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Original Article

Diversity among 3 cultivars (RRIM600, RRIT251, and PB350) of *Hevea brasiliensis* and secondary metabolite production

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Abstract

Hevea brasiliensis was introduced to Thailand for the production of natural rubber. RRIT251 and RRIM600 are high yield varieties while PB350 produces a high yield of latex and resistance to diseases. The cultivar identity must be ascertained prior to plantation establishment because it is important to relate the genetic background with the production of certain secondary metabolites. In this study, the internal transcribed spacer region was used to evaluate the varieties among these 3 cultivars. Callus induction was initiated with 2 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) and 2 mg/L 6-benzylaminopurine (BAP). Hexadecanoic acid, methyl ester and 9-octadecanoic acid (Z), methyl ester were detected in RRIM600 calli. 2-Furancarboxaldehyde-5-(hydroxymethyl) was dominant in the RRIT251 and PB350 calli. These secondary metabolites are relevant in various high performance commercial sectors.

Keywords: *Hevea brasiliensis*, internal transcribed spacer (ITS), 9-octadecanoic acid (Z), methyl ester, hexadecanoic acid, methyl ester, 2-furancarboxaldehyde-5-(hydroxymethyl)

1. Introduction

The commercial production of natural rubber has reached 10 million tons per year globally. It is primarily derived from *Hevea brasiliensis* Müell. Arg., (Rivano *et al.*, 2013). This plant is grown for cultivation in many countries in the Asia Pacific region including Thailand. The resilience, abrasion, efficient heat dispersion and cold-temperature malleability of natural rubber cannot be matched by synthetic rubber (Cornish, 2001). Therefore, the natural rubber tree has become an economically important crop. According to the Rubber Research Institute of Thailand, cultivar of *H. brasiliensis* can categorized into 3 types: high yield, high wood quality and high yield with high wood quality. RRIM600 and RRIT251 are recommended high-yield cultivar. RRIM600 has been identified as a moderately resistant cultivar. The latex production of this cultivar is 289 kilograms per year per rai. The RRIT251 cultivar, has a high-yield production of 457 kilograms per year per rai, grows rapidly and is disease resistant. Another clone PB350 gives also high latex yield (450 kilograms per year per rai) and *Phytophthora* leaf fall and dryness tolerance have been widely cultivated by planters.

The recent demand of the global market for rubber latex has fluctuated and caused uncontrollable prices, which affects the income of farmers. Therefore, it is necessary to find ways to add value in a rubber plantation. Secondary metabolites from plant are one of the remarkably rich source of medically and industrial useful compounds. Plant genomes have evolved to code for a number of difference enzymes involved in the biosynthesis of novel secondary metabolites. Most secondary metabolites are produced at very low levels

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and are difficult to detect or identify. Some secondary metabolites are difficult to synthesize and are produced in wild plants in limited quantities (Ali *et al.*, 2013). Plant cell culture can enable the increased production of these metabolites (Antognoni *et al.*, 2012). Information on secondary metabolites production by *H. brasiliensis* calli is limited, but several studies have reviewed the syntheses of these compounds in other plants. For instance, Ahmad *et al.* (2013) identified a new steroid, albosteroid, with anti-ulcer and antioxidant activities in *Morus alba*. Ankad *et al.* (2013) identified antimicrobial activity in *Achyranthes coynei*-Sant. leaves. Other authors have reported that rosmarinic acid and flavonoids with antioxidant and anticancer activities are produced by hairy root cultures of *Dracocephalum kotschyi* Boiss (Fattahi *et al.*, 2013). The pharmacological effects and medicinal applications of these compounds have become the subject of intense study in recent years. Gas chromatography–mass spectrometry (GC–MS) is a useful analysis method owing to the volatility of the compounds of interest (Prosen *et al.*, 2007). Therefore, GC-MS was chosen for phytochemical compositions analysis for potential pharmaceutical applications.

