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Screening of Rubber Rootstock by the Assessment of Root Growth and Genetic Background

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ABSTRACT

Rubber seedlings from two early-introduced clones (EIRpsu 1 and EIRpsu 2) in southern Thailand were evaluated for root growth performance under field conditions. The two clones had been screened for white root disease tolerance. Genetic analysis of EIRpsu 1 and EIRpsu 2 was compared to RRIM 600 (the major cultivated variety of *Hevea* rubber in Thailand) using random amplified polymorphic DNA markers with 10 primers. Results from cluster analysis indicated that EIRpsu 2 was closer to RRIM 600 than EIRpsu 1 with similarity coefficients of 0.850, 0.860 and 0.890, respectively. Root growth of EIRpsu1 and EIRpsu2 was monitored at a rubber plantation in Langsuan district, Chumphon province during 2012–2013. Six-month-old seedlings of each clone were transplanted into a rhizobox. The experiment was designed as a randomized complete block design with four replications, with one plant per rhizobox. Shoot growth and root proliferation of the seedlings were recorded at two-monthly intervals from October 2012 to March 2013. The shoot growth was monitored by measuring plant height, trunk diameter and the number of compound leaves. It was found that RRIM 600 exhibited the highest shoot growth under field conditions, and it was significantly different from the other two clones. The root length density was measured by scanning the root systems of each seedling from a panel in the rhizobox. Results showed that the seedlings of EIRpsu 1 and EIRpsu 2 had better root growth than RRIM 600. The spatial distribution of roots indicated a rather deeper root system for the two selected clones.

Keywords: random amplified polymorphic DNA (RAPD) markers, root dynamic, distribution, rhizobox, rubber rootstock

INTRODUCTION

The rubber tree (*Hevea brasiliensis* Muell. Arg.) is a major crop in Thailand and it is also the most important economic crop in Southeast Asia (Gianessi and Williams, 2011). In the past, the most common rootstocks for planting material production in Thailand were seeds collected from any early-introduced clone. Nowadays, RRIM 600 is the major cultivated variety of *Hevea* rubber in Thailand, constituting about 80% of plantings (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

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germplasm (Priyadarshan and Goncalves, 2003). Hence, 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). 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). Wherever rubber is grown, it is threatened by white root disease, particularly, in Southern Thailand. (Prasetyo *et al*., 2009). Seedlings of two rubber clones (EIRpsu1 and EIRpsu2) have reported tolerance to the white root disease (Wattanasilakorn *et al*., 2012).No prior genetic background information of these two clones has been reported.

Several methods have been used to study rubber's genetic variability (Feng *et al*., 2012), including isoenzyme analysis and other molecular techniques such as restriction fragment length polymorphisms (RFLPs), random amplified polymorphic DNA (RAPD) and simple sequence repeat (SSR). These studies have revealed a degree of clustering according to geographical origin in wild and clonally selected populations. The RAPD technique has already been applied in research with several aims (Hernández *et al*., 2006). RAPD has been used to determine genetic relationships for several plant species including the rubber tree (Nurhaimi - Haris and Darusamin, 1998; Venkatachalam *et al*., 2002; Nakkanong *et al*., 2008; Oktavia *et al*., 2011). Khonglao (2006) reported that there was more vigorous root development of early-introduced clones than in RRIM 600. Nowadays, almost all of the early-introduced clones have been gradually lost because of replanting with elite clones and about 75% of rubber production grown in Thailand uses RRIM 600. Consequently, seedlings for current rootstock come from the RRIM 600 clone that is susceptible to the white root disease. Furthermore, the ability of seedlings as elite rubber clones for rootstock must be investigated.

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, such as soil core sampling, in growth core measurements, monolith construction, excavation and minirhizotrons have been used for root development information (Caldwell and Virginia, 1989). Important information about root systems by those methods, usually involves harvesting roots from the soil. These methods are destructive, time consuming and labor intensive and they cannot be used for time course studies and in addition, root loss is unavoidable, especially for fine roots during the root harvesting and soil excavation/washing processes (Doussean *et al*., 2006). A transparent-wall technique for observation of plant root growth dynamics in a rhizobox is another option to investigate root development. Rhizobox observations are nondestructive, allow repeated observations of roots to measure root elongation, branching and turn-over, as well as allowing study of the 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. Translating qualitative information from the recorded images to quantitative data is a tedious, time-consuming process.

