Selection of Rubber (*Hevea brasiliensis* Muell. Arg.) Rootstocks for the White Root Disease Tolerance

Suneerat Wattanasilakorn

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy in Plant Science

Prince of Songkla University

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I hereby certify that this work has not been accepted in substance for any degree, and is not being currently submitted in candidature for any degree.

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Candidate

ชื่อวิทยานิพนธ์ การคัดเลือกต้นตอยางพารา (*Hevea brasiliensis* Muell. Arg.) ที่ทนทานต่อโรครากขาว
ผู้เขียน นางสาวสุณีรัตน์ วัฒนาศิลากรณ์
หลักสูตร พืชศาสตร์
ปีการศึกษา 2558

บทคัดย่อ

งานวิจัยมีวัตถุประสงค์เพื่อศึกษาพันธุกรรมของต้นตอยางพาราพันธุ์ดั้งเดิม การเจริญเติบโต การพัฒนาของราก และการคัดเลือกต้นตอยางพาราพันธุ์ที่ทนทานต่อโรครากขาว จำนวน 8 โคลน จากแหล่งต่างๆ ในจังหวัดตรัง นครศรีธรรมราช และสงขลา ทดสอบร่วมกับยางพาราพันธุ์ RRIM 600 ซึ่งใช้เป็นพันธุ์เปรียบเทียบ โดยจัดการทดลองแบบแฟกทอเรียลในแผนการทดลองแบบสุ่มสมบูรณ์ (CRD) จำนวน 3 ซ้ำ โดยการทดลองมี 2 ปัจจัยคือ ต้นกล้ายางพารา จำนวน 8 โคลน และวิธีการปลูกเชื้อรากขาว 2 วิธี ได้แก่ ไม่ปลูกเชื้อ (ต้นควบคุม) และปลูกเชื้อปลูกต้นกล้ายางพาราในไรโซบ็อกซ์ ทำการเก็บข้อมูลในช่วง 24 สัปดาห์ หลังปลูกเชื้อ และศึกษาพันธุกรรม การตอบสนองทางสรีรวิทยาของต้นและรากกล้ายางพารา ผลทดลองพบว่า ในการวิเคราะห์พันธุกรรม สามารถแยกกลุ่มยางพาราได้เป็น 5 กลุ่ม โดยมีค่าความสัมพันธ์ใกล้ชิดทางพันธุกรรม 0.877–1.000 พบว่าต้นกล้ายางพาราโคลนที่ไม่ปลูกเชื้อ EIRpsu 5 มหาวิทยาลัยสงขลานครินทร์ มีการเจริญเติบโตของต้นสูงเมื่อเทียบกับต้นที่ปลูกเชื้อ ส่วนการกระจายของราก พบว่าต้นยางพาราโคลนที่ไม่ปลูกเชื้อ EIRpsu 1, EIRpsu 3, EIRpsu 4 และ EIRpark มีการกระจายของรากที่ระดับความลึก 20–40 เซนติเมตร มีการกระจายของรากหนาแน่นกว่าที่ระดับความลึกอื่นๆ ขณะที่ต้นยางพาราโคลนที่มีเชื้อ EIRpsu 1, EIRpsu 2, EIRpsu 3 และ RRIM 600 มีการกระจายของรากสูงที่ระดับความลึก 0–20 เซนติเมตร และพบว่ายางพาราโคลน EIRpsu 5 มีค่าเฉลี่ยของรากสูง การตอบสนองทางสรีรวิทยา พบว่าต้นกล้ายางพาราโคลน EIRpsu 5 มีอัตราการสังเคราะห์แสง และการชักนำปากใบสูงกว่าโคลนอื่นๆ สำหรับการประเมินดัชนีของโรค พบว่ายางพาราในโคลน EIRpsu 4 อ่อนแอต่อโรครากขาวสูงที่สุด โดยมีคะแนนเท่ากับ 54.09 เปอร์เซ็นต์ ส่วนยางพาราโคลน EIRpsu 5 มีความทนทานต่อการเกิดโรครากขาวมากที่สุด เมื่อเทียบกับพันธุ์อื่นๆ โดยมีคะแนนเท่ากับ 12.12 เปอร์เซ็นต์ ซึ่งชี้ให้เห็นว่ายางพาราในมหาวิทยาลัยสงขลานครินทร์ (EIRpsu 5) มีแนวโน้มทนทานต่อโรครากขาว

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Author Miss Suneerat Wattanasilakorn

Major Program Plant Science

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ABSTRACT

The aims of the present study were to assess the genetic variation in early–introduced rubber (EIR) clones and investigate the growth of their root systems and clonal selection for rubber rootstock tolerant to the white root disease. Assessment of rubber clonal rootstocks for tolerating to white root disease in 8 clones collected from the different areas in southern Thailand such as Trang, Nakhon Si Thammarat, and Songkhla provinces. Seven selected clonal rootstocks and the RRIM 600 were compared and they were grown in rhizoboxes (1 plant/1 rhizobox). The experiment was conducted as an 8×2 factorial laid out in completely randomized design (CRD) with 3 replications. The treatments comprised of eight clones and two treatments (control and inoculation treatments). A total of 48 rubber seedlings were planted in rhizoboxes. The experimental period was during 24 weeks, the data of root distributions, shoot growth, physiological responses and symptom of rubber seedlings were recorded at 2–week intervals. Genetic analysis was studied by RAPD with 7 primers. The results revealed that 48 rubber seedlings could be separated into 5 groups with similarity coefficients ranging from 0.877–1.000. After inoculation, shoot response of the controlled clone from EIRpsu 5 exhibited the significantly highest shoot growth comparing with the inoculation treatment clones. Root length density was accessed by scanning the root profile of each seedling from the panel of rhizobox, it was found that the control treatment had the clones EIRpsu 1, EIRpsu 3, EIRpsu 4 and EIRpark exhibited high portion of root proliferation in the layer 20–40 cm depth from the soil surface and inoculation treatment had the clones EIRpsu 1, EIRpsu 2, EIRpsu 3 and RRIM 600 exhibited high extension root growth to deeper layer of 0−20 cm. The clone from EIRpsu 5 exhibited the highest average root length density from the soil surface. For in the case of physiological responses, the clone EIRpsu 5 showed the highest efficiency of photosynthetic rate and high stomatal conductance.

According to the assessment of disease index, it was found that the clone EIRpsu 4 exhibited highest susceptibility score (54.09 %). Whereas, the lowest score was found the clone EIRpsu 5 (12.12 %). This indicated that the clone from EIRpsu 5 tended to be tolerant to white root disease with good performance of plant growth.

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Suneerat Wattanasilakorn

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LIST OF ABBREVIATIONS

Abbreviations

RAPD	Random Amplified Polymorphic DNA
EIR	Early Introduced Rubber Clone
RRIM 600	Rubber Research Institute Malaysia's rubber clone number 600
PDA	Potato Dextrose Agar
TEM	Transmission Electron Microscopy

CHAPTER 1

INTRODUCTION

Background and Rationale

Rubber tree (*Hevea brasiliensis* Muell. Arg.) is a major crop in Thailand and it is also the most important economic crops in Southeast Asia (Gianessi and Williams, 2011). Planting materials for rubber plantation establishment are budded stump and polybag plastic. They are consist of 2 parts: rootstock and scion. The rootstock is unselected planted in ground nurseries and bud of scion will be grafted when seedling are about 8–12 months. In the past, the most common rootstocks for planting material production in Thailand were seeds collected from any early–introduced clones which had high heterozygosity based on cross–pollination in nature (Nakkanong, 2008) and had higher vigorous root development and there is tolerance clone of rubber available. Therefore, those were suitable for using as rootstocks in the disease area. Nowadays, RRIM 600 is the major cultivated variety of rubber tree in Thailand, constituting about 80% of plantings material grown (Sangsing *et al*., 2004). Almost all of the commercially cultivated clones of *H. brasiliensis* represent a very narrow genetic base since they originated through hybridization or selection from a few seedlings of Wickham germplasm (Priyadarshan and Goncalves, 2003). Hence, the commercial rubber cultivation, due to its genetic vulnerability, is under a constant threat of attack by native as well as exotic diseases and insects (Narayanan and Mydin, 2011). Wherever the rubber is grown, it is threatened by white root disease, particularly, in southern Thailand (Prasetyo *et al*., 2009). From many preliminary studies, it was found that RRIM 600 is sensitive to the white root disease and there is no resistant clone of rubber available (Holiday, 1980). The white root disease causes economic loss not only for the loss of production, it also persists on dead or living root debris for a long time (Kaewchai *et al*., 2010). It forms many white, flattened mycelial strands which grow and extends rapidly through the soil in the absence of any woody substrate (Nandris *et al*., 1987; Kaewchai and Soytong, 2010). Khonglao (2006) reported that seedling of early–introduced clones had higher vigorous root development of than RRIM 600 and no more prior genetic background information of rootstock seedlings has been reported to be tolerated against to the white root disease

(Wattanasilakorn *et al.*, 2012). Furthermore, the ability of seedlings to be the elite rubber clone for rootstock must be investigated.

CHAPTER 2

REVIEW OF LITERATURE

1. Importance of rubber tree (*Hevea brasiliensis*)

Rubber was cultivated in Asia for more than 100 years. Nowadays, Thailand is the leading producer of rubber followed by Malaysia, Indonesia, India, Sri Lanka and China, respectively (Yasen and Koedsin, 2015). Southeast Asia supplies more than 95 % of the world's natural rubber. The economic product of the tree is latex, which is extracted by slicing off a thin layer of bark on the trunk. Cutting exposes the fresh ends of latex vessels and is known as the "tapping cut" (Chee, 1990). The rubber tree is the source of natural rubber, wood products, and rubber products such as rubber smoked sheet, block rubber, concentrated latex, tyres, rubber gloves, medical products, etc. In 2012, 11.3 million tons of natural rubbers were produced worldwide to meet a demand of 11.0 million tons (International Rubber Study Group, 2013). Thailand produces approximately 4.4 million tons of nature rubber per year. In 2015, Office of Agricultural Economics reported that there are more than 7.4 million acres of rubber plantations in Thailand.

2. Taxonomy

Rubber tree is belong to the family Euphorbiaceae. Scientific classification of *H. brasiliensis* are as follow:

Kingdom: Plantae

Subkingdom: Tracheobionta

Division: Magnoliophyta

Class: Magnoliopsida

Subclass: Rosidae

Order: Euphorbiales

Family: Euphorbiaceae

Genus: *Hevea*

Species: *brasiliensis*

Rubber tree is a native species of the Amazon rainforests, south America. The genus *Hevea* exhibits much morphological variability, with nine species now being recognized,

ranging from large forest trees to little more than shrubs. All of them contain latex in their parts. Other *Hevea* spp. is tapped in a wild state, but is of little economic value. Some of them may, however, be important for breeding such as

– *H. benthamiana*: occurs only north of the Amazon river in the north-western part of Amazon and upper Orinoco basins; it grows in low alluvial areas and bogs and, thus, supports hydromorphic soils; it has a pure white latex which is lower in yield than *H. brasiliensis*.

– *H. camporum*: the native of open savannas in the headwaters of the Madeira river, Brazil.

– *H. guianensis* and its variety latea: 30 m high or more; it prefers well-drained upland soils; its yellowish latex yields generally inferior rubber.

– *H. microphylla*: endemic in uppermost Rio Negro basin in Brazil, Colombia and Venezuela; up to 20 m high; grows in low–lying, often permanently flooded land; its white watery latex almost completely lacks rubber resin content.

– *H. nitida*: occurs throughout most of the Amazon valley and the upper Orinoco; the tree is medium–sized and usually grows on sandy forest soils; the thin white latex acts as an anticoagulant with that of other species.

– *H. pauciflora*: occurs in Rio Negro and the upper Orinoco basins and in Guyana; the medium–sized tree grows on rocky hillsides and high well–drained river banks; its white latex has a low rubber and high resin content.

– *H. rigidifolia*: endemic to the uppermost Rio Negro basin of Brazil, Colombia, and Venezuela; the 20–meter high tree grows on high, well–drained soils; its cream–colored latex is poor in rubber and high in resin content.

– *H. spruceana*: abundant in lower Amazon basis; it grows on low and flooded river banks; its watery latex is almost devoid of rubber. (Verheye, 2010).

The rubber tree is a quick–growing tree, rarely exceeding 25 m in height in plantations, where the plant density is optimal for light interception; wild trees might be up to 40 m high in search for sunlight above the dense tree canopy. The tree has a well–developed taproot, 2–5 m long after 3 years, with of laterals roots ones. The lateral roots emerge from the taproot below the collar. They can reach up to 10 m and can make a dense network of feeder roots and root hairs in the upper soil layers. Some 30–60 % of feeder roots are found in the top 10 cm of the soil.

3. Importance of rubber rootstock

During the early years of the rubber crops, propagation was made through seeds only. After 1917, vegetative propagation by budding became very common. At the present, seeds are utilized mainly for the production of rootstocks (Cardinal *et al*., 2007). Rubber clones are screened for resistance to certain diseases before being recommended for large scale planting. In addition to this genetic resistance, fungicides have been found to be highly effective in controlling key diseases of the rubber tree. In the past, the most common rootstocks for planting material production in Thailand were seeds collected from any early–introduced clones. Nowadays, almost all of the early–introduced clones have been gradually lost because of replanting of RRIM 600 elite clones. Approximately 80 % of rubber tree grown in Thailand. It had been reported that RRIM 600 is very sensitive to fungal diseases such as phytophthora, leaf fall and root disease (Crop Protection Research Institute, 2011). Therefore, the ability of seedlings to be the elite rubber clone for rootstock must be investigated.

4. Study of root growth

The root is an important organ for plant growth and development. However, the study of roots in soils is extremely difficult due to the complexity of root structure and distribution, and the scarcity of accurate methods to measure their growth and activity (Dong *et al*., 2003). Traditional methods for root developmental studies are as followings: soil core sampling, in growth core measurements, monolith construction, excavation and minirhizotron (Caldwell and Virginia, 1989). Important information of root systems by methods mentioned above usually involves the harvest of roots from soils, destructive, time–consuming and labor intensive. They cannot be used for time course studies. In addition, root loss is unavoidable, especially for fine roots during the root harvesting and soil excavation/washing processes (Doussan *et al*., 2006). A transparent wall technique for observation of plant root growth dynamics in rhizobox is another option to investigate root development. Rhizobox observations are nondestructive, allowing repeated observations of roots to measure root elongation, branching, and turn–over, as well as root distribution through the soil profile. This technique usually involves installing a transparent tube in the soil and using a miniature digital camera to record root images. However, translating qualitative information from the recorded images to quantitative data is a tedious, time–consuming processes.

A scanner–based technique was developed that allowed the direct capture still digital root images in conjunction with a simple and quick method for root measurements using a computer image analysis system. Measurements were made from rubber trees grew in rhizobox. The advantages of this technique over digital rhizotron include its ability to estimate rootstock effects on the distribution of root length density of rubber tree and roots for physiological measurements.

5. White root disease

The white root disease is caused by *Rigidoporus microporus* (Sw.) Overeem syn *R. microporus* (Klotzsch) Imazeki. White root disease was first by reported on rubber in 1904 from Botanical Gardens, Singapore. *R. microporus* belongs to the order–Basidiomycete in the family Polyporaceae. This fungus causes white root disease in many economically important crops including *H. brasiliensis* (rubber tree) (Liyanage *et al*., 1997; Semangun, 2000; Suwandi, 2007; Jayasuriya and Thennakoon, 2007), *Cocos nucifera* (Coconut), *Anana comosus* (Pineapple), *Delonix regia* (Flamboyant tree), *Tectona grandis* (Teak), *Triplochiton scleroxylon* (Obeche), *Greenwayodendron suaveolens* (Nandris *et al*., 1987; Begho and Ekpo, 1987; Oghenekaro *et al*., 2014), *Camellia sinensis* (Tea), *Artocarpus heterophyllus* (Jackfruit), Artocarpus altilis (Breadfruit), *Mangifera indica* (Mango), *Anacardium occidentale* (Cashew nuts), *Averrhoa carambola* (Carambola), *Persea americana* (Avacado), Manihot esculenta (Cassava), *Cinnamomum verum* (Cinnamon), *Theobroma cacao* (Cocoa), *Salix babylonica* (Weeping willows), *Ficus religiosa* (Bo trees), *Mesua ferrea* (Na tree) (Fernandez–Fueyo *et al*., 2012), *Acacia nilotica* (Gum arabic tree), *Elaeis guineensis* (African oil palm), *Coffea* sp.(Coffee), *Ipomoea batatas* (Sweet potato or Yams), *Nephelium lappaceum* (Rambutan), *Solanum melongena* (Eggplant), and *Piper nigrum* (Black pepper) (Suwandi, 2003). This disease caused significant mortality to rubber tree irrespective of age or health status, causing economic losses to the latex industry in many countries such as Cameroon, Ivory Coast, Ghana, Nigeria, Gabon, India, Indonesia, Malaysia, Sri Lanka, Thailand, West and Central Africa (Nandris *et al*., 1987; Liyanage *et al*., 1997; Semangun, 2000; Guyot and Flari, 2002; Hashim and Malik, 2006; Jayasuriya and Thennakoon, 2007; Kaewchai *et al*., 2010) as shown in Figure 1. In India, Prasetyo and Aeny (2013)

reported that the white root rot causes a loss of rubber production about 3–15 % and financial loss approximately 2.1 billion rupiahs.

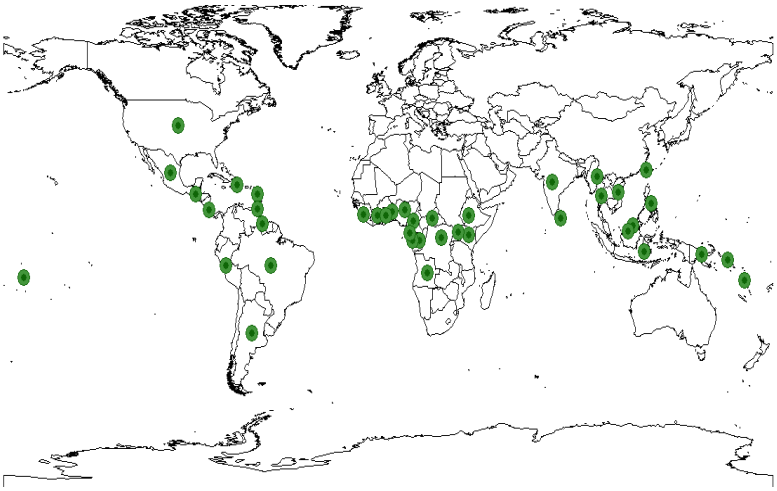

Figure 1 Distribution of white root disease in the world

Source: Plantwise Knowledge Bank (n.d.)

The causative agent (*R. microporus*) persists on dead or living root debris for a long time while causing new infections on healthy plants. The fungus attacks roots and collar region of taproot causing white root rot. It forms many white, flattened mycelial strands which grow and extend rapidly through the soil in the absence of any woody substrate (Nandris *et al*., 1987; Kaewchai and Soytong, 2010). The root of healthy rubber tree can be infected by contact with disease sources, such as rhizomorphs, infected root, dead stump, or wood debris (Nandris *et al*., 1987; Guyot and Flori, 2002; Kaewchai *et al*., 2010). It results in the substantial death of trees and sometimes losses of a whole stand (Guyot and Flori, 2002). The fruiting bodies of this fungus form at the collar of the dead stem which produces a large number of basidiospores and eventually kills trees at any growth stage, but it has a limited role in the dissemination of this disease (Nandris *et al*., 1987).

6. Characteristics of white root disease

The fungus *R. microporus* forms many white, somewhat flattened mycelial strands 1–2 mm thick that grow on and adhere strongly to the surface of the root bark. These rhizomorphs grow rapidly and may extend several meters through the soil in the absence of any woody substrate.

(Kaewchai *et al*., 2010). Thus, healthy rubber trees can be infected by free rhizomorphs growing from stumps or infected woody debris buried in the ground as well as by roots contacting those of a diseased neighboring tree (Jayasuriya and Thennakoon, 2007). Internal progression of the fungus in root tissues lags well behind the front of epiphytic growth of the mycelium of the root. In this respect, the mode of development of *R. microporus* is characteristic of an ectotrophic growth habit. After rhizomorphs infect the roots (Figure 2A), the fungus preferentially penetrates the taproot, deep in the soil. First, however, the rhizomorphs must change morphogenetically into infectious hyphae, characterized by degrading extracellular enzymes able to decay the wood (Boisson, 1972). This mechanism is strictly regulated by conditions of partial anoxia in the soil, at a depth determined by whether the texture is sandy or clayey soil.

Wood colonization inside the taproot spreads up to the collar and to other portions of the root system. A controlled and effective method for artificial infecting young rubber trees has been developed by reproducing the conditions of soil anoxia in the greenhouse (Nandris *et al*., 1983) (Figure 2).

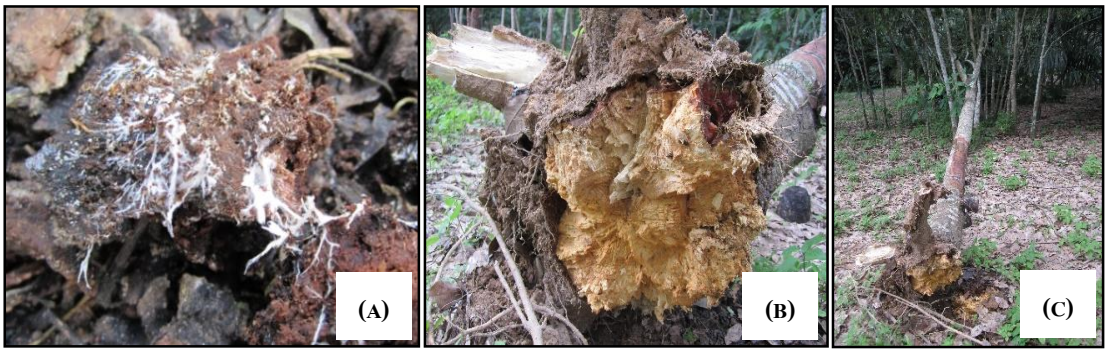

Figure 2 Characteristics of white root disease (A) rhizomorph on the root (B) a large rubber with wood decay and (C) a rubber tree killed by *R. microporus* in the rubber plantation.

