Chapter 2

MATERIALS AND METHODS

1 Interaction between zoospores of *Phytophthora palmivora* and *Hevea brasiliensis* leaves

1.1 *Phytophthora palmivora*

1.1.1 Isolation of *P. palmivora*

P. palmivora isolated from *H. brasiliensis* in Krabi (No.KBNM9) was kindly provided by The Rubber Research Institute of Thailand (RRIT). *P. palmivora* isolated from *H. brasiliensis* was identified in the laboratory at Prince of Songkla University and reconfirmed by Professor André Drenth from the University of Queensland, Australia by the PCR based diagnostic method using *Phytophthora* genus primer. The fungus was further isolated for monospore prior to being stimulated for sporangium formation. It was subcultured in sterile condition every week on potato dextrose agar (PDA) at 25°C and kept in the dark (Fig. 10).

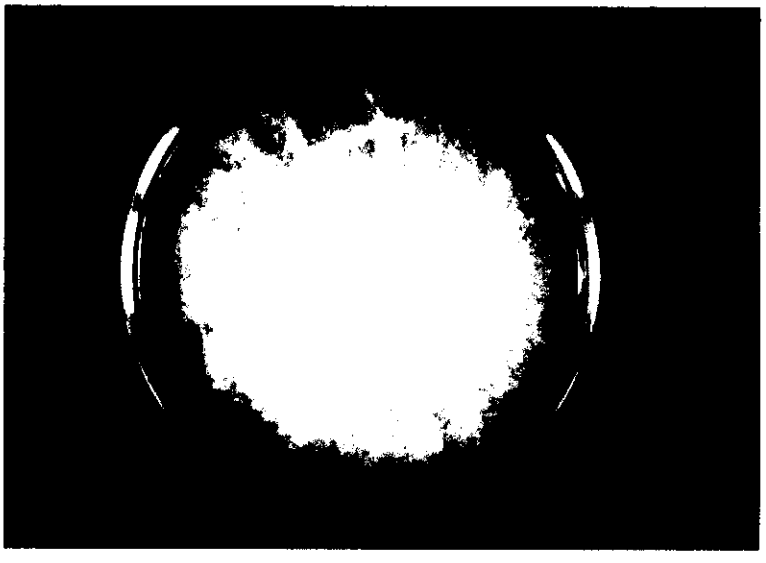


Fig. 10 The one-week mycelium of *Phytophthora palmivora* on potato dextrose agar

1.1.2 Preparation of zoospores of *Phytophthora palmivora*

The growing mycelium of *P. palmivora*, (No.KBNM9) after cultured in PDA for 5 days, was cut with a 5 mm-diameter cork borer and transferred to a V_8 agar. After 5 days, the growing mycelium of *P. palmivora* on the V_8 agar was added with 10 ml of sterile distilled water and kept at 4°C for 15 minutes. Zoospores were released from sporangium after the V_8 agar plate was brought to 25°C for 30 minutes. The suspension of zoospores was dropped on Petroff Hauser and counted under light microscope (Fig. 11). The concentration of zoospores was calculated by the following formula :

$$\text{Zoospores concentration in 1 ml} = \frac{\text{(No. of zoospores in field) X 1000}}{\text{volume on slide}}$$

$$\text{(Volume on slide} = 4 \times 10^{-3}\ \mu\text{l)}$$

Three dilutions of zoospore suspension were adjusted by sterile distilled water. The zoospore suspensions were prepared to the concentrations of $5x10^5$, $5x10^6$ and $5x10^7$ zoospores/ml which were considered as low, medium and high concentration, respectively. The suspensions were used within 30 minutes before the germination could take place.

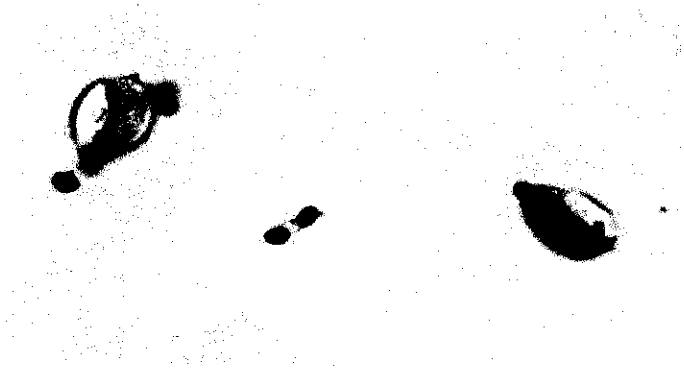


Fig. 11 Micrograph of sporangium and zoospores of *Phytophthora palmivora.* (x10)

1.2 Plant Material

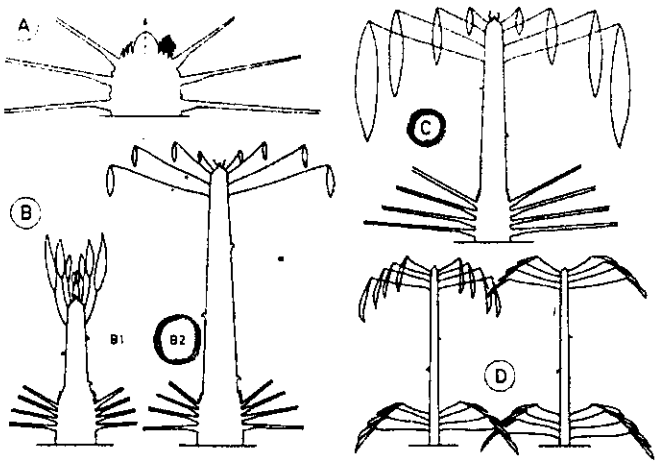
1.2.1 Selection of *Hevea brasiliensis* clones

The *H. brasiliensis* clones were selected by the degree of resistance which was classified by the RRIT. Those are BPM-24 which is considered to be the resistance clone; the PB235 and RRIT251, the partially resistant clones; and RRIM600, the susceptible one. All budding scions were obtained from bud implanted to the stocks of wild–type plant and grown in 10 inches diameter bag with clean soil. They were treated every two weeks with organic and inorganic fertilizers.

1.2.2 Preparation of rubber leaves for zoospores inoculation

Four clones of 6-month-old rubber trees, RRIM600, RRIT251, PB-235 and BPM-24 which differ in degree of resistance to *P. palmivora* were planted. The 6- to 8-day-old leaflets or B_2 – C stage of rubber leaves as described by Halle' and Martin, (1968) of selected clones were detached (Fig. 12). The healthy leaves weighing 0.7 – 1 gram, were selected for each experiment. All were rinsed with sterile distilled water to remove dirts, then placed and turned abaxial surface upward on the whatman paper. The paper was moistened with 5 ml of sterile distilled water in an 11 cm-diameter Petri dish. After the inoculation was performed as designed, the Petri dish was kept in a clear plastic bag and maintained at 25°C in 12 hours daylight (Phillips daylight 40 Watts).

a



b

Fig. 12 The diagram of leaf patterns and various stages of rubber leaves.

(a) B_2 - C stage leaves (arrow).

(b) A, B (B_1, B_2), C and D represent each stage of rubber leaves.

1.3 Necrosis on *Hevea brasiliensis*

1.3.1 Classification of the degree of resistance of rubber clones by zoospores inoculation

The leaves at B_2 – C stage of four rubber clones : BPM-24, PB-235, RRIT255 and RRIM600 which differ in degree of resistance were selected. Then, 20 µl of distilled water (control) and 20 µl of $5x10^6$ zoospores/ml suspension which was prepared as described in 1.1.2 were inoculated on the abaxial surface of rubber leaves (1 drop/ inch ^{2}) which were detached and prepared as in 1.2.2. After 12 hours, all droplets of distilled water and zoospores suspension were blotted with sterile paper. The leaves were kept at 25°C under 12 hours daylight and observed for the necrotic size every 24 hours for 96 hours.