The aims of this study were to identify the varieties among the 3 recommended cultivars of *H. brasiliensis* and identify a suitable medium for callus induction to produce secondary metabolites. The synthesized secondary metabolites were identified, and the abilities of the calli to produce these metabolites for applications as high-quality pharmaceuticals and in food were investigated.

2. Materials and Methods

2.1 Plant materials and sampling sites

All clones of RRIM600, RRIT251 and PB350 were identified by the taxonomist and planted at the Agriculture demonstration plot at Princess of Naradhiwas University.

Young leaves and young shoots from each cultivar of *H.brasiliensis* age 90 days were harvested. Young leaves were stored at -80°C until used. Young shoots were used immediately after harvesting.

2.2 Cloning of ITS

2.2.1 DNA extraction

Thirty gram of young leaves from 3 cultivars were thoroughly washed with tap water and rinsed with distilled water, dried. DNA was isolated using the DNA Mini Kit (Geneaid Biotech Ltd., Taiwan) according to the manufacturer's protocol.

The quality and quantity of the extracted genomic DNA was estimated by measuring the fluorescence emission.

2.2.2 PCR amplification

A primer set (5′-GTTTCCGTAGGTGAACCT-3′ and 5′GCTTATTAATATGCTTAAATCAGCG-3′) was used. The reactions were performed in a volume of 50 µl. The thermal cycling protocol consisted of an initial denaturation of 5 min at 94°C, followed by 40 cycles of 95°C for 0.30 sec, 54°C for 30 sec and 72°C for 10 min, and a final extension at 72°C for 10 min.

2.2.3 ITS DNA cloning

PCR products were purified using a commercial kit (QIA quick PCR Purification kit; QIAGEN, Hilden, Germany) and ligated into the pGEM®-T Easy vector (Promega, USA). The ligation product was transformed into *E. coli* TOP10F′ competent cells. The transformants were grown on LB plates containing 100 mg/ ml ampicillin. Plasmid DNA was extracted using STET buffer and purified using Presto™ Mini Plasmid Kit (Geneaid Biotech Ltd., Taiwan).

2.2.4 Sequencing

Individual clones with an insert were subjected to sequencing using the T7 primer. Homology searches were performed using the BLAST program with default settings (available at http://www.ncbi.nlm.nih.gov/blast/). Sequences were aligned using CLUSTALX 1.81. and BLAST X. Alignments were edited using GeneDoc.

2.3 Callus induction

2.3.1 Screening of optimal sterilization reagents

Fresh, young shoots were rinsed with tap water for 20 min, followed by distilled water. All explants were sterilized in various concentration of commercial detergent listed in Table 1 to investigate surface sterilization. The explants were finally washed three times with sterile distilled water and aseptically cut in 1 cm. segments.

2.3.2 Callus induction and culture medium

Thirty explants in each treatment were incubated in Murashige and Skoog medium (MS) (Murashige and Skoog, 1962) supplemented with 0.5, 1.0, 2.0 and 4.0 mg/l 2,4-D and a constant concentration of 2 mg/l of BAP. The cultures were then incubated in a sterile room with 16 hr of light in 24 hr cycle at a controlled temperature of 25±2°C. The initiated calli were subcultured onto fresh medium after 1 month. The fresh weight of 1 month old calli was measured to monitor their development.

Table 1. The different sterilizing reagents and conditions used on young shoot explants

Treatment	Sterilizing Reagents	Holding Time
1	Commercial Detergent	10 min
	70% Alcohol	30 sec
	50% Clorox +Tween 20	15min
2	Commercial Detergent	5 min
	70% Alcohol	15sec
	50% Clorox +Tween 20	15min
3	Commercial Detergent	10 min
	70% Alcohol	30 sec
	20% Clorox +Tween 20	15min
4	Commercial Detergent	10 min
	70% Alcohol	30 sec
	20% Clorox +Tween 20	5 min

2.4 Secondary metabolites detection

2.4.1 Callus extraction

Phytochemicals were extracted from 0.5 g callus in 1 ml of 70% methanol (HPLC grade) for 24 hr with shaking at room temperature. The supernatant was removed to a new 1.5 ml microcentrifuge tube and stored at 4°C until analysis.