In this study, a scanner-based technique was developed that allowed the direct capture of still, digital, root images in conjunction with a simple and quick method for root measurement using a computer image analysis system. Measurements were made on rubber trees grown in the rhizobox. The advantages of this technique over a digital rhizotron include its ability to estimate rootstock effects on the distribution of root length density of the rubber tree and roots for physiological measurements. Therefore, the objectives of study were to assess genetic information of selected rubber clones for rootstock using the RAPD technique and to monitor their

root system using a rhizobox.

MATERIALS AND METHODS

Experimental materials

Seedlings from the early-introduced rubber clones—EIRpsu 1, and EIRpsu 2—were used in this study. EIRpsu 1, and EIRpsu 2 had been evaluated for white root disease tolerance under greenhouse conditions. The collection locations are indicated in Table 1. Seedlings of RRIM 600 were included as controls. Six-month-old rubber seedlings were grown in rhizoboxes. The experiment was designed as a randomized complete block design with four replications, one plant per each rhizobox. Each seedling (1 month old) was grown in a rhizobox (30 × 90 × 8 cm) as shown in Figure 1.

The experiment was conducted during October 2012 to May 2013 at a rubber plantation, in Langsuan district, Chumphon province, Thailand (9° 56′ 42″ N, 99° 4′ 42″ E). The soil texture was analyzed using standard laboratory procedures (Onthong, 2002). The soil type of the site was sandy clay loam and the pH (5.31) of the soil was acidic.

Table 1 Collection locations of early-introduced clones

Treatment	Geographic coordinates	Place of collection
EIRpsu 1	7° 0′ 23.1″ N 100° 29′ 52.8″ E	Prince of Songkla University, Hat Yai, Songkhla
EIRpsu 2	7° 0′ 31.7″ N 100° 29′ 40.3″ E	Prince of Songkla University, Hat Yai, Songkhla
RRIM 600	6° 53′ 57″ N 100° 23′ 19″ E	Thunglung rubber plantation, Songkhla

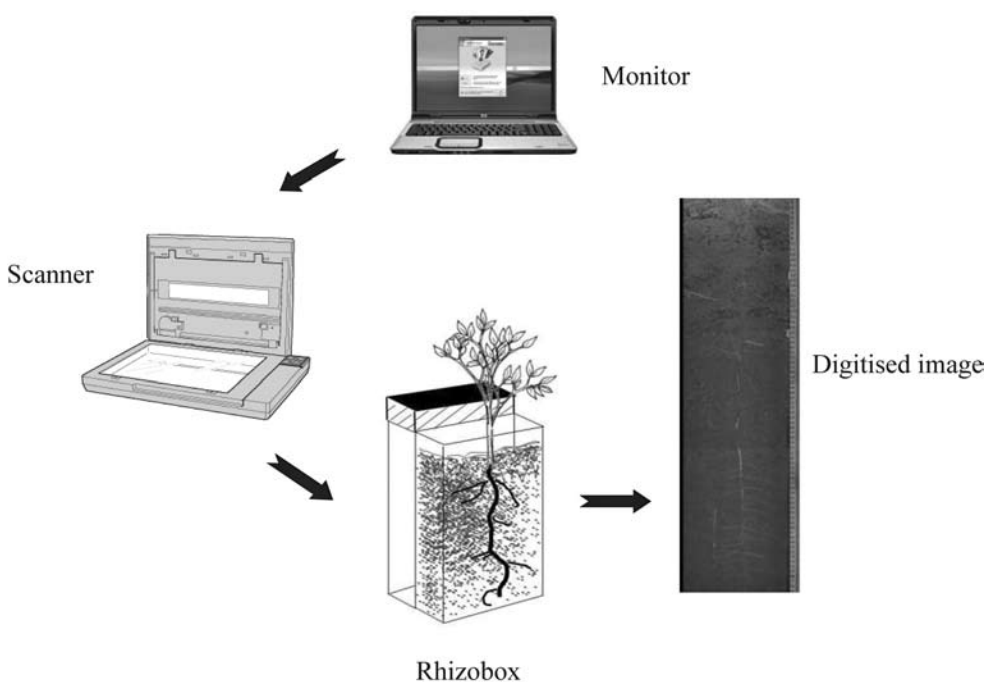

Figure 1 Rhizobox used for root investigation in the field and image acquisition system.