1.3.2 Effect of zoospore concentration on necrotic lesion of rubber leaves

Two rubber clones, BPM-24 and RRIM600, which were very different in their degrees of resistance to *P. palmivora*, were selected for this experiment, the former as the most resistant and the latter was the most susceptible one. The rubber leaves were prepared in the Petri dish as in 1.2.2. then, 20 µl droplets of $5x10^5$, $5x10^6$ and $5x10^7$ zoospores/ml suspension were applied on the right sides of the abaxial surfaces of rubber leaves (4 drops/ inch^2) and droplets of sterile distilled water were placed on the left side as control. The inoculated leaves were kept at 25°C under 12 hours daylight for development of necrotic lesions. The necrotic size was measured every 24 hours for 72 hours

1.3.3 Lignification

The C–stage rubber leaves of BPM-24 and RRIM600 were detached and cut in square. The 2 ml of distilled water was poured into the

small Petri dish as control whereas the 2 ml suspension of $5x10^7$ zoospores/ml was poured into the other Petri dish. The leaves were gently shaken and kept at room temperature. The pieces of leaf were taken at 12, 24, 48 and 72 hours after inoculation. All were immersed in 2 ml of 2% phloroglucinol in 95 % ethanol immediately. After 24 hours in the solution, each leaf piece was placed on a glass slide, HCl was dropped over the sample prior to visualization under the light microscope.

1.4 Scopoletin (Scp)

1.4.1 Identification and analysis of scopoletin

The resistant clone, BPM-24, was used for this experiment. The $5x10^6$ zoospores/ml suspension was prepared with sterile water as described in 1.1.2. The 20 μl of zoospores were applied on the right sides of abaxial surfaces of selected rubber leaves which were prepared as in described1.2.2. The water droplets with or without spores (control) were removed from leaflets 24 hours after inoculation, then 20 μl of new water droplets (without zoospores) were applied to the same inoculated sites. Twenty-four hours later (48 hours after inoculation), the newly synthesized scopoletin was collected for analysis and new water droplets were reapplied to the same inoculated sites. The newly synthesized scopoletin was recovered every 24 hours up to 144 hours after inoculation, then filtered through a membrane (0.22μm, millipores), freeze-dried, resuspended in methanol and analyzed by Thin Layer Chromatography (TLC) on silica gel (Kieselgel-60 thin-layer plate) with 12% acetic acid. The chromatogram was observed under UV light (366nm), and a single, blue fluorescent compound was characterized by comparison with the standard Scp (Sigma). Identification and quantification of

scopoletin were less ascertained by High Performance Liquid Chromatography (HPLC) (Churngchow and Rattarasarn, 2001).

1.4.2 Standard curve of scopoletin

Stock solution of 5 μM Scp was prepared by dissolving 0.96 mg Scp in 1 ml of distilled water. The Scp stock solution was diluted to 0.01, 0.02, 0.03, 0.04 and 0.05 μM and were measured with spectrofluorophotometer using excitation wavelength at 340 nm and emission wavelength at 440 nm. The intensity of standard Scp measured by spectrofluorophotometer had linear correlation with the concentration of standard Scp (Fig. 13).

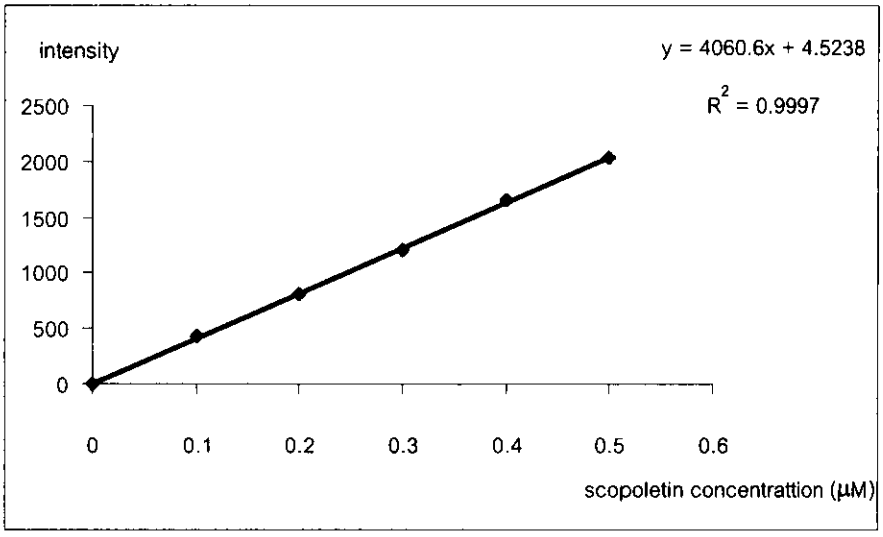


Fig. 13 Plot of standard scopoletin versus intensity using spectrofluorophotometer excitation wavelength at 340 nm and emission wavelength at 440 nm.

1.4.3 Scopoletin synthesis in rubber leaves

The detached rubber leaves, RRIM600, RRIT251, PB-235 and BPM–24 which are different in the degree of resistance to *P. palmivora* were placed on moistened paper in Petri dish as described in 1.2.2. The 20 μl of

$5x10^6$ zoospores/ml were prepared as described in 1.1.2 and placed on the right sides of rubber leaves (abaxial surface, 2 drops/inch^2). The inoculum droplets were removed from leaflets at 24, 48, 72, 96, 120 and 144 hours and sterile distilled water was placed on the left sides of the leaves as control. Each time the inoculum droplets were recovered, the inoculated sites were reapplied with 20 µl of new sterile water droplet. The volume of collected samples at every time point were adjusted to the same total volume which were firstly applied on the leaves. The collected droplets were determined for scopoletin by spectrofluorophotometer at excitation wavelength of 340 nm and emission wavelength of 440 nm.

1.4.4 Effect of zoospores concentration to scopoletin synthesis in rubber leaves

The resistant (BPM-24) and susceptible (RRIM600) rubber clones classified by necrotic lesions and scopoletin synthesis as described in 1.3.1 and 1.4.3 were used. The B_2 – C stage detached leaves were placed in the Petri dishes as described in 1.2.2. The zoospores concentration; $5x10^5$, $5x10^6$ and $5x10^7$ zoospores/ml were prepared as in 1.1.2 and applied on abaxial surfaces of the leaflets which were detached from the same petiole. The sterile water was used as control. The inoculum droplets were collected and determined for scopoletin as described in 1.4.3.

1.4.5 Effect of scopoletin on inhibition of mycelium growth

The growth inhibition of Scp was tested on mycelial cultures of *Phytophthora palmivora*, *P. botryosa*, *Corynespora cassiicola* and *Colletotrichum gloeosporioides*. Four concentrations of Scp, 0.5, 1, 1.5 and 2.0 mmol/l (stock solution of Scp at 50 mmol/l was dissolved in 90 % ethanol) were used for *P. palmivora* and *P. botryosa*. Higher concentrations of Scp 1, 2, 3 and 4 mmol/l were used for the other two leaf pathogens. The final

concentrations of ethanol were the same in the tests and the controls. Growth inhibitions were examined in Petri dishes by subculturing a mycelium plug (diameter of 5 mm) onto PDA medium containing different concentration of Scp. Fungal cultures were incubated for 5 days in the dark at 25°C. Growth was evaluated by measuring daily diameter of mycelial colony on the PDA plate. Values are percentage inhibitions of mycelial growth measured in comparison with the control. The Scp concentration which inhibits mycelium growth by 50% at day 5 was taken as I_{50} value.