2.4.2 Detection of secondary metabolites by GC-MS

Bioactive compounds detection was performed as described by Bowman *et al.* (1977). GC-analysis was conducted using a capillary gas chromatograph (Hewlett-Packard 5840A) equipped with a 60 m X 0.25mm- ID fused-silica column (Supelco SP-2331, Bellefonte, PA) and mass spectrometer. Helium was used as the carrier gas. The temperature program was 50°C for 10 min, followed by a 5°C / min ramp to 200°C and a hold at 200°C for 5 min. The temperatures of the injector and detector were 250°C. A 2 µl of injection was performed with a 100:1 split ratio.

2.5 Statistical analyses

Three replicate measurements were performed for each experiment. The data were analyzed by one-way ANOVA test with a threshold of significant of $P<0.01$.

3. Results

3.1 Cloning of the ITS region

The varieties of *H.brasiliensis* has been studied using various molecular markers such as simple sequence repeats (SSR) (Salgoda *et al.*, 2014), EST-SSR marker (Curby *et al.*, 2014), inter- simple sequence repeats (ISSRs) (Kitijuntaropas and Wansomnuk, 2007) and AFLP (Roy *et al.*, 2012). The ITS region which located on either side of the *5.8S* rDNA is relevant for evolutionary and phylogenetic investigations because the highly conserved flanking region permits the use of a universal PCR primer (Zhongyu *et al.*, 2009). In addition, the ability to easily amplify sequences from various organisms and the high-copy number and moderate size of this regions make it a good molecular marker in phylogenetics (Tippery and Les, 2008; Hribova′ *et al.*, 2011; Ghada *et al.*, 2013; Shama *et al.*, 2012). Here, genomic DNA amplification was performed in a single band encompassing the ITS region, which varied in size 684 bp for RRIM600 and 690 bp for RRIT251 and PB350. Variations of DNA sequences among 3 cultivars of *H.brasiliensis* were identified as shown in Figure 1. The sequence of RRIM600 is homology with RRIT251 92% and 93% with PB350. The homology between RRIT251 and PB350 is 93%. Polymorphic positions of substitutions, insertion and deletion are summarized in Table 2. There were 46 SNP; many insertions and deletions were identified.

3.2 Callus induction

3.2.1 Screening optimal sterilization reagents

Explant survival rate varied with the concentration of sterilizing reagents and the sterilization duration. The use of commercial detergent in treatment 4 (Table 1) resulted in

Table 2. Polymorphic position of ITS region among 3 cultivars

Mutation	Position
Transition	66, 68, 79, 113, 118, 150, 162, 163, 183, 193, 212, 230, 278, 290, 340, 350, 351, 396, 410, 422, 434, 447, 463, 470, 496, 518, 527, 575, 605, 626, 666, 667, 674
Transversion	82, 101, 115, 171, 233, 311, 398, 437, 580, 582, 591, 663, 664
Insertion	108, 109, 585, 614, 628, 629, 630, 631, 679
Deletion	177, 178, 179, 180, 439, 440, 584, 676, 677, 678