7. Symptom expression and development of white root disease

The appearance of a symptom of white root disease is usually at the late stages of development when affected tree becomes untreatable and eventual death eminent.

The fungus mycelia strand or the white rhizomorph grows along and attach firmly to the secondary or tertiary roots and moves towards the main root. The fungus kills the cells ahead of the rot. The destroyed lesions on the roots become discolored, initially turning brownish and

later chalky whitish. The rhizomorphs further penetrate towards the trunk tissue affecting the whole collar of the tree trunk. The spread of the rhizomorph is usually fast.

The foliage symptoms are yellow appearing at the late stages of development of the disease. Initially, the discoloration of the foliage may affect only one branch, but it later spreads to the whole canopy. Infected trees may flower and fruit off–season. Eventually, leaves drop, branches die back and infected trees die, leaving large vacant spaces in severely attacked fields. On roots, networks of rhizomorphs are firmly attached. The growing ends of rhizomorphs form whitish fans. Mature rhizomorphs are brownish or may assume the color of the surrounding soil. Severely infected roots are soft and watery with a creamy color (Omorusi, 2012).

R. microporus causes a white rot of the wood characterized by degradation of lignin at the cell walls. The orange–yellow sporophores are formed mainly during the rainy season at the base of heavily attacked trees. The bracket form is most common, but a resupinate form also exists. These sporophores produce a large number of basidiospores, even during the dry season, but they seem to have a limited role in disseminating the disease. This has been one of the most controversial points in the biology of *R. microporus* since the beginning of this century. The spores are viable, but there is the agreement now that the probability of a spore germinating *in situ* on a receptive substrate is extremely low (John, 1965). In *Hevea* plantations established immediately after a forest is cleared, mycelial filaments of *R. microporus* cause infection the second plantings. However, spores can constitute inoculum for infecting the stump surfaces of old rubber trees remaining between the planting rows. The white root rot disease cycle is illustrated in Figure 3.

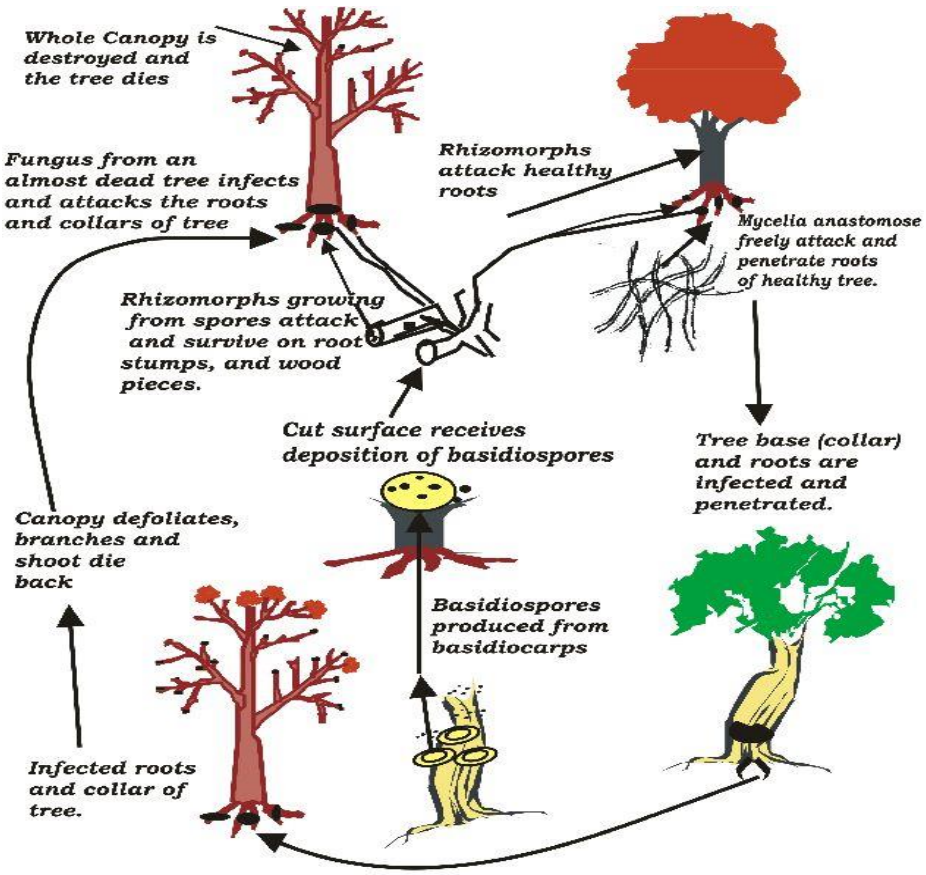

Figure 3 Diagrammatic illustration of white root disease cycle.

Source: Omorusi (2012).

8. Prevention and control of white root disease

All root diseases spread by root contact with a source of inoculum such as infected woody debris in the soil. The pathogen spreads both internally and externally along the roots, which decay in the process, and eventually reaches the collar and tap root, whereupon the tree soon dies. A recent approach to the white root disease control is to drench the soil around the tree with a fungicide. Research has demonstrated that applying the most effective fungicides as protectants for white root disease of rubber tree resulted in no infection compared with 95 % infection in the untreated plots (Tan and Hashim, 1992; Crop Protection Research Institute, 2011).

Control of white root disease is highly necessary to prevent it from spreading to neighboring trees. Disease control should commence as early as possible, normally about 1 year after planting, and be regularly continued thereafter. White root disease incidence should be eradicated, or be at its very minimum, by the time that the rubber trees are opened for tapping at 5–

6 years old. Nowadays, the white root disease can be controlled by using an integration of cultural methods and chemical fungicides.

For cultural methods, a major component of control, when a rubber area is to be replanted, the methods used to clear old trees from the land determine the residual level of inoculum. Full mechanical clearing is uprooting the trees, ploughing and raking the land to collect and dispose of the rubber roots. This procedure offers the least incidence of root disease in a replanting (Newsam, 1967) but expensive and cannot be adopted by smallholders (Plantwise Knowledge Bank, n. d.).

In most chemical fungicides instances, collar protectants containing fungicides and sulphur amendments (Peries, 1969), systemic active ingredients such as propiconazole, hexaconazole (Lam and Chiu, 1993), and other triazoles (Lim *et al*., 1990; Gohet *et al*., 1991), triadimenol, PCNB, triadimefon (Jollands, 1983; Ng and Yap, 1990), pentachlorophenol (PCP) (Jayasinghe *et al*., 1995) and phenol (Jayaratne *et al*., 1997) but chemical fungicides have been known to have a negative effect on human health, cause environmental pollution and leave residues in the agricultural soil (Soytong *et al*., 2005; Haggag and Mohamed, 2007). Moreover, several plant pathogenic fungi have developed resistance to chemical fungicides (Benítez *et al*., 2004; Kim and Hwang, 2007). To avoid the negative or harmful effect of chemical use, biological control would be an alternative method to save and sound measure for controlling disease by reducing the inoculum sources, as well as inhibiting the disease spread. Numerous kinds of fungal species have been found in soil and reported to be biological control agent against plant diseases (Kaewchai and Soytong, 2010).

Biological control may result from direct or indirect interactions between biological control agent and pathogen (Viterbo *et al*., 2007) such as physical contact and synthesis of hydrolytic enzyme, toxic compound or antibiotic, competition, and induce resistance in plant host (Benítez *et al*., 2004). Many antagonistic fungi produce the toxic compound or antibiotic. Some antibiotics have been shown to play the role in impeding spore germination or killing the cells (Handelsman and Stabb, 1996; Benítez *et al*., 2004; Haggag and Mohamed, 2007; Kaewchai and Soytong, 2010).

One way to minimize the amount of fungicide required for a single application is an integration of such application with chemical resistant antagonists, which are capable of

suppressing the weakened pathogen in soil. However, results from integrated applications of systemic fungicides along with *Trichoderma koningii* were not consistent (Hashim and Chew, 1997). In addition, weakening the effect of some chemicals such as furfuraldehyde on *R. microporus* (Jayasuriya and Deacon, 1996; Jayasuriya *et al*., 1996) has been discussed and this effect was considered as a trigger for biological control (Katan *et al*., 1992). Therefore, controlling the disease using biological preparations could be important in terms of cost, environmental concerns, and health hazards. The possibility of using antagonistic organisms to control *R. microporus* has been discussed previously (Jayasuriya, 1998). Research has been undertaken to explore the efficacy of antagonistic organisms against *R. microporus* (Jayasuriya and Deacon, 1996; Jayasuriya *et al*., 1996; Jayasuriya, 1997) and for other fungi causing diseases in agricultural crops (Schoeman *et al*., 1996; Yang *et al*., 1992) including pine in Europe (Rishbeth, 1963). In the majority of incidences *Trichoderma* sp. were highly effective against many pathogens of agricultural crops. (Jayasuriya and Thennakoon, 2007). However, if rubber tree has been severely infected by disease biological control of plant are ineffective (Prasetyo and Aeny, 2013). The pathogenicity tests of *R. microporus* was done in RRIM 600, GT 1, PR 255, PR 26 and KRS 156 (Songkhla 36) by Chantarapratin *et al*., (2004). It was found that seedlings of RRIM 600 sensitive to the white root disease, while low sensitive was found in the PR 261. Omorusi (2012) studied the incidence and severity of white root rot disease on rubber tree such as GT 1, PR 107, RRIM 600 and rubber tree from Nigeria: NIG 800, NIG 801, NIG 802 clones. The results showed that the highest susceptibility score for the white root rot disease was recorded in PR 107 (30.55 %) and the lowest score was in GT 1 (14.71 %). Kaewchai and Soytong (2010) also noted that white root disease is a severe epidemic in southern Thailand. Wattanasilakorn *et al*., (2012) reported that some early–introduced rubber clones were collected for rootstock screening of rootstock white root disease resistance and it was found that there were some clones that exhibited tentative tolerance to white root disease.

Therefore, selection of rubber tree rootstocks for the white root disease tolerance will benefit to the farmers resulting in sustainable rubber production in Thailand.

9. Identification and application of molecular markers

Molecular markers have served as useful aids in understanding the genetics of *H. brasiliensis*. It can play an important role in assisting rubber clonal identification and relationships among clones. For the last two decades, a large number of molecular markers and techniques have been applied in *Hevea* breeding. Nowadays, many types of molecular marker techniques are available, and most widely used include RAPD (random amplified polymorphic DNA), RFLP (restriction fragment length polymorphism), AFLP (amplified fragment length polymorphism), minisatellites and microsatellites or SSR (simple sequence repeats) (Kashi and King, 2006).

9.1 RAPD technique

RAPD technique is a PCR–based method with employs single primers of an arbitrary nucleotide sequence with 10 nucleotides to amplify anonymous PCR fragments from genomic template DNA (Williams *et al*., 1990). RAPD has been used a decade ago as an alternative in genetic analysis such as genetic relationship among species, plant identification. The key advantage of RAPDs is the ability to generate DNA fragments without prior knowledge of the target sequence (Hadrys *et al*., 1992), making this marker system universally applicable in the study of any organism. Additional advantages include ease of use, low start-up costs, low DNA quantity needed, generation of a large number of DNA fragments, non–radioactive visualization, simple interpretation of data, and a simple primer development protocol with few design constraints (Williams *et al*., 1990; Gupta *et al*., 1999; Godwin *et al*., 2001; Ribaut *et al*., 2002; Semagn *et al*., 2006; Zhang *et al*., 2006). RAPDs have since been found to have disadvantages. The greatest drawback is the low stringency PCR conditions (low annealing temperature and to a certain extent the short–length primer), which can increase the likelihood of nonspecific binding and primer mismatch (Williams *et al*., 1990; Tyler *et al*., 1997; Perez *et al*., 1998; Zhang *et al*., 2006; Honig, 2011). RAPD markers have been developed in *Hevea* for varietal identification and genetic analysis (Varghese *et al*., 1997). Research related to the rubber tree using RAPD marker for genetic studies such as selection of rubber clones for rootstock and genetically analysis using DNA markers (Cherngchalard, 2012). Genetic analysis and stock–scion compatibility between RRIM 600 rubber clone by DNA marker and isozyme (Kaewjullakan, 2013). For early–introduced rubber clone,

Nakkanong *et al*., (2008) studied in the genetic analysis 87 rubber clones using 8 primers (OPB–17, OPN–16, OPR–02, OPR–11, OPZ–04, OPAD–01, OPAD–10 and OPAD–12). Seventy amplification fragments were obtained from the 8 primers with an average of 8.75 fragments for each primer. From all fragments were polymorphic fragments (78.57 %). One RAPD primer (OPAD–01) yielded a 700–bp fragment that was found specific to Tjir 1. The results from phenogram showed that the 87 rubber clones could be clustered into 6 groups with similarity coefficients ranging from 0.541–1.000. Cultivated clones revealed more narrow genetic diversity compared to the early introduced clones. The clustering was not correlated with the geographical location of the collected samples. So, the assessment of genetic information of selected rubber clones for rootstock is important.

OBJECTIVES

The main objectives of this research are:

1. To assess genetic information of selected rubber clones for rootstock using the RAPD technique.

2. To assess the effect of white root disease after inoculation on the shoot, root growth and physiological responses of the rubber clones.

3. To assess the survival and diagnosis of plant diseases of selected rubber clones for using as rootstocks.

SCOPE OF THE RESEARCH

The investigation focused on screening early–introduced rubber clones tolerance to disease by identification of rubber clones using RAPD marker, pathogen, and pathogenicity test, and effect of white root disease after inoculation on shoot, root growth, physiological responses, and diagnosis of plant disease and selected early introduced rubber clones in southern Thailand of good rootstocks that exhibit white root disease tolerance.

CHAPTER 3

MATERIALS AND METHODS

1. Materials and equipments

1.1 Plant materials

– Seedling from early–introduced rubber clones of rubber tree and recommended clone, RRIM 600 as a control.

1.2 Equipments

1.2.1 Equipments for sample collection

– Plastic bag

– Scissors

– Foam box

– Pen for glass writing

1.2.2 Equipments for shoot and root growth studies of rubber tree

– Plastic for growing rubber seedlings

– Rhizoboxes (size 30.48×119.38 cm)

– Drip irrigation system

– Nylon mesh

– Black plastic sheets

– Measure tape

– Clear plastic sheets

– Permanent marker

– Pruning scissors

– Two decimal weighing

– Oven

– Computer scanner (Epson Perfection V330 Photo, Seiko Epson Corp., Japan.)

– Portable photosynthesis system model Li–6400 (LCi Ultra Complex Photosynthesis System, USA)

1.2.3 Equipments for DNA extraction, electrophoresis and PCR technique

– Refrigerator and freezer

– Microcentrifuge

– Four decimal weighing

– pH meter

– Hotplate and magnetic stirrer

– Magnetic stir bar

– Micropipette

– Vortex mixer

– Autoclave

– Electrophoresis equipment

– PCR machine

– Mortar and pestle

– Micro centrifuge tube

– Microwave oven

– Fume hood

– Gel documentation system

1.2.4 Equipments for collection and isolation of fungi

– Petri dish

– Wood pieces (size 1×2 inch)

– Flask

– Pot

– Colander

– Alcohol burner

– Hot air oven

– Autoclave

– Laminar air flow cabinet

1.2.5 Equipments for histological study

1.2.5.1 Casting wax block

– Hot plate

– Forceps

– Plastic or metal molds

– Embedding rings

– A small beaker with molten paraplast plus

– A tray with a thin layer of cold water

– An ice bucket

– Flat ice packet or brass plate

1.2.5.2 Sectioning

– Rotary microtome (we are using American Optical AO Spencer No. 820)

– Disposable microtome blades

– A small brush

– A forceps with sharp ends

– A single–edged razor blade

– Dark cardboard plate (for putting the sections on and observing them)

– A stereomicroscope (optional but recommended)

– Hot plate set on 40 ºC

– A small jar with distilled water

– Cleaned objective microscope slides, frosted on one edge

– A pencil

– Slide warmer

– Binocular and trinocular light microscopes

– Blade

– Microtome knife

– Microtome knife sharpener

– Mold or embedding molds

– Paraffin

– Fume hood

– Floating bath or tissue floatation water bath

– pH meter

– Four decimal weighing

– Alcohol burner

– Stirrer

– Refrigerator

– Hot air oven

– Slide glass

– Cover glass/ cover slip or microscope slide coverslips

– Slide saver box

– Cylinder

– Flask

– Reagent bottle

– Pipette

– Coplin Jar

– Vial

– Dropper

– Teasing needle

– Glass reagent bottle

– Glass sample bottles

– Beaker

– Tissue embedding station

– Waxed sheet

1.2.6 Equipments for Transmission electron microscopy (TEM)

– Bakelite mounting materials

– Sandpapers

– Electropolished and etching apparatus

– Stainless sheet

1.3 Chemicals

1.3.1 Chemicals for DNA extraction

– CTAB (Hexadecyl trimethyl–ammonium bromide)

– β–mercaptoethanol

– PVP–40 (Polyvinyl pyrrolidone)

– NaCl (Sodium chloride)

– Na_2EDTA (Disodium ethylenediaminetetraacetate)

– Tris HCl pH 8.0

– Chloroform

– Isopropanol

– TE buffer

– Ethanol

1.3.2 Chemicals for electrophoresis

– Agarose

– LE agarose (FMC Bioproduct, USA)

– Boric acid

– Tris–base

– Ethidium bromide

– Lamda DNA (λ DNA)

– 100 bp and 500 bp DNA Ladder (Operon, U.S.A.)

1.3.3 Chemicals for PCR

– dNTP (dATP, dTTP, dCTP and dGTP) (Promega, USA)

– RAPD Primer

– $MgCl_2$

– *Taq* DNA Polymerase B (Promaga, USA)

– 10X *Taq* buffer

1.3.4 Chemicals for collection and isolation of fungi

– Streptomycin

– Agar

– Glucose powder

– Lactophenol cotton blue

– Ethanol 95 %, 70 %

– Clorox

1.3.5 Chemicals for histological study

– Wax or paraffin

– Liquid paraffin

– Formaldehyde

– Alcohol

  – Absolute ethanol

  – Ethanol 95 %, 70 %, 50 %

– Acetic acid

– Aluminium hydroxide

– Glycerin

– Distilled water

– Mounting media

– Xylene

– FAA II

– Absolute ethanol: xylene (ratio 1:1)

– Permount

– Clove oil

– Used clove oil fast green

– Fast green

– Safranin

2. Methods

2.1 Collection and preparing rubber rootstock seedlings

This experiment was carried out from December 2012 to March 2014, in the glasshouse of Faculty of Natural Resources, Prince of Songkla University, Songkhla Province, Thailand. Early–introduced clones of rubber tree were collected from different sites in Songkhla, Trang, and Nakhon Si Thammarat provinces (Table 1). Those rubber clones were identified verified by their big trunk indicating an age of more than 50 years and their random location outside established rubber plantation areas. All rubber seeds were grown in a plastic basket (size 32.5×40×9.5 cm) and contained mixed soil: sand: coconut dust at the ratio of 1: 1: 1. Watering daily until seed germination.

Table 1 Locations of sampling clones and cultivated clones in Songkhla, Trang and Nakhon Si Thammarat provinces, Thailand.

Clone	Geographic coordinate	Places of collection
EIRpsu 1	7° 0' 31.7" N 100° 29' 40.3"	Faculty of Natural Resources, Prince of Songkla University, Kho Hong, Hat Yai, Songkhla. (PSU)
EIRpsu 2	7° 0' 23.1" N 100° 29' 52.8" E	Faculty of Environmental Management, Prince of Songkla University, Kho Hong, Hat Yai, Songkhla. (PSU)
EIRpsu 3	7° 0' 31.7" N 7° 0' 31.7" N	Drugstore, Prince of Songkla University, Kho Hong, Hat Yai, Songkhla. (PSU)
EIRpsu 4	7° 0' 29.8" N 100° 29' 58.6" E	Faculty of Science, Prince of Songkla University, Kho Hong, Hat Yai, Songkhla. (PSU)
EIRpsu 5	7° 0' 29.6" N 100° 30' 2.2" E	Faculty of Engineering, Prince of Songkla University, Kho Hong, Hat Yai, Songkhla. (PSU)
EIRpark	7° 0' 6" N 100° 27' 24" E	Hat Yai central park, Kho Hong, Hat Yai, Songkhla.
EIRrak	7° 32' 33.1' N 99° 34' 35.6" E	Bang Rak, Rubber plantation, Muang Trang, Trang

Table 1 (Cont.) Locations of sampling clones and cultivated clones in Songkhla, Trang and Nakhon Si Thammarat provinces, Thailand.

Clone	Geographic coordinate	Places of collection
RRIM 600	8°15'42" N 99° 35'42" E	Thung Song, Rubber plantation, Nabon, Nakhon Si Thammarat.

2.2 Genetic identification of rubber rootstock seedlings

Young fully expanded leaves of rubber seedlings from the 8 rubber clones were collected from different locations of Songkhla, Trang and Nakhon Si Thammarat province, Thailand and brought to the laboratory.

2.2.1 DNA extraction and random amplified polymorphic DNA protocol

Young fully expanded leaves of rubber seedlings were collected for DNA extraction according to the procedure modified from Doyle and Doyle (1990). Young leaves were ground to a fine powder in liquid nitrogen and DNA was isolated using CTAB extraction buffer [2 % hexadecyltrimethyl–ammonium bromide (CTAB), 20 mM EDTA, 100 mM Tris–HCl pH 8.0, 1.4 M NaCl]. The quantity and quality of the isolated DNA were determined before storage at 4 °C for further use in polymerase chain reaction (PCR) analysis.

Random amplification reactions analysis was performed according to the methodology of Williams *et al*., (1990) and Nakkanong *et al*., (2008). Seven RAPD primers (OPAD–01, OPAD–10, OPAD–12, OPR–02, OPR–11, OPZ–04 and OPB–17) were used for RAPD–PCR reactions. The reaction was performed in the total volume of 25 µl contained 25 mM $MgCl_2$, 10x *Taq* buffer, 100 µM of each dNTP, 0.3 mM of primer, 1.5 units of *Taq* polymerase and 60 ng of template DNA. PCR amplification was placed in a thermal cycle started at 94 °C for 2 min and subjected to 41 repeats of the following cycle: 94 °C for 30 sec 37 °C for 1 min 72 °C for 2 min and finally 72 °C for 5 min.

All amplification products were analyzed by electrophoresis in 1.5 % (w/v) agarose gels in 0.5X TBE buffer at 100 V. The gels were stained with ethidium bromide for 15 min and viewed under ultraviolet light with gel documentation.