1.4.6 Fungitoxicity of scopoletin for zoospore germination

Zoospore suspension of concentration of $4X10^7$ zoospores/ml was prepared from *P. palmivora* which was cultured in PDA. Stock of Scp solution was made from 2000 μg dissolved in 10 ml sterile distilled water, then diluted to 1000 and 200 μM. Each 1 ml of the respective solutions was mixed with 1 ml of the zoospore suspension of $2X10^7$ zoospores/ml in respective Scp concentration 1000, 500 and 100 μM, each of which was then dropped on Petroff Hauser, closed with cover slip and incubated in moist Petri dish at 25°C for 2 hour prior to counting for germinated zoospores under light microscope. Zoospore concentration of $2X10^7$ zoospore/ml in sterile distilled water was used as a control. GI_{50} was Scp concentration at which 50% spore germination was inhibited at 2 hours after incubation was taken as I50 value.

1.5 Experimental design for total protein and PR-proteins determinations

1.5.1 Preparation of rubber leaves for protein and enzyme assay

Two rubber clones which differed in their degrees of resistance were used. Twelve B_2 – C stage leaves of BPM-24 (R) and RRIM600 (S) were detached and cut the midrib to separate the leaf into right and left parts. Each was placed in a separate moistened Petri dish. The right part was inoculated with the zoospores and the other was control. The high concentration of

P. palmivora zoospores ($5x10^7$ zoospores/ml) was prepared as described in method 1.2.2. The 20 µl droplet of zoospore suspension was placed on the abaxial surface at inoculation parts (2 droplet/inch^2). The left part of rubber leaf was used as control by placing with 20 µl of sterile distilled water. Each leaf was cut into small strips at 24, 48, 72 and 96 hours after inoculation and weighed to 1 gram. All were kept in plastic bag at –20°C for further assay.

1.5.2 Crude protein extraction

One gram of control or inoculated leaves was ground in 5 ml of liquid nitrogen with a mortar and pestle then mixed with 0.5 ml of 50 mM sodium acetate buffer pH 5.0. The ground leaves were put into syringes plugged with a small cotton pad. The syringe was squeezed with our designed equipment. The homogenate was centrifuged at 12,000 rpm and 4°C, for 15 minutes to remove the insoluble materials. The total volume of the supernatant was recorded and stored at -20°C for further analysis of protein content and enzyme activities.

Fig. 14 The syringe was put into the adapter (b). The leaf extract was squeezed through the tip of syringe (arrow) by turning the knob clockwise. (a) The instrument before assembly.

1.5.3 Elimination of internal sugar

Since sugar was the product of substrate digestion by β-1,3–glucanase and chitinase, therefore it should be removed prior to analysing enzyme activity either by a PD–10 column which contained 9 ml of Sephadex G-25 or precipitation with $(NH_4)_2SO_4$ salt. For the PD-10 column, 2.5 ml of crude leaf extract was loaded onto the column and eluted with distilled water, colllecting fractions of 1 ml. The first 3 fractions were discarded and fractions number 4 to 6 were kept for further analysis. For precipitation with $(NH_4)_2SO_4$, the crude leaf extract was brought to 70% saturation of ammonium sulphate at 4°C. The solution was allowed to stand for 30 minutes at 4°C then centrifuged at 10,000 rpm and 4°C for 20 minutes. The pellet was resuspended with sterile distilled water and stored at –20°C for further analysis of enzyme activities.

1.6 Bioassays of total protein and PR-proteins of rubber leaves

1.6.1 Standard curve of protein by Bradford method

The stock solution of Bovine Serum Albumin or BSA (0.5 mg/1ml) was diluted to 5, 10, 15, 20 and 25 µg/100 µl. The diluent was reacted with 1 ml of Bradford solution and stood for 10 minutes at room temperature. The absorbance at wavelength 595 nm was used to perform standard curve of BSA. The absorbance had linear correlation with the concentrations of standard BSA and the obtained slope was 0.0273 A/µg (Fig. 15).

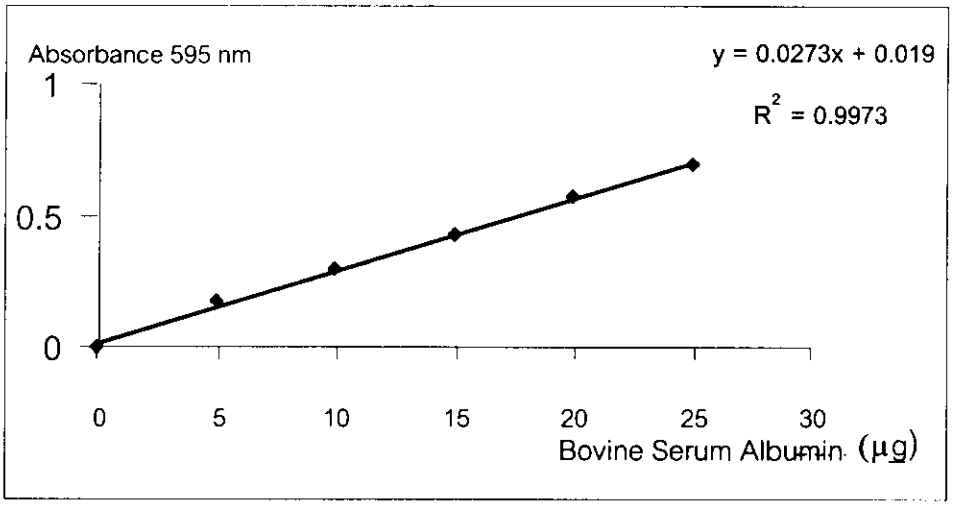


Fig. 15 Standard curve of Bovine Serum Albumin at absorbance 595 nm using the Bradford technique.

1.6.2 Measurement of crude leaf extract.

The amount of protein in crude leaf extract was examined by Bradford method. Ten µl of crude leaf extract, the internal sugar of which had already been eliminated as described in 1.5.2 and 90 µl of 0.1 M sodium acetate buffer, was added with 1 ml of Bradford reagent (appendix) in a test tube. The solution was well mixed and stood at room temperature for 10 minutes prior to measuring the absorbance at wavelength of 595 nm. The amount of protein in the tube was calculated by comparing its absorbance with the standard curve of BSA (Fig. 15).

1.6.3 Semi - sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis for protein (SDS-PAGE)

Sodium dodecyl sulphate (SDS) polyacrylamide gel was prepared with 0.1% SDS in 5% stacking gel using 1 M Tris-HCl pH 6.8 as a buffer and 0.1% SDS in 15% separating gel using 1.5 M Tris-HCl pH 8.8 as a buffer. The electrode buffer contained 0.1% SDS and 250 mM glycine in 25

mM Tris-HCl pH 8.3. The crude leaf extracts of rubber were mixed with the sample buffer (appendix) in the ratio 3:1 (crude extract: sample buffer) and then loaded (without boiling) about 20 µl (150-200 µg of protein) in each lane. The gel was electrophoresed with 60 volts at 4°C for 1–1.5 hours, then stained with Coomassie Brilliant Blue R-250 for 10 minutes and destained with 7% methanol and 7% glacial acetic acid in distilled water until the protein bands were clearly appeared. Photograph was taken using a Nikon camera with blue filter and Kodak Gold II 200 film.

1.7 PR-proteins quantification

1.7.1 Measurement of β-1,3-glucanase activity

The measurement of β-1,3-glucanase activity was that modified from Burner (1964). Ten µl of sample was mixed with 90 µl of 4 mg/ml laminarin. The mixture was immersed and shaken in water bath at 35°C for 30 minutes prior to boiling for another 2 – 3 minutes to denature the enzyme. Then, 0.2 ml of dinitrosalicylic acid (DNS) solution and 0.2 ml of 0.1 M acetate buffer pH 5 were added and boiled for 5 minutes; followed by 5 minutes of cooling down at room temperature. Thereafter 0.9 ml of distilled water was added prior to reading with spectrophotometer at 540 nm. The enzyme activity was calculated by comparing the absorbance values with those of standard glucose. One unit of enzyme activity was equivalent to one micromole of reducing sugar derived from laminarin degradation in one minute. The amount and rate of production of enzyme were studied at 0, 24, 48, 72 and 96 hours after inoculation of rubber leaves with zoospores of *P. palmivora*.