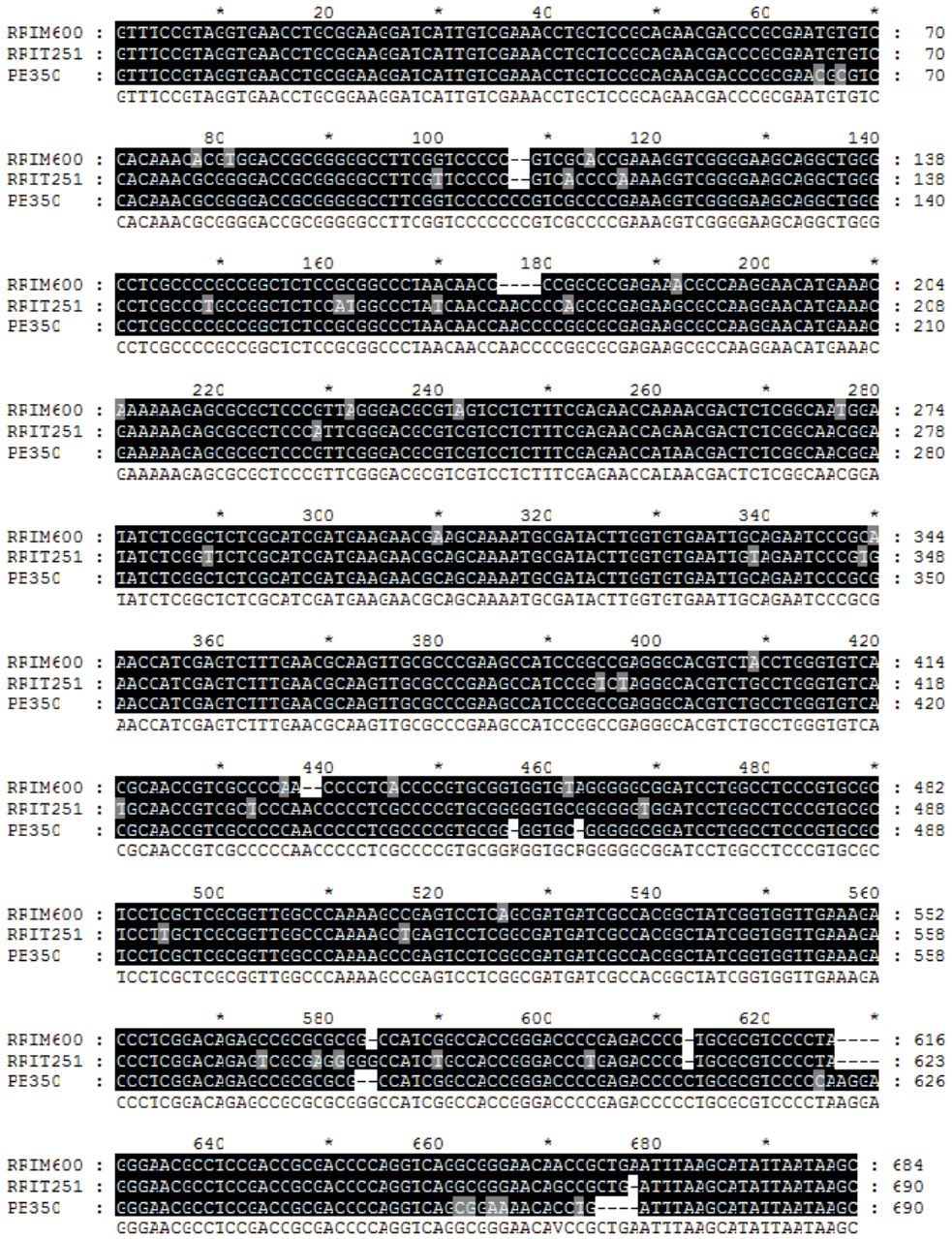

Figure 1. Alignment of the ITS1, *5.8S*, and ITS2 region

the highest survival rate (75.67%) of young shoot explants (Table 3). This treatment was selected for sterilization for callus culture experiment.

3.2.2 Callus induction

Development was observed over 1 month to screen optimal and effective media for the induction. Callus growth on MS medium with 2 mg/l 2,4-D and 2 mg/l BAP was superior to that of the control (MS1) culture. All calli were compact calli and ranged in color from yellow to brown. Young shoot explants began to form yellowish compact calli (Figure 2). After 9 days, this medium initiated dedifferentiation and produced the best development, as assessed by measuring the fresh weight of calli of the 3 cultivars. Significant differences in callus fresh weight were observed among the various types of media ($P<0.01$). The effect of hormone concentrations on the fresh weight of young shoots is presented in Figure 3. The result demonstrates the significant differences in the fresh weight ($p<0.01$) of three cultivars calli in various MS (MS1- MS4). MS4 was MS supplemented with 2 mg/l 2,4-D and 2 mg/l BAP exhibited a superior ability to induce callus formation.