DNA extraction and random amplified polymorphic DNA protocol

DNA extractions were performed according to the procedure modified by Doyle and Doyle (1990). Young leaves were macerated to a fine power 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). Ten RAPD primers (OPAD–01, OPAD–10, OPAD–12, OPR–02, OPR–11, OPZ–04, OPB–17, OPN–08, OPC–05 and OPB–12) were used for RAPD-PCR reactions. The reaction was performed in a total volume of 25 µL containing 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 occurred in a thermal cycler, starting at 94 ^oC for 2 min and then subjected to 41 repeats of the following cycle: 94 ^oC for 30 s, 37 ^oC for 1 min, 72 ^oC for 2 min and finally 72 ^oC for 5 min.

All amplification products were analyzed using electrophoresis in 1.5% (weight per volume) 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.

Data analysis

The DNA fragments generated by RAPD-PCR were analyzed by determining presence (1) or absence (0). Based on polymorphic DNA fragments, genetic distances were estimated based on Nei and Li (1979) and a dendrogram was constructed using the unweighted pair group method with arithmetic average (UPGMA) method in the computer package NTSYSpc 2.1 (Rohlf, 1998).

Rhizobox and plant culture

Six month-old seedlings of the screened rubber clones and RRIM 600 were used as the experimental materials; the collection locations are shown in Table 1. Uniform seedlings were selected according to average height and trunk diameter. Open-top rhizoboxes (30 × 90 × 9 cm and 30 × 100 × 8 cm) were constructed from 5 mm-thick acrylic sheet (Figure 2). Seedlings were planted in the center of each rhizobox and the rhizoboxes were then placed under the soil. All plants were grown under normal field conditions for 6 months.

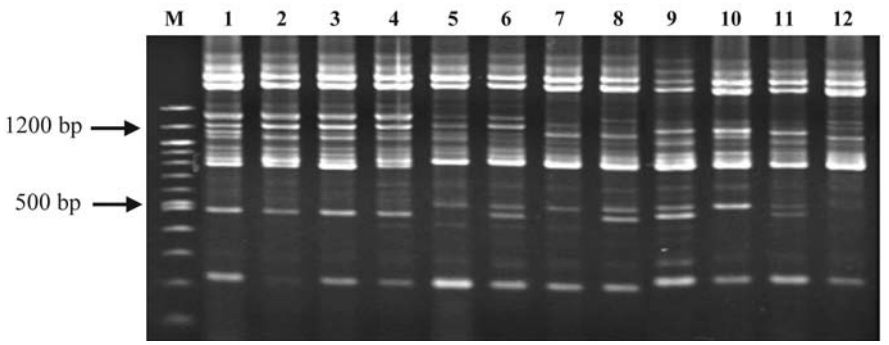

Figure 2 Random amplified polymorphic DNA patterns of rootstock from: Prince of Songkla University, Hat Yai, Songkhla. (EIRpsu 1) (lanes1–4); Prince of Songkla University, Hat Yai, Songkhla (EIRpsu 2) (lanes 5–8); and RRIM600 (lanes 9–12) amplified by primer OPB–17. Lane M = 100 bp ladder.

The vertical distribution of roots in the rhizobox at soil depths of 0–19, 20–39, 40–59 and 60–80 cm were determined.