1.7.2 Standard curve of glucose

The dilutions of 1, 2, 3, 4 and 5 µM of glucose solution were prepared from 0.1 M glucose in 0.1 M sodium acetate buffer pH 5.0. The

100 µl of each concentration of glucose solution (which consist of 0.1, 0.2, 0.3, 0.4 and 0.5 µmole of glucose, respectively) was added with 0.2 ml of DNS solution and 0.2 ml of 0.1 M sodium acetate buffer pH 5. The mixture was boiled in a water bath at 100°C for 5 minutes, stood for to cool down, and 0.9 ml of distilled water was added prior to reading with a spectrophotometer at 540 nm (Fig. 16).

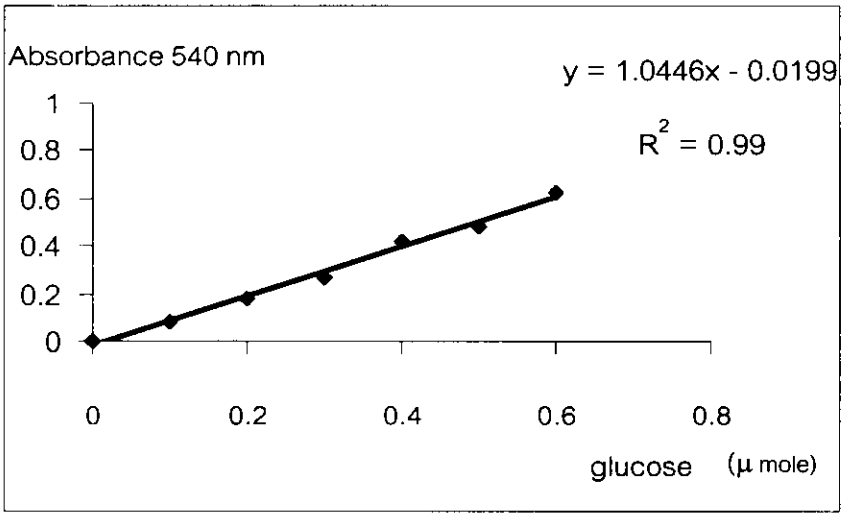


Fig. 16 Standard curve of glucose using the absorbance at 540 nm.

1.7.3 Measurement of chitinase enzyme activity

The chitinase activity was measured by the modified method of Sujibo, *et al.* (1991). One hundred µl of sample was mixed with 100 µl of colloidal chitin which was prepared as described in appendix in the presence of 800 µl of 0.1 M sodium acetate buffer pH 5.0. The mixture was shaken in a water bath at 37°C for 1 hour and then boiled for 10 minutes to denature the enzyme. After cooling down, the mixture was centrifuged at 10,000 rpm for 10 minutes. An aliquat of 750 µl of the supernatant was collected and mixed with 1 ml of Schales reagent, then boiled for 10 minutes. After cooling down, the

absorbance of sample was measured with spectrophotometer at 420 nm. Enzyme activity was calculated by comparing with the absorbance values of N-acetyl-D-glucosamine standard curve (Fig. 17). One unit of enzyme activity was equivalent to 1 μmole of reducing sugar derived from colloidal chitin degradation in one minute. The amount and rate of production of chitinase enzyme were studied at 0, 24, 48, 72 and 96 hours after inoculation of rubber leaves with *P. phytophthora* zoospores as described in 1.5.1.

1.7.4 Standard curve of N-acetyl-D-glucosamine

The dilutions of 0.5, 1, 2, 3 and 4 μM of N-acetyl-D-glucosamine were prepared from a stock solution of 50 μM of N-acetyl-D-glucosamine. The 100 μl of each concentration of N-acetyl-D-glucosamine (consisting of 0.05, 0.1, 0.2, 0.3 and 0.4 μmole of N-acetyl-D-glucosamine, respectively) was mixed with 650 μl of 0.1 M sodium acetate buffer pH 5.0 and 1 ml of Schales' reagent. The mixture was boiled in water bath for 10 minutes. After cooling down, the absorbance was measured with spectrophotometer at 420 nm and compared with standard curve as shown in Fig. 17.

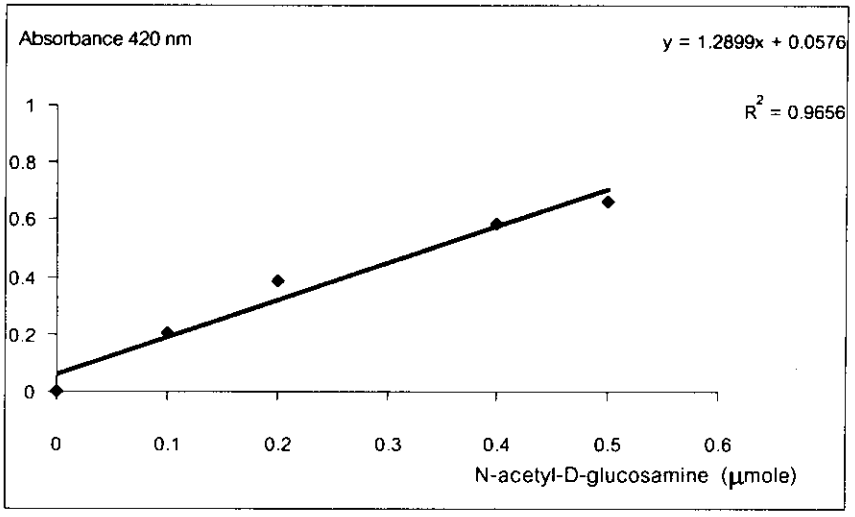


Fig. 17 Standard curve of N-acetyl-D-glucosamine using the absorbance at 420 nm.

1.7.5 Measurement of chitinase enzyme activity by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was prepared as followed; the separating gel consisted of 10% acrylamide, 0.01% glycol chitin, 0.1% SDS and 1.5 M Tris-HCl buffer pH 8.8; the stacking gel consisted of 5% acrylamide, 0.1% SDS and 1.0 M Tris-HCl buffer pH 6.8; the reservoir buffer was 0.1% SDS, 250 mM glycine in 25 mM Tris-HCl buffer pH 8.3. The SDS gel was electrophored with 30 mA at 4°C for 8-10 hours using electrophoresis apparatus (ATTA Cooperation). Then, the gel was shaken in 0.1 M sodium acetate buffer pH 5.0 for 5 minutes prior to shaking in 100 ml of 1% Triton X –100 in 0.1 M sodium acetate buffer pH 5.0 at 37°C for 2 hours to remove SDS from the gel. After digestion with chitinase, the digested chitin could be seen as a clear zone after staining with 0.01% fluorescent brightener 28 (Calcoflour white M2R) in a dark room for 10 minutes or as a violet strip under UV light. The stained gel was photographed using a red filter and kept for later isozyme study.

2. Interaction between elicitin and rubber leaves

2.1 Plant Materials

2.1.1 Rubber tree

The rubber, *H. brasiliensis*, clones were selected according to the degree of resistance to zoospores of *P. palmivora* as described in 1.2.1. The resistant clone, BPM-24, and the susceptible clone, RRIM600, were used and grown as the same method as described in 1.2.1.

2.1.2 Tobacco

Tobacco, *Nicotiana tabacum*, was grown in 10 inches-diameter plastic bag with sterile soil and fertilized every 2 weeks. The 6–8 weeks old tobacco leaves were detached and used for the following experiments.