The DNA fragments generated by RAPD–PCR were analyzed by determining its presence (1) or absence (0). Based on polymorphic of DNA fragments, genetic distances were estimated based on Nei and Li (1979) and a dendrogram was constructed by UPGMA (Unweighted Pair Group Method with Arithmetic average) method using the computer package NTSYSpc 2.1 (Rohlf, 1998).

2.3 Soil sampling and analysis

The soils used for the experiments were obtained from agricultural fields located in Songkhla province. Soil samples were air-dried at room temperature (Carter, 1993). The samples were then analyzed for physical and chemical properties. The soil physical properties were soil texture. Soil texture was determined by the pipette method (dispersion, sedimentation, and decantation). Soil chemical properties included pH using 1: 5 soil: water solution by a pH meter (Bantex, Digital pH meter, A 300), total nitrogen (N), available phosphorus (P), exchangeable potassium (K). The total nitrogen in soil was determined using the block digester technique as detailed by Kjeldahl (1883). Available phosphorus was determined by the Bray (1945). Determination of plant available potassium (P) was performed after extraction with ascorbic acid and exchangeable phosphorus (K) was determined by ammonium acetate extraction.

2.4 Effect of pathogen on growth of rubber rootstock seedlings.

2.4.1 Root growth analysis

Rubber seeds of each genotype as verified by the method described in experiment 2.2 were germinated in the sand for 4 weeks, a total of 48 seedlings was planted. To evaluate the growth of both root and shoot rhizobox and monitor system as shown in Figure 4 were employed. Each rhizobox was made of a storm water pipe (30.48 cm diameter) with 119.38 cm long. The panel of each rhizobox was made of clear acrylic and covered with the black plastic sheet to avoid light exposure. All seedlings were transferred to a rhizobox containing soil: manure: husk at the ratio of 3: 2: 2. At 8 weeks after inoculation, the 24 rubber seedlings of each treatment were inoculated with *R. microporus* using ten inoculum blocks (size 2.54×5.08 cm) placed in contact with the taproot at the depth of 10 cm below soil surface. To investigate root distribution, root image was scanned from the panel in each 20 cm depth intervals by a scanner (Epson Perfection

V330 Photo, Seiko Epson Corp., Japan.). The total length of the sample roots was determined by using Image Rootfly Software which is a free, open–source software application to aid researchers in rhizobox image analysis by GNU General Public License (Stanley and Christina, 2011). The length of roots, as well as the alive and death rates, were recorded every two weeks. After 24 weeks of inoculation and statistically root growth compared among different clones of rubber by Duncan's new multiple range test (DMRT) at $P < 0.05$ was employed for mean comparison. All the experimental data were stored in a single file using the Rootfly software format as shown in Figure 4.

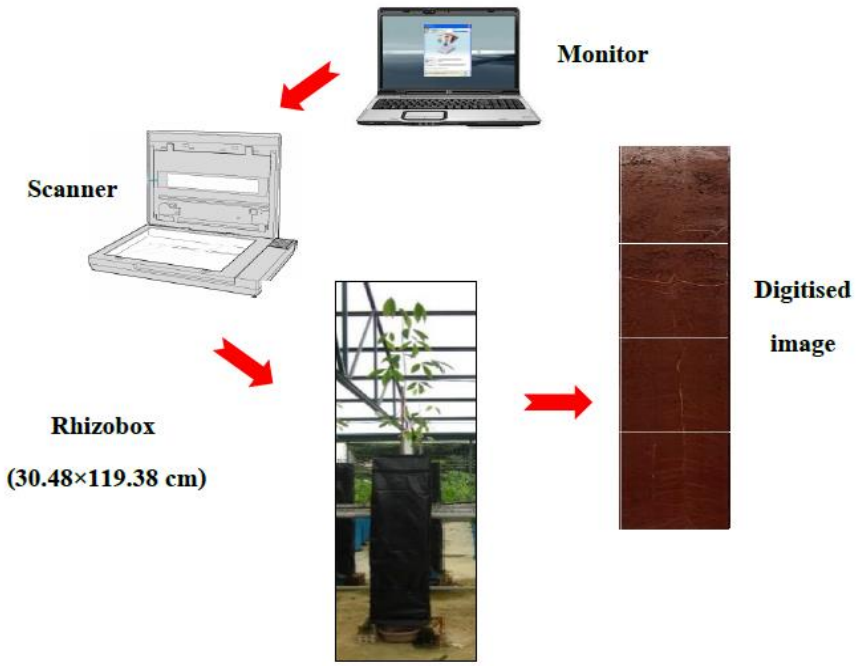

Figure 4 The rhizobox used for root investigation and image acquisition system.

2.4.2 Shoot growth analysis

After growing seedlings of each clone in rhizobox and inoculation the pathogen according to the methods described in 2.4.1 for 8 weeks plant height, trunk diameter and a number of leaves were recorded. The plant height was measured at 10 cm above soil level to the top of the plant using measured tape. The plant trunk diameter was measured at 10 cm above the soil level with a vernier caliper. The number of leaves was determined by counting the number of compound leaves per plant. All shoot growth parameters from each clone of rubber seedlings were statically

compared. A least significant difference (LSD) test at $P < 0.05$ was employed for mean comparisons.

2.5 Influence of pathogen on physiological responses of rubber rootstock seedlings

In this experiment, two important physiological characters, photosynthetic rate, and stomatal conductant were evaluated. After growing seedlings of each clone in rhizobox and inoculation the pathogen according to the methods described in 2.4.1 for 8 weeks photosynthetic rate (A) and stomatal conductance (g_s) was measured from 10.00–12.00 h using a portable photosynthesis system (LICOR–6400; LI-COR; Lincoln, NE, USA). Measurements were made on three fully expanded leaves in each treatment. Both physiological parameters from each clone of rubber seedlings were statically compared. A least significant difference (LSD) test at $P < 0.05$ was employed for mean comparisons.

Statistically analysis

All experiments were arranged in 8×2 factorial in completely randomized design (CRD) comprising 8 rubber clones (EIRpsu 1, EIRpsu 2, EIRpsu 3, EIRpsu 4, EIRpsu 5, EIRpark, EIRrak and RRIM 600) and two methods of treatments, control (no inoculation) and inoculation with the pathogen. Analysis of variance (ANOVA) of data was performed using R Gui software (version 2.12.0).

2.6 Pathogenicity test of *R. microporus* in rubber rootstock seedlings infected with white root disease

The fungus mycelium of white root disease supported by the Department of Pest Management, Faculty of Natural Resources, Prince of Songkla University, Songkhla, Thailand. The white root rot isolated were collected on in potato dextrose agar medium (PDA) for 4–5 days drill with a 5 mm cork borer in PDA, and incubated at room temperature (28±2 ºC) for 4–5 days. Pieces of wood (size 2.54×5.08 cm) were placed on culture medium for 5 days or until the fungus mycelium spread on whole wood pieces (Figure 5).

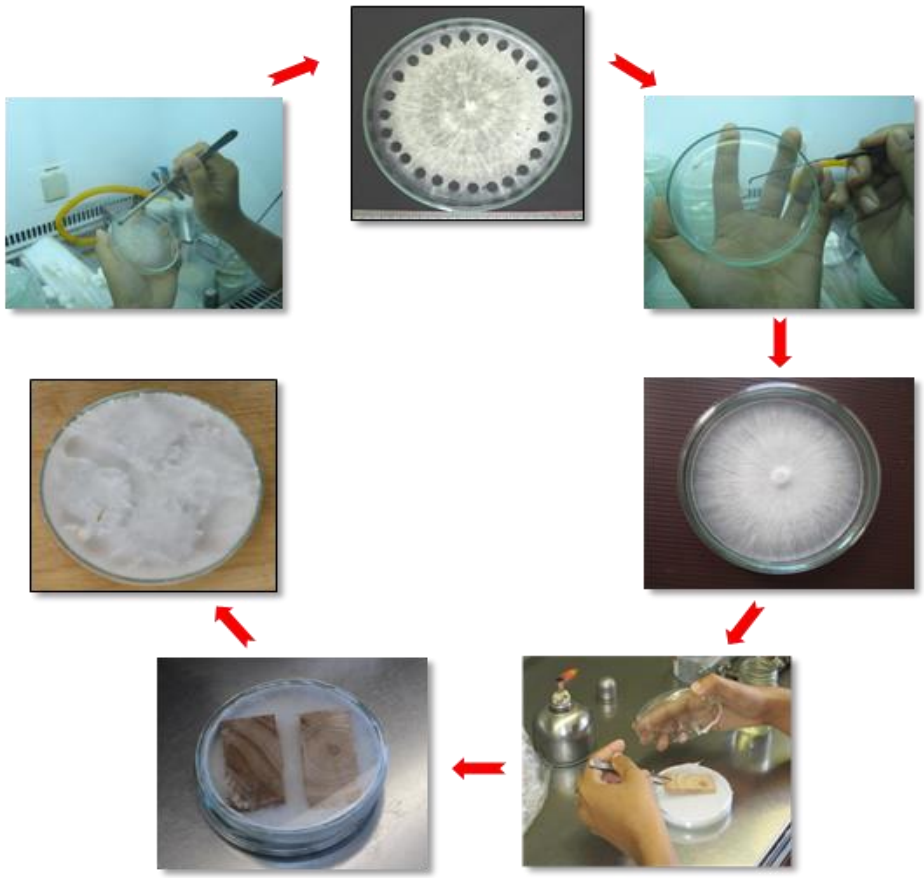

Figure 5 Preparation of culture media for fungi growth.

After growing seedlings of each clone in rhizobox according to the methods described in 2.4.1 for 8 weeks pathogenicity study was conducted. Assessment of the severity of the white root rot disease was based on pathogenicity test and histological/ultrastructure observation.

For pathogenicity test disease index method described by Soytong and Kaewchai (2014) was used. The data collection as disease index (DI) was recorded at 8 weeks after inoculation. The disease index was categorized as the below equation and the level of disease was as follows:

Level 1 = healthy with 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

The symptom of seedlings was recorded every two weeks. After 24 weeks of inoculation the severity of the white root disease parameters produced from each clone of rubber seedlings were statically compared.

$$\text{Disease index (DI)} = \frac{(0 \times a) + (1 \times b) + (2 \times c)}{a + b + c} \times \frac{100}{x}$$

where,

0, 1 and 2 are infection categories

a, b and c are plants that fall into the infection categories

x is the maximum disease category which is 3

For histological and ultrastructural observation seedling roots of each inoculated clone grown in rhizobox were collected at 24 weeks of planting.

For histological study root samples (0.5×0.5 cm) were washed with distilled water and fixed in FAA II (formaldehyde: glacial acetic acid: 70% ethyl alcohol; 5: 5: 90 v/v/v) for at least 48 h (Ruzin, 1999). Fixed samples were dehydrated through a tertiary–butyl alcohol series and infiltrated in Histoplast. Sections were cut with a rotary microtome and affixed on slides. Sections were stained with safranin and fast green and viewed under a light microscope to observe the general structures.

For ultrastructural observation samples of infected root were excised and fixed overnight in a cold (4 °C) solution of 2.5% glutaraldehyde (v/v) in 0.1 M phosphate buffer (pH 7.2). Pre–fixed samples were washed 3 times with 0.1 M phosphate buffer (pH 7.2) and post–fixed with 1% osmium tetroxide in 0.1 M phosphate buffer (pH 7.2) for 1 h at room temperature. The post–fixed samples were then washed 3 times with distilled water, dehydrated through a graded ethanol series and infiltrated with Embed 812. Ultrathin sections were cut and stained with 2% uranyl acetate for 10 min followed by lead citrate for 5 min. Sections were viewed with a JEM 2010 Transmission electron microscope (JEOL, Japan) operating at 160 kV. Samples derived from healthy rubber trees were used as the control.

CHAPTER 4

RESULTS

1. Identification of rubber rootstock using RAPD markers

Genetic analysis and relatedness of early–introduced rubber clones and RRIM 600 were studied by RAPD technique with 7 primers used (Table 2). All produced reproducible with polymorphic bands. The clear visible bands of the individuals were there used for future analysis. From the 7 primers, a total of 65 fragments were obtained, of which 54 fragments or about 83.08 % showed polymorphisms. The highest polymorphic fragment was found in primer OPAD–10 and the lowest was observed in primers OPR–11. The number of polymorphic DNA fragments per primer ranged from 5 to 14, with an average of 6 bands per primer (Table 2).

Table 2 Primers producing polymorphic DNA bands in RAPD patterns of the 8 rubber clones collected from difference places.

Primer	Sequence (5' →3')	Amplified fragments	Monomorphic fragments	Polymorphic fragments	Polymorphism (%)
OPAD–01	CAAAGGGCGG	9	2	7	77.78
OPAD–10	AAGAGGCCAG	15	1	14	93.33
OPAD–12	AAGAGGGCGT	9	1	8	88.89
OPR–02	CACAGCTGCC	7	1	6	85.71
OPR–11	GTAGCCGTCT	8	3	5	62.50
OPZ–04	AGGCTGTGCT	10	1	9	90.00
OPB–17	AGGGAACGAG	7	2	5	71.43
Total		65	11	54	

The results showed different primers generated different fragment numbers and length of DNA amplification products. DNA patterns of each seedling with 7 primers are shown in Figure 5–11.

The size of bands generated by OPAD–01, OPAD–10, OPAD–12, OPR–02, OPR–11, OPZ–04 and OPB–17 ranged from 200 bp to 1,500 bp (Appendix of Table 3). Eight DNA

fragments, 1,300 bp, 1,250 bp, 900 bp, 800 bp, 550 bp, 500 bp, 420 bp and 320 bp amplified by primer OPAD–01 (Figure 6). Fourteen DNA fragments, 1,500 bp, 1,420 bp, 1,390 bp, 1,300 bp, 1,290 bp, 1,000 bp, 810 bp, 700 bp, 600 bp, 450 bp, 400 bp, 300 bp, 260 bp and 250 bp amplified by primer OPAD–10 (Figure 7). Eight DNA fragments, 1,400 bp, 1,100 bp, 900 bp, 800 bp, 480 bp, 350 bp, 290 bp and 200 bp amplified by primer OPR–12 (Figure 8). Six DNA fragments, 1,000 bp, 890 bp, 700 bp, 400 bp, 350 bp and 300 bp amplified by primer OPR–02 (Figure 9). Five DNA fragments, 1,150 bp, 1,000 bp, 800 bp, 700 bp, 450 bp amplified by primer OPR–11 (Figure 10). Nine DNA fragments, 1,400 bp, 1,250 bp, 1,150 bp, 1,100 bp, 1,000 bp, 800 bp, 650 bp, 420 bp, 350 bp amplified by primer OPZ–04 (Figure 11). Five DNA fragments, 1,500 bp, 1,400 bp, 1,000 bp, 900 bp and 650 bp amplified by primer OPB–17 (Figure 12). Examples of the amplification of RAPD markers are shown in Appendix (Table 4).

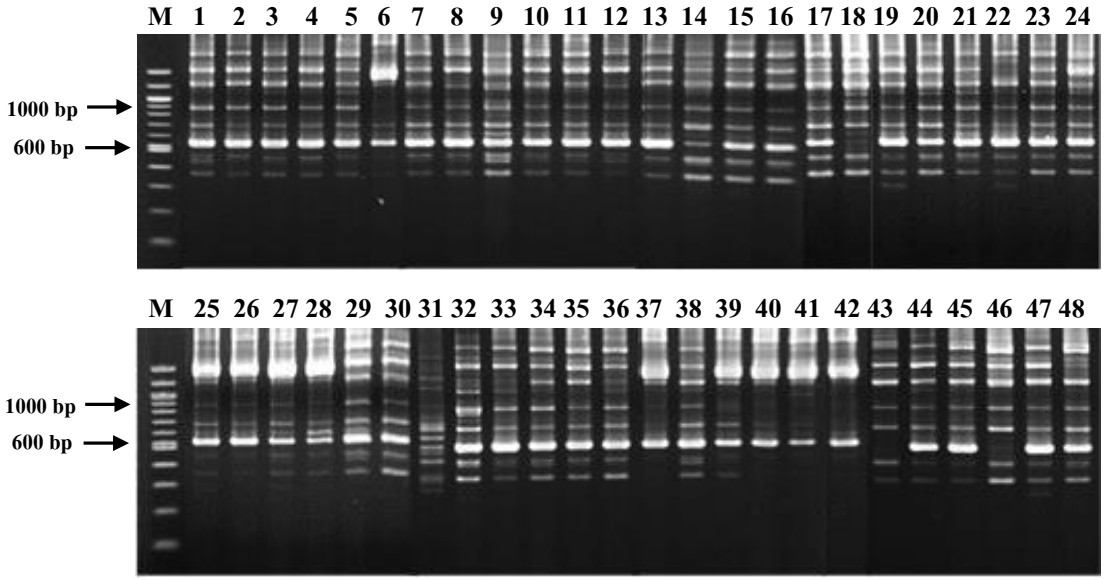

Figure 6 RAPD patterns of rootstock seedlings from EIRpsu 1 (lane 1–6), EIRpsu 2 (lane 7–12), EIRpsu 3 (lane 13–18), EIRpsu 4 (lane 19–24), EIRpsu 5 (lane 25–30), EIRpark (lane 31–36), EIRrak (lane 37–42) and consist of RRIM 600 (lane 43–48) amplified by primer OPAD–01 Lane M = 100 bp ladder.

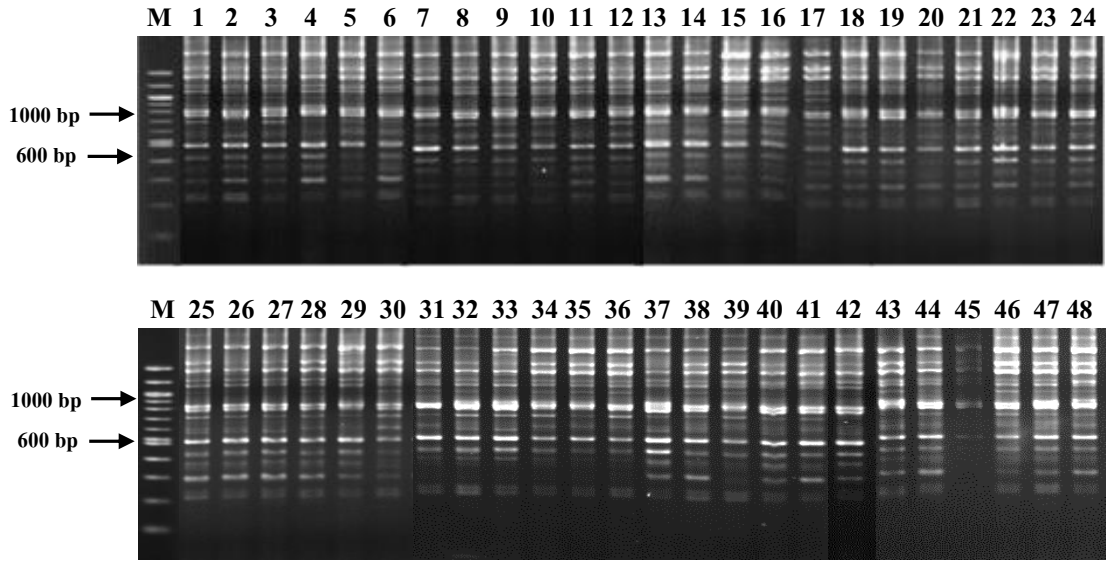

Figure 7 RAPD patterns of rootstock seedlings from EIRpsu 1 (lane 1–6), EIRpsu 2 (lane 7–12), EIRpsu 3 (lane 13–18), EIRpsu 4 (lane 19–24), EIRpsu 5 (lane 25–30), EIRpark (lane 31–36), EIRrak (lane 37–42) and consist of RRIM 600 (lane 43–48) amplified by primer OPAD–10 Lane M = 100 bp ladder.

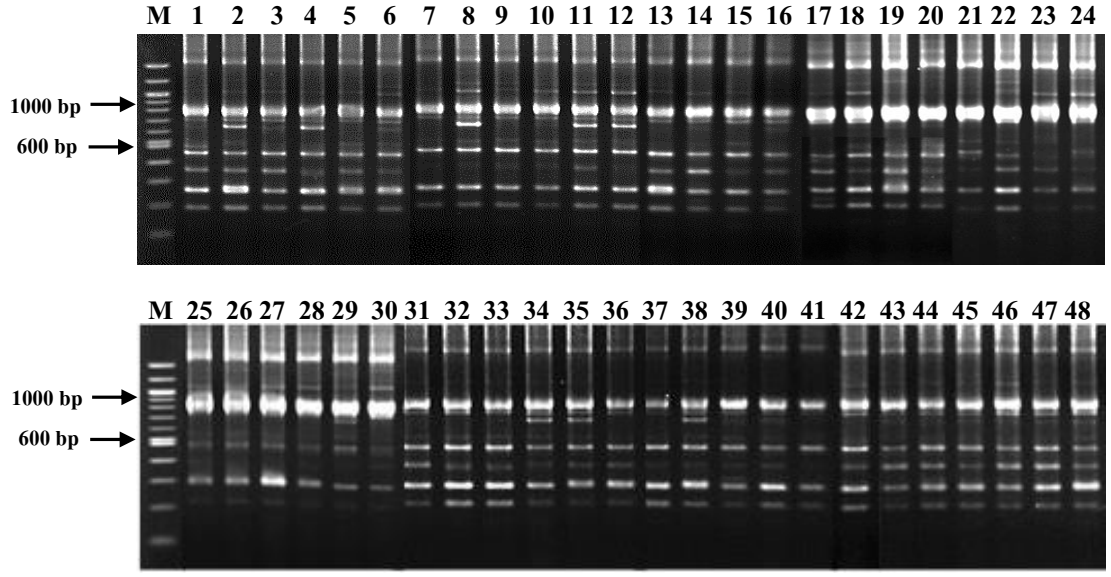

Figure 8 RAPD patterns of rootstock seedlings from EIRpsu 1 (lane 1–6), EIRpsu 2 (lane 7–12), EIRpsu 3 (lane 13–18), EIRpsu 4 (lane 19–24), EIRpsu 5 (lane 25–30), EIRpark (lane 31–36), EIRrak (lane 37–42) and consist of RRIM 600 (lane 43–48) amplified by primer OPAD–12 Lane M = 100 bp ladder.