Table 3. Sterilization test results

Treatment	Total no. explants	No. of surviving explants				% Survival
		Rep1st	Rep 2nd	Rep 3rd	Mean	
1	30	0	0	0	0	0
2	30	0	0	0	0	0
3	30	5	4	5	4.7	15.67
4	30	25	23	20	22.7	75.67

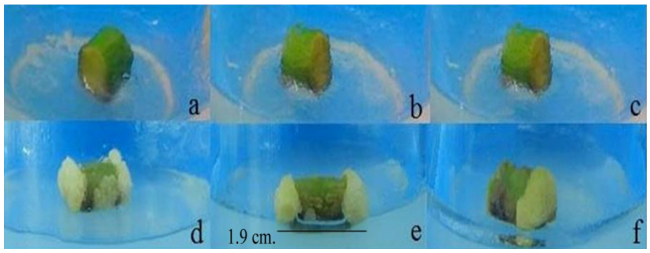

Figure 2. Callus induction of *H. brasiliensis* incubated with MS medium supplemented with 2 mg/l 2,4-D and 2 mg/l BAP. Figure a,b,c the culture of 1 week of incubation whereas figure d,e,f were 1 month of incubation (a: RRIM600, b:RRIT251, c:PB350, d: RRIM600, e:RRIT251 and f: PB350)

3.3 Identification of secondary metabolites

Methanol extract of the calli from each of the 3 cultivars was fractionated to identify the secondary metabolites and the data of their retention time are summarized in Table 4.

The diversity of secondary metabolites among these cultivars can be noticed from GC-MS phytochemicals profile with the high peak areas. 2-Furancarboxaldehyde and hexadecanoic acid were found in RRIT251 and PB350, whereas 9- octadecenoic acid (Z) and 4H-pyran-4 -one,2,3-dihydro-3,5-dihydroxy-6-methyl were only produced from RRIM600 callus (Figure 4).

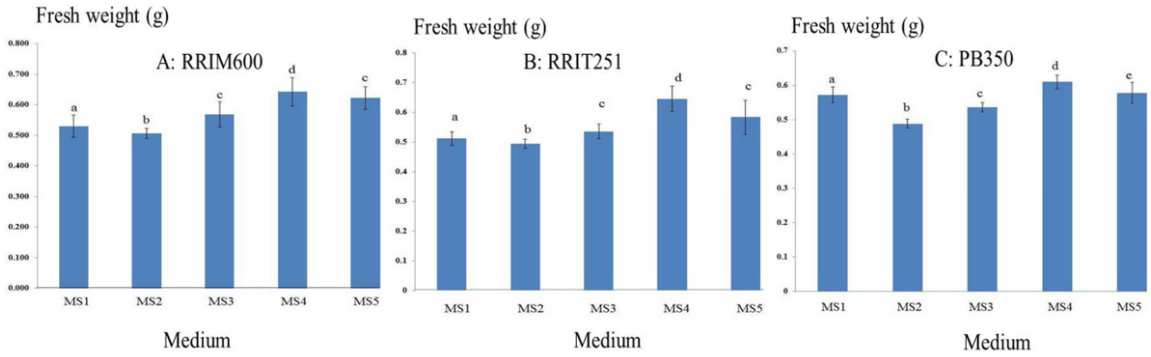

Figure 3. Effect of various media on 1 month of incubation fresh callus (a; RRIM600, b: RRIT251, c:PB350). The ratio of 2,4-D and BAP (mg/l): MS1 = 0:2, MS2 = 0.5:2, MS3 = 1:2, MS4 = 2:2, MS5 = 4:2)