2.2 Elicitin

2.2.1 The culture filtrate of *P. palmivora*

Mycelium of *P. palmivora* grown on PDA for 5-7 days was cut with 5 mm diameter cork borer, twenty-five pieces of which were transferred into 150 ml sterile Potato Dextrose Broth (PDB) in 500 ml conical flask and subsequently shaken at 100 rpm at 25°C in the dark condition for three weeks. Thereafter, small amount of mycelium in PDB culture was transferred to PDA in Petri dish for checking the contamination in the culture filtrate from other microorganisms. The mycelium was filtered by Whatman paper No.4 and the filtrate was then kept at –20°C for several months or immediately purified for elicitin.

2.2.2 The growth curve of *P. palmivora*

The culture filtrate of *P. palmivora* was prepared as described in 2.2.1. An aliquat of about 1.5 ml was collected in the Bioharzard at 7, 10, 15, 20, 25, 30 and 40 days and kept at –20°C for protein determination. The

protein was quantified by using bicinchoninic acid (BCA) method and SDS-PAGE electrophoresis was performed on the aliquot, then followed by silver staining. For the BCA method, 100 µl of culture filtrate was mixed with BCA reagent and quantified as described in 2.2.6 (Smith, *et al.*, 1985). 25 µl of each sample was subsequently loaded in each lane of SDS-PAGE and run with 65 volts for 1 hour. Thereafter, the gel was stained with silver nitrate as described in 2.2.7.

2.2.3 Purification and percent yield of elicitin

One litre of culture filtrate was slowly added with gentle stirring with 657 grams of $(NH_4)_2SO_4$ (ammonium sulphate) to bring to 90% saturation of solution at 4°C. The solution was allowed to stand for 30-60 minutes at the same temperature, then centrifuged at 10,000 rpm at 4°C for 20 minutes. The supernatant was discarded and the pellet was resuspended with distilled water. The 2.5 ml of solution was desalted by loading onto PD-10 column and eluted with distilled water. The first 2.5 ml fraction was discarded and the next 3 ml fraction was kept. The fraction from PD-10 column was further purified by chromatography on 20 ml column of DEAE cellulose in 20 mM Tris-HCl buffer (pH 7.0) at 4°C. The DEAE cellulose column was washed with 20 mM Tris-HCl buffer (pH 7.0), 3 ml fractions were monitored for protein at the absorbance of 280 nm until the washed fraction had no protein. Elution was achieved with 0.1 M NaCl in the same buffer, 1 ml/fractions were collected and measured for protein with the same procedure as the washed fraction. The eluted fractions with high protein content were pooled and precipitated with ammonium sulphate to 90% saturation at 4°C, then let stand for 30-60 minutes before centrifugation at 10,000 rpm at 4°C for 20 minutes. The supernatant was discarded and the pellet was resuspended with distilled water, the 5 ml of which was loaded onto Sephadex G-50 column in 20 mM Tris-HCl buffer

(pH7.0) and eluted by the same buffer after loading the elicitin solution. The fractions of 2 ml were collected and measure protein content was measured at 280 nm absorbance. The high protein fractions were pooled and lyophilized. The dried elicitin was resuspended with sterile water and measured for protein content by the BCA method as described in 2.2.6. The solution was stable for several months at 20°C.

The yield of protein from each step of purification by ammonium sulphate, PD-10 column, DEAE column, Sephadex G-50 column and lyophilization, was measured for the concentration by Bradford and BCA methods and identified by the silver staining of 16.5% polyacrylamide gel (Tricine-SDS-PAGE) according to 2.2.7.

2.2.4 Amino acid compositions

Elicitin was analysed in microgram quantities using a System 6300 High Performance Analyser following the methods of the manufacturer. The values in Table 6 are the mean of three analyses. The cysteine content was determined as cysteic acid after performic oxidation. Results were rounded to proximal integer values in order to compare with amino acid compositions of other known elicitins.

2.2.5 N-terminal amino acid sequencing.

Amino acid sequencing was performed by methods described by Applied Biosystems company. Automated cycles of Edman degradation were performed by gas phase protein sequencer (Applied Biosystems model 476A) and phenylthiohydantoin (PTH) amino acid derivatives were automatically identified by data analysis apparatus (Applied Biosystems model 610 A).

2.2.6 Protein quantification BCA method

The method was modified from that of Smith *et al.*, (1985). The 100 µl of sample was reacted with 2 ml of solution C (appendix) and shaken for a while. The mixture was left standing for 30 minutes at 34°C and the absorbance at 562 nm was measured. The amount of protein was calculated by comparing with standard curve of BSA.

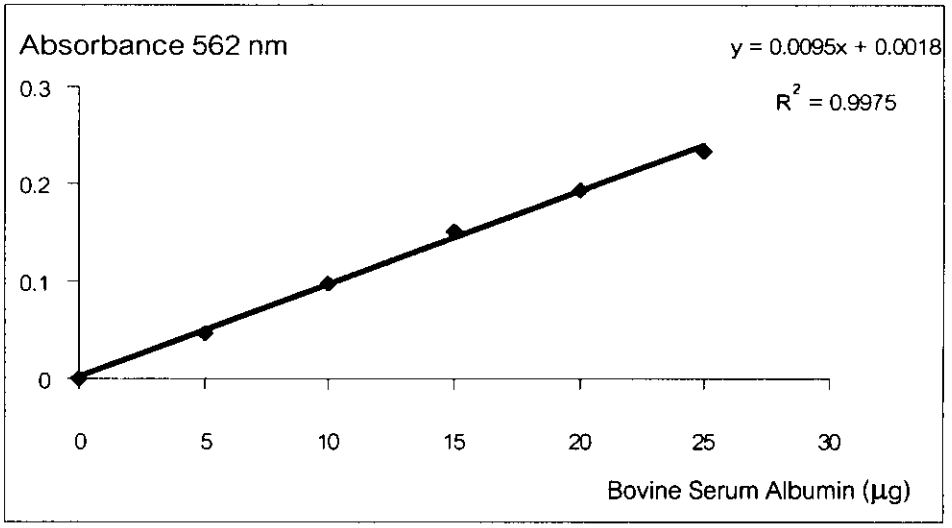


Fig. 18 Standard curve of Bovine Serum Albumin at absorbance 562 nm using the Bicinchoninic acid method.

2.2.7 Characterization of elicitin by Tricine-SDS-PAGE

SDS-PAGE was prepared according to 1.6.3. The sample and sample buffer in the ratio of 3:1 was applied in each lane of slab gel. The electrophoresis was run with 60 volts at 4°C for 1-1.5 hour. The electrophoresed gel was stained with silver nitrate according to the procedure of Pharmacia Biotech. Firstly, the gel was fixed in 40% ethanol and 10% glacial acetic acid for 30 minutes and sensitized with 30% ethanol, 25% w/v glutardialdehyde, 0.2% sodium thiosulphate and 6.8% sodium acetate for 30

minutes. After washing with distilled water 3 times, 5 minutes each, the gel was reacted with 37% w/v formaldehyde and 2.5% w/v silver nitrate solution followed by washing 2 times with distilled water, 1 minute each. The gel was developed by 2.5% sodium carbonate and 37% w/v formaldehyde then gently shaking for 2-5 minutes. When the dark-brown bands appeared, the gel was immediately immersed in stopping solution that contained 1.46% Na_2-EDTA solution for 10 minutes. Thereafter, the gel was washed with distilled water 3 times for 5 minutes and preserved with 30% ethanol and 87% w/v glycerol. The photograph was taken by Nikon camera Kodak Gold II 200 film.