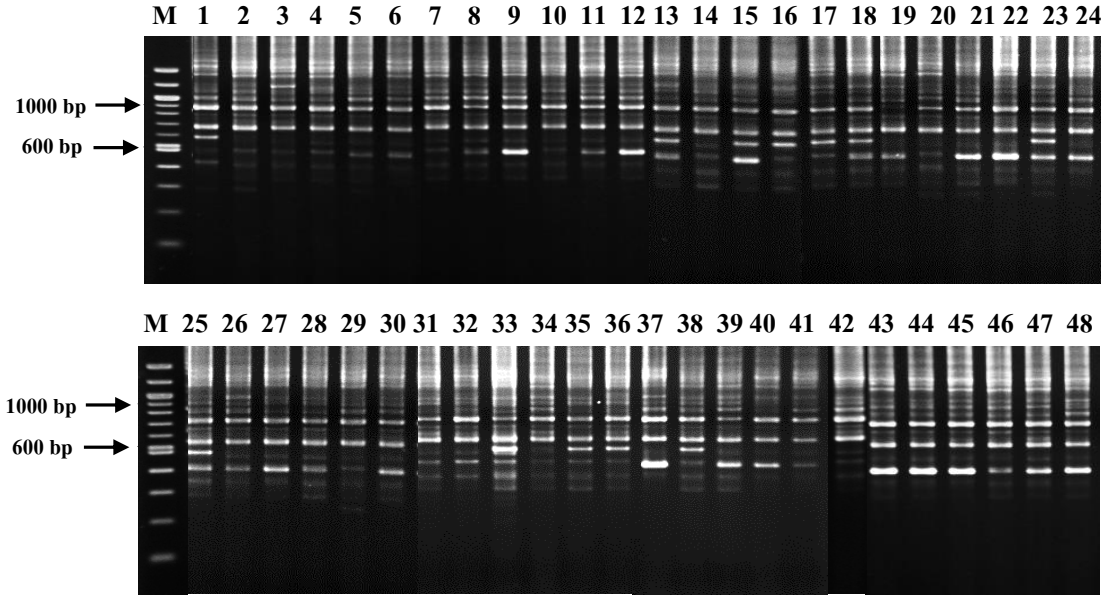

Figure 9 RAPD patterns of rootstock seedlings from EIRpsu 1 (lane 1–6), EIRpsu 2 (lane 7–12), EIRpsu 3 (lane 13–18), EIRpsu 4 (lane 19–24), EIRpsu 5 (lane 25–30), EIRpark (lane 31–36), EIRrak (lane 37–42) and consist of RRIM 600 (lane 43–48) amplified by primer OPR–02 Lane M = 100 bp ladder.

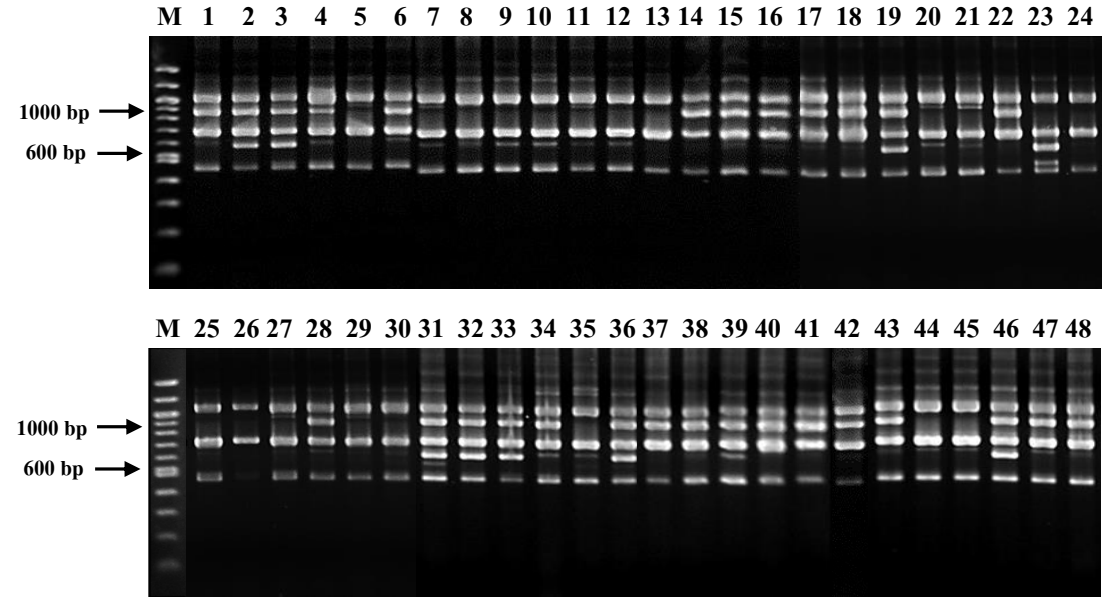

Figure 10 RAPD patterns of rootstock seedlings from EIRpsu 1 (lane 1–6), EIRpsu 2 (lane 7–12), EIRpsu 3 (lane 13–18), EIRpsu 4 (lane 19–24), EIRpsu 5 (lane 25–30), EIRpark (lane 31–36), EIRrak (lane 37–42) and consist of RRIM 600 (lane 43–48) amplified by primer OPR–11 Lane M = 100 bp ladder.

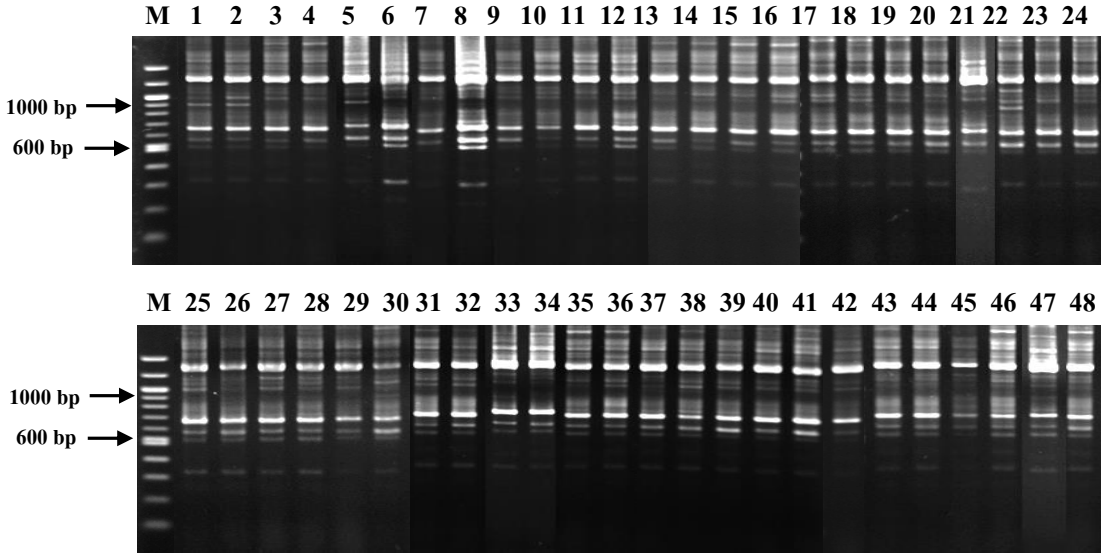

Figure 11 RAPD patterns of rootstock seedlings from EIRpsu 1 (lane 1–6), EIRpsu 2 (lane 7–12), EIRpsu 3 (lane 13–18), EIRpsu 4 (lane 19–24), EIRpsu 5 (lane 25–30), EIRpark (lane 31–36), EIRrak (lane 37–42) and consist of RRIM 600 (lane 43–48) amplified by primer OPZ–04 Lane M = 100 bp ladder.

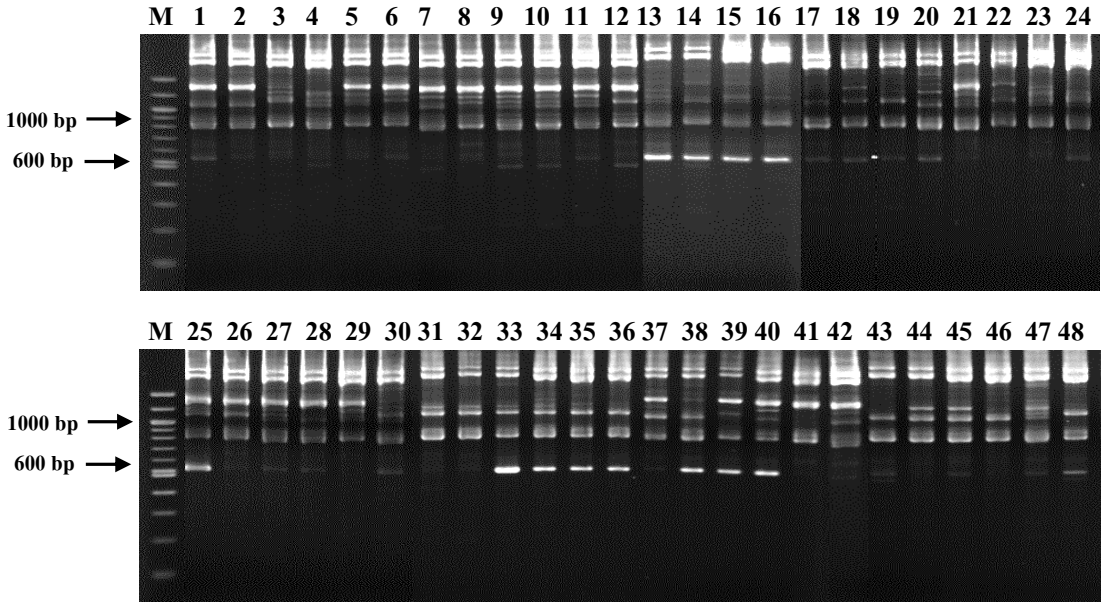

Figure 12 RAPD patterns of rootstock seedlings from EIRpsu 1(lane 1–6),EIRpsu 2 (lane 7–12), EIRpsu 3 (lane 13–18), EIRpsu 4 (lane 19–24), EIRpsu 5 (lane 25–30), EIRpark (lane 31–36), EIRrak (lane 37–42) and consist of RRIM 600 (lane 43–48) amplified by primer OPB–17 Lane M = 100 bp ladder.

1.1 Analysis of similarity coefficients and dendrogram construction of rubber seedlings

The genetic relationships among the early– introduced rubber seedling populations, and RRIM 600 was determined by the polymorphism of DNA fragment patterns using RAPD and polythetic cluster analysis. Results showed that the 48 rubber seedlings were clustered into 5 groups. The composition and description of each group are given in Figure 13.

The first group consists of 2 samples from Prince of Songkla University, Hat Yai, Songkhla (EIRpsu 5–1, EIRpsu 5–4) and 2 samples of RRIM 600 (RRIM 600–1, RRIM 600–4).

The second group consists of 5 samples (EIRpsu 1–1, EIRpsu 1–3, EIRpsu 1–4, EIRpsu 1–5, EIRpsu 1–6), 6 samples (EIRpsu 2–1, EIRpsu 2–2, EIRpsu 2–3, EIRpsu 2–4, EIRpsu 2–5, EIRpsu 2–6), 6 samples (EIRpsu 3–1, EIRpsu 3–2, EIRpsu 3–3, EIRpsu 3–4, EIRpsu 3–5, EIRpsu 3–6), 6 samples (EIRpsu 4–1, EIRpsu 4–2, EIRpsu 4–3, EIRpsu 4–4, EIRpsu 4–5, EIRpsu 4–6), and 3 samples (EIRpsu 5–2, EIRpsu 5–3, EIRpsu 5–6) from Prince of Songkla University, Hat Yai, Songkhla, 5 samples from Bang Rak, Rubber plantation, Muang Trang, Trang (EIRrak–1, EIRrak–3, EIRrak–4, EIRrak–5, EIRrak–6) and 4 samples from RRIM 600 (RRIM 600–2, RRIM 600–3, RRIM 600–5, RRIM 600–6).

The third group consists of 1 sample from Bang Rak, Rubber plantation, Muang Trang, Trang (EIRrak–2).

The fourth group consists of 1 sample from Prince of Songkla University, Hat Yai, Songkhla (EIRpsu 5–5).

The fifth group consists of 6 samples from Hat Yai central park, Hat Yai, Songkhla (EIRpark: EIRpark–1, EIRpark–2, EIRpark–3, EIRpark–4, EIRpark–5, EIRpark–6).

The genetic similarity coefficient of the analyzed seedlings varied from 0.877–1.000 with average 0.952 (Appendix of Table 4). The lowest similarity coefficient was found between the EIRpsu 5–5 (Faculty of Engineering, Prince of Songkla University, Songkhla) and EIRpark–4 (Faculty of Science, Prince of Songkla University, Songkhla) with similarity coefficient 0.877. While the highest was recorded between of EIRpsu 1–6 (Faculty of Natural Resources, Prince of Songkla University, Hat Yai, Songkhla) and EIRrak–6 (Bang Rak, Rubber plantation, Trang), EIRpsu 5–2 (Faculty of Engineering, Prince of Songkla University, Songkhla) and EIRpsu 1–4 Faculty of Natural Resources, Prince of Songkla University, Hat Yai, Songkhla), EIRpsu 4–6 (Faculty of Science, Prince of Songkla University, Songkhla) and EIRpsu 5–6 (Faculty of

Engineering, Prince of Songkla University, Songkhla). Small variation was found among seedling within the same clone (Table 3). From table 3, seedlings of EIRpsu 2 was almost identical similarity coefficients vary from 0.985–1.000 with among 0.991.

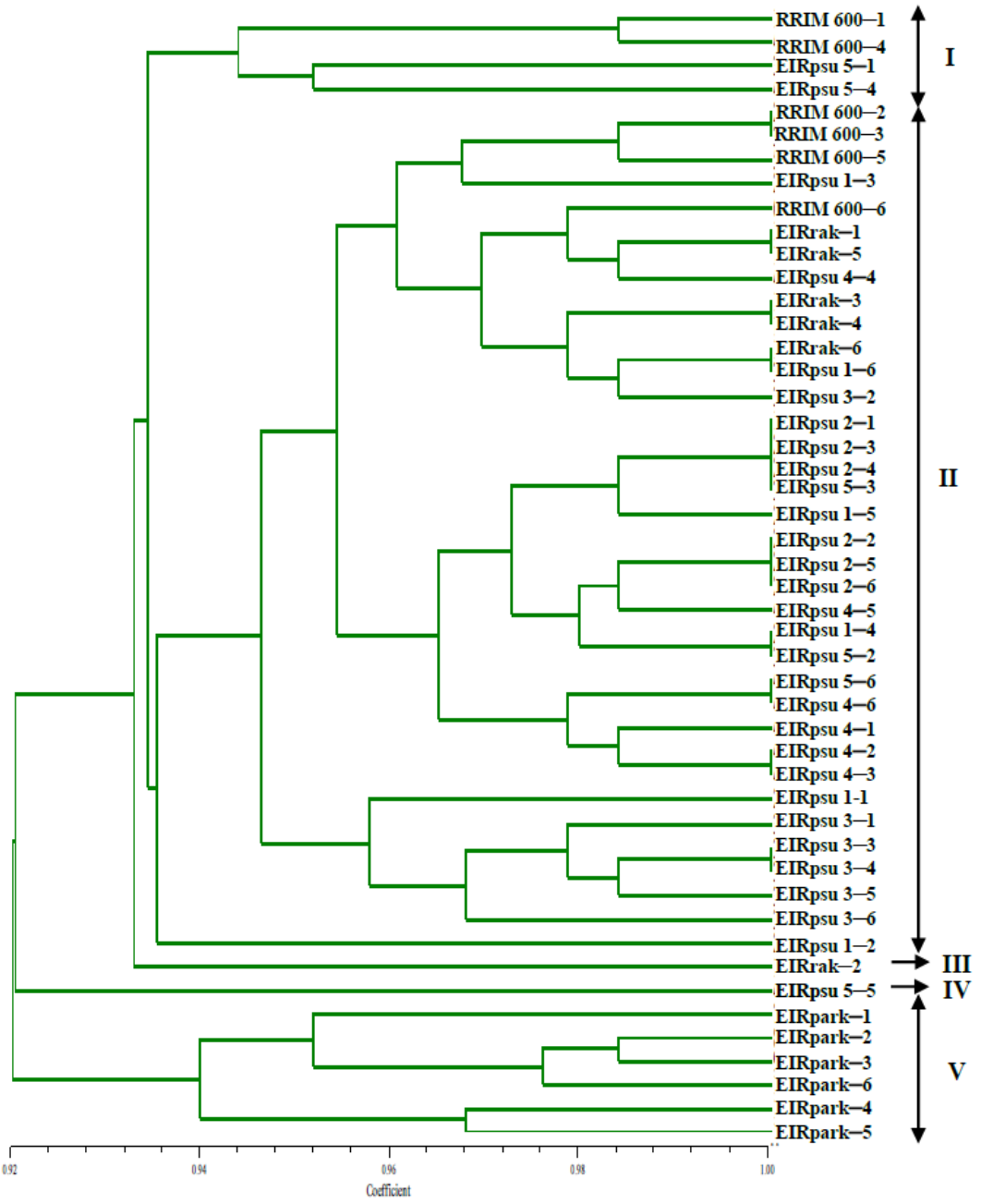

Figure 13 Dendrogram showing the relationship between 8 rubber clones based on RAPD with 7 primers.

Table 3 Similarity coefficients of the seedlings among clones of 8 rubber clones based on RAPD with 7 primers.

Clones	Range of the similarity coefficients	Mean of the similarity coefficients
EIRpsu 1	0.938–0.984	0.960
EIRpsu 2	0.985–1.000	0.991
EIRpsu 3	0.954–1.000	0.980
EIRpsu 4	0.969–1.000	0.980
EIRpsu 5	0.923–0.969	0.947
EIRpark	0.908–0.989	0.954
EIRrak	0.938–1.000	0.976
RRIM 600	0.938–1.000	0.969

2. Soil properties

The soil in the experiment was sandy clay loam in texture with moderate compaction. It was characterized by low pH (5.45) and contained the reasonable amount of total nitrogen, Available P, and Available K. Soil total nitrogen was 0.06 %. The level of available P was low content (7.07 mg/kg) but high exchangeable K (65.03 mg/kg). The results indicated that soil pH, total nitrogen, available P and available K were optimal ranges for rubber (Table 4).

Table 4 Analysis of the soil used in the experiment.

Soil properties	Characteristics
pH (1:5 soil/water)	5.45
Nitrogen (%)	0.06
Available P (mg/kg)	7.07
Exchangeable K (mg/kg)	65.03

3. Influence of pathogen on growth of rubber rootstock seedlings.

3.1 Root growth

Root growth of the rubber seedling was assessed in each 20 cm–interval depth. It was found that in the control treatment of EIRpsu 1, EIRpsu 3, EIRpsu 4 and EIRpark, the plants exhibited high portion of root proliferation in the layer 20–40 cm depth from the soil surface. Whereas the plants in inoculation treatment of EIRpsu 1, EIRpsu 2, EIRpsu 3 and RRIM 600 exhibited high extension root growth at 0–20 cm depth (Figure 14). The clone EIRpsu 5 exhibited the highest total average root length density compared with the other clones. While the lowest root length density of clone RRIM 600 was found at various depths. All clones of the control treatment were better in root growth compared with the inoculated treatment (Figure 15).

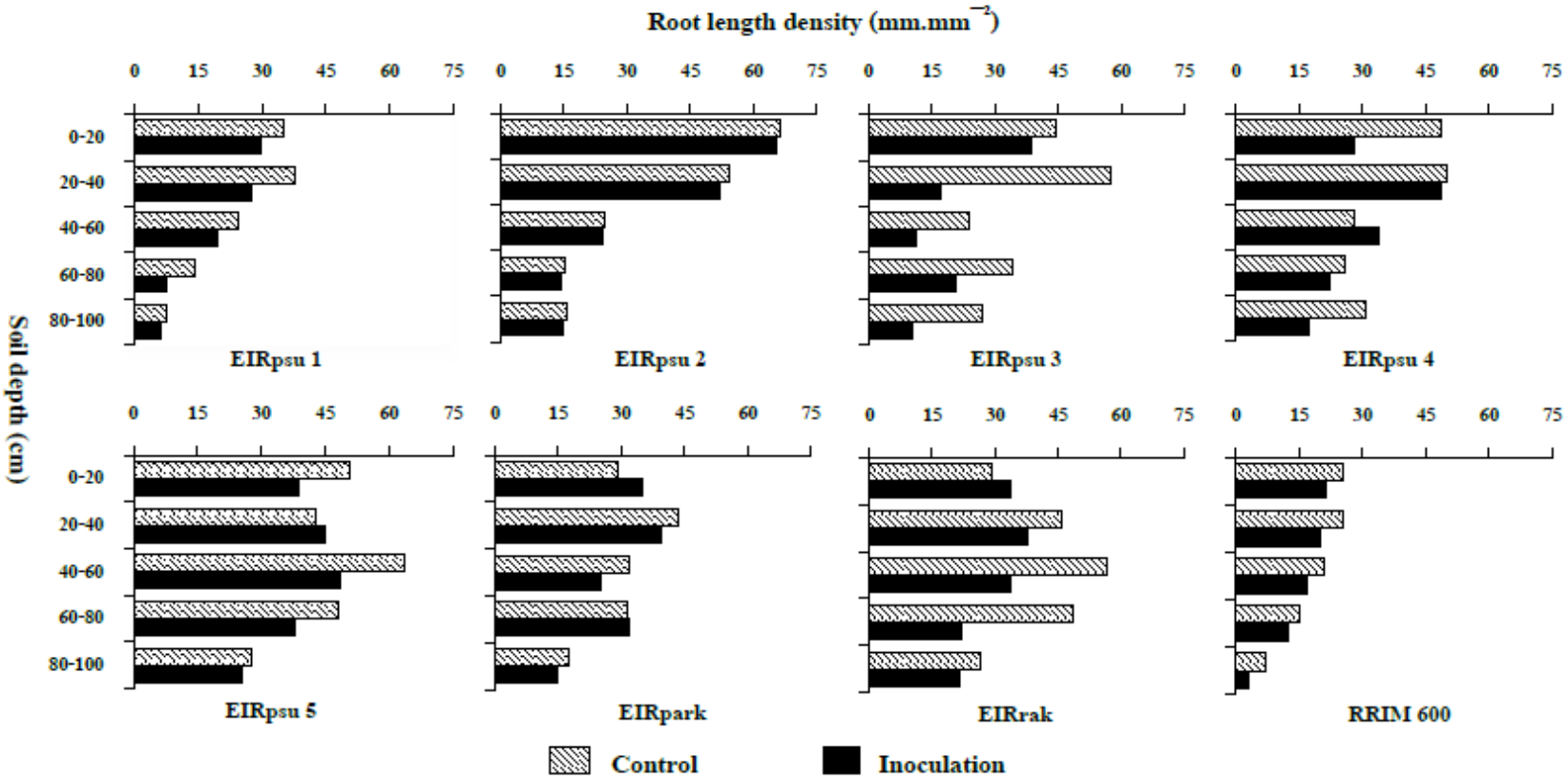

Figure 14 Comparison of the root profile between the control and inoculation treatments of the eight rubber clones.