Experimental design and data analysis

The study was conducted in a randomized complete block design with four replications. Each treatment was grown in a separate rhizobox. Analysis of variance on data obtained was performed using the R Gui software (version 2.12.0). A least significant difference (LSD) test at $P < 0.05$ was employed for mean comparisons. The following data were recorded:

Root Growth: on the transparent wall of the rhizobox was periodically measured manually and the digital root images were collected with an adapted color image scanner (CanoScan LiDE110; Canon U.S.A. Inc.; Melville, NY, USA), as shown in Figure 1. The total length of the sample roots was measured using the Image Rootfly Software—a free, open-source software application to aid researchers in rhizobox image analysis under a GNU General Public License. The length, diameter and color of roots, as well as living and mortality rates were recorded. All the experimental data were stored in a single RFY file.

Height: was measured at 10 cm from the soil level to the top of the plant shoot.

Trunk diameter: was measured 10 cm from the soil level.

Number of compound leaves: was determined by counting the number of compound leaves per plant.

RESULTS AND DISCUSSION

Random amplified polymorphic DNA analysis

The genetic relationships among the early-introduced clone populations and RRIM 600 were determined by the polymorphism of DNA fragment patterns using RAPD. Results are presented in Table 2 and indicate low genetic variation in each population. The similarity coefficient for the three sets of seedlings of EIRpsu 1, EIRpsu 2 and RRIM 600 were 0.890, 0.850 and 0.860, respectively. The polymorphisms showed different characters among the clones and can be used to determine genetic relationships among the analyzed clones. Examples of the amplification of RAPD markers are shown in Figure 2.

Table 2 Primers producing polymorphic DNA bands in random amplified polymorphic DNA patterns of rubber clones in the present study.

Primer	Sequence (5' → 3')	Amplified fragments	Monomorphic fragments	Polymorphic fragments	Polymorphism (%)
OPR–11	GTAGCCGTCT	6	3	3	50.00
OPAD–01	CAAAGGGCGG	14	7	7	50.00
OPAD–12	AAGAGGGCGT	9	3	6	66.67
OPAD–10	AAGAGGCCAG	14	6	8	57.14
OPB–17	AGGGAACGAG	11	7	4	36.36
OPR–02	CACAGCTGCC	8	3	5	62.50
OPZ–04	AGGCTGTGCT	10	5	5	50.00
OPN–08	ACCTCAGCTC	8	6	2	25.00
OPC–05	GATGACCGCC	12	7	5	41.67
OPB–12	CCTTGACGCA	13	6	7	52.85
Total		105	53	52	

Cluster analysis revealed a closer relationship between EIRpsu 2 and RRIM 600 than for EIRpsu 1.

The results showed that EIRpsu 1 and EIRpsu 2 were clones originating from seed of early-introduced rubber clones from Malasia almost 80 years ago. Khonglao (2006) reported that in the past, most rootstock for high yielding rubber trees came from seedlings of early-introduced clones. It is important to select for rubber clone tolerance to white root from the early-introduced clone population and there was no prior knowledge of the genetic background of either clone. Therefore, the RAPD technique was useful for detecting genetic variations within and among the populations (Nayanakantha *et al*., 2010). However, detailed morphological study is also desirable in order to understand all aspects of this variation.

Shoot-root development

Shoot development

All shoot growth parameters in the three clones responded differently with interactive effects for all growth parameters, and the growth response to season differed among clones. Table 3 shows the differences in the development of rubber shoots by the number of compound leaves, plant height and trunk diameter at 10 cm from the soil surface for the rubber seedlings. The data obtained in this experiment indicated that the shoots among the rubber clones were significantly different. In comparing the growth response of EIRpsu 1, EIRpsu 2, and RRIM 600, RRIM 600 had higher values that were statistically significant for the average number of compound leaves, plant height and trunk diameter than the other clones at March 2013. In addition, it was found that seedlings of EIRpsu 1, EIRpsu 2 and RRIM 600 decreased their numbers of compound leaves from January to March because of the dry period. Nahar and Gretzmacher (2011) reported the response of shoot growth parameters in different cultivars with increasing stress periods.

Table 3 Growth and development of rubber seedling tree in the three treatments.