2.3 Toxicity test

2.3.1 Toxicity of elicitin on tobacco and rubber leaves.

BPM-24 and RRIM600 were selected as resistant and susceptible clones to *P. palmivora,* respectively. Ten samples of rubber leaflets at B_2 - C stage as described by Halle' and Martin (1968) were weighed about 2 grams each, then detached and washed with sterile distilled water. The ten of two weeks-old *N. tabacum* leaflets were selected as a non-host for fungal-plant interaction. Tobacco leaves, weighing about 4 grams each, were selected for elicitin treatment and prepared as similar to the rubber leaves. All of tobacco and rubber leaves were exactly weighed and recorded before performing the experiment. The elicitin solutions were prepared in the concentrations of 1, 10 and 50 μg in 100 μl of sterile distilled water concentration of protein was measured by the BCA method in comparision with sterile distilled water was used as a control. The tobacco and rubber leaves were dipped into the elicitin solution using 1, 10 and 50 ug/1 gram of leaves. After the solution was taken up by the petioles, the tobacco and rubber leaves were dipped into sterile distilled water at room temperature in

the dark condition to allow necrosis to develop. All of treated leaves were weighed every 24 hours for 96 hours. Necrotizing effects were assessed by the changes in leaf fresh weight and expressed as percentages of initial leaf weight, modified according to the method of Rusterúcci *et al.* (1996)

2.3.2 The toxicity tests of the culture filtrate and purified fractions of *P. palmivora* on rubber leaves

Firstly the culture filtrate of *P. palmivora* was precipitated with $(NH_4)_2SO_4$ and desalted with PD-10 column. All of the fractions from PD-10 were checked for the toxicity of elicitin using the method as described in 2.3.1 but only the susceptible rubber clone, RRIM600 which was rapidly and strongly response to elicitin was selected. Then the fractions which had toxicity were pooled and loaded onto DEAE column. The toxicity of elicitin was also checked in each steps of purification such as the bound and unbound fraction of DEAE column, the fraction of Sephadex G-50 column and the purified form of elicitin. The unbound fractions from DEAE column were pooled and desalted by PD-10 column, the fractions no. 4-6 of which were tested for the toxicity of elicitin as described above. For the bound fractions, the fractions of the same peak of protein were pooled as a group. Each group of protein was desalted and checked for the toxicity as done on the unbound fractions. Thereafter, the fractions which had toxicity were precipitated, and loaded onto Sephadex G-50 column and washed by 20 mM Tris-HCl pH 7.0. The fractions of high protein content were checked for the toxicity of elicitin. Similarly, the fractions of high protein content were pooled and precipitated with $(NH_4)_2SO_4$. Finally the precipitate was resuspened and desalted by PD-10 column. The fractions no. 4-6 which contained purified elicitin were pooled and kept for further analysis. The fractions of 100 µl from precipitated filtrate and purified elicitin as described above were aliquoted in eppendorf tubes

whereas 100 µl of distilled water and 100 µl of 20 mM Tris HCl pH 7.0 was used as control for the fractions collected from column, respectively. The RRIM600 leaves were detached and weighed before performing the experiment. The petiole was cut with a sharp blade. The rubber leaves were then dipped into the prepared Eppendorf at room temperature and weighed 72 hours later. The toxicity of solution was revealed by the leaf weight loss compared with that of the control leaves.

2.3.3 Lignification

The C-stage of rubber leaves (BPM-24 and RRIM600) were detached and placed on the moist ened paper in a Petri dish. The leaves were wounded with fine sterile needle on both sides of abaxial leaf surfaces. The 10 µg/10 µl of elicitin solution was dropped over the lesion on the right side of leaf, while 10 µl of distilled water was dropped in the same manner on the left side as control. After 12 hours, the leaves were pad dried and kept at room temperature. Leaf area surrounding droplet of elicitin was cut in square every 24 hours after elicitin application and immersed in 2% phloroglucinol / 95% ethanol and kept at room temperature for 24 hours. Thereafter, the sample was placed on a glass slide and added 40 µl of concentrated HCl in the fume hood. After 2 minutes, the slide was observed under light microscope.

2.3.4 Effect of elicitin on necrosis of rubber leaves

The resistant rubber clone, BPM-24 (R), and the susceptible one, RRIM600 (S), were selected. The B_2 - C stage leaves were detached, cleaned and placed with abaxial side up on the moist paper in a Petri dish. The leaf was cut along the petiole and scraped on both right and left sides. Ten µl of solution which contained 10 µg elicitin was dropped on the scraped leaf surface on the left side whereas distilled water was dropped on the other

side as control. The Petri dish was kept at 25°C for the development of necrosis. The photograph was taken by a Nikon camera and micrograph was taken by Olympus light microscope equiped with camera after 24 hours of elicitin application.

2.4 Scopoletin synthesis in rubber leaves

To examine the Scp synthesis induced with elicitin in BPM-24, the resistant rubber clone and RRIM600, the susceptible rubber clone, five B_2-C stage leaves of both clones, weighed about 2 grams each, were detached and thoroughly washed with sterile distilled water. All leaves were cut in half, the upper parts of which were placed on the moist paper in the Petri dish and used as controls, the lower parts were dipped in the Eppendorf tubes containing 12.5 μg of elicitin in 30 μl solution. After all of the solution was taken up by petioles, the procedure was repeated twice using 30 μl of the sterile distilled water instead. Thereafter, small wounds were made on the abaxial surfaces of each treated and control leaves which were placed on moist paper in the Petri dish. The 50 μl of the sterile distilled water was dropped over every wound as a control. All were kept for 12 hours at 25°C using 40W Phillip daylight lamps. The droplets were collected after 8, 12, 16 and 24 hours and stored at –20°C. All samples were measured for the fluorescence by spectrofluorophotometer using emission wavelength at 340 nm and excitation wavelength at 440 nm. Amount of Scp was calculated by standard curve of Scp.

2.5 Total protein and PR-proteins

2.5.1 Determination of protein content and enzyme assays induced by elicitin

Five rubber leaves of the respective resistant and susceptible clones; BPM-24 and RRIM600, weight about 2 grams each, were detached

and selected at the stage as described in 1.2.1. The leaves were cross-sectioned into half, upper and lower parts. The small strip was cut for control at 0 hour. The lower half of leaves were dipped into 1.5 μg / 5 μl of elicitin solution in Eppendorf tubes, the upper half were used as controls and placed on the moist paper in the Petri dish at room temperature under light condition for 12 hours (using Phillip daylight 40 watt). After all solution was absorbed by petioles, the 20 μl sterile distilled water waas added into Eppendrof tubes. When they were taken all of water, the leaves were transferred to place on the moist paper in Petri dish at the same condition as control. Each group of leaves was cut into small strips, weight about 0.25 gram each and removed the petiole before weighting together. The samples were then quick frozen and stored at –20°C. One gram of each samples was grounded and prepared the crude leaf extract for protein and enzymes assays as described in 1.5.2 and 1.5.3.

2.5.2 Total protein estimation and enzyme activities of crude leaf extract.

The amount of total protein in crude leaf extract was examined by the Bradford method and gel electrophoresis as described in 1.6.1 and 1.6.2, respectively. The enzyme activities of glucanase and chitinase were measured according to the same methods as described in 1.7.1, 1.7.3, 1.7.4 and 1.7.5. The measurements of protein and enzyme activities were carried out at 0, 12, 24, 48 and 72 hours after elicitin treatment.

2.6 Transcription of chitinase gene after elicitin treatment

2.6.1 Northern blot analysis

2.6.1.1 Preparation for DNA probes

The first cDNA probes used for the study of β-1,3-glucanase and chitinase mRNAs were from tobacco (kindly donated by Prof.

Meins. Jr., Friedrich Miescher Institute, Switzerland) which had less homology with the two mRNAs, therefore, the second cDNA probes from rubber (Hglu and QHb-16) were used instead (kindly provided from Prof. Herve' Chrestin, ORSTOM, France).