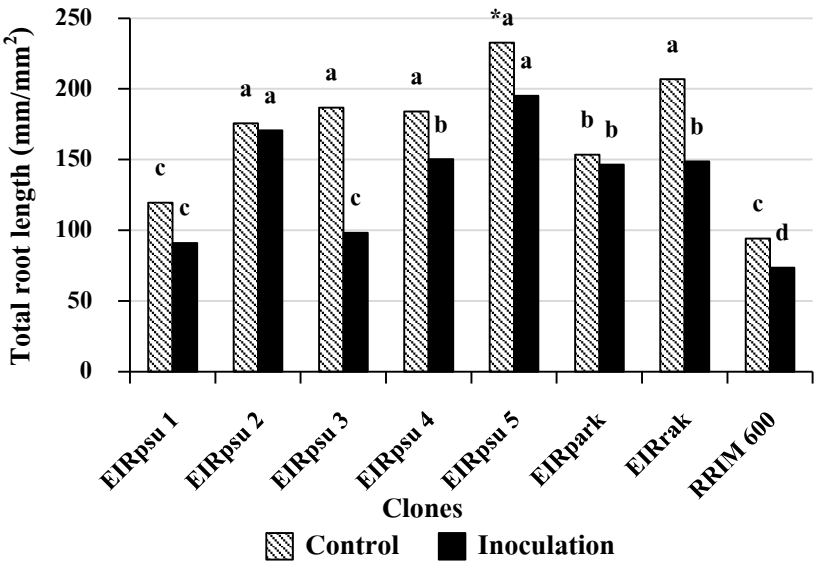

Figure 15 Comparison of average total root length density (mm/mm^2) among the eight clones in the soil profile observed from rhizobox panel in the control (▨) and inoculation (■) treatments.

*Different letters in each column indicate the highly significant difference ($P < 0.05$) by Duncan's new multiple range test.

3.2 Shoot growth

The difference of shoot growth among the eight rubber clones was found (Figure 16), there was the significant difference in the plant height, leaf number, and diameter. Comparing shoot growth response between the control and inoculation treatments, it was shown that the shoot growth of the control treatment was higher than the inoculation treatment. In the control treatment, the clone EIRpsu 5 had the highest plant height (130.45 cm), diameter (13.11 mm) and leaf number per plant (24.74 leaves per plant), which was the significant different from the other clones. Meanwhile, it was found that the clone EIRpsu 1 had low plant height, diameter and leaf number as shown in Figure 16. The inoculated EIRpsu 3 clone showed a sensitive response to the white root disease with the decrease of the diameter and leaf number. It was found that the pathogen affected shoot growth and plant responses were different among the clones. Comparing shoot growth response between the control and inoculation treatments, it was shown that most of the shoot growth in the control treatment was higher than the inoculation treatment. In addition, it was found that some clone in the inoculation treatment was higher than the control treatment. The clone

EIRpsu 2 had high plant height, which the clones EIRpsu 1, EIRpsu 2, and EIRpsu 4 had the high diameter. The high leaf number was found only in the clone EIRpsu 4 (Figure 17).

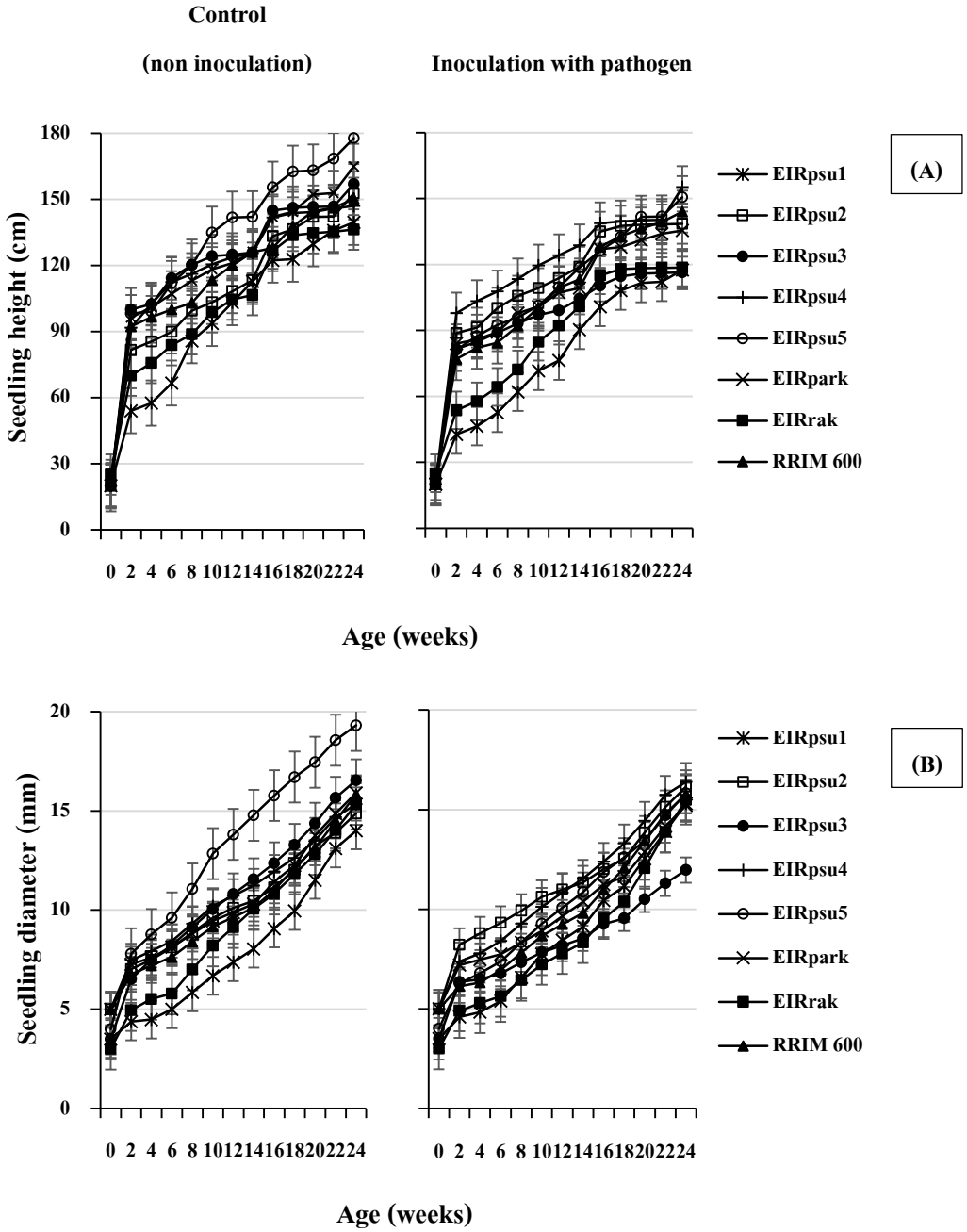

Figure 16 Shoot growth of the 8 clones in the control and inoculated rubber seedlings during the experimental period (A) increment of plant height (B) increment of diameter and (C) increment of leaf number (error bars indicate mean ± SE).

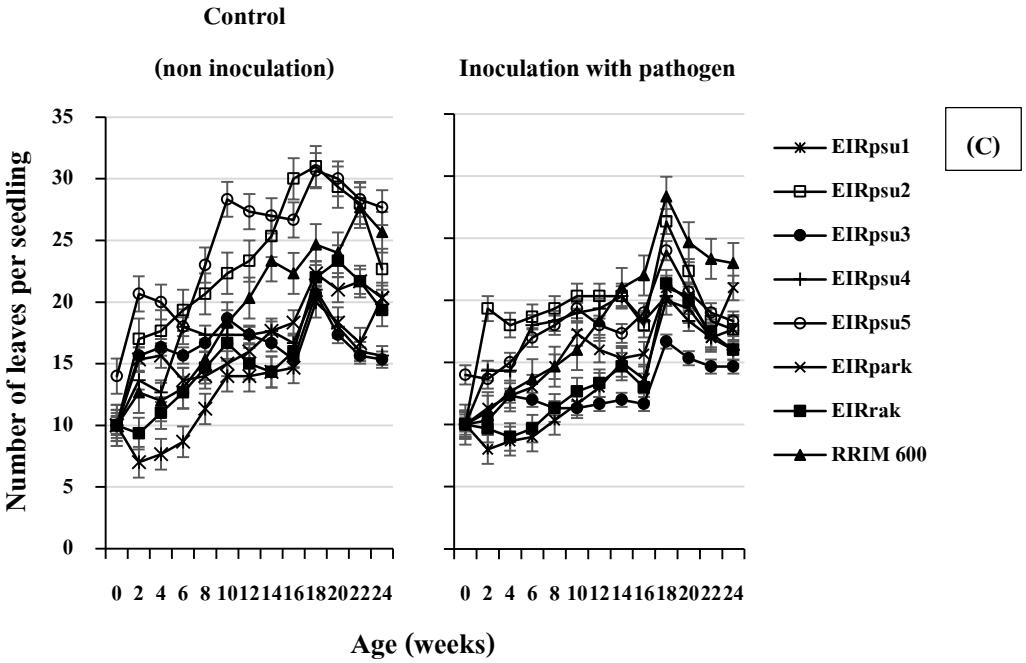

Figure 16 (Cont.) Shoot growth of the 8 clones in the control and inoculated rubber seedlings during the experimental period (A) increment of plant height (B) increment of diameter and (C) increment of leaf number (error bars indicate mean ± SE).

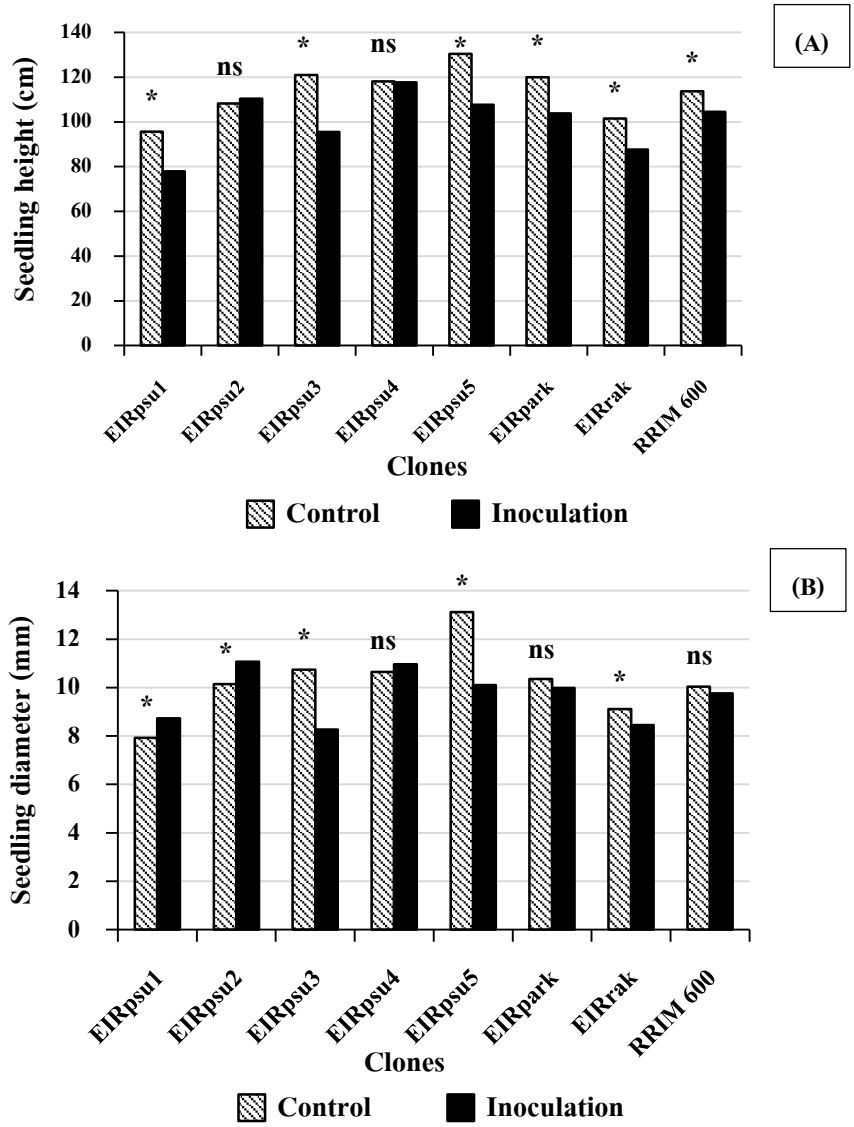

Figure 17 Comparison of average shoot growth of the 8 clones (A) increment of plant height (B) increment of diameter and (C) increment of leaf number in the control and inoculated rubber seedlings.

* = significant difference between the control and inoculation of each clone at $P < 0.05$ by Least significant difference *(*LSD*)* test.

ns = non-significant difference between the control and inoculation of each clone.

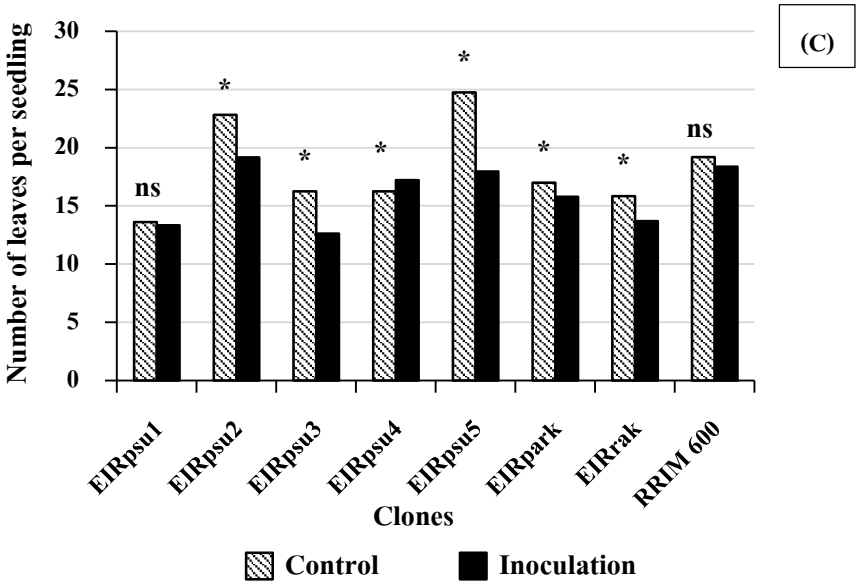

Figure 17 (Cont.) Comparison of average shoot growth of the 8 clones (A) increment of plant height (B) increment of diameter and (C) increment of leaf number in the control and inoculated rubber seedlings.

* = significant difference between the control and inoculation of each clone at $P < 0.05$ by Least significant difference (LSD) test.

ns = non-significant difference between the control and inoculation of each clone.

4. Influence of pathogen on physiological responses of rubber rootstock seedlings

Figure 18 showed that the control treatment of EIRpsu 5 clone exhibited the highest average photosynthetic rate (7.38 μmol/m^2/s), and stomatal conductance (293.33 mmol/m^2/s). While the clones EIRpsu 1 had the lowest photosynthetic rate (6.52 μmol/m^2/s) and clone EIRrak had low stomatal conductance followed by other clones. In the inoculated treatment, it showed that the clone EIRpsu 5 exhibited the highest average photosynthetic rate (7.22 μmol/m^2/s), and stomatal conductance (289.58 mmol/m^2/s) and the clones of EIRpsu 4 had the lowest photosynthetic rate (6.31 μmol/m^2/s) and stomatal conductance (219.58 mmol/m^2/s). The photosynthetic rate and the stomatal conductance of each rubber clone were in the range 4.52–11.92 mmol/m^2/s and 110–440 mmol/m^2/s, respectively. Comparing the photosynthetic response of the control and inoculated treatments, it was found that the rubber clones in the control treatment tended to exhibit the highest average of photosynthetic rate and stomatal conductance than the inoculated treatment. The lowest average of photosynthetic rate and stomatal conductance was found EIRpsu

1 clone in the control treatment. However, there were significant differences in the photosynthetic rates in the control and inoculum treatment of EIRpsu 1, EIRpsu 3, EIRpsu 4 and RRIM 600 clones, however, it was not significantly different in the stomatal conductance of EIRpsu 1, EIRpsu 5 and EIRpark clones (Figure 19).

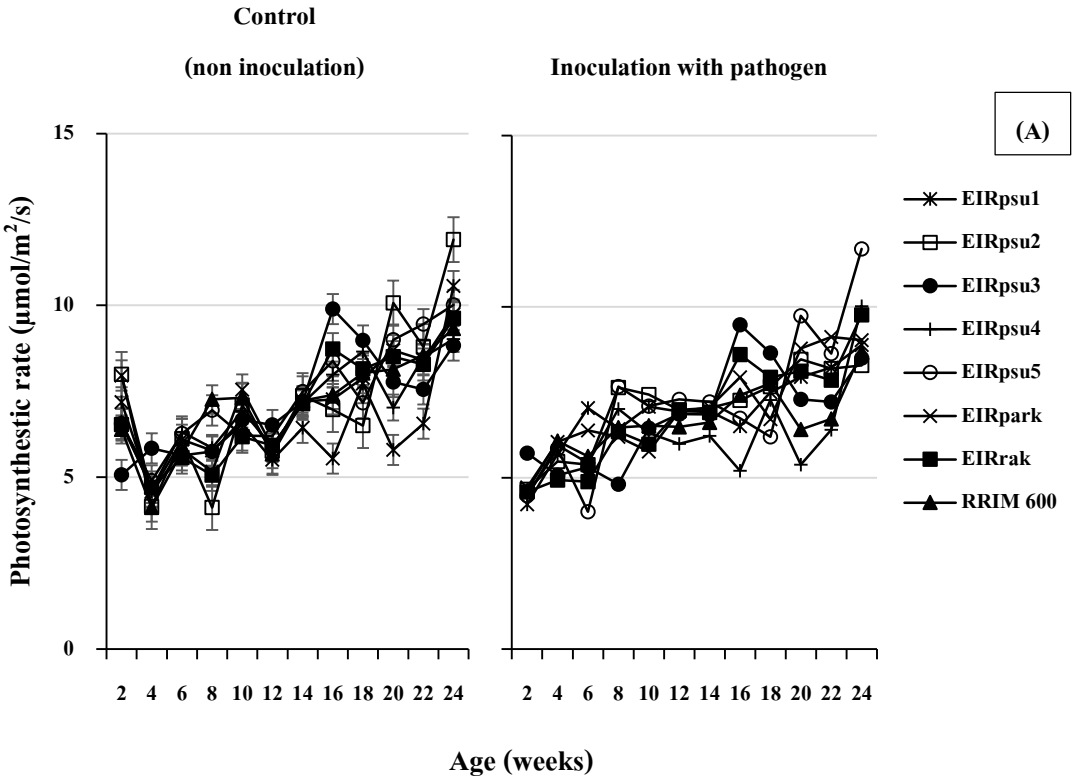

Figure 18 Physiological responses of the 8 clones in the control and inoculated rubber seedlings during the experimental period (A) photosynthetic rate and (B) stomatal conductance (error bars indicate mean ± SE).

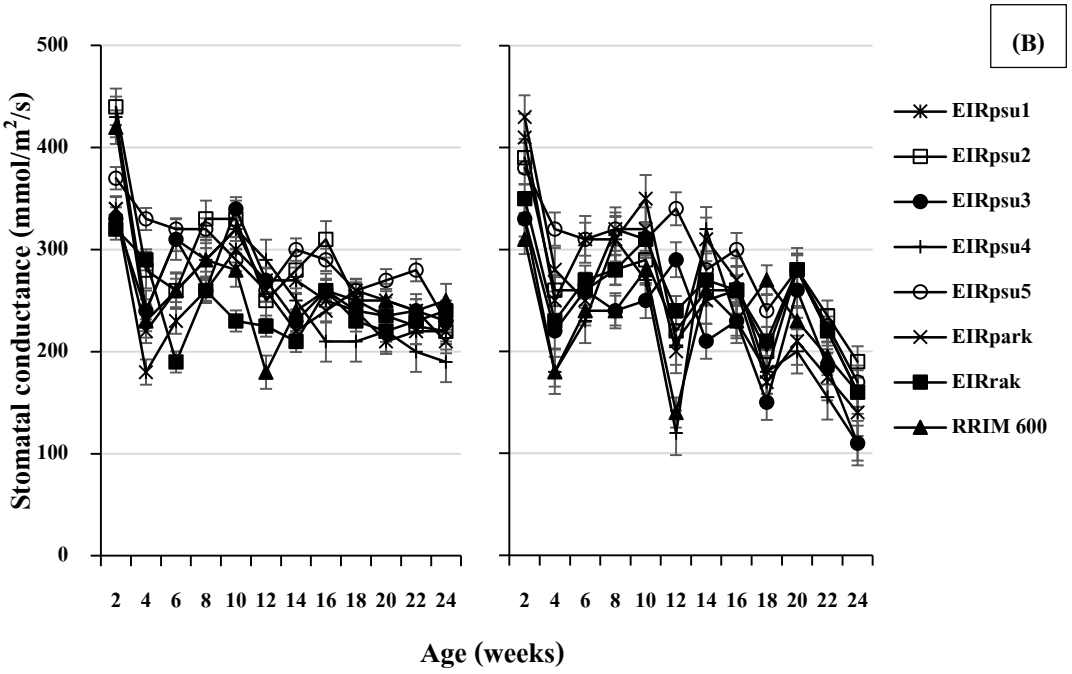

Figure 18 (Cont.) Physiological responses of the 8 clones in the control and inoculated rubber seedlings during the experimental period (A) photosynthetic rate and (B) stomatal conductance (error bars indicate mean ± SE).