Treatment	Trunk diameter (cm)			Height (cm)			Number of compound leaves		
	Nov	Jan	Mar	Nov	Jan	Mar	Nov	Jan	Mar
EIRpsu 1	0.71^{ab}	0.88^{ab}	0.97^{a}	72.23	85.00	90.40^{b}	16.50	15.25^{a}	12.75^{a}
EIRpsu 2	0.60^{b}	0.68^{b}	0.74^{b}	82.45	73.05	92.50^{ab}	12.25	9.75^{b}	5.50^{b}
RRIM 600	0.79^{a}	0.97^{a}	1.04^{a}	95.05	95.88	115.60^{a}	18.00	14.50^{a}	9.00^{ab}
F-test	*	*	*	ns	ns	*	ns	*	*
CV (%)	16.37	13.82	13.76	18.80	18.89	14.41	19.87	13.69	41.72
$LSD_{0.05}$	0.20	0.20	0.22	27.08	27.66	24.81	5.36	3.12	6.56

Means with the same lowercase letter in each column are not significantly different by least significant difference test at $P < 0.05$ ($LSD_{0.05}$); ns = Not significantly different at $P > 0.05$; * = Significantly different at $P \leq 0.05$; CV = Coefficient of variation.

Root development

Figure 4 shows rubber root development at a depth of 0–80 cm during the experimental period. Figure 5 shows the different root profiles among seedlings of EIRpsu 1, EIPpsu 2 and RRIM 600 with regard to root growth dynamics. The dry period was during February and March with high evaporation and high temperature. Seedlings of EIRpsu 1 and EIRpsu 2 developed deeper roots at 60–80 cm in the soil profile and their root distribution in the subsoil was greater than for the RRIM 600 clone. The rooting density was significantly higher for EIRpsu 2 (40–59 cm) than for EIRpsu 1 and RRIM 600. The high root distribution values of EIRpsu 1 and EIRpsu 2 were 0.773 and 0.839 $cm.cm^{-2}$, and the lowest root length density was observed at the 40–59 cm and 0–19 cm depths, respectively, while the lowest root length density of RRIM 600 was observed at a depth of 40–59 cm (0.772 $cm.cm^{-2}$) and the highest root distribution was at 20–39 cm soil depth (0.836 $cm.cm^{-2}$). The results revealed that among clones (Figure 6), EIRpsu 2 exhibited the highest total root length density during November 2012–March 2013. Liedgens *et al*. (2000) reported that the root density increased to a maximum with time and then decreased under all conditions. Hamblin (1985) suggested that root development in any plant is governed by various factors such as nutrient availability, soil physical properties and genetic characters. From the results, it was concluded that seedlings of EIRpsu 2 and EIRpsu 1 had better root growth than seedlings of RRIM 600. The results showed that the two selected clones exhibited good performance of tentative tolerance to white root disease and had a high proliferation of deep roots. Furthermore, they showed a similar genetic background to clone RRIM 600. This may lead to good compatibility when grafted with the RRIM 600 clone as a scion. A correlation between the genetic relationship and graft compatibility has been suggested for grafting of peach-peach (Bregger,1948). Schmitling (1991) studied *Pinus taeda* and reported that genetic relatedness between the scion and rootstock enhanced the survival of clones with incompatibility problems.