2.6.1.2 Hglu (cDNA of *Hevea* β-1,3-glucanase)

cDNA of *Hevea* β-1,3-glucanase (976 bp) was inserted in carrier DNA. The carrier DNA was pMOS-Blue T (Amersham kit) which was the ampicillin resistant plasmid (Fig. 19)

2.6.1.3 QHb-16 (cDNA of *Hevea* chitinase)

cDNA of *Hevea* chitinase (160 bp) was inserted in carrier DNA. The carrier DNA was pMOS–BlueT which was the ampicillin resistant plasmid (Fig. 19).

2.6.1.4 Plasmid DNA preparation

E. coli were cultured in 50 ml of LB (appendix) medium for 14-16 hours and centrifuged at 12,000 rpm at 4°C for 10 minutes to collect bacterial cells. One ml of lysis solution was added, mixed together and left at 37°C for 10 minutes, then added 2 ml of NaOH/SDS, mixed and put on ice for 5 minutes. Thereafter, 1-5 ml of potassium acetate buffer pH 5.0 was added and left on ice for another 15 minutes prior to centrifugation at 12,000 rpm at 4°C for 10 minutes to precipitate proteins. The clear supernatant was recollected and transferred to a fresh tube; 10 ml of absolute ethanol was added and kept at -70°C for 10 minutes or at -20°C for 1 hour prior to centrifugation at 12,000 rpm at 4°C for 10 minutes. The precipitate of plasmid was washed with 70% ethanol, dried and dissolved with 0.5 ml of TE (10 mM Tris-HCl pH 7.6, 1mM EDTA) buffer solution. Contaminated RNA in plasmid was removed by RNase enzyme which was made to a final

concentration of 0.1 mg/ml and incubated at 37°C for 30-45 minutes. DNA concentration was analysed by measuring an absorbance at 260 nm (50 μg / ml of DNA had OD_{260} =1).

2.6.1.5 Treatment of plasmid DNA with restriction endonuclease enzyme

Fifteen μg of Hglu and QHb-16 plasmid was digested with 20 units of *Hin*dIII (Hybaid Limited) and 20 units of *Bam*HI (Hybaid Limited) in 3 μl of assay buffer (which supplied with the enzymes by Hybaid Limited.) and 18 μl of distilled water and incubated at 37°C overnight.

2.6.1.6 Separation of insert DNA from carrier DNA

Plasmid DNAs (from 2.6.1.4) and standard DNA (Lamda DNA cut with *Hin*dIII) were run in submerse gel electrophoresis using 0.8% agarose in TAE buffer (40 mM Tris-acetate and 2 mM EDTA). Orange bands appeared under UV light after staining with ethidium bromide (Fig. 19). DNA bands of interest were then cut from agarose gel (Hglu had 976 bp, QHb-16 had 160 bp) and added with sodium iodide (NaI), the volume of which was 3 times of agarose gel weight. Gel was melted by incubation at 45-55°C for 5 minutes. Glassmilk suspension (from The Geneclean II kit) was added in the ratio of 1 μl per 1μg of DNA and gently shaken to let DNA binded with the glassmilk (silica matrix) prior to precipitatien by centrifugation for 5 seconds. The precipitation was washed with NEW WASH (from kit) for 3 times. DNA was washed from the glassmilk with distilled water and collected for further labelling.

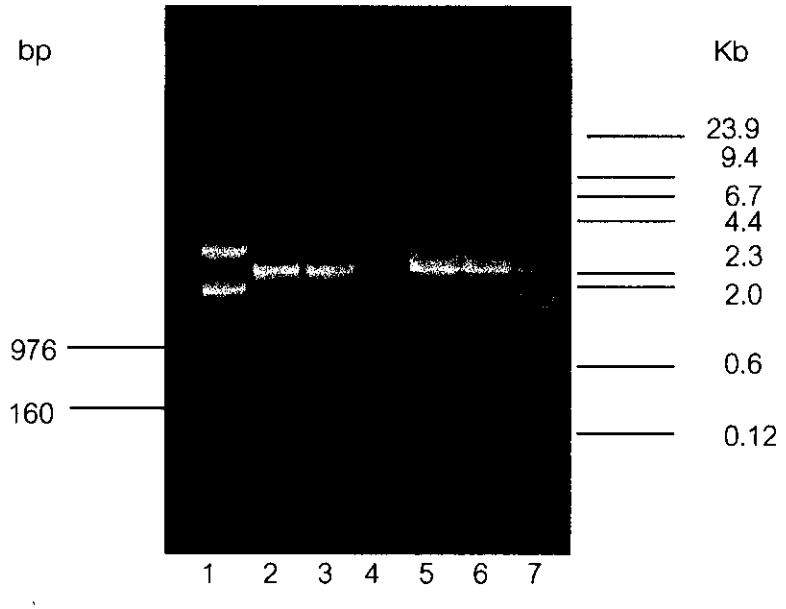


Fig. 19 The agarose gel electrophoresis pattern of *Hevea* glucanase and chitinase cDNAs digested with *Bam*HI and *Hin*dIII.

Lane 1 : plasmid of *Hevea* glucanase before digestion

Lane 2, 3 : inserted cDNA from plasmid of *Hevea* glucanase after digestion

Lane 4 : standard DNA (Lamda DNA + *Hin*dIII)

Lane 5, 6 : inserted cDNA from plasmid of *Hevea* chitinase after digestion

Lane 7 : plasmid of *Hevea* chitinase before digestion

2.6.1.7 DNA probe labelling by Biotin Random Prime kit (Pierce Company)

By this method, DNA from 2.6.1.5 was used as template; primer from kit was added to bind randomly with template by using

Biotin-N4-dCTP as one of deoxynucleotide mix, new DNA strand was synthesized after adding DNA polymerase (Klenow fragment). The 500 ng of DNA template was boiled with 10 µl of heptanucleotide mix for 5 minutes. After frozen and thawed, the mixture was brought on ice bath and added 10 µl of dNTP mix, 5 µl reaction buffer and 1 µl of Klenow fragment (the reagents were supplied with Kit). After incubation at 37°C for 1 hour, the enzyme was inactivated and unincorporated nucleotides were eliminated. The synthesized probe was quantified by measuring the OD_{260}.

2.6.1.8 Preparation of rubber leaves and fungal inoculation

Leaves of rubber clones, RRIM 600 and BPM-24, at B_2-C stage (as shown in 1.2.1) were elicited with elicitin 2 µg/10 µl/1 gram leaf weight by dip assay (as in 2.5.1). The distilled water was used as control. Both control and inoculated leaves were collected for RNA extraction after 0, 48 and 72 hours, respectively.