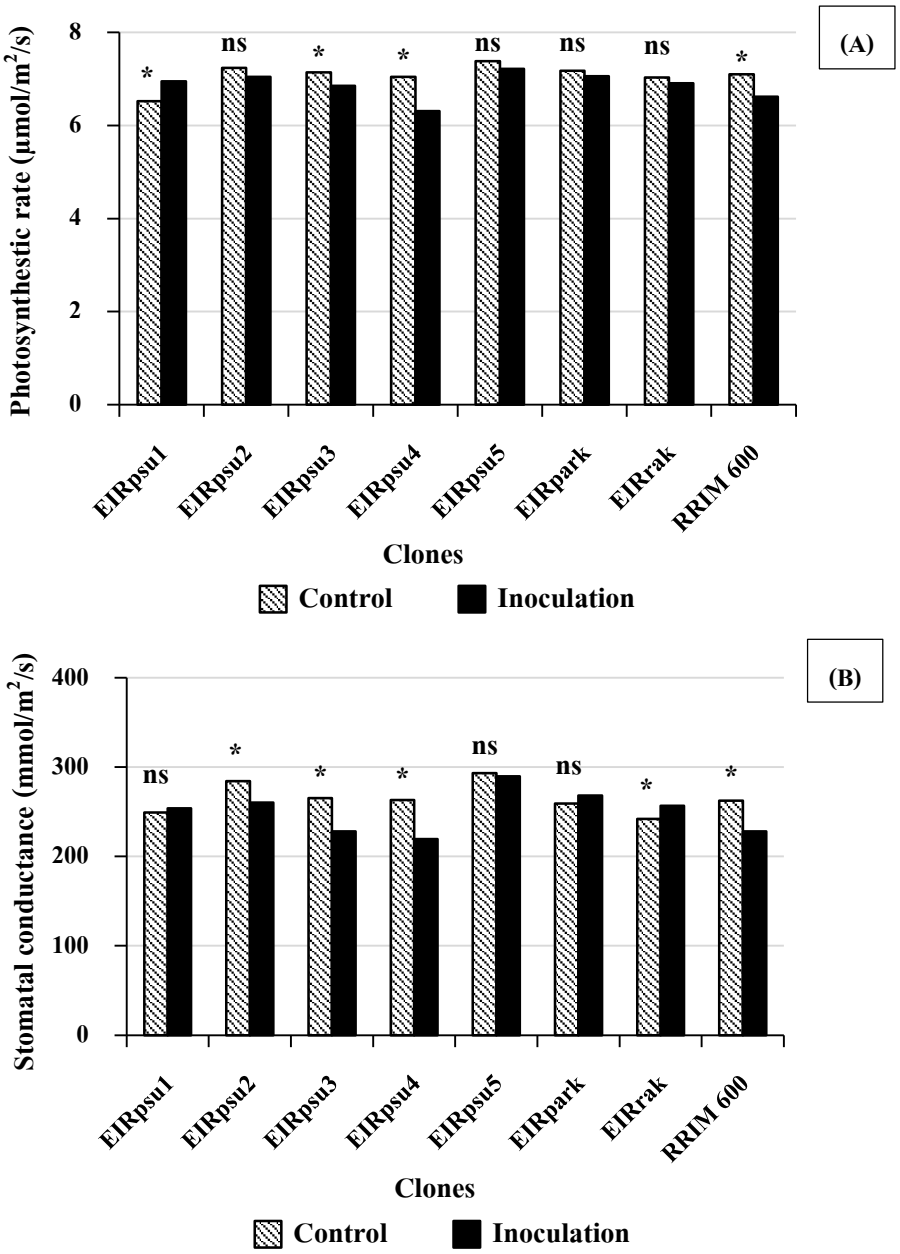

Figure 19 Comparison of average physiological responses among the eight clones in the control and inoculated rubber seedlings (A) photosynthetic rate (µmol/m^2/s) and (B) stomatal conductance (mmol/m^2/s).

* = significant difference between the control and inoculation treatments of each clone at $P < 0.05$ by Least significant difference *(*LSD*)* test.

ns = non-significant difference between the control and inoculation treatments of each clone.

5. Evaluation of rubber rootstock seedlings for the white root disease tolerance

5.1 Characteristic mycelium of *R. microporus*

The characteristic of *R. microporus* which isolated from the infected root of rubber trees by tissue transplanting technique. The colony on PDA at 6 days showed white and flattened mycelium (Figure 20A). The hypha showed hyaline, septum, no clamp connection, and possess many branches (Figure 20B). The fruiting bodies were broad shape, leathery, and no stalk. The upper surface was orange–red–brown, smooth and the lower surface orange–brown, fine pores (Figure 20C).

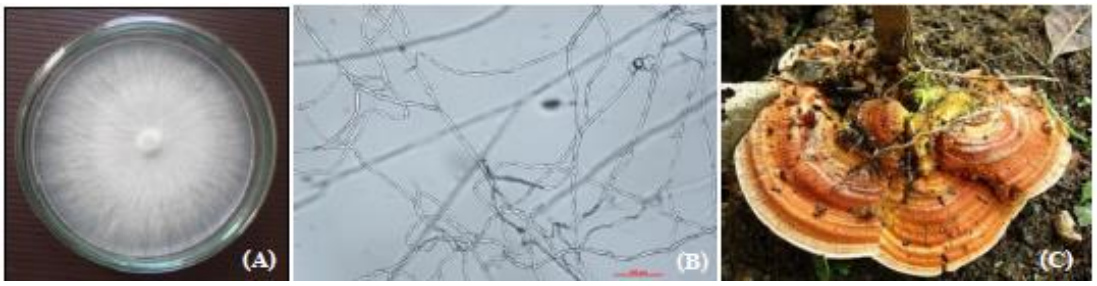

Figure 20 Characteristic of *R. microporus* hyphae (A) colony on PDA at 6 days (B) hypha and (C) fruiting body.

5.2 Symptom development of white root disease in rubber rootstock seedlings

Evaluation of the effects of white root disease on the seven selected clones and clone RRIM 600 clone at 24 weeks, 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 the whole canopy depends on the severity of the disease (Figure 21). Table 6, it was found that each clone exhibited different response to the white root disease. The clones of EIRpsu 3 (49.49 %), EIRpsu 4 (54.09 %), EIRrak (41.16 %) and RRIM 600 (51.76 %) had the high impact from the white root disease chlorosis was observed in the leaves with falling-off at 24 weeks after inoculation. In addition, it was found that clones EIRpsu 1, EIRpsu 2, EIRpsu 5 and EIRpark showed fewer symptoms of the white root disease comparing with the other clones. For disease index recorded, the clone EIRpsu 4 (54.09 %) showed the highest susceptibility score, whereas the lowest score was found in the clone EIRpsu 5 (12.12 %).

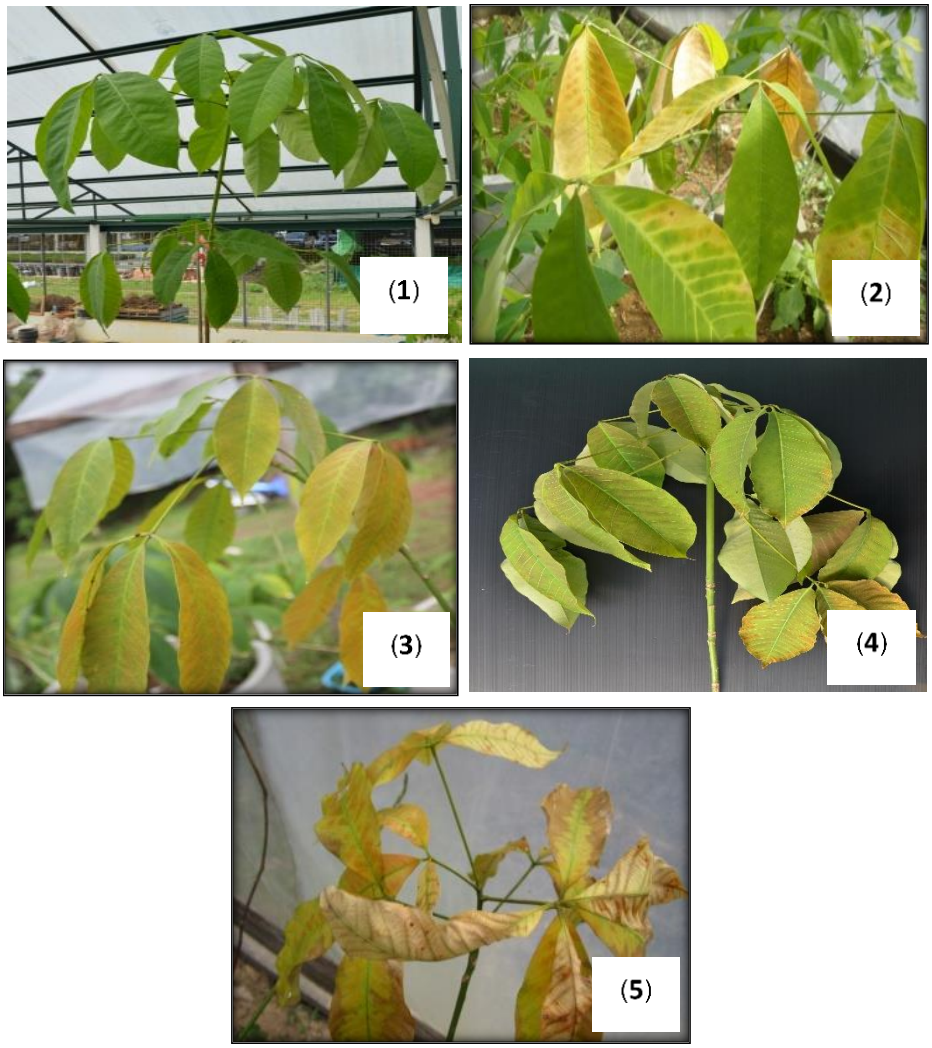

Figure 21 Development of symptoms on samplings of *H. brasiliensis*.

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 5 Disease index (DI) after inoculated rubber clone seedlings with *R. microporus* for 24 weeks.

Clone	% Survival	% DI
EIRpsu 1	100	36.36
EIRpsu 2	100	30.30
EIRpsu 3	33.33	49.49
EIRpsu 4	0	54.09
EIRpsu 5	100	12.12
EIRpark	100	27.27
EIRrak	66.66	41.16
RRIM 600	33.33	51.76

6. Influence of response rubber rootstock seedlings infected by *R. microporus*

6.1 Histological observation of rubber seedling root disease infected by *R. microporus*

Three seedlings with foliar symptom were randomly harvested the EIRpsu 4, RRIM 600 and EIRpsu 5 at 24 weeks after inoculation. A thin layer of fungal mycelia was observed growing on the root surface of the seedling (Figure 22). For cross–section and seen under a microscope, the hyphae had already infected the epidermis layer and some of the hyphae had advanced into the cortex cells. Closer observation revealed that the hyphae had invaded the root cells, especially in the epidermis. Colonized of the epidermis cells by the pathogen was even more extensive, causing some of the heavily colonized periderm and epidermis cells to rupture (Figure 22).

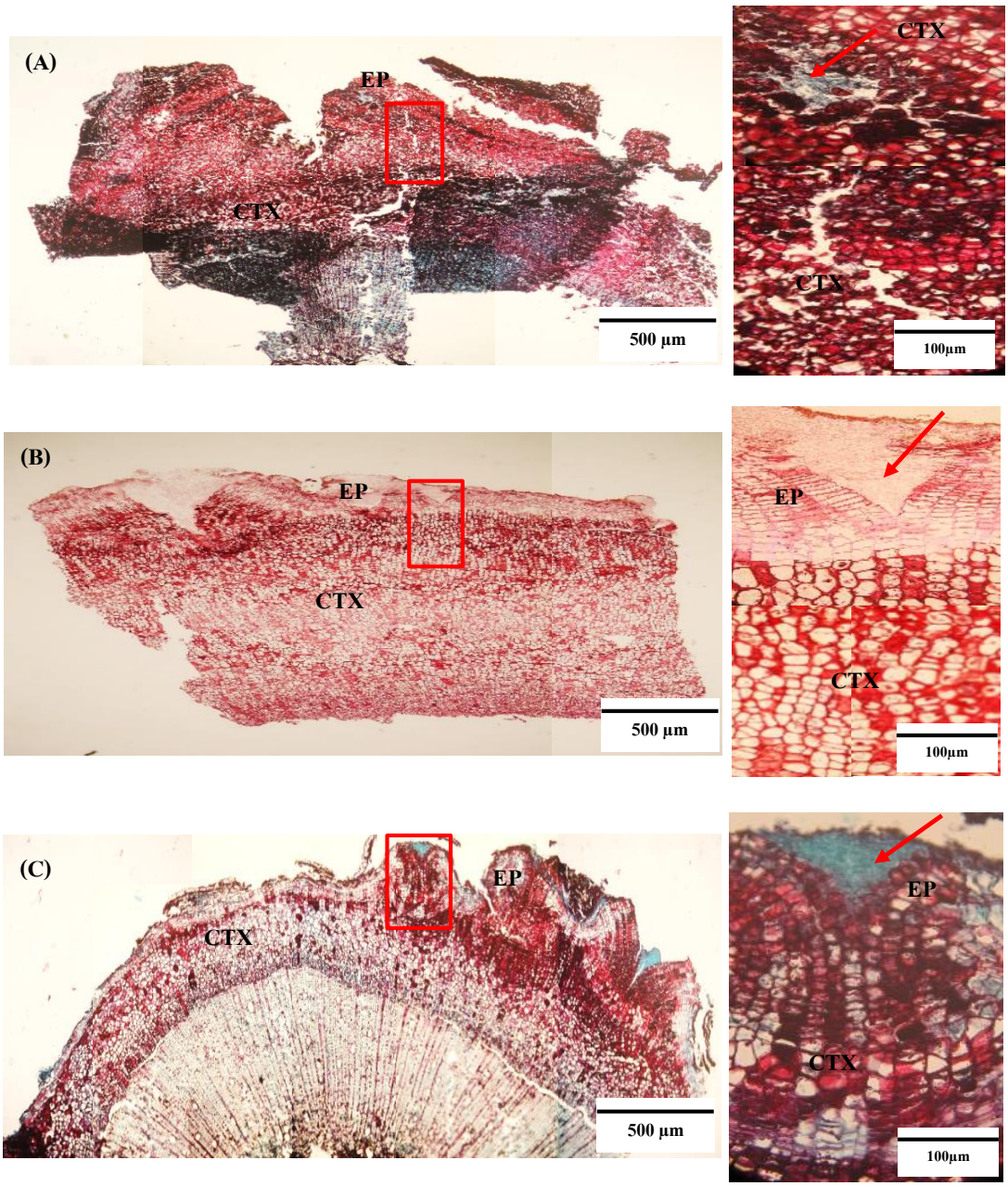

Figure 22 Cross–section of seedling root inoculated with *R. microporus* after inoculation for 24 weeks of (A) EIRpsu 4 (B) RRIM 600 and (C) EIRpsu 5 clones. Arrows showed growing hyphae on the root tissue.

EP= Epidermis CTX= Cortex

6.2 Ultrastructural observations of rubber seedlings root disease infected by *R. microporus*

TEM observation revealed that root tissues of the healthy control seedlings (no inoculated) were free from fungal infection throughout the study period and the cells of healthy plants are normal with thick and smooth walls (Figure 23A, 23B, and 23C). No fungal hyphae were observed in the cells of control (no inoculation) plants.

After inoculated with *R. microporus* for 24 weeks, TEM observations exhibited the presence of the fungal hyphae in the root cells. Penetration of hyphae was decay observed (Figure 23A, 23B, and 23C). These hyphae penetrated the cells intercellular and intracellular (Figure 23)

Cross–section in control (in the left) and inoculation (in the right) tissue showed that cell walls were extensively degraded with large amounts of extracellular materials present in front of thin–walled hyphae. In addition, the highest amount of hyphae was observed in clone EIRpsu 4, whereas the lowest hyphae infected was found in the clone EIRpsu 5.

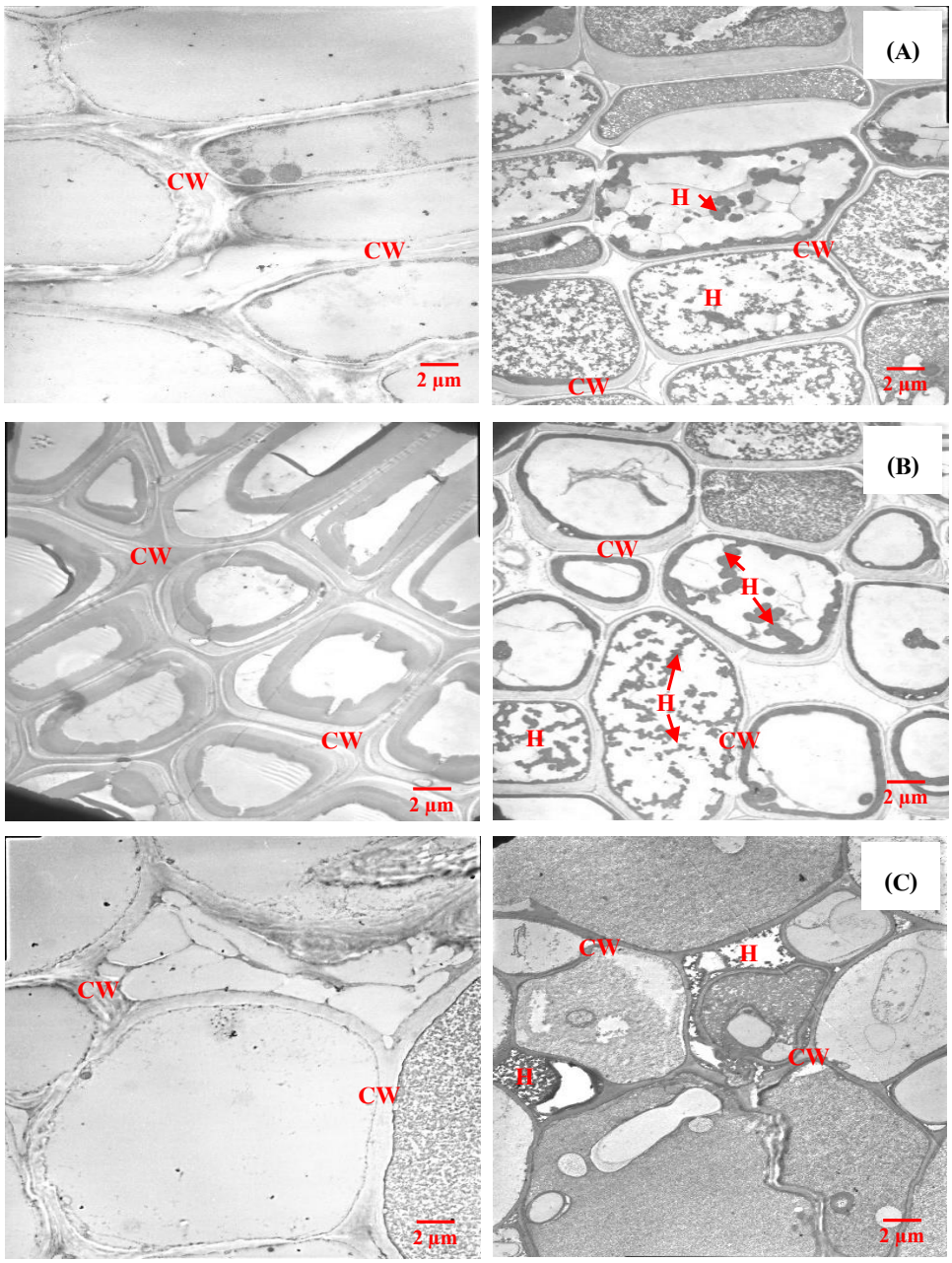

Figure 23 Transmission electron microscopy (TEM) observation of root section in the control and inoculated of (A) EIRpsu 4 (B) RRIM 600 and (C) EIRpsu 5 clones. Arrows showed hyphae penetrated the cell at 24 weeks.

CW= Cell Wall H= Haphae

CHAPTER 5

DISCUSSION

In the past, the most common rootstocks for planting material production in Thailand were seeds collected from any early–introduced rubber clones which had high heterozygosity based on cross–pollination in nature (Nakkanong, 2008). Those seeds adapt well to a climatic condition such as heat, drought, and cold, should be resistant or tolerance to disease and pest insects. They should possess good germination and exhibit to a high degree of compatibility (Dahla, 2013). At present, rubber seeds are collected from the smallholder's rubber plantation and therefore, most seeds are from rubber clone RRIM 600, which is mainly grown in southern Thailand and is sensitive to white root disease (Wattanasilakorn *et al*., 2012). Many years ago, Rubber plantations have been damaged by the white root disease sensitive economic losses in the rubber plantation in southern Thailand and the distribution of the white root disease in rubber plantation was recorded in every province (Nachapong and Chuenchit, 2011).

Wattanasilakorn *et al*., (2012) reported that some early–introduced rubber clones tended to exhibit to white root disease. Khonglao (2006) reported that seedling of early–introduced clones higher had vigorous root development of than RRIM 600. No prior genetic background information of rootstock seedlings has been reported tolerance to the white root disease.

To assess genetic information of selected rubber clones for rootstock and screening of rubber rootstock for the white root disease tolerance is important.