Table 4. Detection of secondary metabolites (indicate by ✓) from the calli of the 3 cultivars of *Hevea brasiliensis*

Rt	Compounds	Cultivar		
		RRIM 600	RRIT 251	PB350
28.68	2-Pyrrolidinone	✓		
32.31	2,3-Dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one	✓	✓	✓
32.98	Hexadecanoic acid, methyl ester	✓	✓	✓
36.65	9-Octadecanoic acid (Z), methyl ester	✓	✓	✓
44.39	Hexadecanoic acid	✓	✓	✓
36.38	2-Furancarboxaldehyde,5(hydroxymethyl)		✓	✓

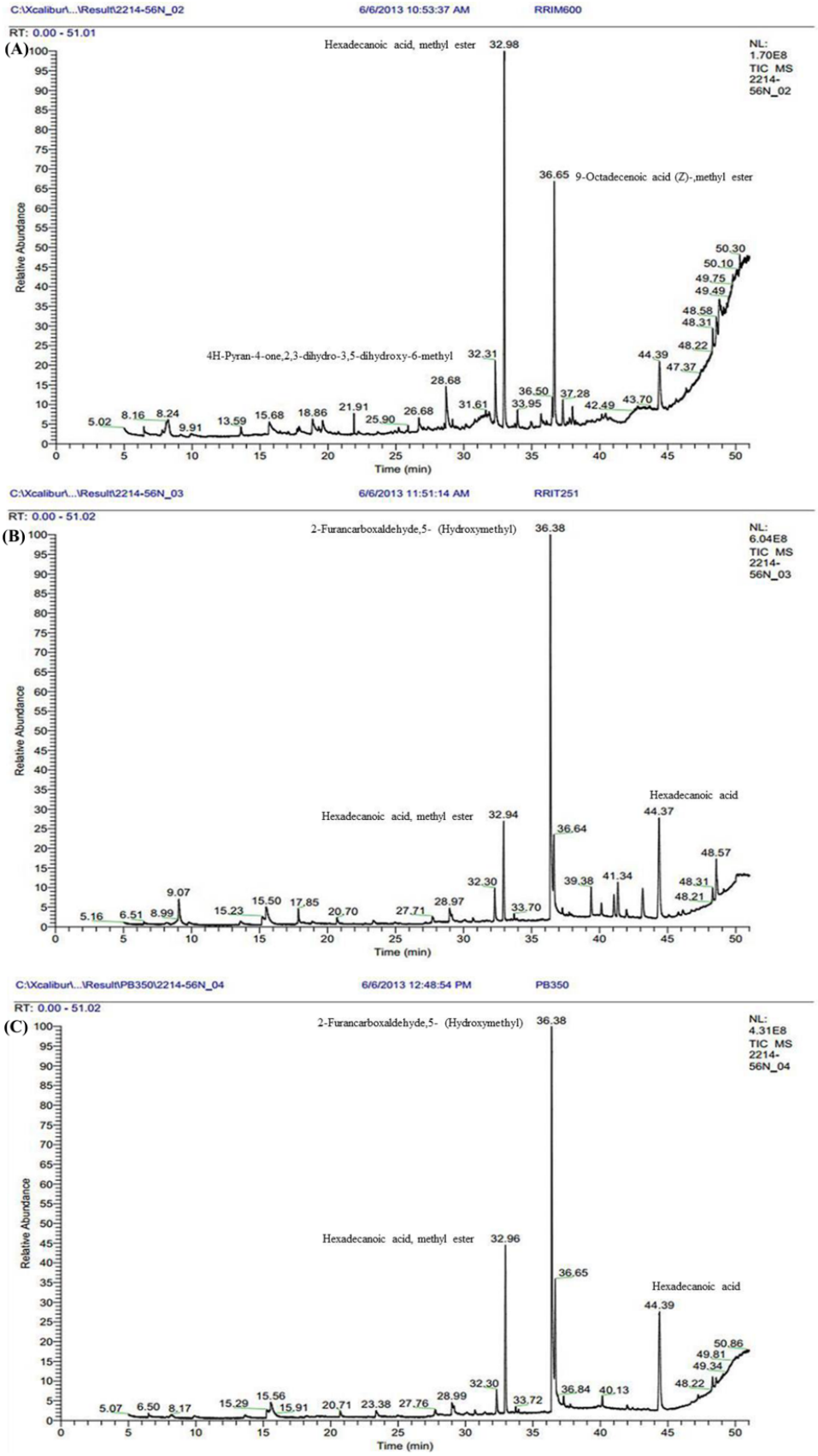

Figure 4. Detection of secondary metabolites in calli of the 3 cultivars of *Hevea brasiliensis* by GC-MS. (A: RRIM600, B: RRIT251, and C: PB350)

4. Discussion

In this study, polymorphisms at the ITS region were used to characterize the diversity of the 3 cultivars of *H. brasiliensis*. The result of correct cultivar identification is very important because the genetic background might affect the production of certain secondary metabolites or the estimated yields and performance on the disease resistance of *H.brasiliensis*. After the detection of the cultivars, proliferation of this plant should be carried out with *in vitro* micropropagation. Callus induction is the good technique to synthesize various phytochemicals. The most suitable treatment for young shoot rubber callus induction was the medium supplemented with 2 mg/l 2,4-D and 2 mg/l BAP in agreement with Roy and Banerjee (2003). The 2,4-D induces high quality calli (Bhalla and Xu, 1999) and is important for callus induction (Haliloglu, 2006) . It was also used to induce callus formation by *Camellia sinensis* because of its ability to promote active proliferation (Nikalaeva *et al.*, 2008). Cytokinins are related to the control of the balance between auxin and cytokinin which regulate cell division and prevent callus necrosis in some species (Roy and Banerjee, 2003). Our results are in agreement with those of Haque *et al.* (2003), who reported the highest callus induction performance of G-103 cultivar of garlic in MS supplemented with 2 mg/l 2,4-D and 0.5 mg/l BAP.

