsRNA mycovirus as found in other polypore, *H. mompa* (Ikeda et al., 2003). Our attempts to detect dsRNA elements in these isolates were not success (data not shown).

Laccase activity of isolates of *R. microporus* as qualitatively determined by RBBR decolorization intensity was varied regardless their MCG. Most of strongly pathogenic isolates decolorized RBBR more intense than the less pathogenic ones. RBBR decolorization by moderately pathogenic isolates was varied, but generally less intense than strongly pathogenic isolates (Table 1). As our future study will focus on exploration hypovirulence in the white root fungus, therefore, RBBR decolorization method may be useful in preliminary selection of reduced pathogenic isolates.

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