1. Identification of rubber rootstock using RAPD markers

Early–introduced rubber clones were recognized by their big trunk indicating an age of more than 50 years and their random location outside established rubber plantation areas. It is believed that most of those clones originated from seeds introduced from Malasia almost 80 years ago. Genetic variation of 48 plants belonging to eight clones from this study was investigated using RAPD technique. Seven primers (OPB–17, OPR–02, OPR–11, OPZ–04, OPAD–01, OPAD–10 and OPAD–12) were chosen to assess genetic variation of 48 individual plants. Sixty–five amplification fragments were obtained from 7 primers with an average of 9.28 fragments for each primer. The highest polymorphic fragments were obtained from primer OPAD–10 (15 bands),

while the lowest polymorphic fragments were obtained from primer OPR–02, and OPB–17 (7 bands). The DNA pattern from 7 primers of RAPD marker was shown in Figure 6–12. It was found that genetic variation between clones was low. The high similarity coefficient between clones varied from 0.877–1.000 and each rubber clone had high polymorphic similarity because we used too little primer. So, it must also be add primer to detect polymorphisms between rubber clones. In contrast, Varghese *et al*., (1997) studied in 24 clones from various countries in Southeast Asia by RAPD with 43 primers and they reported that high genetic variability among clones (mean genetic distance of 0.5). Nakkanong, (2008) studied in 14 early rubber introduced clones and 23 cultivated clones using RAPD with 8 primers and microsatellite with 4 primers and reported narrow pairs genetic diversity among cultivated clones compared to those among early introduced clones. It is also found that seedlings from the same mother clone have low high genetic variability. The variability is much or less depending on the variety and a source of pollen. In this study, most early–introduced rubber clones come from Prince of Songkla University. It is believed that those clones originated from seed collected in the same plantation. From UPGMA cluster, indicated the presence of distinct geographical grouping.

2. Influence of pathogen on growth of rubber rootstock seedlings.

2.1 Root growth

According to root profile study in the control treatment, it indicated that the clones EIRpsu 1, EIRpsu 3, EIRpsu 4 and EIRpark exhibited high root proliferation in the layer of 20–40 cm depth. The inoculation treatment, it was found that clones of EIRpsu 1, EIRpsu 2, EIRpsu 3 and RRIM 600 had high extension root growth in the shallow layer of 0–20 cm. The clone EIRpsu 5 exhibited the highest total average root length density compared with the other clones. While the lowest root length density of clone RRIM 600 was found. The resulting similarity with Cherngchalard (2012) reported that the rubber seedling grown in a minirhizotron had high root proliferation at 20–40 cm depth from the soil surface. Whereas the of root proliferation the seedlings of clone RRIM 600 and GT 1 were located within 0–15 cm and 20–40 cm depth from the soil surface, respectively. Root activity declined with increasing depths (George *et al*., 2009). Nares and Sayan (2551) evaluated the growth of rubber tree roots by using a minirhizotron, and it was found that the high root density was at soil depth 0–10 cm. soil depth. However, Hamblin (1985)

suggested that root development in any plant is governed by factors such as nutrient availability, soil physical properties, and genetic characters. One problem of rhizobox observation is the overestimating root length density at depth, which may be due to roots channeled down the vertical tube to soil interface. Besides, the pattern of root development and distribution are normally affected by many factors such as nutrient availability, soil physical properties, and genetic characters. (Hamblin, 1985; Liedgens *et al*., 2000).

2.2 Shoot growth

The fungus attacked the rubber roots causing the decrease of shoot growth. It was evident that the shoot growths of the control clones were higher than the inoculated clones. The results of control treatment showed that the height, diameter and leaf number per plant continuously increased. In the control treatment, the clone EIRpsu 5 had the highest height (130.45 cm), diameter (13.11 mm) and leaf number per plant (24.74 leaves per plant) and the highest root length density was found in clone EIRpsu 5. Russell (1977) reported that shoot and root growth related with an environmental condition and when changing environmental conditions could affect the dry weight of roots and trees.Thus, analyzing the growth is determined using the principles of the relationship between the source and sink. Meanwhile, it was found that the inoculated clone EIRpsu 4 had plant height (117.64 cm). The EIRpsu 2 clone had the highest diameter (11.06 mm) and leaf number (19.15 leaves per plant) and it was significantly different from the other clones. Zaini and Halimoon (2013) reported that the changes of diameter are influenced by many factors and one of the major factor is water content. Water tension inside the rubber plants and the fungal attack to the plant root causes the loss of their function to uptake water and nutrients from the soil. The fungus harmed the lifespan of the plants. This might be due to the impact of *R. microporus* on root growth leading a limitation of water uptake. Nahar and Gretzmacher (2011) also reported that plant growth was limited because of increasing stress.

3. Influence of pathogen on physiological responses of rubber rootstock seedlings

The investigation of physiological parameters could support the plant response to the white root disease because the photosynthetic rate and stomatal conductance of the inoculated plant were decreased. While the plants in the control treatment showed high physiological

responses. According to Lee and Noraini, (1999); Zaini and Halimoon (2013) studied the ability of *Catharanthus roseus* stem extract to control white root rot disease of rubber trees, it was found that the fungus will start to attack plant roots at 2 weeks after inoculation and it depends on the plants as every plant have its own defense to the disease. Since the *R. microporus* cannot produce their own food and they need to rely on their host (rubber plants). When the leaves were old and there was a damage on the leaves, it might turn yellow or orange color. The chlorophyll was translocated out of the leaves and appeared yellow before death (Zaini and Halimoon, 2013). Zaini and Halimoon (2013) reported that chlorophyll contents in the leaves also influence the leaves performance and when the level of chlorophyll decrease, the leaves turn chlorosis leading high leave falling. Sudden changes in temperature can lead the leaves to turn yellow or brown and thus cause the leaves to drop. The reductions on stomata conductance of guard cells force the stomata to close so that it will reduce the transpiration rate and photosynthetic rate (Vijayakumar *et al*., 1998; Mokhatar *et al*., 2011). Somjun (2009) also supported that physiological response of the rubber could be assessed by the assessment of stomatal conductance. It was found that there was a bit difference of stomatal conductance and photosynthesis rate among the clones. Kröber *et al*., (2015) reported that stomatal conductance and stomatal regulation were found to be related to morphological, anatomical and chemical leaf traits. Supacharoenkun (2008) reported that the difference in photosynthesis rate and stomatal conductance also depend on the different clones. The photosynthetic rate of the leaves was decreased as the decreased of leaf water potential or water stress (Sittichai and Sdoodee, 2014).

4. Evaluation of rubber rootstock seedlings for the white root disease tolerance

4.1 Pathogen and pathogenicity test

The characteristic of *R. microporus* which isolated from the infected root of rubber trees by tissue transplanting technique. The colony on PDA at 6 days showed white and flattened mycelium. The hypha showed hyaline, septate and possession many branches but no clamp connection. The fruiting body showed broad, thin, and orange–red. Basidiospores showed globose, colorless, thin–walled, and smooth. This result was similar to the report of Nandris *et al*., (1987) and Kaewchai *et al*., (2010) who stated that the fungus formed many white and flattened mycelium but the colony on malt medium formed superficial, white mycelial felt.

R. microporus was isolated from the infected root of the rubber tree and causes white root disease. This disease is an important of rubber trees which causing economically important losses in the rubber plantation in Thailand and many countries. Nandris *et al*., (1987) reported that this fungus infects the roots by free rhizomorphs growing from the stumps or infected woody debris remaining on the ground and by contacting with the infected root as also supported by Nandris *et al*., (1987). The visible symptom is changed in the color of the leaves from green to yellow (Guyot and Flori, 2002).

According to white root disease, it was found that *R. microporus* could infect all stage of the plant from the seedling. The clone of EIRpsu 4 showed yellowing leaves on one or a few branches at 12 weeks and the symptoms appeared wilting and yellowing of the leaves, defoliation and white mycelium on the root system after infection leading to the tree death. While clone of EIRpsu 1, EIRpsu 2, EIRpsu 5, and EIRpark exhibited high survival and lowest susceptibility score until the end of the observation and the clones EIRpsu 3, EIRrak and RRIM 600 were affected by the white root disease and the leaves were chlorosis with falling after 12 weeks. This result was similar to the report of Kaewchai and Soytong (2010) who found that RRIM 600 showed the symptom of yellowing leaves at 70 days and the root of the dead tree was possessed with rhizomorph of the pathogen and it produced fruiting body at the collar of the dead stem. Wattanasilakorn *et al*., (2012) studied screening of rubber rootstocks for the white root disease resistance and compared with RRIM 600 and GT 1, it was found that GT 1 seedlings were sensitive to the white root disease.

The symptom development of the white root disease also depended on the environmental factors (Joko, 2009). Most commonly, the symptoms would start after the infection with the *R. microporus*, it appeared to exhibit almost similar foliar symptoms. The progress of the disease was firstly observed as yellowing followed by wilting, defoliation and finally death of the host. In addition, the progress of these symptoms was similar to the report by Mohd Farid *et al*., (2001, 2006), and roots of samplings inoculated with *R. microporus* had white rhizomorphs on their surface. Nissapa and Chuenchit (2011) reported that clones of RRIM 600 and BPM 24 are highly susceptible to diseases caused by the white root disease. In this study, the result also showed that clone RRIM 600 was sensitive to the white root disease. Khonglao (2006) reported that most rootstocks from the seed of early introduced clones to Thailand, and it is suggested that rubber

clones that are tolerant of the white root are needed to be selected from early introduced clone population.

5. Influence of response rubber rootstock seedlings infected by *R. microporus*

5.1 Histological and ultrastructural observation of rubber seedling root disease infected by *R. microporus*

At 8 weeks after inoculation, hyphae of *R. microporus* were the widespread surface of the taproot and after inoculation for 24 weeks were produced hyphae that penetrated inner root tissue. The highest amount of hyphae was observed in clone EIRpsu 4, whereas the lowest hyphae infected was found in the clone EIRpsu 5. In contrast to Nicole and Benhamou (1991) studied the infection process of rubber root seedling at 1 month. Collected root samples at 2, 4, 10, and 15 weeks after inoculation by *R. microporus* at a depth of 20 cm in the soil, it was found that rhizomorphs start growing along the root surface 2 weeks after inoculation until at 15 weeks after inoculation, hyphae penetrated in xylem cells. According to Mohd Farid *et al*., (2001) the pathogen can infect the host root system within a week after inoculation although no wound was made and the fungal hyphae penetrated the epidermis by colonizing the cells and subsequently breaks down the cell wall.

For root tissue of infection *H. brasiliensis* seedlings by *R. microporus*, preparation of root sections with attached rhizomorphs was difficult and a clear observation of the means of external hyphae penetration into the root system could not be observed.

Cross–section of the infected root tissue provided evidence that *R. microporus* was capable of both penetrating and colonizing rubber roots within a short period of time and also found in *Azadirachta excels* seedlings infected with *R. microporus* white root disease by Mohd *et al*., (2009). Nicole *et al*., (1986) also reported that this pathogen normally develops two types of mycelium which are different from each other morphologically and metabolically including their enzymatic secretions. However, it is suggested that the infection process starts with the development of white fungal strand on the root surface and further histological studies are required to clearly reveal the connection between hyphae.

The presence of the rubber clones showed that the plants have the ability to adapt after infection referring to a positive result of shoot growth (height, diameter, and leaf number), root growth, physiological response as shown in this study.

However, this experiment was observed in a short period, therefore, it needs to be investigated further in a long term. It should be considered for the response of selected scions grafted or budded on these rubber rootstocks with the investigation of growth performance under field condition.

CHAPTER 6

CONCLUSION

Tentative tolerance to the white root disease was investigated by monitoring the genetic background of seedlings from seven clones of early–introduced rubber tree and 1 cultivated clone (RRIM 600) using RAPD with 7 primers. The genetic similarity coefficient of analyzed seedlings varied from 0.877–1.000. The 48 rubber seedlings were clustered into 5 groups.

The investigation of shoot growth, root development and physiological responses of the seven early–introduced clones and RRIM 600 was carried out in rhizoboxes at 24 weeks and indicated that the control plants exhibited higher performance than the inoculated plants. The clone EIRpsu 5 showed the significantly highest shoot growth with the highest average root length density. The physiological responses of the clone EIRpsu 5 showed the highest efficiency of photosynthetic rate and high stomatal conductance. According to the assessment of the symptom development of the white root disease, it showed that clone EIRpsu 4 exhibited the highest susceptibility score, whereas the lowest score was found in the clone EIRpsu 5. This suggested that the clone EIRpsu 5 tended to be tolerant of the white root disease with good performance of plant growth.

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APPENDICES

APPENDIX A

Component of FAA II solution

Formaldehyde	5 % (v/v)
Glacial acetic acid	5 % (v/v)
70 % alcohol	90 % (v/v)

Dehydration solution

Table 1 Preparation of ethyl–butyl alcohol series (Johansen, 1940).

No.	Total alcohol (%)	Composition (mL)				
		TBA	Ethanol		Water	Other
			95 % alcohol	100 % alcohol		
1	5	–	5	–	95	
2	10	–	10	–	90	
3	20	–	20	–	80	
4	30	–	30	–	70	
5	50	10	40	–	50	
6	70	20	50	–	30	–
7	85	35	50	–	15	–
8	95	55	40	–	5	–
9	100	75	–	25	–	–
10	–	100	–	–	–	Eosin
11	–	100	–	–	–	–
12	–	100	–	–	–	Paraffin oil

Note TBA: tert–butyl alcohol

*Equal of TBA: paraffin oil = 1: 1

For sample fixed in FAA II should be start step 5

Dehydrate tissue in each step for an hour to one day, depending on tissue size. Steps 1–5 should be at room temperature, steps 6–8 in the incubation oven at 56–60 °C

Preparation of tissue section slides

Xylene I	2	min	**Deparaffinization**
Xylene II	2	min	
Absolute ethanol : xylene	2	min	
Absolute ethanol I	2	min	**Hydration**
Absolute ethanol II	2	min	
Ethanol 95 % I	2	min	
Ethanol 95 % II	2	min	
Ethanol 70 % I	2	min	
Ethanol 70 % II	2	min	
Ethanol 50 % I	2	min	
Ethanol 50 % II	2	min	
Tap water	2	min	
Safranin	15	min	→ **Staining**
Tap water	2	min	
Picric acid in ethanol 95 %	5–10	sec	**Dehydration**
Ammonium hydroxide in ethanol 95 %	10 sec–1 min		
Absolute ethanol I	10	sec	
Absolute ethanol II	10	sec	
Used clove oil fast green	1–2	sec	**Staining**
Fast green	10–15	sec	
Used clove oil fast green	1–2	sec	
Absolute ethanol : xylene	2	min	**Clearing**
Xylene I	2	min	
Xylene II	2	min	
Mount			

Preparation of Safranin O and Fast Green staining solution

Staining & dye (Johansen's Safranin and Fast Green method)

Method involving additions to the stain (e.g. dehydrating and clearing agents) to enhance and differentiate tissue structure.

1) Safranin O ($C_{20}H_{19}N_4Cl$) (Ruzin, 1999)

Safranin O-brilliant red in chromosomes, nuclei, lignified, suberized, or cutinized cell walls

Safranin O	2	g
Methyl cellusolve	100	mL
(ethylene glycol monoethyl ether)		
Ethanol 95 %	50	mL
Sodium acetate	2	g
Formalin	4	mL

Transfer 100 mL of methyl cellusolve (ethylene glycol monoethyl ether) with cylinder to one vial into the beaker. Add 2 g of safranin O and stir on a magnetic stirrer for several hours to ensure that the solution has dissolved. Finally add 50 ml of 95 % ethanol, 2 g of sodium acetate and add 4 mL of formalin (use in hood).

2) Fast Green ($C_{37}H_{34}O_{10}N_2Na_2S_3$)

Fast Green–brilliant green in cytoplasm and cellulosic cell wall; blue to bluish-green in the stems and leaves of aquatic plants and most gymnosperms

Methyl cellusolve	100	mL
Absolute ethanol	100	mL
Cove oil	100	mL
Fast green	1.5	g

Take 100 mL of methyl cellosolve, absolute ethanol, clove oil and add 1.5 g of fast green. Stir for several hours to ensure that the solution has dissolved.

Note: It is recommended that the Safranin O solution is used within a month.

Transmission electron microscopy observation

Fixation reagents:

Pre−fixed solution

Glutaraldehyde	2.5 % (v/v)
Caffeine	1 % (w/v)
Phosphate buffer (pH 7.2)	0.1 M

Pre−fixed solution

Osmium tetroxide	1 %
Phosphate buffer (pH 7.2)	0.2 M

APPENDIX B

1. Preparation of 0.1 M Sodium phosphate buffer (pH 7.2)

Stock solution

1) Solution A

Sodium phosphate dibasic (Na_2HPO_4)	2.84	g
Distilled water	100	mL

2) Solution B

Sodium phosphate monobasic ($NaH_2PO_4.2H_2O$)	3.12	g
Distilled water	100	mL

0.2 M Sodium phosphate buffer

Mixing solution A with solution B

Solution A	72	mL
Solution B	28	mL

0.1 M Sodium phosphate buffer

0.2 M Sodium phosphate buffer	50	mL
Distilled water	50	mL

2. Preparation of solution for DNA extraction

CTAB buffer (Cetyltrimethyl ammonium bromide), 100 mL

PVP–40	1	g
$NaCl_2$	8.12	g
0.5 M Na_2EDTA (pH 8.0)	4	mL
1.0 M Tris–HCl (pH 8.0)	10	mL
CTAB	2	g

Adjust volume to 100 mL. Add 2 g of CTAB and heat at 65 °C until dissolved in a water bath. Sterilize by autoclaving. This solution is stable and can be stored indefinitely at room temperature. Just before use, add β–mercaptoethanol to a final concentration of 2 % in the buffer.

1.0 M Tris–HCl (pH 8.0)

Tris–HCl	121.1	g

Add 121.1 g of tris base was dissolved in 800 mL of distilled water, pH was adjusted to 8.0 with 1 N HCl and the volume was made up to 100 mL with distilled water.

TE buffer (Tris–EDTA), 500 mL

1.0 M Tris–HCl (pH 7.5)	500	µl
0.25M Na_2EDTA (pH 7.0)	200	µl

Deionizer water was added to make the final volume to 500 mL and sterilize by autoclaving.

3. Preparation of solution for agarose gel electrophoresis

5x TAE (Tris–Acetate Buffer)

Tris Base	121.1	g
Acetic acid	28.5	mL
0.5M Na_2EDTA (pH 8.0)	50	mL

Dissolve 121.1 g of Tris base, 28.5 mL of glacial acetic acid, and 100 mL of 0.5 M EDTA (pH 8.0) in H_2O up to 500 mL. The 50x TAE is the concentrated stock solution. Use 1x TAE as working solution.

5x TBE (Tris–Borate/EDTA buffer)

Tris base	216	g
Boric acid	110	g
0.5 M Na_2EDTA (pH 8.0)	80	mL

Dissolve 216.0 g of Tris base, 110.0 g of boric acid, and 80 mL of 0.5 M Na_2EDTA (pH 8.0) in H_2O up to 4 liter. Stir until dissolved. The 5x TBE is the concentrated stock solution. Use 0.5x TBE as electrophoresis buffer.

DNA sample buffer

Bromophenol blue	125	mg
Xylene cyanol FF	125	mg
Glycerol	15	mL

Dissolve 125 mg of Bromophenol blue, 125 mg of Xylene cyanol FF, and 15 mL of Glycerol in H_2O up to 50 mL. Sterilize by autoclaving.

Ethidium bromide solution (10 mg/mL)

Add 200 mg of ethidium bromide to 20 mL of H_2O. Stir on a magnetic stirrer for several hours to ensure that the dye has dissolved. Store in a light−proof container (e.g., in a falcon tube wrapped in aluminum foil) at room temperature.

4. Preparation of potato dextrose agar (PDA) (Rodesuchit, 1998)

Potato	200	g
Dextrose	20	g
Agar	17	g
Distilled water	1,000	mL

Table 2 *Hevea* germplasm used for the study.

Clone	**Pedigree**	**Source**
PB 5/51	PB 56 × PB 24	Malaysia
RRIM 600	Tjir 1× PB 86	Malaysia
GT 1	Primary clone	Malaysia

Table 3 Fragment size (bp) created by RAPD in each rubber seedlings.

Primer	Fragment size (bp)	DNA fragment							
		EIRpsu1	**EIRpsu2**	**EIRpsu3**	**EIRpsu4**	**EIRpsu5**	**EIRpark**	**EIRrak**	**RRIM 600**
OPAD-01	1,300	+	+	+	+	+	+	+	+
	1,250	+	+	+	+	+	+	+	+
	900	+	+	+	+	+	+	+	+
	800	+	+	+	+	+	+	+	+
	600	+	-	+	+	+	-	-	+
	550	+	+	+	+	+	+	+	+
	500	+	+	+	+	+	+	+	+
	420	+	+	+	+	+	+	+	+
	320	+	+	+	+	+	+	+	+
OPAD-10	1,500	+	+	+	+	+	+	+	+
	1,420	+	+	+	+	+	+	+	+
	1,390	+	+	+	+	+	+	+	+
	1,300	+	+	+	+	+	+	+	+
	1,290	+	+	+	+	+	+	+	+
	1,000	+	+	+	+	+	+	+	+
	810	+	+	+	+	+	+	+	+
	700	+	+	+	+	+	+	+	+
	600	+	+	+	+	+	+	+	+
	450	+	+	+	+	+	+	+	+
	400	+	+	+	+	+	+	+	+
	350	+	+	+	+	+	+	-	+
	300	+	+	+	+	+	+	+	+
	260	+	+	+	+	+	+	+	+
	250	+	+	+	+	+	+	+	+
OPAD-12	1,400	+	+	+	+	+	+	+	+
	1,100	+	+	+	+	+	+	+	+
	900	+	+	+	+	+	+	+	+

Notes + = Presence

- = Absence

Table 3 (Cont.) Fragment size (bp) created by RAPD in each rubber seedlings.

Primer	Fragment size (bp)	DNA fragment							
		EIRpsu1	**EIRpsu2**	**EIRpsu3**	**EIRpsu4**	**EIRpsu5**	**EIRpark**	**EIRrak**	**RRIM 600**
	800	+	+	+	+	+	+	+	+
	500	-	-	-	-	-	-	-	-
	480	+	+	+	+	+	+	+	+
	350	+	+	+	+	+	+	+	+
	290	+	+	+	+	+	+	+	+
OPAD-12	200	+	+	+	+	+	+	+	+
OPR-02	1,000	+	+	+	+	+	+	+	+
	890	+	+	+	+	+	+	+	+
	700	+	+	+	+	+	+	+	+
	550	-	-	-	+	-	-	+	-
	400	+	+	+	+	+	+	+	+
	350	+	+	+	+	+	+	+	+
	300	+	+	+	+	+	+	+	+
OPR-11	1,150	+	+	+	+	+	+	+	+
	1,000	+	+	+	+	+	+	+	+
	900	-	+	+	+	-	+	+	-
	800	+	+	+	+	+	+	+	+
	700	+	+	+	+	+	+	+	+
	600	-	-	-	-	-	-	-	-
	500	-	-	-	-	-	-	-	-
	450	+	+	+	+	+	+	+	+
OPZ-04	1,400	+	+	+	+	+	+	+	+
	1,250	+	+	+	+	+	+	+	+
	1,150	+	+	+	+	+	+	+	+
	1,100	+	+	+	+	+	+	+	+
	1,000	+	+	+	+	+	+	+	+
	900	-	-	-	-	-	-	-	-
	800	+	+	+	+	+	+	+	+
	650	+	+	+	+	+	+	+	+
	450	+	+	+	+	+	+	+	+
	350	+	+	+	+	+	+	+	+

Notes + = Presence

- = Absence

Table 3 (Cont.) Fragment size (bp) created by RAPD in each rubber seedlings.