Several potential useful bioactive compounds were identified in three cultivars' calli by GC-MS analysis. 2,3-Dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one; hexadecanoic acid, methyl ester; 9-octadecanoic acid (Z), methyl ester and hexadecanoic acid were found in all cultivars. These compounds exhibit anti-inflammatory, anti-oxidant and anti-carcinogenic activity (Lerma *et al.*, 2010; Mathuravalli and Eswara, 2012; Selvamangai and Bhaskar, 2012; Soussi *et al.*, 2014). It is interesting to note that 2-pyrrolidinone was found only in RRIM600 and 2-furancarboxaldehyde, 5- (hydroxymethyl) was detected in the RRIT251 and PB350, two cultivars with show high yield and resistance to the *Phytophthora*, whereas 2-pyrrolidinone was found only in RRIM600. 2-Furancarboxaldehyde, 5-hydroxy methyl is a major chemical constituent present in the ethanoic leaf extract of *Tobebuia rosea*. This plant has been used traditionally for treating gastrointestinal problems, cancer, diabetes and allergies (Ramalakshmi and Muthuchelian, 2011). Thangam *et al.* (2013) identified 2-pyrrolidinone as an active compound from *Brassica oleracea* exhibiting antioxidant and *in vitro* anticancer activities and used 2-pyrrolidinone to induce apoptosis in human cancer cells.

In conclusion, the results of this study clearly indicated that the ITS region is a good tool for studying diversity of three cultivars. MS medium supplemented with 2 mg/l 2,4-D and 2 mg/l BAP produced the best callus induction. In addition, various secondary metabolites were synthesized with properties beneficial to humans. Future work aims to improve our understanding of the regulation of secondary metabolites biosynthesis and apply these molecules to medical and industrial uses.

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