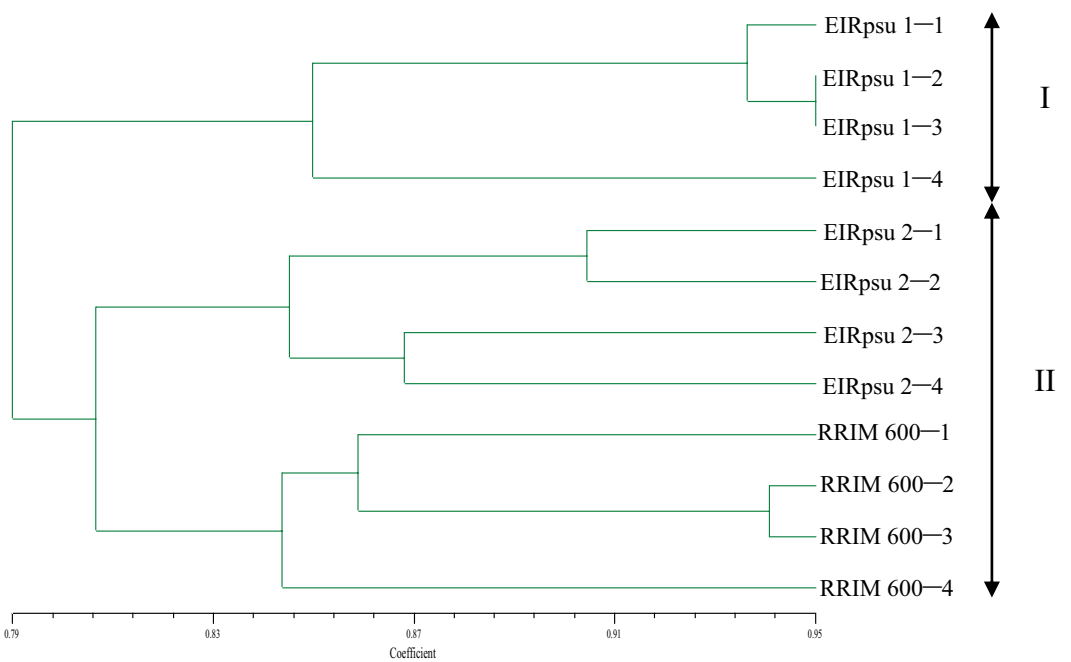

Figure 3 Dendrogram showing the relationship between 12 rubber clones based on random amplified polymorphic DNA with 10 primers.

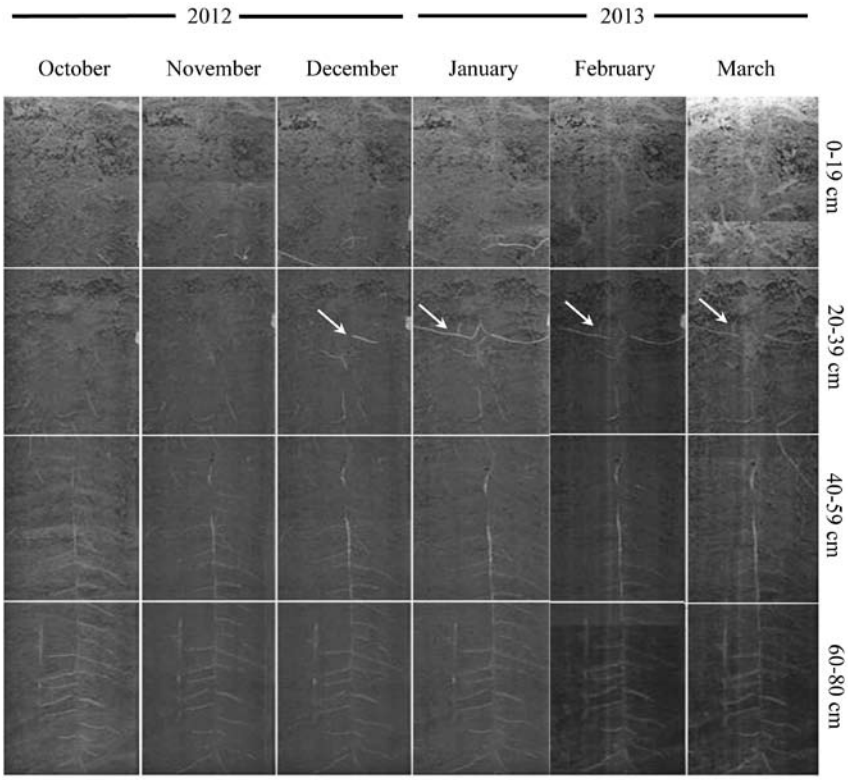

Figure 4 Rhizobox images collected with the adapted scanner from October 2012 to March 2013and pieced together in vertical columns. Images show root proliferation through the soil from 0–80 cm depth. Arrows indicate newly appearing roots based on the previous month's images.