Primer	Fragment size (bp)	DNA fragment							
		EIRpsu1	**EIRpsu2**	**EIRpsu3**	**EIRpsu4**	**EIRpsu5**	**EIRpark**	**EIRrak**	**RRIM 600**
OPB-17	1,500	+	+	+	+	+	+	+	+
	1,400	+	+	+	+	+	+	+	+
	1,250	+	+	+	-	-	-	-	+
	1,200	+	+	+	+	-	+	+	-
	1,000	+	+	+	+	+	+	+	+
	900	+	+	+	+	+	+	+	+
	650	+	+	+	+	+	+	+	+

Notes + = Presence

- = Absence

Table 4 Genetic similarity matrix of 48 individual rubber seedlings obtained from RAPD markers with 7 primers. Genetic distances were calculated on the basis of the Nei similarity index (Nei, 1972).

	RRIM 600 –1	**RRIM 600–2**	**RRIM 600–3**	**RRIM 600–4**	**RRIM 600–5**	**RRIM 600–6**	**EIR park–1**	**EIR park–2**	**EIR park–3**	**EIR park–4**	**EIR park–5**	**EIR park–6**
RRIM 600–1	1.000											
RRIM 600–2	0.954	1.000										
RRIM 600–3	0.954	1.000	1.000									
RRIM 600–4	0.985	0.938	0.938	1.000								
RRIM 600–5	0.969	0.985	0.985	0.954	1.000							
RRIM 600–6	0.985	0.969	0.969	0.969	0.985	1.000						
EIRpark–1	0.908	0.923	0.923	0.923	0.938	0.923	1.000					
EIRpark–2	0.938	0.954	0.954	0.954	0.969	0.954	0.969	1.000				
EIRpark–3	0.923	0.938	0.938	0.938	0.954	0.938	0.954	0.985	1.000			
EIRpark–4	0.938	0.954	0.954	0.923	0.969	0.954	0.938	0.969	0.954	1.000		
EIRpark–5	0.908	0.954	0.954	0.892	0.938	0.923	0.908	0.938	0.954	0.969	1.000	
EIRpark–6	0.938	0.923	0.923	0.954	0.938	0.954	0.938	0.969	0.985	0.938	0.938	1.000

Table 4 (Cont.) Genetic similarity matrix of 48 individual rubber seedlings obtained from RAPD markers with 7 primers. Genetic distances were calculated on the basis of the Nei similarity index (Nei, 1972).

	RRIM 600 –1	RRIM 600–2	RRIM 600–3	RRIM 600–4	RRIM 600–5	RRIM 600–6	EIR park–1	EIR park–2	EIR park–3	EIR park–4	EIR park–5	EIR park–6
EIRrak–1	0.969	0.954	0.954	0.954	0.969	0.985	0.908	0.938	0.923	0.938	0.908	0.938
EIRrak–2	0.938	0.923	0.923	0.923	0.938	0.954	0.877	0.908	0.923	0.938	0.938	0.938
EIRrak–3	0.954	0.969	0.969	0.938	0.985	0.969	0.923	0.954	0.938	0.954	0.923	0.923
EIRrak–4	0.954	0.969	0.969	0.938	0.985	0.969	0.923	0.954	0.938	0.954	0.923	0.923
EIRrak–5	0.969	0.954	0.954	0.954	0.969	0.985	0.908	0.938	0.923	0.938	0.908	0.938
EIRrak–6	0.938	0.954	0.954	0.923	0.969	0.954	0.938	0.938	0.923	0.938	0.908	0.908
EIRpsu 2–1	0.923	0.969	0.969	0.908	0.954	0.938	0.923	0.923	0.908	0.923	0.923	0.892
EIRpsu 2–2	0.908	0.954	0.954	0.892	0.938	0.923	0.908	0.908	0.892	0.938	0.938	0.877
EIRpsu 2–3	0.923	0.969	0.969	0.908	0.954	0.938	0.923	0.923	0.908	0.923	0.923	0.892
EIRpsu 2–4	0.923	0.969	0.969	0.908	0.954	0.938	0.923	0.923	0.908	0.923	0.923	0.892
EIRpsu 2–5	0.908	0.954	0.954	0.892	0.938	0.923	0.908	0.908	0.892	0.938	0.938	0.877
EIRpsu 2–6	0.908	0.954	0.954	0.892	0.938	0.923	0.908	0.908	0.892	0.938	0.938	0.877

Table 4 (Cont.) Genetic similarity matrix of 48 individual rubber seedlings obtained from RAPD markers with 7 primers. Genetic distances were calculated on the basis of the Nei similarity index (Nei, 1972).

	RRIM 600 –1	RRIM 600–2	RRIM 600–3	RRIM 600–4	RRIM 600–5	RRIM 600–6	EIR park–1	EIR park–2	EIR park–3	EIR park–4	EIR park–5	EIR park–6
EIRpsu 1–1	0.906	0.938	0.938	0.891	0.938	0.922	0.906	0.906	0.922	0.906	0.922	0.906
EIRpsu 1–2	0.891	0.922	0.922	0.906	0.922	0.906	0.922	0.922	0.906	0.922	0.906	0.891
EIRpsu 1–3	0.938	0.969	0.969	0.953	0.969	0.953	0.969	0.969	0.953	0.938	0.922	0.938
EIRpsu 1–4	0.938	0.969	0.969	0.922	0.969	0.953	0.938	0.938	0.922	0.969	0.953	0.906
EIRpsu 1–5	0.908	0.954	0.954	0.892	0.938	0.923	0.908	0.908	0.892	0.908	0.908	0.877
EIRpsu 1–6	0.938	0.954	0.954	0.923	0.969	0.954	0.938	0.938	0.923	0.938	0.908	0.908
EIRpsu 3–1	0.923	0.969	0.969	0.908	0.954	0.938	0.923	0.923	0.938	0.923	0.954	0.923
EIRpsu 3–2	0.954	0.969	0.969	0.938	0.985	0.969	0.954	0.954	0.938	0.954	0.923	0.923
EIRpsu 3–3	0.938	0.954	0.954	0.923	0.969	0.954	0.938	0.938	0.954	0.938	0.938	0.938
EIRpsu 3–4	0.938	0.954	0.954	0.923	0.969	0.954	0.938	0.938	0.954	0.938	0.938	0.938
EIRpsu 3–5	0.954	0.938	0.938	0.938	0.954	0.969	0.923	0.923	0.938	0.923	0.923	0.954
EIRpsu 3–6	0.969	0.923	0.923	0.954	0.938	0.954	0.908	0.908	0.923	0.908	0.908	0.938

Table 4 (Cont.) Genetic similarity matrix of 48 individual rubber seedlings obtained from RAPD markers with 7 primers. Genetic distances were calculated on the basis of the Nei similarity index (Nei, 1972).

	RRIM 600 –1	RRIM 600–2	RRIM 600–3	RRIM 600–4	RRIM 600–5	RRIM 600–6	EIR park–1	EIR park–2	EIR park–3	EIR park–4	EIR park–5	EIR park–6
EIRpsu 5–1	0.938	0.923	0.923	0.954	0.938	0.954	0.938	0.938	0.923	0.938	0.908	0.938
EIRpsu 5–2	0.923	0.969	0.969	0.908	0.954	0.938	0.923	0.923	0.908	0.954	0.954	0.892
EIRpsu 5–3	0.923	0.969	0.969	0.908	0.954	0.938	0.923	0.923	0.908	0.923	0.923	0.892
EIRpsu 5–4	0.954	0.938	0.938	0.938	0.954	0.969	0.923	0.923	0.908	0.923	0.892	0.923
EIRpsu 5–5	0.908	0.923	0.923	0.923	0.908	0.923	0.938	0.908	0.923	0.877	0.908	0.938
EIRpsu 5–6	0.954	0.969	0.969	0.938	0.954	0.969	0.923	0.923	0.908	0.923	0.923	0.923
EIRpsu 4–1	0.923	0.938	0.938	0.908	0.923	0.938	0.892	0.892	0.908	0.892	0.923	0.923
EIRpsu 4–2	0.938	0.954	0.954	0.923	0.938	0.954	0.908	0.908	0.892	0.908	0.908	0.908
EIRpsu 4–3	0.938	0.954	0.954	0.923	0.938	0.954	0.908	0.908	0.892	0.908	0.908	0.908
EIRpsu 4–4	0.954	0.938	0.938	0.938	0.954	0.969	0.923	0.923	0.908	0.923	0.892	0.923
EIRpsu 4–5	0.923	0.938	0.938	0.908	0.923	0.938	0.892	0.892	0.877	0.923	0.923	0.892
EIRpsu 4–6	0.954	0.969	0.969	0.938	0.954	0.969	0.923	0.923	0.908	0.923	0.923	0.923

Table 4 (Cont.) Genetic similarity matrix of 48 individual rubber seedlings obtained from RAPD markers with 7 primers. Genetic distances were calculated on the basis of the Nei similarity index (Nei, 1972).

	EIRrak–1	**EIRrak–2**	**EIRrak–3**	**EIRrak–4**	**EIRrak–5**	**EIRrak–6**	**EIRpsu 2–1**	**EIRpsu 2–2**	**EIRpsu 2–3**	**EIRpsu 2–4**	**EIRpsu 2–5**	**EIRpsu 2–6**
EIRrak–1	1.000											
EIRrak–2	0.969	1.000										
EIRrak–3	0.985	0.954	1.000									
EIRrak–4	0.985	0.954	1.000	1.000								
EIRrak–5	1.000	0.969	0.985	0.985	1.000							
EIRrak–6	0.969	0.938	0.985	0.985	0.969	1.000						
EIRpsu 2–1	0.954	0.923	0.969	0.969	0.954	0.985	1.000					
EIRpsu 2–2	0.938	0.938	0.954	0.954	0.938	0.969	0.985	1.000				
EIRpsu 2–3	0.954	0.923	0.969	0.969	0.954	0.985	1.000	0.985	1.000			
EIRpsu 2–4	0.954	0.923	0.969	0.969	0.954	0.985	1.000	0.985	1.000	1.000		
EIRpsu 2–5	0.938	0.938	0.954	0.954	0.938	0.969	0.985	1.000	0.985	0.985	1.000	
EIRpsu 2–6	0.938	0.938	0.954	0.954	0.938	0.969	0.985	1.000	0.985	0.985	1.000	1.000

Table 4 (Cont.) Genetic similarity matrix of 48 individual rubber seedlings obtained from RAPD markers with 7 primers. Genetic distances were calculated on the basis of the Nei similarity index (Nei, 1972).

	EIRrak–1	**EIRrak–2**	**EIRrak–3**	**EIRrak–4**	**EIRrak–5**	**EIRrak–6**	**EIRpsu 2–1**	**EIRpsu 2–2**	**EIRpsu 2–3**	**EIRpsu 2–4**	**EIRpsu 2–5**	**EIRpsu 2–6**
EIRpsu 1–1	0.938	0.938	0.953	0.953	0.938	0.969	0.969	0.953	0.969	0.969	0.953	0.953
EIRpsu 1–2	0.922	0.922	0.938	0.938	0.922	0.953	0.953	0.969	0.953	0.953	0.969	0.969
EIRpsu 1–3	0.938	0.906	0.953	0.953	0.938	0.969	0.969	0.953	0.969	0.969	0.953	0.953
EIRpsu 1–4	0.938	0.938	0.953	0.953	0.938	0.969	0.969	0.984	0.969	0.969	0.984	0.984
EIRpsu 1–5	0.938	0.908	0.954	0.954	0.938	0.969	0.985	0.969	0.985	0.985	0.969	0.969
EIRpsu 1–6	0.969	0.938	0.985	0.985	0.969	1.000	0.985	0.969	0.985	0.985	0.969	0.969
EIRpsu 3–1	0.923	0.923	0.938	0.938	0.923	0.954	0.969	0.954	0.969	0.969	0.954	0.954
EIRpsu 3–2	0.954	0.923	0.969	0.969	0.954	0.985	0.969	0.954	0.969	0.969	0.954	0.954
EIRpsu 3–3	0.938	0.938	0.954	0.954	0.938	0.969	0.954	0.938	0.954	0.954	0.938	0.938
EIRpsu 3–4	0.938	0.938	0.954	0.954	0.938	0.969	0.954	0.938	0.954	0.954	0.938	0.938
EIRpsu 3–5	0.954	0.954	0.938	0.938	0.954	0.954	0.938	0.923	0.938	0.938	0.923	0.923
EIRpsu 3–6	0.938	0.938	0.923	0.923	0.938	0.938	0.923	0.908	0.923	0.923	0.908	0.908

Table 4 (Cont.) Genetic similarity matrix of 48 individual rubber seedlings obtained from RAPD markers with 7 primers. Genetic distances were calculated on the basis of the Nei similarity index (Nei, 1972).

	EIRrak–1	**EIRrak–2**	**EIRrak–3**	**EIRrak–4**	**EIRrak–5**	**EIRrak–6**	**EIRpsu 2–1**	**EIRpsu 2–2**	**EIRpsu 2–3**	**EIRpsu 2–4**	**EIRpsu 2–5**	**EIRpsu 2–6**
EIRpsu 2–1	0.938	0.938	0.923	0.923	0.938	0.938	0.923	0.938	0.923	0.923	0.938	0.938
EIRpsu 2–2	0.923	0.923	0.938	0.938	0.923	0.954	0.969	0.985	0.969	0.969	0.985	0.985
EIRpsu 2–3	0.954	0.923	0.969	0.969	0.954	0.985	1.000	0.985	1.000	1.000	0.985	0.985
EIRpsu 2–4	0.954	0.923	0.938	0.938	0.954	0.954	0.938	0.923	0.938	0.938	0.923	0.923
EIRpsu 2–5	0.908	0.908	0.892	0.892	0.908	0.908	0.923	0.908	0.923	0.923	0.908	0.908
EIRpsu 2–6	0.954	0.923	0.938	0.938	0.954	0.954	0.969	0.954	0.969	0.969	0.954	0.954
EIRpsu 4–1	0.954	0.954	0.938	0.938	0.954	0.954	0.969	0.954	0.969	0.969	0.954	0.954
EIRpsu 4–2	0.969	0.938	0.954	0.954	0.969	0.969	0.985	0.969	0.985	0.985	0.969	0.969
EIRpsu 4–3	0.969	0.938	0.954	0.954	0.969	0.969	0.985	0.969	0.985	0.985	0.969	0.969
EIRpsu 4–4	0.985	0.954	0.969	0.969	0.985	0.985	0.969	0.954	0.969	0.969	0.954	0.954
EIRpsu 4–5	0.954	0.954	0.938	0.938	0.954	0.954	0.969	0.985	0.969	0.969	0.985	0.985
EIRpsu 4–6	0.954	0.923	0.938	0.938	0.954	0.954	0.969	0.954	0.969	0.969	0.954	0.954

Table 4 (Cont.) Genetic similarity matrix of 48 individual rubber seedlings obtained from RAPD markers with 7 primers. Genetic distances were calculated on the basis of the Nei similarity index (Nei, 1972).

	EIR psu 1–1	**EIR psu 1–2**	**EIR psu 1–3**	**EIR psu 1–4**	**EIR psu 1–5**	**EIR psu 1–6**	**EIR psu 3–1**	**EIR psu 3–2**	**EIR psu 3–3**	**EIR psu 3–4**	**EIR psu 3–5**	**EIR psu 3–6**
EIRpsu 1–1	1.000											
EIRpsu 1–2	0.953	1.000										
EIRpsu 1–3	0.938	0.953	1.000									
EIRpsu 1–4	0.938	0.953	0.969	1.000								
EIRpsu 1–5	0.984	0.969	0.953	0.953	1.000							
EIRpsu 1–6	0.969	0.953	0.969	0.969	0.969	1.000						
EIRpsu 3–1	0.969	0.922	0.969	0.969	0.954	0.954	1.000					
EIRpsu 3–2	0.953	0.938	0.984	0.984	0.954	0.985	0.969	1.000				
EIRpsu 3–3	0.969	0.922	0.969	0.969	0.938	0.969	0.985	0.985	1.000			
EIRpsu 3–4	0.969	0.922	0.969	0.969	0.938	0.969	0.985	0.985	1.000	1.000		
EIRpsu 3–5	0.953	0.906	0.953	0.953	0.923	0.954	0.969	0.969	0.985	0.985	1.000	
EIRpsu 3–6	0.938	0.891	0.938	0.938	0.908	0.938	0.954	0.954	0.969	0.969	0.985	1.000

Table 4 (Cont.) Genetic similarity matrix of 48 individual rubber seedlings obtained from RAPD markers with 7 primers. Genetic distances were calculated on the basis of the Nei similarity index (Nei, 1972).

	EIR psu 1–1	EIR psu 1–2	EIR psu 1–3	EIR psu 1–4	EIR psu 1–5	EIR psu 1–6	EIR psu 3–1	EIR psu 3–2	EIR psu 3–3	EIR psu 3–4	EIR psu 3–5	EIR psu 3–6
EIRpsu 5–1	0.906	0.953	0.969	0.969	0.908	0.938	0.923	0.954	0.938	0.938	0.954	0.938
EIRpsu 5–2	0.938	0.953	0.969	1.000	0.954	0.954	0.969	0.969	0.954	0.954	0.938	0.923
EIRpsu 5–3	0.969	0.953	0.969	0.969	0.985	0.985	0.969	0.969	0.954	0.954	0.938	0.923
EIRpsu 5–4	0.953	0.938	0.953	0.953	0.954	0.954	0.938	0.969	0.954	0.954	0.969	0.954
EIRpsu 5–5	0.922	0.906	0.953	0.922	0.908	0.908	0.954	0.923	0.938	0.938	0.954	0.938
EIRpsu 5–6	0.938	0.922	0.969	0.969	0.954	0.954	0.969	0.969	0.954	0.954	0.969	0.954
EIRpsu 4–1	0.969	0.922	0.938	0.938	0.954	0.954	0.969	0.938	0.954	0.954	0.969	0.954
EIRpsu 4–2	0.953	0.938	0.953	0.953	0.969	0.969	0.954	0.954	0.938	0.938	0.954	0.938
EIRpsu 4–3	0.953	0.938	0.953	0.953	0.969	0.969	0.954	0.954	0.938	0.938	0.954	0.938
EIRpsu 4–4	0.953	0.938	0.953	0.953	0.954	0.985	0.938	0.969	0.954	0.954	0.969	0.954
EIRpsu 4–5	0.938	0.953	0.938	0.969	0.954	0.954	0.938	0.938	0.923	0.923	0.938	0.923
EIRpsu 4–6	0.938	0.922	0.969	0.969	0.954	0.954	0.969	0.969	0.954	0.954	0.969	0.954

Table 4 (Cont.) Genetic similarity matrix of 48 individual rubber seedlings obtained from RAPD markers with 7 primers. Genetic distances were calculated on the basis of the Nei similarity index (Nei, 1972).

	EIR psu 5–1	**EIR psu 5–2**	**EIR psu 5–3**	**EIR psu 5–4**	**EIR psu 5–5**	**EIR psu 5–6**	**EIR psu 4–1**	**EIR psu 4–2**	**EIR psu 4–3**	**EIR psu 4–4**	**EIR psu 4–5**	**EIR psu 4–6**
EIRpsu 5–1	1.000											
EIRpsu 5–2	0.954	1.000										
EIRpsu 5–3	0.923	0.969	1.000									
EIRpsu 5–4	0.954	0.938	0.938	1.000								
EIRpsu 5–5	0.938	0.923	0.923	0.923	1.000							
EIRpsu 5–6	0.954	0.969	0.969	0.969	0.954	1.000						
EIRpsu 4–1	0.923	0.938	0.969	0.938	0.954	0.969	1.000					
EIRpsu 4–2	0.938	0.954	0.985	0.954	0.938	0.985	0.985	1.000				
EIRpsu 4–3	0.938	0.954	0.985	0.954	0.938	0.985	0.985	1.000	1.000			
EIRpsu 4–4	0.954	0.938	0.969	0.969	0.923	0.969	0.969	0.985	0.985	1.000		
EIRpsu 4–5	0.954	0.969	0.969	0.938	0.923	0.969	0.969	0.985	0.985	0.969	1.000	
EIRpsu 4–6	0.954	0.969	0.969	0.969	0.954	1.000	0.969	0.985	0.985	0.969	0.969	1.000

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2. The Scholarship for Ph.D. Program from the Higher Education Research Promotion and National Research University Project of Thailand, Office of the Higher Education Commission (2011–2013).
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List of Publication and Proceeding

Publication

Wattanasilakorn, S., S. Sdoodee, C. Nualsri and S. Chuenchit. 2011. Preliminary test of the white root disease tolerance in early introduced clones of rubber tree. Agricultural Science Journal 42 3/1 (suppl): 311–314. [in Thai].

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Wattanasilakorn, S., S. Sdoodee, C. Nualsri and B. Bunratchoo. 2015. Screening of rubber rootstock by the assessment of root growth and genetic background. Kasetsart Journal (Natural Science) 49: 821–831.

Wattanasilakorn, S., S. Sdoodee, C. Nualsri, S. Chuenchit, U. Meesawat and J. Sopharat. 2017. Assessment of rubber clonal rootstocks for the tolerance of white root disease (*Rigidoporus microporus*) in southern Thailand. Walailak Journal of Science and Technology : (in press).

Proceeding

Wattanasilakorn, S. Sdoodee, S. Chuenchit and C. Nualsri. 2012. Selection of rubber (*Hevea brasiliensis* Muell. Arg.) rootstocks for the white root disease resistance. The 5th AGBIO /PERDO Graduate Conference on Agricultural Biotechnology and KU–UT Joint Seminar II, Kasetsart University, Kamphaeng Saen Campus, Nakhon Pathom, Thailand, 8–9 December 2012, pp. 9.