2.6.1.9 RNA isolation from rubber leaves

One gram of the control and treated rubber leaves were ground by using 5 ml of liquid nitrogen and homogenated with 6 ml of solution D (consisted of 4 M gaunidium thiocyanate, 25 mM sodium citrate pH 7.0, 0.5% sarcosyl and 0.1 M 2-mercaptoethanol). After cooling on ice for 15 minutes, the solution was centrifuged at 10,000 rpm for 20 minutes at 4°C. After discarding the supernatant was transferred to a fresh tube then added 0.18 ml of 3 M sodium acetate and 4.5 ml of absolute ethanol. The mixture was placed at –20°C for at least 1 hour and centrifugated at 10,000 rpm for 20 minutes at 4°C. After discarding the supernatant, the pellet was resuspended in 1.2 ml of solution D and sequentially added 0.6 ml of phenol and 0.6 ml of chloroform/isoamyl alcohol (24:1); the sample was mixed by tube inversion

after each addition and shaken vigorously for 10 seconds. Thereafter, the mixture was centrifuged at 5,000 rpm for 10 minutes at 20°C. The supernatant was again added 0.6 ml of phenol and 0.6 ml of chloroform/ isoamyl alcohol (24:1) then centrifuged at 5,000 rpm for 10 minutes at 20°C. The upper phase was transferred to a fresh tube and added 133 µl of 2 M NaCl, 177 µl of 3 M sodium acetate and 2 ml of isopropanol. The solution was kept at –20°C for 2 hours and recentrifuged at 15,000 rpm for 30 minutes at 4°C. The pellet was air dried at room temperature for 5 to 10 minutes and resuspended in 300 µl of sterile distilled water. The solution was brought to final concentration of 0.1% SDS, 0.001 M $MgCl_2$ and 2 M LiCl and kept at 4°C for overnight. The incubated sample was centrifuged at 15,000 rpm for 30 minutes at 4°C. The RNA pellet was air dried for 10 minutes, resuspended with 425 µl of sterile distilled water and precipitated with 75 µl of 3 M sodium acetate and 1,250 µl of absolute ethanol for 2 hours at -20°C. The RNA pellet was recollected by centrifugation at 15,000 rpm for 30 minutes at 4°C, rinsed with 70% ethanol and centrifuged at 15,000 rpm for 30 minutes at 4°C. The RNA pellet was dried and resuspended with sterile distilled water which could be kept at -20° C for several months. The concentration of RNA was determined by measuring the absorbance at 260 nm. This measurement permits the calculation of the RNA 's concentration in a sample by using the following formula

$$[RNA] = 40\ D \times A_{260}\ \mu g/ml$$

Where A_{260} = The optical densities (OD) or absorbance at 260 nm

D = dilution factor of the sample

40 = extinction coefficient of RNA

The purity of sample could be calculated from the ratio between OD of absorbances at 260 nm and 280 nm. The purified RNA had a A_{260} /A_{280} ratio of 2± 0.05.

2.6.1.10 Agarose gel electrophoresis

Gel electrophoresis was used for determining the size of RNA from rubber leaves. The 1.5% (w/v) agarose solution was prepared by using TAE buffer (appendix) and autoclaved at 60°C in order to eliminate the ribonuclease, before being poured into plastic block. The plastic comb was placed into one end of the gel. The comb was carefully removed after the agarose gel was completely polymerized which took about 30–45 minutes at room temperature. The gel was installed on the platform of the electrophoresis tank, which contained TAE buffer. The 15 µl of RNA sample was mixed with the 25 % (v/v) gel loading buffer (appendix) was carefully loaded into the slots of submerged gel by using a micropipette. Electrophoresis was carried out at a constant 60 volts for 1 hour and then at 95 volts until the dye was migrated at a necessary distance. After electric power was turned off, the gel was brought to determine RNA patterns under UV light box and photographed by using polaroid camera.

2.6.1.11 RNA preparation for gel electrophoresis

Ten µg of each RNA sample was precipitated with ethanol, then centrifuged and dried prior to dissolving in 2.2 µl of buffer A (appendix). Thereafter, 4.8 µl of 2.2M/50% formaldehyde/formamide (appendix) was added and warmed up to 70°C for 10 minutes, then immersed on ice at once. RNA was separated with 1.5% agarose in MOPS/EDTA buffer (1X) after adding 1.5 µl of gel loading buffer.

2.6.1.12 Transfer of RNA from agarose to nylon

After electrophoresis, gel was washed 1-2 times in deionized DEPC (Diethyl pyrocarbonate) H_2O in order to remove formaldehyde and then respectively put in 0.05 M NaOH for 20 minutes to partial hydrolyse RNA and neutralizing solution (0.1 M Tris-HCl, pH 7.5) for 30–45 minutes. To transfer RNA by capillary process, wet nylon was put on top of gel in 10X SSC (1.5 M NaCl, 0.15 M Tris sodium citrate pH 4.0). RNA from gel ascended to inner side of nylon as well as buffer solution (10X SSC) which required 6 – 24 hours to diffuse. After completion of transfer process, nylon was washed with 2X SSC and incubated at 80°C for 2 hours before being used for Northern hybridization.

2.6.1.13 Northern hybridization

Nylon (from 2.6.1.12) was immersed in hybridization buffer (0.1 ml/1 cm^2) at 55°C for 30 minutes for pre-hybridization. To denature, Biotinylated DNA probe (from 2.6.1.7) was denatured by boiled at 100°C for 10 minutes and left on ice for 5 minutes, then added to bind with pre-hybridized RNA on nylon and left overnight at 55°C for complete hybridization. Nylon was washed with stringency wash buffer (2X SSC and 1% SDS) at 55°C for 3 times, 15-20 minutes each (Reagents were supplied in North2South Chemiluminescent Nucleic Acid Hybridization and Detection Kit).

2.6.1.14 Chemiluminescent detection

After draining of stringency wash buffer, blocking buffer was added and shaken gently for 15 minutes at room temperature prior to mixing with streptavidin–HRP conjugate in a tube and made to 1:300 final dilution. This solution was put back on nylon for another 15 minutes. Thereafter, nylon was washed with washing buffer 4 times, 5

minutes each; then immersed in substrate solution (luminol/enhancer solution and peroxide solution) for 5–10 minutes prior to X-ray exposure. RNA was demonstrated as a black strip on X-ray film (substrate working solution produced luminescence for 6–8 hours after reacting with HRP).

2.6.2 RT-PCR

2.6.2.1 RT-PCR

If the Northern analysis was not successful, the RT-PCR was attempted instead. To obtain a chitinase cDNA (CHN) for expression studies both forward and reverse oligonucleotide primers was designed as described in 2.6.2.2 from the *H. brasiliensis* chitinase gene. The *H. brasiliensis* chitinase gene (or Hevamine B was searched from Gene Bank at www.ncbi.n/m.nih.gov. which cDNA sequences were reported by Bokma, E. (1998). RT-PCR reactions were performed with the QIAGEN OneStep RT-PCR kit using 4 μl of total RNA isolated from leaves (as described in RNA preparation) of the resistant clone, 4 hours after elicitation with elicitin (1 μg elicitin/ 1 g leaf weight). RT-PCR was performed in thin walled PCR tube which contained the following reaction mixture: 10 μl of 5× QIAGEN OneStep RT-PCR buffer, 2 μl dNTP Mixture, 10 μl of 3 pmol/μl of primer A, 10 μl of 3 pmol/μl of primer B and 2 μl QIAGEN OneStep RT-PCR Enzyme. Mix and using the following PCR cycle: Reverse transcription at 50°C for 30 minutes, activated DNA polymerase at 95°C for 15 minutes, and amplified with denaturation at 94°C for 1 minute, annealing at 51°C for 1 minute, extension at 72°C for 1 minute for 40 cycles with a final extension at 72°C for 10 minutes. The amplified DNA were electrophored on 1.5% agarose gel with TAE buffer at 150 volts for 1.5 hours and stained with ethidium bromide.

2.6.2.2 Primer CHN for RT-PCR

The forward and reverse primers were designed for 19 and 20 bps using primerchecker v 0.07. The forward primer (d5'-GGGATT GGAAGCTACACCCC-3') corresponded to nucleotides (nt) 361 to 379 and the reverse primer (d5'-ACCAAAGCATAACACCTCCG-3') corresponded to nt 886 to 867 in the chitinase gene of *H. brasiliensis* were employed. The melting temperature, %GC and self dimer of primers were shown in table 4.

Table 4 Sequences and properties of the forward and reverse primers designed from *Hevea brasiliensis* chitinase gene.

nt	Sequence (5'→3')	Melting temp. (Tm)	% GC	Self Dimer
361-379	GGGATTGGAAGCTACACCCC	59.4	57.9	-6.3
886-867	ACCAAAGCATAACACCTCCG	60.0	50.0	-3.6