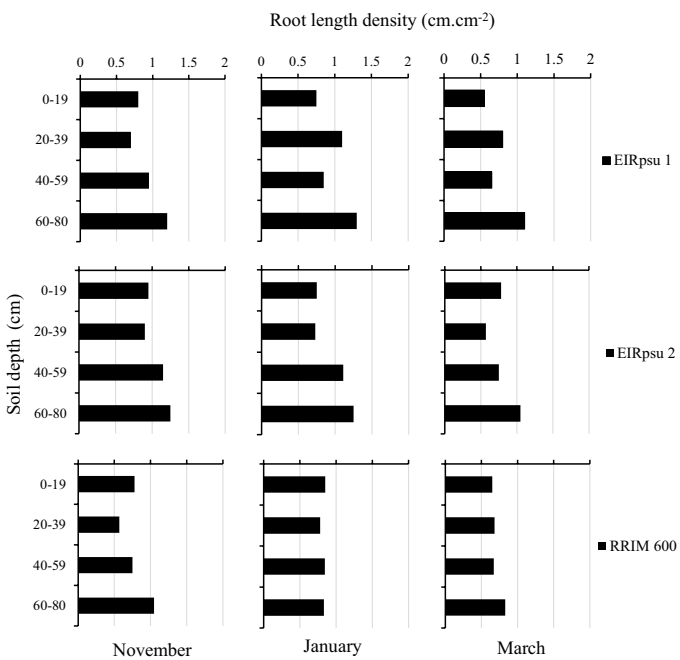

Figure 5 Comparison of root growth among the three rubber clones November 2012 to March 2013.

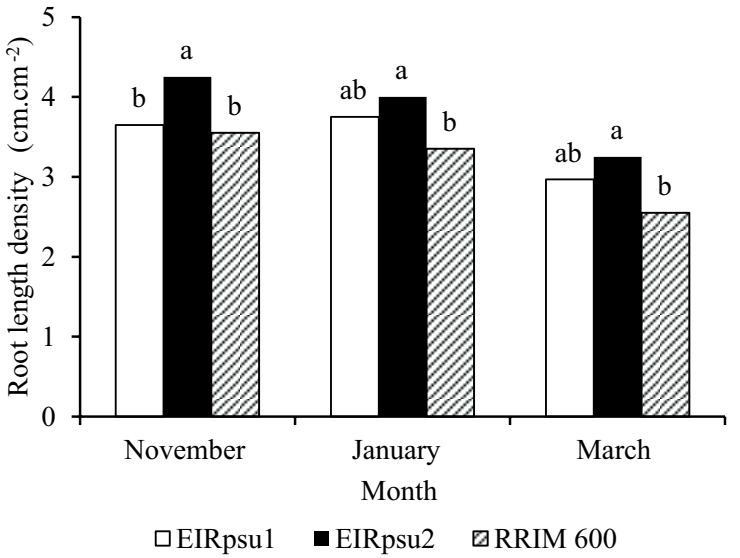

Figure 6 Average total root length density of three rubber clones in the soil profile (November 2012–March 2013) observed from rhizoboxes.

However, further research is needed to investigate the response of selected scions grafted or budded onto these rubber rootstocks with the investigation of growth performance under field conditions.

CONCLUSION

Tentative tolerance to the white root disease was investigated by monitoring the genetic background of seedlings from two clones of early-introduced rubber tree. EIRpsu 2 had a closer relationship to RRIM 600 than EIRpsu 1 with similarity coefficients of 0.850, 0.860 and 0.890, respectively. The results implied a close relationship of both clones to RRIM 600.

The investigation under field conditions of dynamics shoot and root growth of the two early-introduced clones and RRIM 600 was carried out in rhizoboxes over 6 mth and indicated that RRIM 600 exhibited the highest shoot growth while high root growth was recorded in seedlings of EIRpsu 1 and EIRpsu 2. The root growth of RRIM 600 was the least with shallow root proliferation. The results confirmed the good ability of EIRpsu 1 and EIRpsu 2 as elite rootstock for bud grafting with RRIM 600.

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